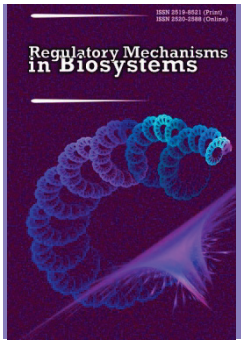

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The effect of water-soluble pristine C_{60} fullerene on 6-OHDA-induced Parkinson's disease in rats

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Oxidative stress is thought to be one of the mechanisms that leads to the dysfunction and degeneration of dopaminergic neurons in Parkinson's disease pathogenesis and presumed to be underway during the prodromal phase. Therefore, therapy, which is effective against premotor symptoms, might be effective in preventing or delaying the development and progression of Parkinson's disease. The aim of our study was to investigate the therapeutic efficiency of pristine C_{60} fullerene aqueous solution (C_{60}FAS) during Parkinson's disease in rats. The unilateral dopamine deficiency was induced in male Wistar rats (220–250 g) by stereotaxic microinjection of neurotoxin 6-hydroxydopamine (6-OHDA, 12 μg). C_{60}FAS was injected to rats intraperitoneally daily for 10 days (0.65 mg/kg per day). The percentage of destroyed dopaminergic neurons was determined by the apomorphine test and by IHC staining of tyrosine hydroxylase-positive neurons in substantia nigra. We evaluated the rat body weight, the water and food intake, Open Field behavioural test, the level of biochemical antioxidant system, the activity of peritoneal macrophages. Levels of spontaneous and carbachol-stimulated colon motility were estimated by ballonographic method *in vivo*. C_{60}FAS showed a positive tendency to increase the number of tyrosine hydroxylase-positive cells in the midbrain, which was associated with more profound improvement in apomorphine-rotation behaviour and slight relief of the anxiety level in Open Field test. Furthermore, C_{60}FAS treatment increased the index of stimulated distal colon motor activity while it did not have a significant effect on water content in feces and total gastrointestinal transit time. C_{60}FAS treatment did not affect water intake behaviour or body weight changes while it induced an increase of glutathione level and decrease activity of glutathione peroxidase in the brain as well as an increase in activity of peritoneal macrophages in 6-OHDA-Parkinson's disease rats. These findings confirmed the potential therapeutic effectiveness of water-soluble pristine C_{60} fullerene in Parkinson's disease pathogenesis, though there is ground for caution because of its systemic mild toxic effect.

Keywords: motility; non-motor symptoms; antioxidant system; gastrointestinal transit time.

Introduction

Parkinson's disease (PD) is most prevalent in older people (>65 y. o.), but in inherited cases it can begin in age 30–40 y. o. (Williams-Gray, 2016). The main feature of PD pathogenesis is a loss of dopaminergic neurons in the substantia nigra (SN) with the deposition of intraneuronal aggregates of α-synuclein (Lewy bodies) (Siderowf & Lang, 2012). The spreading of α-synuclein through peripheral factors such as the olfactory nerve and gastrointestinal (GI) tract is one of the famous hypothesis of PD pathogenesis (Braak et al., 2003). Diagnosis of PD occurs with the onset of motor symptoms (e.g. bradykinesia, muscular rigidity, rest tremor, and postural and gait impairment) which are preceded by 20 years or more with numerous non-motor symptoms (Kalia & Lang, 2015). Constipation and motility dysfunction are the most common non-motor symptoms in PD patients which are characterized by colonic and anorectal symptoms. It is observed in up to 66% of all PD-patients thereby effecting psychological and social distress and consequently reducing quality of life (Carrasco et al., 2018) and related to disordered central nervous system (Stocchi & Torti, 2017). The pathomechanism of delayed colonic transit in PD relates to a disordered central nervous system as well as age-related loss of excitatory cholinergic neurons in the colon and impaired cholinergic function (Amani et al., 2017; Ferreira et al., 2018). Therefore, the PD treatment is complicated (Pirtošek et al., 2020).

