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Differences in Antioxidant/Protective Efficacy of Hydrated C_{60} Fullerene Nanostructures in Liver and Brain of Rats with Streptozotocin-Induced Diabetes

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Abstract

Background: Diabetes mellitus causes damage to many vital organs, including brain and liver, mostly due to excessive free radical generation and development of oxidative stress. Some water-soluble forms of C_{60} fullerene and their hydrated nanostructures are proposed for prevention as well as treatment of various pathological conditions caused by oxidative stress.

Aim of the study: Assessment and comparison of antioxidant effects of hydrated C_{60} fullerene (C_{60}HyFn) in brain and liver of rats with experimental streptozotocin (STZ)-induced hyperglycemia and evaluation of possible neuroprotective capacity of C_{60}HyFn acting as potent agent to suppress reactive astrocytosis.

Materials and methods: To induce hyperglycemia, male Wistar rats received single intraperitoneal (i.p.) injection of STZ in a dose of 45 mg/kg body weight (b.w). Thirty five rats were divided into 5 groups (7 animals per group): Group I (control, saline-injected rats); Group II (STZ-diabetic rats); Group III (rats injected with C_{60}HyFn in a dose of 0.3 mg/kg b.w–C_{60}HyFn control); Group IV (rats received single i.p. injection of C_{60}HyFn in the same dose one week prior to STZ injection – prophylactic regime); Group V (rats received single i.p. injection of C_{60}HyFn in the same dose one week after development of stable hyperglycemia–therapeutic regime). The following parameters were assessed in the groups of control and experimental animals: blood glucose concentration, levels of end-products of lipid peroxidation (LPO) and carbonylated proteins as markers of protein oxidative modifications (POM) in liver and brain tissues. Levels of astrogliosis in various sections of rat brain were monitored as additional parameter of C_{60}HyFn neuroprotection. Immunochemical and immunohystochemical determination of glial fibrillary acidic protein (GFAP) as sensitive marker of astrocyte response were applied to evaluate intensity of astrocyte reactivity.

Results: STZ induced consistent hyperglycemia accompanied by extended oxidative damage of lipids and proteins in rats' central nervous system (CNS) and liver. Beneficial effects of C_{60}HyFn on oxidative status in liver of rats with hyperglycemia were observed in both regimes of administration. However, C_{60}HyFn markedly reduced abnormally high levels of macromolecule oxidation in diabetic brain tissues only when used in therapeutic regime. In addition, diabetic rats were characterized by significant elevation of GFAP levels due to increase of astrocyte reactivity in brain cortex and hippocampus. C_{60}HyFn treatment restored GFAP levels near to normal supposedly by lowering of astrogliosis caused by hyperglycemia. Though, C_{60}HyFn, serving as high-performance antioxidant and alleviating harmful side-effects of diabetes such as oxidative stress, does not affect the blood glucose levels in diabetic rats and therefore not remove the prime cause of metabolic disturbance.

Conclusion: Nanostructures of hydrated C_{60} fullerene have been shown to exert beneficial effects in brain and liver of rats with STZ-induced diabetes mainly through the diminution of oxidative stress and can be applied for prevention and treatment of diabetic complications such as liver injury and neuropathy.

Keywords: Hydrated C_{60} fullerene; Streptozotozin (STZ)-induced diabetes; Oxidative stress; Liver; Brain; Astrogliosis; Glial fibrillary acidic protein (GFAP)

Introduction

Oxidative stress accompanies development of numerous complications of diabetes mellitus [1]. Hyperglycemia induces redox reaction disturbances, which is resulted in alterations in nerves, as well as neuronal degeneration, leading, on one hand, to dysfunction of peripheral nerve conductivity, and, on the other hand, neuronal damage in central nervous system (CNS), which may contribute to behavioral impairments and memory disturbances [2,3]. Liver is also affected due to free radical injuring action, and resulted fatty changes in the liver is another contributing factor in constellation of signs of metabolic syndrome in diabetes [4]. It is well known that normal liver function is essential to sustain CNS health. Altered metabolism products accumulated due to hepatocyte dysfunction may cause disturbances in brain and contribute to CNS damage. It is important to note that liver failure induced by various factors causes significant changes to supporting neurons cells called astrocytes [5]. Astrocytes are the most abundant cell type presented in the CNS. They provide metabolic and trophic support to neural cells, maintain the blood-brain barrier

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(BBB), modulate synaptic activity, and protect neurons against injury [6]. Several studies show that astrocytes are affected during diabetes [7,8]. The glial cells may exhibit the earliest and potent cellular reaction within the CNS in response to various damaging factors, well known phenomenon of so called reactive gliosis. Reactive astroglial cells are characterized by hypertrophy, hyperplasia and intense fibrillogenesis [9]. A key indicator of glial reactivity is an increased synthesis of glial fibrillary acidic protein (GFAP), an intermediate filament protein of astrocytic cytoskeleton [10,11]. This sensitive biomarker of neuronal injury is a parameter for standard neurotoxicity assessment [12,13]. It has been earlier demonstrated that in uncontrolled hyperglycemia during diabetes, chronic reactive gliosis exacerbates diabetic neuropathy [14,15]. The positive correlation between GFAP levels and excessive generation of reactive oxygen species (ROS) has been clearly documented [7]. The last fact makes possible to utilize GFAP as an additional parameter for evaluation of CNS injury extent, as well to assess efficacy of beneficial effect of potential neuroprotective agent tested in our experiment.

