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C60 FULLERENE NANOPARTICLES PREVENT DIABETIC RETINIPOTHY COMPLICATIONS

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Chronic complications of diabetes are progressed in 30-50% patients both 1-st and 2-nd types of diabetes mellitus and are important cause of capacity for work and mortality. The pathogenesis of diabetic complications associated with multifactorial disturbances of the main pathways regulation. Diabetic retinopathy primary is determined as microvessel disease. Recent data show that glial cells play important role in this diabetic complication. Diabetic complications associated with switching over to polyol pathway of glucose utilization, the growth of nonenzimatic glycation products, an activation of proteinkinase C that lead together to disturbance of redox balance. Glial cells of retina provide the nutrition and the defence of neurons from excitotoxic death. Glial reactivation induced metabolic disease and oxidative stress progress in retina during diabetes mellitus is key pathogenetic factor of diabetic retinopathy. Fullerene C_{60} and some watersoluble derivates of this nanoparticles are known as more power antioxidants, which show neuroprotective effects in different kind pathology and damaging factors. In presented work firstly there were observed the effects of nanoparticles hydrated C_{60} fullerene on expression poly(ADP-ribose) polymerase (PARP), caspase-3 and glial fibrillary acidic protein in retina of diabetic rats. Reliable rising of PARP, GFAP and caspase-3 levels determinated in retina of diabetic rat compare with control. Obtained data show that hyperglycemia induces the reactivation of glial cells in retina. The consuming a solution C_{60} (60 нМ) with drinking water during 12 weeks leads to significant decreasing every observed parameters in treated with fullerene diabetic rat group compared with untreated diabetic rats. Thus, obtained results evidence of neuroprotective effect of C_{60} fullerene for diabetic retinopathy and realization this effect through inhibition of gliocytes overactivity in retina.

Keywords: diabetic retinopathy, glial fibrillary acidic protein (GFAP), poly(ADP-ribose) polymerase (PARP), apoptosis, hydrated C_{60} fullerene.

Diabetes mellitus belongs to is a group of metabolic disorder characterized by hyperglycemia and often leads to numerous complications, including retinopathy. Diabetic retinopathy (DR) is a multifactorial disease, and persistent hyperglycemia appears to be a major cause of its progression. DR is the most-feared complication of diabetes mellitus and the most frequent cause of new cases of blindness among adults and aged patients [1]. Diabetes is retinal neuropathy is associated with enhanced oxidative stress resulting of excess generation of ROS that often leads to retinal microvascular cell death. Recent data suggest that diabetic retinopathy is a progressive neurodegenerative disease associated with disturbance the main metabolic pathways and oxidative stress generation. Administration of antioxidants to diabetic rats protects retina from oxidative damage, as well as the development of retinopathy [2].

Oxidative damages of the macromolecules, a specially DNA, are critical for retinal pathogenesis. High level of ROS induces DNA strand breaks in the retina by hyperglycemia [3], and

ROS-induced DNA single-strand breakages were considered an obligatory step for poly(ADP-ribose) polymerase (PARP) activation. PARP is a nuclear enzyme that regulates several cellular events including DNA repair, cellular division and differentiation, gene expression, mitochondrial function, and cell death. However, there are no studies has showing the cross - talk between PARP activation and glial cells reactivation in diabetic retina. Glial cells have powerful antioxidant systems and provide a surviving of neurons during oxidative stress. Astroglia also produce a host of trophic factors, which are crucial for the survival of neurons. However, activated astroglia become hypertrophic, exhibit increased production of glial fibrillary acidic protein (GFAP). Different kind factors induce typical reactivation of astrocytes. This universal cell response named astrogliosis and associated with cytoskeleton protein GFAP overexpression [4]. Pristine C_{60} fullerene has recently gained considerable attention as a promising candidate for many biomedical applications [5].

The aim of this study was to explore the effect of C_{60} fullerene on astrocyte reactivity, oxidative stress generation and apoptosis activation. To test this neuroprotective effect of C_{60} fullerene, we measured the levels of ROS generation, an expression PARP, GFAP and cleaved caspase-3 in the retina of diabetic animals.