A recent systematic review by Carrasco et al. (2018) based on MEDLINE, EMBASE, PsycINFO databases revealed the necessity for new effective medications for constipation intervention in PD, since the available treatment options provide no strong recommendations about effectiveness.

Oxidative stress is one of the key pathological triggers of PD pathogenesis, inducing mitochondrial dysfunction, with further activation of neuroglia cells, the infiltration of T-lymphocytes, which is followed by chronic inflammation and α-synuclein aggregation, which eventually results in the degeneration of dopaminergic neurons (Sun et al., 2019). In light of the multiple injury cascades to which free radicals contribute, an antioxidant therapy, including the development of novel nanoscale free radical scavengers, remains attractive strategy (Ferreira et al., 2018).

C_{60} fullerene, as the third allotropic form of carbon materials, has a stable spherical-like hollow structure with a diameter of 0.72 nm, which is close to that of the polypeptides' α-helix and steroid molecules. Thus, the steric compatibility of C_{60} fullerene with biological structures such as the receptor recognizing sites or enzyme active centers may be suggested (Prylutska et al., 2017; Skivka et al., 2018). The surface of the C_{60} molecule consists of 60 carbon atoms, which are connected by sp^2 single and double bonds. Pristine C_{60} fullerene has very low solubility in water. One of the main physicochemical properties of C_{60} fullerene responsible of its various biomedical (Goodarzi et al., 2017; Moussa, 2018; Zhang et al., 2018), in particular to protect biological systems against cell damage and tissue abnormalities, is the ability to scavenge a large number of free radicals (Gharbi et al., 2005). Accordingly, C_{60} fullerene acts as a highly efficient "free radical sponge" (Eswaran, 2018). Furthermore, Wang et al. (1999) reported that C_{60} fullerene and some of its derivatives (covalently modified C_{60} molecules) can efficiently prevent peroxidation and mem-

brane breakdown triggered by free radical species, and were also more effective in inhibiting lipid peroxidation than vitamin E, which is a natural antioxidant. They could weaken certain inflammatory processes (e.g. arthritis and acute inflammation in rats) and possibly facilitate the recovery of damaged tissue (Yudoh et al., 2009; Dragojevic-Simic et al., 2011) However, it can form aggregates in aqueous solutions and make stable colloid solutions which contain both individual C_{60} fullerene and its nanoclusters (Skamrova et al., 2014; Ritter et al., 2015). As is known, C_{60} fullerene is active only in a soluble form when its carbon double bounds are freely accessible (Gharbi et al., 2005).

Despite the fact that C_{60} fullerene has been extensively probed for a number of biomedical applications using *in vivo* models (Gharbi et al., 2005; Halenova et al., 2016; Gonchar et al., 2018; Vereshchaka et al., 2018), the number of studies dedicated to the carbon nanomaterials in pathologies of the central nervous system e.g. PD is substantially lower (Baldrighi et al., 2016). C_{60} fullerene-based antioxidants demonstrate the robust neuroprotection against excitotoxic, apoptotic and metabolic insults in cortical cell cultures (Dugan et al., 2001). Zha et al. (2012) found that only at low concentrations did water-soluble C_{60} fullerene derivatives exhibit neuroprotective effect and increase hippocampal neuronal viability. The hydrated forms of C_{60} fullerene inhibited the fibrillization of amyloid-β 25–35 peptide in rats with Alzheimer's disease (Podolski et al., 2007). We assumed that water-soluble C_{60} fullerenes might be a promising nanodrug in the therapy of PD, including non-motor symptoms, such as delayed colonic motility. The aim of our study was to investigate the therapeutic efficiency of C_{60}FAS in a rat model of PD as well as estimate its possible side-effects.

Materials and methods

The research was conducted according to the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and was approved by the Bioethical Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, Ukraine. Male Wistar rats (220–250 g, n =50) were bred and housed in standard temperature conditions (21–23 °C), lighting (12/12 h light-dark cycle), at humidity (30–35%). All animals had unlimited access to food and tap water.