Therapeutic strategy and approaches to the pathogenesis of diabetic neuropathy and liver injury is focused on elimination of causes of cell damage, particularly oxidative stress, which has been demonstrated to play a central role [16,17]. In our study, we support the general understanding that deleterious effects induced by hyperglycemia in rats would be alleviated by means of the antioxidant treatment. Due to marked antioxidant activity, C_{60} fullerene, the third natural allotropic form of carbon, and some of its water-soluble chemical derivatives have recently gained considerable attention as promising candidates for many biomedical applications, in particular, at neurodegenerative states as well as at liver failure [18-20]. In this regard, hydrated chemically non-modified C_{60} fullerene (C_{60}HyFn) possesses all necessary characteristics for the treatment of free radical-induced alterations [21,22], including some diabetic complications [23]. In 1995, Andrievsky et al. developed method of C_{60}HyFn water solution (C_{60}FWS) production [24]. Such solutions of C_{60}HyFn contain single hydrated C_{60} fullerene molecules as well as their labile nanoclusters (secondary associates). C_{60}HyFn chemically is characterized as highly hydrophilic and highly stable donor-acceptor complexes of C_{60} associated with molecules of water [25-27] (Figure 1). A great deal of information is accumulated concerning tissue-protective effects of C_{60}HyFn including its neuroprotective, anticancer, anti-inflammatory, antiatherogenic action, mainly based on the powerful antioxidative capacity of C_{60}HyFn, which is displayed even at its super-small concentrations and doses [22,28].

The present study was aimed to examine and compare the potential protective effects of C_{60}HyFn against oxidative stress in the CNS and liver tissues in diabetic rats. As additional parameter of C_{60}HyFn neuroprotection, the levels of astrogliosis in different brain sections of rats have been monitored. The model of streptozotocin (STZ) diabetes that is thought to be the most appropriate animal model to study the etiology, prevention and treatment of diabetic complications, particularly diabetic neuropathy, was chosen [29,30]. To check whether C_{60}HyFn acts as effective protective agent against diabetes-induced oxidative stress, the following parameters were studied in control and experimental animals: i) blood glucose concentration; ii) levels of products of lipid peroxidation (LPO); iii) levels of carbonylated proteins as products of protein oxidative modifications (POM); iv) GFAP levels, and v) astrocyte reactivity in various brain sections.

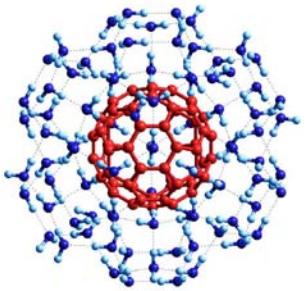


Figure 1: Nanostructure of Hydrated C_{60} Fullerene – stable supramolecular complex C_{60} (red) with water molecules (blue) having unique physical-chemical and biological properties (http://www.ipacom.com/index.php/en/publications-about-c60hyfn/92).

Materials and Methods

C_{60}hyfn production, characterization and preparation of C_{60}FWS

For C_{60}FWS preparation (C_{60}HyFn water solution), C_{60} fullerene samples with purity of more than 99.5% (MER Corporation, Tuscon, AZ, USA) have been used. C_{60}FWS was produced without using of any solubilizers or chemical modification. This method is based on transferring of fullerene from organic solution into the aqueous phase with the help of ultrasonic treatment. Having been modified and optimized the method of C_{60}FWS preparation, now it is possible to obtain such solutions with C_{60} concentration up to 5.5×10^{-3} M (~4 mg/ml). Depending on C_{60} concentration, C_{60}FWS contains both single C_{60}HyFn ($C_{60}@\{H_2O\}_n$, where n=22-24) and their labile nanoclusters (secondary associates) with the size of 3-36 nm [24-26]. Preparation of C_{60}HyFn with concentration of 8.88×10^{-4} M was used as stock solution for preparing C_{60}FWS prior the experiment.

Animals, experimental design and induction of diabetes

Male Wistar rats, 35 individuals in total, aged 12-15 weeks and weighing 150-200 g, were used in this study. During the whole experimental period, the animals were kept in standard laboratory conditions (room temperature (22 ± 3°C), constant humidity, 12/12 h light/dark cycle, water and food were given *ad libitum*. The experimental protocols were reviewed and approved by the Regional Committee for the Ethical Use of Animals (Dniepropetrovsk, Ukraine).

After 1 week of acclimatization to laboratory conditions the rats were randomly divided into 5 groups, each containing 7 animals as follows:

- Group I: intact control (saline);
- Group II: STZ-diabetes;
- Group III: C_{60}HyFn control;
- Group IV: C_{60}HyFn before STZ (prophylactic regime);
- Group V: C_{60}HyFn after STZ (therapeutic regime).

At the onset of the study, blood samples were collected from the tail vein of each rat for the measurements of blood glucose levels by means of glucose-oxidase kit ("Reagent", Dniepropetrovsk, Ukraine). Diabetes was induced by a single intraperitoneal injection of buffered solution (0.1 M citrate, pH 4.5) of streptozotocin (STZ) purchased from Sigma (St. Louis, MO, USA) in a dose of 45 mg/kg body weight. The animals were considered diabetic if blood glucose values were > 10 mM at 72 h after STZ administration. C_{60}HyFn dissolved in sterilized and apyrogenic 0.9% NaCl was administered i.p. in a dose of 0.3 mg/kg

b.w. 1 week before STZ injection or 1 week after development of stable hyperglycemia. The control group (intact animals) received saline i.p. (0.5 ml per animal). One group was treated only with C_{60}HyFn (0.3 mg/kg i.p.). All rats were then euthanized after 3 weeks since STZ injection; trunk blood was collected for glucose measurements, brain tissues were removed and cerebral cortex, hippocampus and cerebellum were dissected and taken for biochemical and immunochemical studies.

