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TEST-SYSTEM FOR ESTIMATION OF ACTIVITY OF GSK-3 INHIBITORS AS ANTIHYPOXANTS AND DIFFERENTIATION OF ENDOTHELIAL PROGENITORS IN VITRO

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Abstract: A model test-system was developed for validation in vitro of antihypoxic and endothelio-differentiating activity of newly created glycogen synthase kinase-3 inhibitors in comparison with the reference drug β4-thymosin.
Keywords: glycogen synthase kinase-3; GSK-3; β4-thymosin; hypoxia; ischemia; endothelium.

Glycogen synthase kinase 3 (GSK-3) is a tyrosine kinase participating in many of the central cell signaling pathways, including proliferation, migration, and apoptosis. The process of protein phosphorylation with the involvement thereof normally inhibits the activity of subsequent elements in the signal transduction chain. GSK-3 activity inhibitors are promising candidates for the role of drugs used in many diseases, including the role of stimulators of tissue repair and neoangiogenesis, and inducers of myelopoiesis in different areas [1, 2].

One of the challenges in the development of new drugs is a screening of biological activity of synthesized candidate molecules. Currently, most of the experimental and preclinical studies in Russia is carried out in laboratory animals, which, in our opinion, is not the best solution. It is difficult to detect and control all body-level chemical and biological processes occurring with a medicament, including strength of initially unknown pharmacokinetic characteristics of new chemical compounds, and inaccurate correspondence between the active substance in an animal and a human. There are also issues of the ethics of using living models, and the lack of model animals applicable for some conditions and diseases. In this regard, pharmacologists and pharmaceutical companies start using more actively the cell cultures in the early stages of development and testing of new drugs [3]. The described approach is free from dangerous shortcomings for animals, otherwise, a wide range of standardized and primary cell lines allows developing an adequate model virtually for any potential medicines and screening of biological activity of the synthesized candidate molecules.

This paper deals with the development of a model cell test system for testing the biological activity of new GSK-3 inhibitors in the experiments in vitro. Any

model requires using certain control substance with known activity to conduct a comparative study of new molecules. For the GSK-3 inhibitors, we chose β4-thymosin studied in sports pharmacology and in some other areas of medicine [4, 5, 6], and available in the form of ready substance.

Materials and methods.

Endothelial progenitors were obtained from bone marrow of 10 Wistar rats aged 3-4 weeks, sacrificed by decapitation under ether anesthesia, by the modified method by Nana Y. et al. [7]. Bone marrow was washed with phosphate-buffered saline (PBS, pH = 7.4; PanEco # R074) using a syringe out of the diaphysis and mechanically crushed epiphysis of tubular bones of limbs. For removal of bone, connective tissue and muscle fragments, the washed bone marrow was filtered through nylon filters (SPL LifeSciences #93100) with a pore diameter of 100 μm.

The filtered cell suspension in PBS was carefully transferred to a ficoll-urografin solution with ρ=1.077 (PanEco #P052) and centrifuged at 2000 rev/min for 20 min at room temperature. A ring-shaped mononuclear fraction on the ficoll-urografin surface was carefully collected and transferred to new centrifuge tubes (SPL LifeSciences # 51115), re-suspended in PBS and centrifuged at 1500 rev/min for 10 minutes at room temperature. The supernatant was aspirated, and the settled cells were again re-suspended in PBS and centrifuged under the same conditions, after which the supernatant was removed. The cell pellet was re-suspended in complete medium containing Medium 199 (PanEco #S210p), 15% fetal bovine serum (HyClone#K052/SV30160.03), 10 ng/ml FGFb (PanEco #FR07010) and 20 ng/ml VEGF (Sigma #V4512), and dissipated on a 24-well plate (Corning Costar #3524) at the rate of 100 thousand cells per well. Further, incubated in a humidified atmosphere of 5% CO_2 at 37°C.

After 2 days, the cells not attached to the well bottom were removed and a new complete growth medium was added to the attached cells. Further, every 3 days, a half of the medium was removed and equal volume of fresh complete medium was added to each well.

After 11 days, the supernatant was completely removed from cells and fresh complete medium was introduced to a portion of the cells – with the addition of β4-thymosin (LCN Bio-Chemicals Limited) at a concentration of 2 mg/ml. The cells were incubated for 3 days in a humidified atmosphere of 5% CO_2 at 37°C. Cell ischemia was simulated by blocking oxygen delivery to cells through the formation of an oil film on the surface of the culture medium in the plate wells, with the use of cell-indifferent mineral oil (Sigma #M5310). The duration of hypoxia was 2, 4, 6 and 8 hours. In addition, some of the cells were subjected to further "starvation", by replacing the medium with the PBS.