A highly stable C_{60}FAS with purity of more than 99.96% was prepared and characterized (Golub et al., 2003; Ritter et al., 2015). This method is based on transferring C_{60} fullerenes from organic solution into the aqueous phase by ultrasonic treatment. The used concentration of C_{60} fullerenes in water obtained by this method was 0.15 mg/mL. The prepared C_{60}FAS is stable within 12 months at temperature +4 ºC.

The dopamine neurons were disrupted by unilateral stereotaxic microinjections of 12 μg of selective neurotoxin 6-OHDA (Sigma, USA) in the medial forebrain bundle (MFB) (Talanov et al., 2006). 6-OHDA was dissolved in 4.0 μL of sterile physiological solution (SPS) (0.9% NaCl, JSC "Infusion", Ukraine) with the addition of 0.1% ascorbic acid (as a stabilizer inhibiting oxidation of 6-OHDA). The sham-lesioned groups of animals were injected 4 μL SPS. Rats were placed on the modified stereotaxic apparatus (SEG-4). Animals were scalped and trepanated by using following coordinates (mm) from the bregma (AP = –2.2; ML = 1.5; DV = 8.8). After that, the animals were given pargilin (40 mg/kg, i.p., Sigma, USA) to inhibit metabolic conversion of 6-OHDA by monoaminooxidase and desipramine (25 mg/kg, i.p., Sigma, USA) to block neurotoxin capture by noradrenergic cells. In 30 min either 6-OHDA or 0.9% SPS were injected into the brain into the burr hole at 8.8 mm depth. All microinfusions were completed at a rate 1 μL/15 s. After that the microinjector remained in the brain for 5 min.

The rats were randomly divided into four groups according to the treatment protocol (Fig. 1): I group (n = 13) – the sham-lesioned rats were daily injected 0.1 mL SPS (i.p., 10 days); II group (n = 5) – the sham-lesioned rats were daily injected 1 mL (0.15 mg/mL) C_{60}FAS at a dose 0.65 mg/kg (i.p., 10 days); III group (n = 16) – the 6-OHDA-lesioned rats were injected 0.1 mL SPS (i.p., 10 days); IV group (n = 16) – the 6-OHDA-lesioned rats were treated with 1 mL (0.15 mg/mL) C_{60}FAS at a dose 0.65 mg/kg (i.p., 10 days). All compounds were injected from the 2nd to the 11th day of the experiment.