Measurements of LPO and POM levels

Contents of thiobarbituric acid reactive substances (TBARS) and protein carbonyl bonds for evaluation of lipid peroxidation (LPO) levels and extent of protein oxidation modifications (POM) respectively in brain tissues of control and experimental animals were determined as described elsewhere [21] with minor modifications. Briefly, samples of whole brain tissues were homogenized in 25 mM Tris-HCl (pH 7.4), 2 ml of homogenates were taken and total protein fraction was separated from homogenates by precipitation with trichloroacetic acid (TCA) and further centrifugation for 15 min at 5000 g. The amount of LPO products was measured in the supernatants spectrophotometrically at λ=532 nm using the thiobarbituric test and taking molar extinction coefficient $\varepsilon_{532}=1.56\times10^5$ $M^{-1}cm^{-1}$. For estimation of POM levels, intensity of protein carbonyl formation was assessed in protein pellets left after preceding centrifugation of tissue homogenates processed with TCA. 1 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added to the pellets. Having been mixed and intensively stirred, the reaction mixture was incubated for 1 h at 37°C and then centrifuged for 15 min at 5000 g. The pellets were rinsed triply with 1 ml ethanol/ethylacetate mixture (1:1 v/v). Washed pellets were resuspended and dissolved in 6M urea. Non-solubilized material was separated by centrifugation for 15 min at 5000 g. The content of protein carbonyl bonds was estimated in supernatants spectrophotometrically at λ=370 nm taking molar extinction coefficient $\varepsilon_{370}=2.2\times10^4$ $M^{-1}cm^{-1}$.

Protein sample collection

Fresh sections of brain tissues were homogenized (10% w/v) in 25 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 2 mM β-mercaptoethanol and proteinase inhibitors (10 μg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonylfluoride). The homogenates were centrifuged at 30 000 g for 1 h at 4°C. The supernatants contained soluble brain proteins; the pellets were abundant with water non-soluble fibrillary brain proteins. The pellets were resuspended in the same buffer additionally containing 4 M urea for solubilization of cytoskeletal proteins and then the samples were incubated during 12 h at 4°C. The homogenates were centrifuged in the same regime and the supernatants were collected, aliquoted and stored at 20°C. Protein in the samples was measured by the Bradford protein assay [31] using bovine serum albumin (BSA) as a standard.

Western blotting

Samples and standard protein markers were subjected to polyacrilamide gradient gel (7÷17%) electrophoresis in the presence of 0.1% sodium dodecylsulphate (SDS) as described previously [32]. Proteins separated were transferred to nitrocellulose filters (Schleich & Schuell Inc., USA). Nonspecific binding was blocked by incubation with 3% BSA solution. The blots were incubated with primary rabbit antibodies against GFAP (Santa Cruz Biotechnology Inc., USA) at 1:2000 dilution. After overnight incubation (4°C), the blots were rinsed with Triton X-100-containing buffered saline (NaPBS+0.05% Triton X-100). Then blots were incubated for 1 h with a secondary antibody, a goat anti-rabbit Ig G peroxidase conjugate (Sigma Aldrich, USA), at room temperature. Blots were visualized using 0.05% 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 as substrate [33]. The relative immunostaining intensity of bands on Western blots, which is proportional to antigen (GFAP) content, was quantified in arbitrary units by scanning blots and further calculating by computerized software program (LabWorks 4.0; UVP Inc., UK).

Immunohystochemical assay

Brain fragments were fixed in 10% neutral buffered formalin for 12-24 h. After fixation, the tissue blocks were embedded in paraffin, then cut in a microtome to the desired thickness (approximately 5 microns) and affixed onto the slide. Tissue sections were mounted on positively charged or APES (amino-propyl-tri-ethoxy-silane) coated slides. Once mounted, the slides were accurately dried by leaving slides at room temperature overnight. For deparaffinization, slides were placed in a rack, and the following washes were performed: xylene (2×3 min), xylene 1:1 with 100% ethanol (3 min), 100% ethanol (2×3 min), 95% ethanol (3 min), 70% ethanol (3 min), 50% ethanol (3 min), and finally slides were rinsed in running cold tap water. Heat-induced antigen retrieval was carried out in Tris/EDTA buffer (pH 9.0) using a pressure cooker. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in 50 mM Tris buffer saline (TBS) for 15 min. After that, slides were washed 2×5 min in TBS plus 0.025% Triton X-100 with gentle agitation and then incubated in blocking solution (TBS with 3% BSA) for 2 h at room temperature. Sections were incubated overnight at 4°C with the primary antibody anti-GFAP 1/500 diluted in TBS. On the second day, slices were washed with TBS-Triton (3×5 min) and incubated (1 h at room temperature) with 1/300 diluted secondary antibody (peroxidase-conjugate anti-rabbit Ig G). After a rinse with TBS-Triton (3×5 min), antibody binding sites were revealed by DAB and H_2O_2 in TBS (pH 7.2) for 10 min then, after final rinse with TBS, counterstained with hematoxylin to visualize cell nuclei. Finally, sections were mounted on slides and covered with Eukitt balsam before image analysis. The tissue sections were examined using an upright optical microscope (Leika DM 2000, Leika Microsystems AG, Germany) coupled with a color camera.

Statistical analysis

All data is expressed as Means ± SEM. Significance of differences between groups was evaluated using two-way analysis of variances (ANOVA). When ANOVA showed a significant effect on the group on any of dependent variables, the Tukey-Kramer test was used for post hoc multiple comparisons. P-values<0.05 were considered statistically significant.

Results

Administration of C_{60}HyFn does not affect glucose levels

Diabetes inductions was attempted in 23 male Wistar rats, of which 21 demonstrated consistent blood glucose levels greater than 10 mM and were included in the diabetes groups (7 individuals per group). One animal died of renal failure and one failed to become hyperglycemic. Animals were fed *ad libitum* and no dietary modification was used. Age-matched male rats served as intact or C_{60}HyFn controls. All animals survived the entire experimental protocol.