The total number of cells was assessed by counting the cell in 5 adjacent fields of view in each well of the plate by using Hoffman staining with Eclipse Ti-S (Nikon) fluorescence microscope. The number of viable cells was assessed by fluorescence microscopy using fluorochromes: calcein – AM (Sigma #17783) and ethidium bromide (Sigma #E7637) in the same fields of view. Belonging to the endothelial progenitors was evaluated by membrane expression of Flk1/VEGFR1 specific marker stained by double antibody (SantaCruz #sc-505 and #sc-2012) fluorescence method.

Results and discussion.

Subject to the interest in the anti-ischemic and angiogenic effect of the GSK-3 inhibitors, we have studied the β4-thymosin ability to influence these parameters in our model.

Upon assessing the dynamics of the total number of cells in the plate wells under hypoxia (Figure 1), we found a protective effect of β4-thymosin on cells, which persists throughout all hypoxia – even at 6 and 8 h of hypoxia the test wells with a nutrient medium and β4-thymosin retained 22.9 ± 2.7 and 20.2 ± 2.0 cells, respectively, in the 5 fields of vision (hereinafter, M±tm, n=10), while the number in the control continued to decrease progressively up to 1.9 ± 1.2 and 0.9 ± 0.6 cells in these periods of time. The differences between the control and the β4-thymosin-containing medium were significant ($p<0.05$). Under hypoxia and “starvation”, there was a sharp decline in the number of cells at the beginning of the experiment – from 54.4±2.6 to 36.2±1.9 after 2 hours, and to 6.0±0.7 cells after 4 hours, which exceeded the initial values, where the number of cells was decreasing slower – from 30.1±1.3 to 20.0±1.0 after 2 hours, and to 11.8±1.0 after 4 hours.

The dynamics of cell death in the wells against their initial number is shown in Fig. 2.