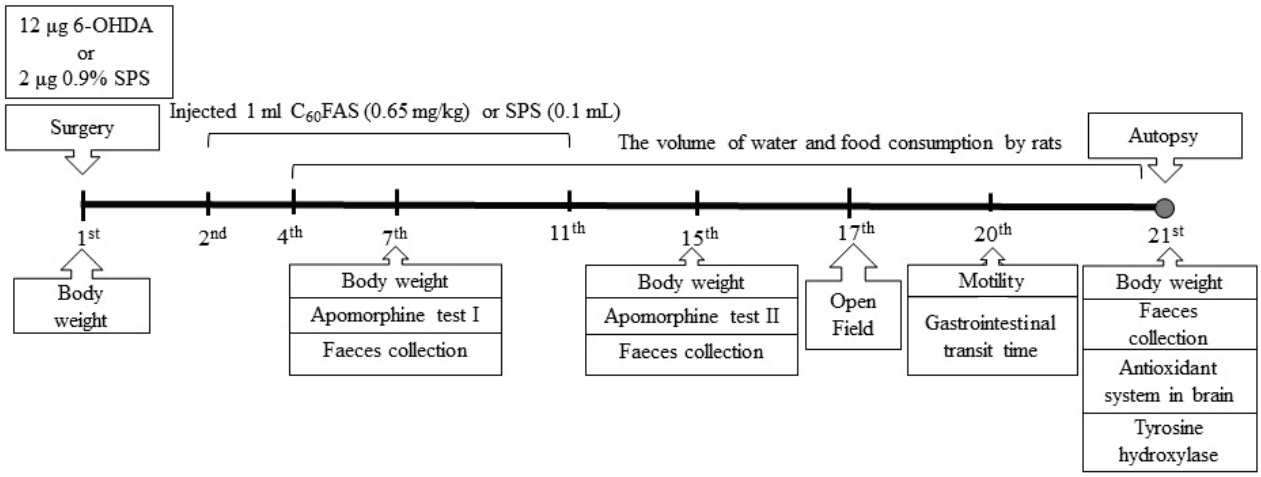

Fig. 1. The scheme of the experiment: doses and solvents in 6-OHDA-induced PD:
12 μg of selective neurotoxin 6-hydroxydopamine (6-OHDA, Sigma, USA); the sham-lesioned groups of animals were injected 4 μL 0.9% SPS

It is important to note that according to a previous study (Prylutska et al., 2019), the maximum tolerated dose of C_{60}FAS is 721 mg/kg for i.p. administration to mice.

The body weight, the apomorphine-induced rotation test, the Open Field test, the water intake rate, the water content in faeces, the motor activity of the distal part of the colon, GI transit time, activity of peritoneal macrophages, antioxidant system in brain were controlled according to the protocol described in Figure 1. Before autopsy (the 21st day) rats were euthanized by CO_2 inhalation with further cervical dislocation. All experiments were repeated three times and all data pooled together.

Full body apomorphine-induced rotations were recorded for 30 min after apomorphine injection (0.5 mg/kg, i.p Sigma, USA) on the 8th (the 1st week) and 15th (the 2nd week) days after surgery. Data are expressed as net turns for 30 min. The intensity of rotation positively correlates with the % of destroyed DA in one hemisphere, so no rotation or rotation less than 180 turns/30 min reflects destruction of 70% or less of dopaminergic neurons; more than 180 turns/30 min – 90–100% destruction (Talanov et al., 2006). Taking this into account, the rats that during the 1st apomorphine test (1 week after surgery) turned more than 180 turns/30 min were excluded from the study.

For immunohistochemistry the rats were anaesthetized with a thiopental (50 mg/kg, i.p.) and transcardially perfused with 100 mL of ice-cold heparinized saline (5000 U/L) followed by 150 mL of 4% paraformaldehyde (pH 7.4). Following transcardial perfusion fixation, the brains were removed, post-fixed in 4% paraformaldehyde. The paraffin-embedded 5 μm sections were incubated overnight (4 ºC) with tyrosine hydroxy-

lase (TH) primary antibodies (1:200, Millipore, AB152, USA), followed by the secondary antibodies (biotinized anti-rabbit, 1:200) for 60 min. Diaminobenzidine (Dako, EnVision Flex, DM821, USA) immunoreactivity detection system was applied for 5 min (Walsh et al., 2011). The intensity of TH-positive staining was graded on a semiquantitative scale by Quantitative Scoring Methods (www.ihcworld.com/ihc_scoring.htm, Table 1). Results are scored by multiplying the percentage of positive cells (P) by the intensity (I) and presented as Quick score (Q): Q = P x I.

Table 1
The semiquantitative scale for grading of the intensity of TH-positive staining by Quantitative Scoring Methods

Score	0	1	2	3	4
Positive cells, %	<10	10–25	25–50	50–75	>75
Intensity of staining	n/a	weak staining	moderate staining	strong staining	n/a

For the behavioural Open Field test the chamber measuring 80 cm (length) x 80 cm (width) x 40 cm (height) and the bottom was lined into 36 squares (the Open Field). During the experiment, each animal was individually landed in the center of the open field and its activity was recorded using a digital camera ("Casio® EX-Z850", China) for 3 min, which was above the center of the field at a height of 1 m. The following behavioural parameters were recorded: 1) the level of anxiety by the latent period of the leaving the center of the field (s) and the number of visits to the field center from periphery; 2) locomotor activity by the total number of square crossings (horizontal activity), the number of rearings (rises up on hind legs), the time of standing on hind legs (s), adopted by the rat with the intention of exploring, during the test period (vertical activity). After each animal was tested, the chamber was washed and allowed to dry.