The results of glucose measurements in animals, performed prior STZ injection and at the end of the study are presented in table 1. At the onset of the experiment, all rats had equivalent blood glucose levels. At the end of the experiment, glucose concentrations were significantly elevated in the blood of the STZ-treated rats (group II). Administration

Treatment	Blood Glucose, mM	
	Onset of Experiment	End of Experiment
Group I (untreated control)	5,7 ± 0.31	5.9 ± 0.42
Group II (STZ)	5.8 ± 0.32	14.6 ± 2.40 *
Group III (C_{60}HyFn control)	5.6 ± 0.30	5.5 ± 0.19
Group IV (C_{60}HyFn prophylactic regime + STZ)	5.3 ± 0.42	13.8 ± 1.70 *
Group V (STZ + C_{60}HyFn therapeutic regime)	6.1 ± 0.53	15.1 ± 2.21 *

*P<0.05 vs. intact control (Group I)

Table 1: Blood glucose levels in control and experimental rats at the onset and at the end of the experiment (n=7).

of C_{60}HyFn alone (group III) did not change this parameter at the conclusion of the study. However, high glucose levels in STZ-injected animals were not altered by C_{60}HyFn used in prophylactic or therapeutic regimes (groups IV and V respectively). Therefore, it is important to note that C_{60}HyFn treatment did not affect the glucose level in diabetic rats, and consequently did not eliminate the origin of metabolic disturbance, probably due to absence of protective effects on insulin-produced pancreatic cells damaged by STZ.

C_{60}HyFn decreases levels of oxidative stress markers in rats with STZ-induced diabetes

Analysis of brain and liver tissues of rats with hyperglycemia showed elevation of oxidative stress indices including TBARS levels as LPO marker and protein carbonyl content as marker of protein oxidation compared to non-diabetic animals. As shown in table 2, the susceptibility of diabetic tissues to oxidative stress appeared to be various, with the most prominent deleterious changes in liver compared with brain. In both organs studied, injection of C_{60}HyFn (group III) did not affect the levels of oxidation markers indicating that pristine fullerene is unlikely to exhibit pro-oxidant capacities *in vivo*. Abnormally high levels of LPO and POM in STZ-affected liver tissues were significantly restored regardless of the regime of antioxidant administration, i.e. preventively or therapeutically. In contrast, C_{60}HyFn had different effectiveness in defending of biological macromolecules against oxidative injury in diabetic brains, depending on the mode of administration. C_{60}HyFn therapeutical treatment reduced TBARS and protein carbonyl content to control levels (Group V), while pre-diabetes C_{60}HyFn injection had no statistically significant influence on levels of oxidative stress markers in brain tissues.

C_{60}HyFn counteracts reactive gliosis (astrocytosis) in brain of rats with experimental diabetes

Immunochemical studies used GFAP monospecific antibody to examine glial reactivity in various brain regions of STZ-induced diabetic rats and determine whether C_{60}HyFn exerts neuroprotective activity at hyperglycemia. Western blot analysis demonstrated statistically significant higher expression of GFAP 49 polypeptide, as well as products of its degradation, in the cortex and hippocampus of STZ-diabetic rats in comparison with the intact control group (Figures 2A and 2B). In contrast, a slight but non-significant elevation of GFAP content was observed in cerebellum of diabetic rats (Figure 2C). C_{60}HyFn only (group III) did not change GFAP content in any brain section taken for study compared with intact control. The beneficial effects of the C_{60}HyFn prophylaxis and therapy on diabetes-induced GFAP overexpression were found in cortex and hippocampus, which are manifested by down-regulation of this astrogliosis protein marker as well as its degradation products at both regimes of administration (Figures 2A and 2B).

These results indicate hydrated C_{60} fullerene to display pronounced protective activity against diabetes-induced alterations in CNS, attenuating oxidative stress and alleviating astrocyte reactivity in STZ-diabetic rat brain. Though, C_{60}HyFn may be beneficial as symptomatic drug at diabetic neuropathy, it does not remove the source of metabolic disorders and it does not affect the abnormally high sugar levels, likely because of absence of protective effects on insulin-produced pancreatic cells damaged by STZ. Moreover, in brain tissues, antioxidant efficacy of C_{60}HyFn depends on the regime of administration. C_{60}HyFn display excellent protective potential against oxidative damage of lipids and proteins when followed by the inducing of hyperglycemia. Unexpectedly, astrocytes affected by STZ-induced diabetes appeared to be less reactive as a result of C_{60}HyFn administration at both regimes. It is obvious that not only single mechanism (i.e. oxidative stress) contributes to the astrocytic reaction during diabetes.

Typical GFAP immunostaining slides of hippocampus of all five groups of animals to evaluate astrocyte reactivity *in situ* are depicted in figure 3. In untreated rats (Figure 3A) or rats obtained C_{60}HyFn only (Figure 3C), GFAP immuhistochemistry revealed astroglial cells with typical stellar-like form and numerous thin cell processes. Robust increase in GFAP immunostaining was observed in brain sections of rats with STZ-induced diabetes compared with that in the control rats. Hypertrophic astrocytes with enlarged cell bodies and elongated overlapping processes were apparent in brain of STZ-diabetic animals, thus signs of glial scar formation are evidently seen (Figure 3B). It is important to note that similar trend in elevation of GFAP immunolabeling in cerebral cortex (data not shown) of STZ-diabetic rats from group II correlates with upregulation of this astrocytosis marker revealed by Western blot. Both C_{60}HyFn prophylaxis and treatment reduced the number of GFAP-immunostained astrocytes as well as extent of their hypertrophy in diabetic animals (Figures 3D and 3E). These data are also in accordance with results obtained by GFAP immunoblotting.