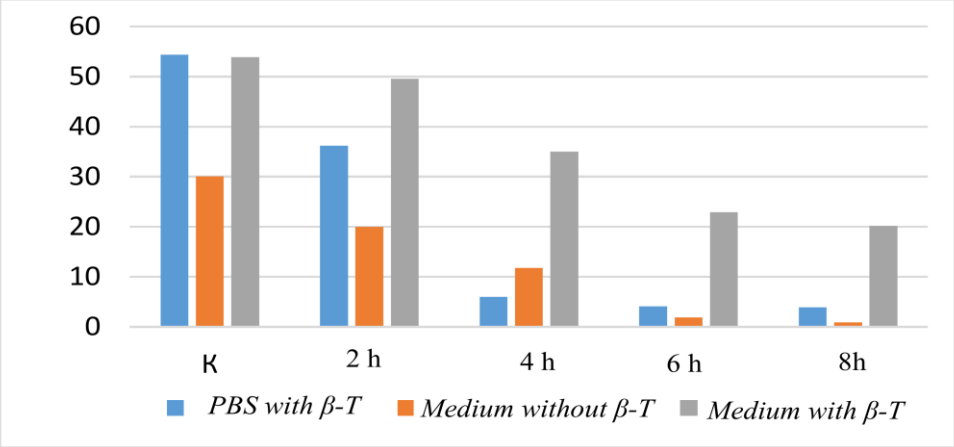

*Figure 1.*The absolute number of cells in 5 fields of view

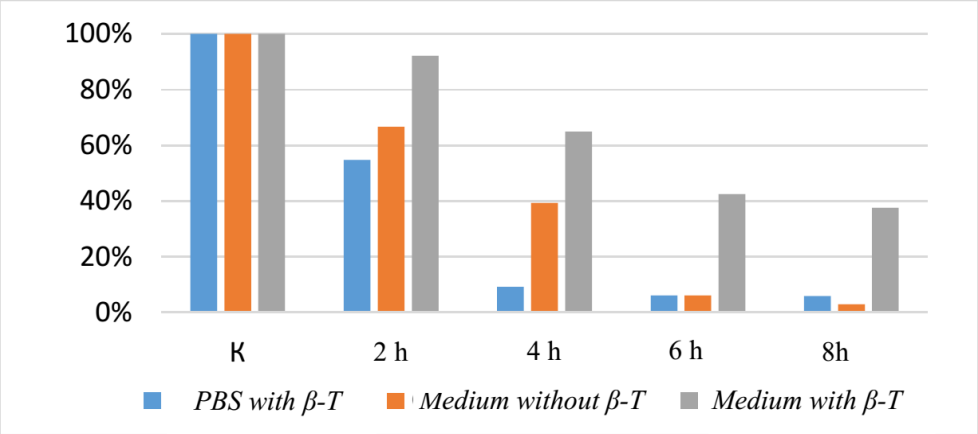

Figure 2. The percentage dynamics of the number of cells in 5 fields of view

According to these graphs, we cannot exclude a protective effect of β4-thymosin expressing at long stay of the cells under hypoxic and "starvation" conditions, because, despite the sharp decline in the number of cells in the early hours of combined ischemia, the wells with the PBS had more cells remained than the control wells at the later stages of the research (4.1±0.9 cells after 6 hours and 3.9±0.6 cells after 8 h against 1.9±1.2 and 0.9±0.6 cells, respectively, – in the control).

More pronounced protective effect of β4-thymosin on cell survivability under hypoxia and combination of hypoxia with "starvation" is shown in Figures 3 and 4, where the number and percentage of living cells in control is much lower than in test samples.

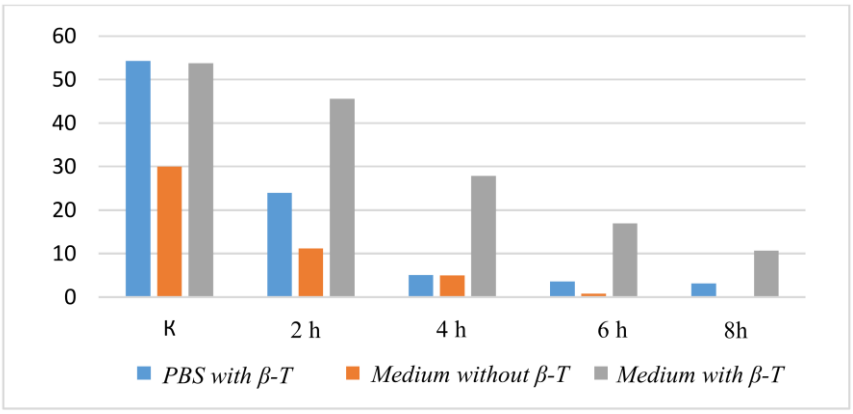

*Figure 3.*The absolute number of living cells in 5 fields of view

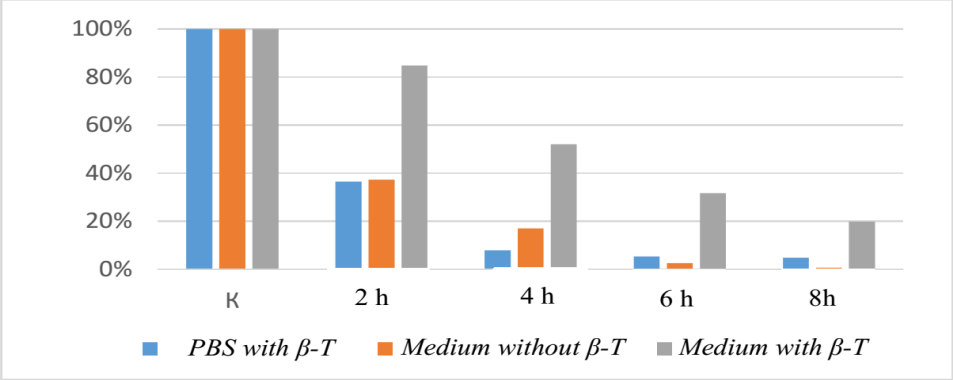

*Figure 4.*The percentage dynamics of the number of living cells in 5 fields of view.

According to the literature it is known that β4-thymosin prevents apoptosis of endothelial progenitor cells in culture [5]. This ability, apparently, explains a large proportion of living cells in the test samples as compared to the control. However, the study found that the initial number of cells in the wells with the cultivation conducted for 3 days in the presence of β4-thymosin is higher than in β4-thymosin-free wells (columns K in Fig. 1). The experiment does not give an answer, to what extent this phenomenon is due to the anti-apoptogenic effect of β4-thymosin and whether the latter is able to stimulate the cell proliferation, since these mechanisms have not been studied yet. There is information the literature about both stimulating and inhibiting effect of β4-thymosin on proliferative activity of the cells through the implementation of Wnt and insulin signaling pathways [1].