For determination of water and food intake, each animal was transferred into separate cages for 3 days (adaptation period for new conditions). In 24 h (4th day) volume of water and amount of food was measured to the 21st day of the experiment. The volume of water drunk and the amount of food, water/per day for each animal was averaged per group from 4th to 11th day (the period during the C_{60}FAS administration) and from 12th to 21st day of the experiment (the period after C60FAS withdrawal).

For determination of colonic motility, the animals were anesthetized with a thiopental (50 mg/kg, i.p., Ind. Stock Company Darnytsya, Ukraine). Tracheotomy was performed. A thin latex rubber balloon was connected with plastic tubing and introduced into colon. The balloons were filled with 0.7 mL water (room temperature) by the level required to induce an intracolonic pressure of 10–11 cm H_2O. After the 20 min adaptation period, the spontaneous motor activity of the colon was recorded during 60 min. After that, carbachol (AlfaAesar, USA) was injected at a dose of 10 μg/kg i.p. for recording stimulated motility during the next 60 min. We used the IMA for characterization of the motor function of the colon. After the experiment, the rats were immediately euthanized by CO_2 inhalation with further cervical dislocation.

For determination of GI assay rats were orally gavaged with 0.5 mL aqueous solution of 3% carmine red (ECO Resourse, Ukraine) and placed in a new cage with no bedding. The time at which gavage took place was recorded as T_0. Starting at 120 min post-gavage, the rats were monitored every 10 min for production of a red faecal pellet. Total GI transit time was considered as the interval between T_0 and the time of the first observation of carmine red in the stool (Yano et al., 2015).

The body weight of rats was determined before surgery (the 1st day) and on the 8th, 15th and 21st days of the experiment. The body weight of rats on the 1st day was taken at 100% and each successive weighing was counted in relation to the 1st day.

The freshly expelled faeces of rats were collected and weighted in 8th, 15th and 21st days of the experiment (mw – wet weight). They were dried in an incubator at T = 60 °C for 24 h (md – dry weight). The calculation of water content (W, %) was carried out according to the following formula: W = 100 – (md * 100% / mw).

The spontaneous and induced metabolic activity of peritoneal macrophages was measured using a nitro blue tetrazolium (NBT) recovery assay. To detect spontaneous (SA) NADPH oxidase activity, NBT was added to the macrophages in the wells of a 96-well plate (0.1 mL of 0.2% NBT was added to 1×10^5 macrophages in 0.1 mL of medium). For the detection of stimulated activity (STA), 20 nm of phorbolmyristate-acetate was added to the NBT solution. Cells were incubated in standard conditions (+37 °C, 5% CO_2) for 1 h. The plates were centrifuged at 300 g for 10 min, the supernatants were removed and 0.2 mL of 100% ethanol was added to each well. The plates were re-centrifuged and the accumulated formazan was released by resuspending the cell pellet in 0.1 mL 100 mm KOH and 0.1 mL dimethyl sulfoxide per well. The optical density was measured with a NanoDrop Multiscan Spectrum 2000 photometer (Thermo Scientific, USA) at a wavelength of 540 nm. The percentage of STA was calculated by the formula: (absorption at STA – absorption at SA) × 100% / absorption at SA.