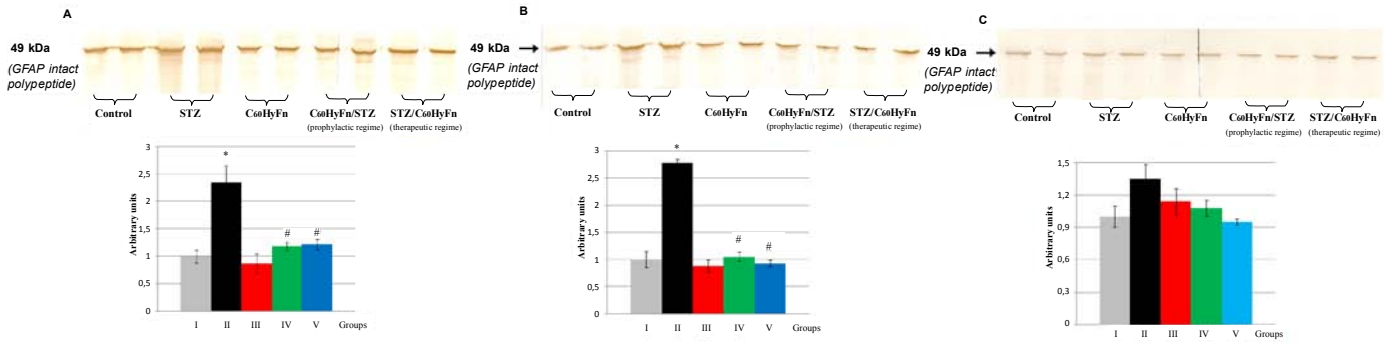
Discussion

Diabetes mellitus causes damage to many vital organs including brain and liver, mostly due to increased free radical generation that is responsible for development of oxidative stress leading to further metabolic abnormalities and development of various complications [34]. Oxidative stress occurs in the cellular system when over-production of free radicals, i.e. ROS, overwhelms anti-oxidative reserve of the system, and then sub-cellular structures undergo destructive changes [35]. Also, oxidative stress represents the cornerstone in the intersection of pathogenetic mechanisms of diabetic neuropathy, serving as a crucial target for therapeutic strategies [36]. In diabetic neuropathy, oxidative

Organ	Treatment				
	Group I (untreated control)	**Group II** (STZ)	**Group III** (C_{60}HyFn control)	**Group IV** (C_{60}HyFn prophylactic regime + STZ)	**Group V** (STZ + C_{60}HyFn therapeutic regime)
	TBARS content, nM/mg protein				
Liver	45 ± 5.2	173 ± 23.0 *	28 ± 6.3	39 ± 9.1 #	32 ± 7.2 #
Brain	27 ± 7.4	59 ± 9.5 *	31 ± 4.8	47 ± 8.2	25 ± 6.8 #
	Carbonylated protein content, nM/mg protein				
Liver	4 ± 0.6	24 ± 1.7 *	3 ± 0.3	5 ± 0.7 #	2 ± 0.5 #
Brain	8 ± 0.4	18 ± 0.6 *	11 ± 0.4	19 ± 0.8 *	7 ± 0.9 #

*P < 0.05 vs. intact control (Group I)
#P < 0.05 vs. STZ-diabetes (Group II)

Table 2: Levels of oxidative stress indices (LPO levels, carbonylated protein content) in brain tissues of control and experimental rats (n=7).



*P<0.05 vs. intact control (Group I)
#P<0.05 vs. STZ-diabetes (Group II)

Figure 2: Representative Western blots and densitometric analysis of total glial fibrillary acidic protein (GFAP) in protein fractions obtained from the three brain regions of control and experimental rats (data expressed as arbitrary units, n=7): cerebral cortex (A), hippocampus (B), and cerebellum (C). A significant increase in GFAP from cortex and hippocampus of STZ-diabetic rats (II group) is observed. Note the appearance of low-molecular weight GFAP polypeptides in the same brain regions of STZ-diabetic rats. C_{60}HyFn leads to normalization of astrogliosis marker content in these two brain regions injured in the conditions of experimental hyperglycemia. C_{60}HyFn has appeared to display similar efficacy against reactive gliosis regardless of regime of administration.

stress mainly results from hyperglycemia-induced impairment of mitochondrial electron transfer, which leads to increased production of superoxide anion by mitochondria [37], through protein kinase C-dependent activation of membrane-associated NADPH oxidase [38], and by glucose auto-oxidation [39]. Sustained hyperglycemia causes depletion of antioxidants furthermore aggravating oxidative stress [4]. Besides these triggering mechanisms, neuronal damage is further worsened by subsequent cascade of pathologic processes such as ischemia, inflammation, advanced glycation end-products formation, changes in transcription factors resulting in modified genes expression [40].

Strategy of treatment of diabetic neuropathy and liver injury is focused on the elimination of causes of cell damage, particularly oxidative stress, which plays a central role. Due to the fact that pathophysiological mechanisms of hyperglycemia are believed to involve the increased generation of free radicals, we hypothesized that oxidative damage over the hyperglycemia would be reduced if antioxidant treatment is applied. C_{60} fullerene is the third natural allotropic form of carbon after graphite and diamond, and has unique physicochemical properties and biological activities [41-43]. Pristine C_{60} fullerene, along with some water-soluble derivatives, has recently gained considerable attention as a promising candidate for many biomedical applications [44]. Series of numerous scientific publications highlight ability of fullerenes to serve as potent anti-oxidant *in vivo*, at the same time demonstrating minimal or no side effects, which makes possible to administer them in conditions associated with oxidative stress [18,20,45]. It is shown that C_{60} is more potent antioxidant than α-tocopherol and ascorbic acid, thus it can be used as free-radical eliminating agent for treating diseases, which are associated with abnormally high levels of membrane lipid peroxidation. Both liposoluble and water-soluble C_{60} derivates could effectively protect membrane lipids from ROS-induced peroxidation, in this way maintaining integrity of cellular membranes [46].

To the best of our knowledge, current study is the first experimental investigation on the pristine chemically non-modified water-soluble C_{60} fullerene as an effective and safe drug for diabetic complication. To date, the only study was performed to test neuroprotection effect of nanoparticles of porphyrin adducts of cyclohexil fullerene C_{60} (^{25}Mg-PMC_{16}) to diminish oxidative stress, attenuate mitochondrial dysfunction and improve metabolic processes in neurons of rats with STZ-induced diabetes [47]. However, it should be mentioned, that protective effects of this fullerene-containing derivative is likely determined by Mg molecule, with known neuroprotective activity through inhibition of neuronal apoptosis and inflammation [48] but not cyclohexyl derivate of C_{60} itself.