Materials and methods

All procedures were carried out in accordance with the national and international guidelines and laws concerning animal welfare and are ethically acceptable. The study was performed on male Wistar rats (210–310 g) 11-12 weeks of age, which were fed a standard diet and had free access to food and water. Diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) solution in citrate buffer (pH 4,5) at 60 mg/kg b.w. The animals were maintained on 12-h light/dark cycle and randomly divided into the following groups (n=7): control group (C); normal control treated with C_{60} fullerene (C60); diabetic group (STZD); diabetic groups treated with C_{60} fullerene (STZD+C60). STZ-induced diabetic rats were divided into 2 groups: the rats in group I received normal drinking water without any supplementation, and those in group II received drinking water with C_{60} fullerene (40 ng/ml) during 12 weeks after establishment of diabetes. After 12 weeks, all experimental rats were sacrificial via cervical dislocation under mild diethyl ether narcosis. The eyes were removed, and retina was isolated and frozen immediately in liquid nitrogen and stored at -80^0C to be analyzed by Western-blot analysis, immunohistochemistry (IHC) or biochemical assay, as described early [5]. Reactive Oxygen Species (ROS) level was measured in retinal tissue homogenates using a 2,7-dichlorofluorescein-diacetate (DCHFDA).

Quantitative results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests. P values <0,05 were considered to indicate statistical significance. SPSS version 12.0 was used for the statistical analyses.

Results and discussion

The body weight of the diabetic rats was significantly lower, and their blood insulin value as lower compared with age-matched normal control rats (243±13,5 g versus 312±17,1 g and 27,8±1,85 μU/ml versus 46,9±2,52 μU/ml, respectively). It should be noted that C_{60} fullerene treatments of the diabetic rats partially restored the insulin and body weight.

Spectrofluorometric analysis demonstrated significant upregulation of ROS level by 49 % in diabetic retinas compared to non-diabetic retinas.

Comparative analyses of the images of fixed retinal slices showed dramatically increased overall GFAP immunostaining in retinas of diabetic rats versus non-diabetic animals (Fig. 1, A). Western-blot analysis demonstrated significant upregulation of PARP-1/2 expression in diabetic retina compared to non-diabetic retinas. The expression of PARP-1/2 protein in the retina of

diabetic rats was upregulated by about 83 % as compared to the retinas of non-diabetic rats (Fig. 1, B). The treatment with C_{60} fullerene did not change the content of PARP-1/2 protein in the retina of control group. These differences between GFAP immunoreactivity in diabetic retinas and healthy control retinas indicates that STZ-induced hyperglycemia caused both astrocytes and Müller cell gliosis while 12 weeks of diabetes.

Western-blot analysis of retinal protein samples confirmed an increased GFAP level detected by the immunohistochemistry. Diabetes-induced elevation of intact subunit 49 kDa GFAP content in comparison with age-match control was shown (1,9±0,17 vs. 1,0±0,01 a.u., respectively, P>0,01).

Cleaved caspase-3, the apoptosis executer enzyme, was significantly (about 79 %) upregulated in the diabetic retinas compared to non-diabetic controls. (Fig. 1, C).

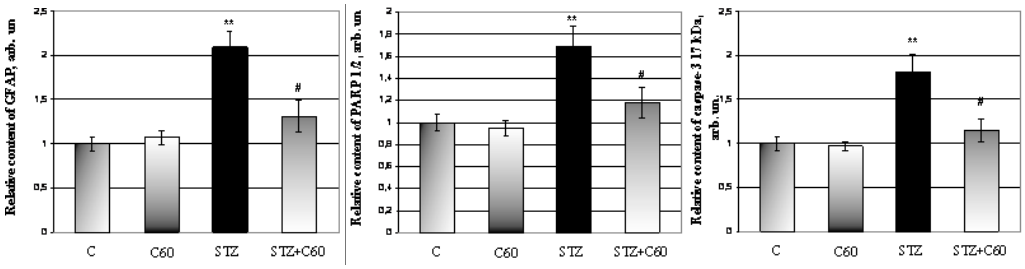

Fig. 1. Relative content of GFAP (A), PARP (B) and cleaved caspase-3 (C) determined in retina of control (C), normal control (C_{60}), diabetic (STZ) and diabetic treated with C_{60} (STZ+C_{60}) rats groups. ** - compared to control; # - compared to diabetic

Taking together, these results show that oxidative stress generation and upregulation of PARP, caspase-3 and GFAP overexpression might be are considered as targets for neuroprotection mechanisms fullerene nanoparticles for diabetic retinopathy therapy.

The level of ROS generation is attenuated by about 41 % in the retina of diabetic rats treated by C60 fullerene, as compared to untreated diabetic rats.