The next stage of the experiment was to study the endothelial differentiation of adhesion fraction of bone marrow mononuclear cells, wherefore we counted the number of cells that express a marker of endothelial progenitor cells – early progenitors of endothelial cells – Flk-1 (VEGFR), which is a receptor of vascular endothelial growth factor. Results in Table 1 show that the presence of β4-thymosin not just increases the total number of cells, but also significantly increases the percentage of cells that express the said endothelial progenitor marker - 50.6±4.7% Flk^+ cells in the control, and 82.8±2.2% upon cultivation for 3 days with β4-thymosin.

Table 1

Expression of endothelial progenitor marker ($Flk\text{-}1^+$) in the culture of bone marrow MSCs

	Without β4-thymosin	With β4-thymosin
Total cells (M±tm)	20.8±1.3	51.0±2.4*
$Flk\text{-}1^+$ cells (M±tm)	10.5±1.1	42.2±2.1*
% $Flk\text{-}1^+$ cells	50.6±4.7	82.8±2.2*

Note: * - $p<0.05$

Along with cardioprotective properties of GSK-3β [8, 9] inhibitors, shown by other researchers, the ability to stimulate the differentiation of MSCs into endothelial ones makes it more promising to study them in terms of prevention and treatment of coronary heart disease and arterial occlusive diseases.

Thus, the experimental findings are consistent with the results obtained by other researchers involved in the study of biological activity of GSK-3 inhibitors and, in particular, β-thymosin. The latter may further serve as a reference agent in comparative studies. Therefore, the proposed experimental cell model can be successfully used in the preliminary testing of new chemical compounds in terms of their curative potential in ischemic disorders without the use of laboratory animals at the early stages of research.

References

1. Osolodkin D.I. Molecular design of potential glycogen synthase kinase 3 inhibitors. Diss. PhD on Chemistry. Moscow: 2011, 205 p. [eLIBRARY]

2. Shamanskaya T.V., Osipova E.Yu., Rumyantsev S.A. Umbilical cord bloods hematopoietic stem cells ex vivo expansion (the literature review). Oncohematology. N 1 (2012): P. 35-44. [eLIBRARY] [Full text]

3. Marx U., Sandig V. Drug Testing In Vitro: Breakthroughs and Trends in Cell Culture Technology. Germany: WILEY-VCH, 2007. 298 p [Open Url]

4. Xu T.J., Wang Q., Ma X.W., et al. A novel dimeric thymosin beta 4 with enhanced activities

accelerates the rate of wound healing. Drug Design, Development and Therapy. Oct 1; Vol. 7 (2013): P. 1075-1088. [PubMed][PMC]

5. Chang Z.T., Hong L., Wang H., et al. Application of peripheral-blood-derived endothelial progenitor cell for treating ischemia-reperfusion injury and infarction: a preclinical study in rat models. Journal of Cardiothoracic Surgery. Vol. 8 (2013): P. 33-43. [PubMed][PMC]

6. Zhao Y., Qiu F., Xu S., et al. Thymosin β4 activates integrin-linked kinase and decreases endothelial progenitor cells apoptosis under serum deprivation. Journal of Cellular Physiology. Vol. 226 (2011): P. 2798-2806. [PubMed]

7. Yang N., Li D., Jiao P. et al. The characteristics of endothelial progenitor cells derived from mononuclear cells of rat bone marrow in different culture conditions. Cytotechnology. Vol. 63 (2011): P. 217-226. [PubMed][PMC]

8. Hinkel R., El-Aouni C., Olson T., et al. Thymosin β4 Is an Essential Paracrine Factor of Embryonic Endothelial Progenitor Cell-Mediated Cardioprotection. Circulation. Vol. 117 (2008): P. 2232-2240. [PubMed][PMC]

9. Miura T., Nishihara M., Miki T. Drug Development Targeting the Glycogen Synthase Kinase-3β (GSK-3β)-Mediated Signal Transduction Pathway: Role of GSK-3β in Myocardial Protection Against Ischemia/Reperfusion Injury. // J. Pharmacol. Sci. V. 109 (2009): P. 162-167. [PubMed][Full text]