In our study, model of STZ-induced diabetes, which is relevant to chronic oxidative stress, has been provided [7,49]. STZ mostly affects insulin-producing β-cells of the islets of Langerhans without influencing directly other organs after systemic administration [29]. In particular, disturbed glucose metabolism is the main cause of diabetic neuropathy, and it was thought that only maintained glucose control within the normal range provides protective action on glial and neural cells. C_{60}HyFn is not involved in glucose metabolism, thus cannot eliminate the main result of STZ action, but it may be beneficial acting as antioxidant and abating side effects of hyperglycemia. Results of the current study parallel to our previous study [23], in which C_{60}HyFn was not shown to be sugar-lowering agent; however it provided excellent protective effects on male gonads, sperm cells and restored reproductive function disrupted by hyperglycemia in rats with STZ-diabetes. Nevertheless, further studies will be performed to investigate influence of C_{60}HyFn on insulin-producing cells in experimental diabetic animals for future pharmacotherapeutic applications of fullerenes.

Diabetic encephalopathy and neuropathy, as well as hepatic injury are being studied intensively and described in scientific literature all over the years [2,50,51]. Considering facts that these organs are extremely susceptible to oxidative impact due to high rate of metabolism and relatively increased oxygen consumption, the aim of the first part of our study was to measure and compare the levels of main oxidative stress markers in rats with STZ-induced diabetes and estimate if C_{60}HyFn would be a beneficial in this pathological condition. Analysis of diabetic rat brains and livers showed elevated oxidative stress markers including lipid peroxidation (LPO) products and protein carbonylated derivates. Diabetes-induced oxidative stress affects liver more than brain due to pro-oxidant-antioxidant imbalance. This observation is in line with earlier obtained data demonstrating that long-term hyperglycemic state disturbs redox state in liver tissue more profoundly in comparison with other organs [4].

C_{60}HyFn administration did not correct hyperglycemia in STZ-treated rats. At the same time, we observed protective effect of fullerenes

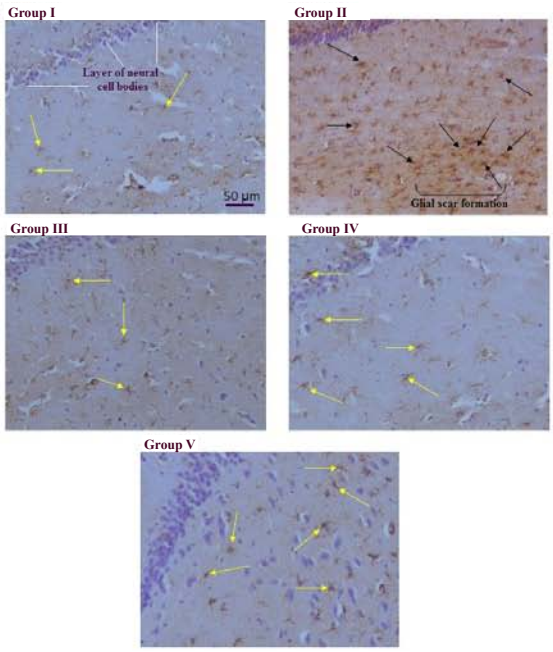


Figure 3: Photomicrographs of sections from hippocampus (CA1 field) of rats from Groups I (intact control), II (STZ-diabetic), III (C_{60}HyFn control), IV (STZ-diabetic animals pre-treated with C_{60}HyFn – prophylactic regime), and V (STZ-diabetic animals treated with C_{60}HyFn – therapeutic regime) stained immunohistochemically for glial fibrillary acidic protein (GFAP). Astrocytes (yellow arrows) are seen as brown-coloured stellar-like cells with numerous processes. Scar-forming astrocytes (black arrows) are visualized as hypertrophic cells with increased number of processes. GFAP immunostaining demonstrates that STZ-diabetes induces global increase in astrocyte size, number and density, which altogether are the hallmark of severely reactive astrogliosis. C_{60}HyFn reduces the number of GFAP-positive astrocytes in diabetic rat brain administered in both regimes. All sections were additionally counterstained with hematoxylin to visualize cell nuclei; layer of neural cell bodies is indicated at the top left. Original magnification ×200.

on liver tissue and brain affected by oxidative damage at the level of sub-cellular macromolecules. Thus, it was demonstrated that C_{60}HyFn expressed potent anti-oxidative effectiveness in these organs. It was shown that hydrated fullerene preserves hepatocytes membrane lipids and intracellular proteins against hyperglycemia-induced oxidative stress in all regimes of administration. In case of observation over the decreased rate of oxidative stress markers in brain tissue (LPO and POM), our study demonstrates that C_{60}HyFn exhibits beneficial effect only after hyperglycemia is managed (group V), as we did not observe lowering of markers of oxidative stress in group IV (STZ-diabetic rats pre-treated with fullerene). Our interpretation of these results is based on experimental proof that liver is an organ, in which accumulation and further metabolization of C_{60} generally occur, and expression of its antioxidant potential could be more pronounced in liver than in other organs [52]. Present study shows that C_{60}HyFn displays hepatoprotective effect, which is in line with previous data obtained in earlier investigation on rodents, showing hepatoprotective effect in a model of ROS-mediated liver injury induced by acute CCl_4 intoxication [20]. These authors have recently obtained important results, which are widely discussed by scientific community, indicating C_{60} dissolved in olive oil almost doubles the lifespan of rats. It has been suggested that the effect on lifespan is mainly due to the attenuation of age-associated increases in oxidative stress [53].