Further, we detected the reduction of macroglial activation in retinas of diabetic rats treated with C_{60} fullerene 12 weeks. Administration of C_{60} fullerene had no effect on intact GFAP levels. However, the rising of amount of cleaved GFAP polypeptides in the range of molecular weights 47–35 kDa were observed in the diabetic retina. Treatment with C_{60} fullerene resulted in decrease of the content of cleaved polypeptides in retina of the diabetic rats. Thus, the neuroprotection effect of C_{60} nanoparticles directs both glial cytoskeleton dynamic reconstruction and modulation macroglial cells reactivity in the diabetic retina.

We determined that chronic intake of C_{60} fullerene with drinking water of diabetic group of animals leads to significantly attenuated diabetes-induced upregulation of ROS level, PARP-1/2, GFAP and caspase-3. The treatment of diabetic rats with C_{60} fullerene resulted in significant decrease (68 %) of cleaved caspase-3, PARP content (70 %) and overexpression of GFAP (about 57 %) in retina compared with group of diabetic rats. It should be marked that consuming of C_{60} fullerene for 12 weeks did not change expression of GFAP, PARP and cleaved caspase-3 in the retina of normal control group.

Retina has a complex structure with a number of layers and cells types. The major cell types in retina are the vascular cells - pericytes and endothelial cells, macroglial cells - Muller cells and astrocytes, neurons - photoreceptors, bipolar cells, amacrine and ganglion cells, and microglia which act as phagocytes. Glial cells are important for providing blood-retinal barrier function and the microenvironment of another retinal cells [6, 7].

In present study, we investigated the role of astrocyte reactivity, oxidative stress, apoptosis activation and the effect of C_{60} fullerene in preventing diabetes-induced retinopathy. We demonstrated that in the diabetic retina, GFAP expression, PARP activation, levels of ROS, and cleaved caspase-3 were significantly increased. The administration of C_{60} fullerene to diabetic rats significantly attenuated diabetes-induced increase in GFAP overexpression, PARP activation, level of ROS and cleaved caspase-3 in retina of the diabetic rats. The consuming of C_{60} with drinking water for 90 days leads to significan protection of GFAP overexpression in retina of the diabetic rats. C_{60} fullerene nanoparticles facilitate stability of astrocytic cytoskeleton in brain under the oxidative stress [5]. The main mechanism by means of which C_{60} can provide survival of astrocytes is its ability to neutralize free radicals and, thus protect cellular membranes against the oxidative damage. The presented results show the immediate anticytotoxicity effectiveness of C_{60} in retina at the hyperglycemic stress. Our results are consistent with previous reports that demonstrated overexpression of PARP in the diabetic retinas [8]. We found that C_{60} fullerene treatment significantly prevented diabetes induced astrogliosis and apoptosis changes as well ROS generation. The mechanism by which the C_{60} fullerene prevents diabetes induced changes in the expression of PARP, GFAP and caspase-3 in the retinas remains unclear. Thus, these effects of C_{60} could be mediated by attenuating ROS generation. An additional mechanism that may contribute to the protective effect of C_{60} could be related to a suppression of ROS level, as initial factors for activation of intracellular signaling molecule that regulates the expression of genes involved in cell survival, apoptosis, and inflammatory response.

During the last decade, the biological effects of water-soluble form of C_{60} that is denoted as hydrated C_{60} fullerene are being studied extensively [5, 9]. Nevertheless, the intrinsic mechanisms of C_{60} antioxidant and tissue-protective activities are not yet completely elucidated.

Presented data suggest that C_{60} fullerene has a striking effect on signaling pathways associated with a control of astrogliosis and apoptosis and might be a novel therapeutic strategy for cellular dysfunction in the diabetic retinopathy. Additional study is required to further investigate this possibility.