Chemically non-modified C_{60} as well its water-soluble derivates have been considered as powerful liver-protective agents in several studies *in vivo*. In particular, it has been demonstrated that polyhydroxylated fullerene (fullerenol) alleviates doxorubicin-induced hepatotoxicity in rats with experimental malignancies [54]. One of the possible reasons for the absence of C_{60}HyFn noticeable antioxidant effects in rats injected with this agent prior development of hyperglycemia (group IV), may be attributed to non-permeability of healthy blood brain barrier (BBB) for hydrated C_{60} molecules and their nano-associates. On the contrary, C_{60}HyFn as highly hydrophilic agent is able to enter hepatocytes due to functional features of liver cells [55]. BBB is considered to form a neurovascular unit, in concert with astrocytes, pericytes, neurons and extracellular matrix that protects brain from circulating neurotoxic substances while maintaining nutrients and ions in brain at levels necessary for neuronal function. It is recognized that BBB is only crossed by the molecules, which are lipid soluble and have molecular mass <400 Da [56,57]. At the same time, C_{60}HyFn is highly hydrophilic supramolecular complex and has more molecular mass (>720 Da), which makes impossible to penetrate BBB. In addition, size of individual C_{60} molecule (approx. 2 nm) with the first stable shell of interfacial water, does not allow such hydrated fullerene to penetrate through plasma membrane of a cell. A number of publications [58-60] have reported that very few or no pristine C_{60} nanoparticles penetrate through lipid membrane and pass the BBB. Although, we reported that antioxidant effects of C_{60}HyFn is observed in diabetic brain only when administered after achievement of hyperglycemic state. Nevertheless, the mechanism of action and biodistribution of C_{60}HyFn are still not explained in whole extent, besides general anti-oxidative effect is satisfactory. It could be suggested that BBB overcoming by C_{60}HyFn and its further probable penetration into diabetic brain could be possible due to specific disease-induced changes in the structure and functionality of this biological system. The observation that STZ-induced diabetes produces a progressive increase in BBB permeability to small molecules [61,62] at least partially may support this hypothesis.

In the scientific literature, C_{60} fullerene and some of its water-soluble derivates are referred as antioxidants and believed to reduce highly reactive harmful chemical species, such as oxygen and nitrogen free radicals, *in vitro* and *in vivo* [18,63,64]. C_{60}HyFn is proposed to treat diseases associated with oxidative stress by virtue of its long-lasting antioxidant properties. It has been shown in previous *in vivo* studies that C_{60}HyFn reduces oxidative stress in CNS of rats chronically exposed to ethyl alcohol [21] also significantly improves reproductive function in case of experimental model of diabetes in rats [23]. Clinical studies have demonstrated that C_{60}HyFn holds promise as antioxidant for effective pathogenetic therapy of bronchial asthma [65]. The results of the recent study [22] indicate that C_{60}HyFn displays significant anti-radical and radioprotective activities both *in vitro* and *in vivo* neutralizing irradiation-generated OH-radicals in aqueous medium and increases survival rate of mice irradiated in the lethal dose of X-rays. Data obtained in this investigation and many other experimental studies cited above shed light on the subtle mechanism of antioxidant mechanism of C_{60}HyFn action. Hydrated C_{60} fullerene is considered as a new class of anti-radical acting drugs with several unique features and advantages, which is rather different compared to other known antioxidants. First of all, fullerene can no longer be interpreted as a free radical scavenger ("radical sponge") for the reason that reactivity of pristine C_{60} and its nano-clusters in aqueous medium against free radicals is very limited [28,66]. Antiradical activity of C_{60}HyFn is most likely realized due to its unique catalytic properties in aqueous medium via enzyme-like mechanism acting as SOD-mimetics [67,68]. Explained above mechanism guarantees long-lasting antioxidant effects of C_{60}HyFn expressed even at low and super-low

concentrations (10^{-9}-10^{-15} M). Another exclusive property of C_{60}HyFn is that it can neutralize only excessive free radicals, while sustains unaffected minimal level, maintaining balance between oxidative and anti-oxidative capacity, which is necessary for normal functioning of biological systems. This basal level of free radicals can be interrupted by any other antioxidant overload that may have deleterious consequences. In contrast, "nanodrug" C_{60}HyFn can self-regulate its radical removing activity–a property lacking in many of current pharmacological antioxidants. It should be mentioned that C_{60}HyFn has rate-limited free-radical regulating activity, which differs it from other antioxidants. Finally, it is important that the main advantage of C_{60}HyFn water solutions (C_{60}FWS) is that they are absolutely non-toxic in wide range of concentrations for mammals and other living organisms [28,69].

The prevalence of diabetes mellitus is increasing worldwide and it is expected that the number of subjects suffering from diabetic neuropathy will grow in the near future [70]. So, seeking for more efficient agents, which might prevent or treat oxidative stress-related diseases and therefore counteract diabetic-induced neuropathy, remains a pressing problem. The second part of our study was aimed to clarify ability of C_{60}HyFn to counteract process of reactive gliosis (astrocytosis) in experimental diabetes. Astrocytes play pivotal role in neuronal metabolism, function and survival, consequently modifications in these cells are important in the pathogenesis of diabetes mellitus neuropathy [6]. Glial reactivity is characteristic feature of brain damages, and excessive astrocytosis can be harmful for neural regeneration through interrupting neurite outgrowth in injured CNS [71].

One of the considerable markers indicating astroglial activation caused by CNS injury, aging and neurodegeneration is increased levels of GFAP, which is astrocyte intermediate filament cytoskeletal protein, however, astroglial response reaction may vary according to the severity and duration of the diabetes [14,72]. Several mechanisms may account for the astrocyte reaction in STZ-induced diabetes. These mechanisms include increases in the polyol pathway, protein glycation, disturbed calcium homeostasis and oxidative stress [40]. Correlations of GFAP levels altered with excessive free radical generation have been clearly documented [7]. Results of our study confirm suggestion that increased levels of GFAP and its degradation products are indicative of astrocyte reactivity caused by diabetes-induced oxidative stress. Due to excessive generation of free radicals is involved in pathophysiological mechanisms at hyperglycemia, we hypothesized that glial reactivity in diabetic rats can be reduced by antioxidant C_{60}HyFn. The current study showed that glial cells respond to the diabetes by overexpression and degradation of GFAP in a few weeks after the onset of diabetes. It has been revealed that C_{60}HyFn displays beneficial effects expressed in prevention of reactive astrogliosis in diabetic brain. Immunological analysis has revealed that elevated levels of GFAP are observed in cerebral cortex and hippocampus compared to cerebellum. Thus, these brain sections, responsible for cognitive functions, can be considered as more vulnerable CNS structures observed to damage during diabetes. These findings are synchronized with well-known observation that cognitive function decline and memory impairment are among main symptoms of diabetic encephalopathy in humans [3]. Limited proteolysis of GFAP intact 49 kDa polypeptide can be induced by Ca^{2+}-dependent enzymes, calpains, with increased activity in some brain regions in STZ-diabetic rats [73]. Cytoskeleton of astrocyte is comprised of GFAP intermediate filaments, and their disruption can lead to malformations of astrocyte morphology. As a result, metabolic and trophic support to neurons, integrity of the BBB as well as synaptic plasticity can be altered. It has been earlier established that fullerene water-soluble polyhydroxilated derivative, fullerenol, is able to attenuate β-induced increase in intracellular free Ca^{2+} level [74]. Based on this observation, we suppose that C_{60}HyFn prevents GFAP cleavage in diabetic brain by diminishing of intracellular Ca^{2+} levels. Neuro- and gliaprotective effects of hydrated C_{60} fullerene are well-documented. It has been just shown that C_{60}HyFn prevents neurotoxicity induced by amyloid-β peptide in rat hippocampus [75]. C_{60}HyFn attenuates astrocyte dysfunction and restores behavioural characteristics in rats after prolonged intoxication with ethyl alcohol [21].

It should be noted that C_{60}HyFn decreased astrocyte reactivity despite the regime of administration, either injected in preventive or therapeutic doses, nonetheless its antioxidative effects in brain tissue were only seen in the case when stable hyperglycemia had been induced (group V). It is likely that several, rather than a single, cellular events account for the reactive gliosis at diabetic neuropathy. Multisystemic alterations occur in many organs, most prominent and significant are liver changes with subsequent development of hepatic encephalopathy with neuronal damage [4]. Complications of diabetes mellitus comprise among others liver injury beginning from hepatocytes vacuolar dystrophy, undergoing hepatic steatosis, steatohepatitis, which can progress to cirrhosis [51]. Liver damage is accompanied by hyperammonemia, which is the main contributing factor of hepatic encephalopathy. It has been shown that abnormally high levels of ammonia in blood have toxic effects on astrocytes and can damage neurons [76]. In clinical studies with human subjects diagnosed with hepatic encephalopathy, significant changes in astrocyte morphology and impairments of GFAP metabolism have been described [77]. Therefore, we assumed that one of the possible causes of glial reactivity in diabetic rats could be partial liver failure induced by oxidative stress, and marked hepatoprotection of C_{60}HyFn in model of STZ-diabetes can indirectly mediate its neuroprotective effects through potent alleviation of glial dysfunction.

Diabetes mellitus type I, or insulin dependent diabetes, is characterized by onset of symptoms at young age group, while type II affects people in their advanced years. Initial symptoms of type I diabetes are very conspicuous, such as high levels of glucose in blood, or extreme hyperglycemia with accompanying keto-acidosis, early development of hepatic steatosis with hepatitis, if occasionally left untreated without the administration of appropriate doses of insulin, even liver failure may occur with fatal outcome [78]. On the contrary, diabetes mellitus type II is characterized by the substantial onset of symptoms. Patients are often diagnosed occasionally during routine medical examination, when applied to hospital for other reasons caused by long standing crippling step-by-step hyperglycemia, e.g. myocardial infarction, hypercholestorelemia, hypertension, angina pectoris, atherosclerosis, retinopathy, cataracts, moderate liver steatosis, peripheral neuropathy and other symptoms and signs [79]. Time and severity of development of complications are different for both types of diabetes. As type I diabetes starts in young age, when patient reaches advanced age, disease is already lasting for several decades. In case of diabetes type II, complications usually occur within 15-20 years. In both cases it is important to control level of glycemia within the near normal range. Due to advancement of new methods to achieve tight control of glucose in blood, fewer complications develop, although lack of full control requires administration of anti-oxidative preparations regularly. Effectiveness of administered drugs also depends upon pathogenesis of complication induced by toxic oxygen species over the years. Everything depends upon state of cell injury, if alterations are at their reversible or irreversible stage, theoretically anti-oxidative drugs may eliminate harmful impact to stop progression of cascade of

oxidative reactions [80]. Thus it is obvious, that duration of disease, as well sustained control of hyperglycemia are crucial factors in the pathogenesis of diabetes mellitus complications, not only administered drugs. Thus, effectiveness of these anti-oxidative drugs is influenced by physiological conditions of the human body. Considering these factors, attention should be focused on clinical expressions of diabetes mellitus, although *in vitro* and *in vivo* studies confirmed that current drug, i.e. hydrated chemically non-modified fullerene has proved to be appropriate drug to overcome oxidative stress at the early stages of disease, when it is possible to prevent development of complications of diabetes. At the same time, studies should be performed at the population level. Also, investigation of influence of fullerene not only on the development of early complications, but also late consequences of diabetes at larger study group of population is required in the nearest future.

Conclusion

In fine, we presume that the present study represents the first attempt to estimate potential beneficial effects of chemically unmodified hydrated C_{60} fullerene (C_{60}HyFn) in the experimental model of diabetes mellitus. Different antioxidative efficacy of C_{60}HyFn in brain and liver, as hyperglycemia-sensitive organs, has been demonstrated in diabetic rats. Also, C_{60}HyFn appeared to be efficient against diabetes-induced reactive astrocytosis, thus facilitating neuronal survival. Based on the results obtained, we assume that C_{60}HyFn does not affect oxidation balance of biomolecules in normalcy, however acting as efficient and safe antioxidant and tissue-protecting agent during diabetes. Nanostructures of hydrated C_{60} fullerene can be recommended to apply separately or with conventional scheme of treatment as additional remedy to prevent and anticipate complications of diabetes in initial stages of disease and to manage diabetic complications such as liver injury and neuropathy.

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