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НАНОЧАСТИЦЫ С60 ФУЛЛЕРЕНА ПРЕДУПРЕЖДАЮТ ОСЛОЖНЕНИЯ ДИАБЕТИЧЕСКОЙ РЕТИНОПАТИИ

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Хронические осложнения диабета развиваются у 30-50 % пациентов 1-го и 2-го типов диабета и являются важной причиной утраты работоспособности и смертности. В патогенез диабетических осложнений вовлечены множественные нарушения регуляции основных метаболических процессов. Диабетическую ретинопатию первично определяют как сосудистое заболевание. Однако данные последних лет указывают на важную роль глиальных клеток в развитии данного осложнения диабета. Диабетические осложнения сопровождаются переключением утилизации глюкозы на полиольный путь, ростом продуктов неферментативной гликации, активации протеинкиназы С, что ведет к нарушению окислительно-восстановительного баланса. Глиальные клетки ретины обеспечивают питание и защиту нейронов от эксайтотоксической гибели. Реактивация глии, индуцированная метаболическими расстройствами и развитием окислительного стресса в сетчатке при сахарном диабете, является ключевым патогенетическим фактором диабетической ретинопатии. Фуллерен C_{60} и некоторые его водорастворимые производные известны как одни из наиболее мощных антиоксидантов, которые проявляют нейропротекторные свойства в условиях широкого спектра патологий и повреждающих факторов. В представленной работе впервые исследовано влияние наноструктур гидратированного C_{60} фуллерена на экспрессию поли(ADP-рибозо) полимеразы (ПАРП), каспазы-3 и глиального фибриллярного кислого белка (ГФКБ) в ретине диабетических крыс. Выявлено достоверное возрастание уровня ПАРП, ГФКБ и активации каспазы-3 в сетчатке крыс с диабетом по сравнению с контролем ($P<0,01$), что указывает на реактивацию ретинальных глиальных клеток в условиях гипергликемии. Потребление диабетическими крысами раствора C_{60} (60 нМ) с питьевой водой в течение 12 недель привело к значительному снижению всех исследованных показателей по сравнению с группой диабетических животных. Таким

образом, полученные результаты свидетельствуют о том, что протекторное действие гидратированного фуллерена в условиях диабетической ретинопатии реализуется благодаря угнетению им избыточной активации глиоцитов сетчатки.

Ключевые слова: диабетическая ретинопатия, глиальный фибриллярный кислый белок (ГФКБ), поли(ADP-рибозо)потилимераза (ПАРП), апоптоз, гидратированный C_{60} фуллерен.

НАНОЧАСТКИ С60 ФУЛЕРЕНУ ЗАПОБІГАЮТЬ УСКЛАДНЕНЯМ ДІАБЕТИЧНОЇ РЕТИНОПАТІЇ

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Хронічні ускладнення діабету розвиваються у 30-50 % пацієнтів 1-го і 2-го типів діабету і є важливою причиною втрати працездатності та смертності. У патогенез діабетичних ускладнень залучаються множинні порушення регуляції центральних метаболічних процесів. Діабетичну ретинопатію первинно визначають як судинне захворювання. Однак дані останніх років вказують на важливу роль гліальних клітин у розвитку даного ускладнення діабету. Діабетичні ускладнення супроводжуються переключенням утилізації глюкози на поліольний шлях, ростом продуктів неензиматичної глікації, активації протеїнкінази С, що веде до порушень відновно-окислювального балансу. Гліальні клітини ретини забезпечують харчування і захист нейронів від ексайтотоксичної загибелі. Реактивація глії, що індукується метаболічними розладами і розвитком окисного стресу в клітинах сітківки при цукровому діабеті, є ключовим патогенетичним фактором діабетичної ретинопатії. Фулерен C_{60} та окремі його водорозчинні похідні відомі як один із найбільш потужних антиоксидантів, що виявляє нейропротекторні властивості в умовах широкого спектра патологій та ушкоджуючих чинників. У роботі вперше досліджено вплив наноструктур гідратованого C_{60} фулерену на експресію полі(ADP-рибозо)полімерази (ПАРП), каспази-3 та гліального фібрилярного кислого білка (ГФКБ) в ретині діабетичних щурів. Виявлено достовірне зростання рівня ПАРП, ГФКБ і активації каспази-3 в сітківці щурів з діабетом порівняно з контролем ($P<0,01$), що свідчить про реактивацію ретинальних гліальних клітин в умовах гіперглікемії. Споживання діабетичними щурами розчину C_{60} (60 нМ) з питною водою протягом 12 тижнів привело до значного зниження всіх досліджених показників порівняно з групою діабетичних тваин. Таким чином, отримані результати свідчать про те, що протекторна дія гідратованого фулерена C_{60} в умовах діабетичної ретинопатії реалізується через запобігання надмірній активації гліоцитів сітківки.

Ключові слова: діабетична ретинопатія, гліальний фібрилярный кислий білок (ГФКБ), полі(ADP-рибозо)полімераза (ПАРП), апоптоз, гідратований C_{60} фулерен.