During the autopsy the right hemisphere of the brain (where 6-OHDA neurotoxin was injected) was isolated and homogenized in liquid nitrogen for assessment of antioxidant system: 1 – with SPS to determine catalase activity, protein thiol (SH)-groups and thiobarbituric acid reactive substances (TBARS) levels; 2 – in 0.01 M phosphate buffer (pH 7.5) for reduced glutathione (GSH), glutathione peroxidase (GP), glutathione-S-transferase (GST) detection. Catalase activity in the brain was assessed colorimetrically in a reaction with 0.03% H_2O_2 solution. Then, samples were kept for 10 minutes at room temperature and reaction was stopped by the 4% molybdate ammonium (Alfarus, Ukraine). The measurement was taken at a wavelength of 410 nm (Koroliuk & Ivanova, 1988). The total level of SH groups in brain was measured after sample incubation with 2.6 mM Ellman's reagent (Merk, Germany) for 30 min at room temperature. For measuring non-protein SH groups, samples were mixed with 10% trichloroacetic acid (Alfarus, Ukraine) and centrifugated at 3,000 rpm for 15 minutes. The supernatant was neutralized with 1 M NaOH. Then, samples were incubated with Ellman's reagent. The measurement was taken at a wavelength of 412 nm. Protein SH groups' level (mmoL/g protein) was calculated using coefficient of trinitrophenol anion molar extinction 14.15 M^{-1} cm^{-1} (Ellman, 1959). For determining the level of TBARS reactive substances, Tris-HCl buffer was added to the samples. Then, the protein was denatured by 20% trichloroacetic acid (Merk, Germany) and was subjected to centrifugation for 15 min at 1000 g. To the supernatant, 0.8% thiobarbituric acid was added (Merk, Germany), and this was incubated in a boiling water bath for 15 min for colour development. The measurement was taken at a wavelength of 532 nm. The content of TBARS reactive compounds was calculated on the basis of the molar extinction coefficient of the malonic dialdehyde complex with 2-thiobarbituric acid ($\varepsilon = 1.56 \times 10^5$ $cm^{-1} \times M^{-1}$) (Orekhovich, 1977). To determine the level of GSH, samples were added sequentially: 0.1 M Tris-HCl buffer with 6 mM EDTA (Sigma, USA) (pH = 8.5), 20% sulfosalicylic acid (Merk, Germany). GSH levels were assessed based on its ability to be oxidized by 5,5-dithiobis-2-nitrobenzoic acid (Merk, Germany) resulting in the formation of glutathione disulfide (Merk, Germany) and 5-thio-2-nitrobenzoic acid (Merk, Germany), with the maximum of absorbance at 412 nm (Tipple & Rogers, 2012). GSH level was quantified using calibration curve. GP activity was assessed by measuring unconsumed GSH after its addition to reaction mixture (0.1 M Tris-HCl buffer with 6 mM EDTA (pH = 8.5), 12 mM of sodium azide and 4.8 mM reduced glutathione (Merk, Germany) and incubation with t-butyl hydroperoxide (Merk, Germany). Reaction was stopped by 20% trichloroacetic acid. The measurement was taken at a wavelength of 412 nm (Faraji & Kang, 1987). Total GST activity was assessed by measuring the conjugation of 2 mM 1-chloro-2,4-dinitrobenzene (Merk, Germany) with GSH, which is accompanied by an increase of absorbance at 340 nm. Molar extinction coefficient was used by Nilsson et al. (2000).

Data are presented as mean (x) ± standard derivation (SD). Homogeneity of variance was assessed using the Levene test. Statistical analysis of the data was performed using Kruskal-Wallis test or one-way analysis of variance ANOVA. The difference was considered statistically significant at $P < 0.05$ (taking into account Bonferroni correction). GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, United States) was used for making graphs.

Results

Sham-lesioned rats did not have apomorphine-induced rotation, which is explained by absence of degeneration of DA in these groups of

rats. On the 8th day of the experiment, 6-OHDA rats treated with saline rotated 61.18 ± 22.92 turns/30 min, whereas treated with C_{60}FAS rotated less intensively – 47.71 ± 31.70 turns/30 min, but the difference did not reach significance. At the 2nd week the number of turns increased to 83.27 ± 61.23 turns/30 min in the saline-treated 6-OHDA rats and to 76.57 ± 46.98 in the C_{60} FAS treated 6-OHDA rats (P < 0.05, Fig. 2a).

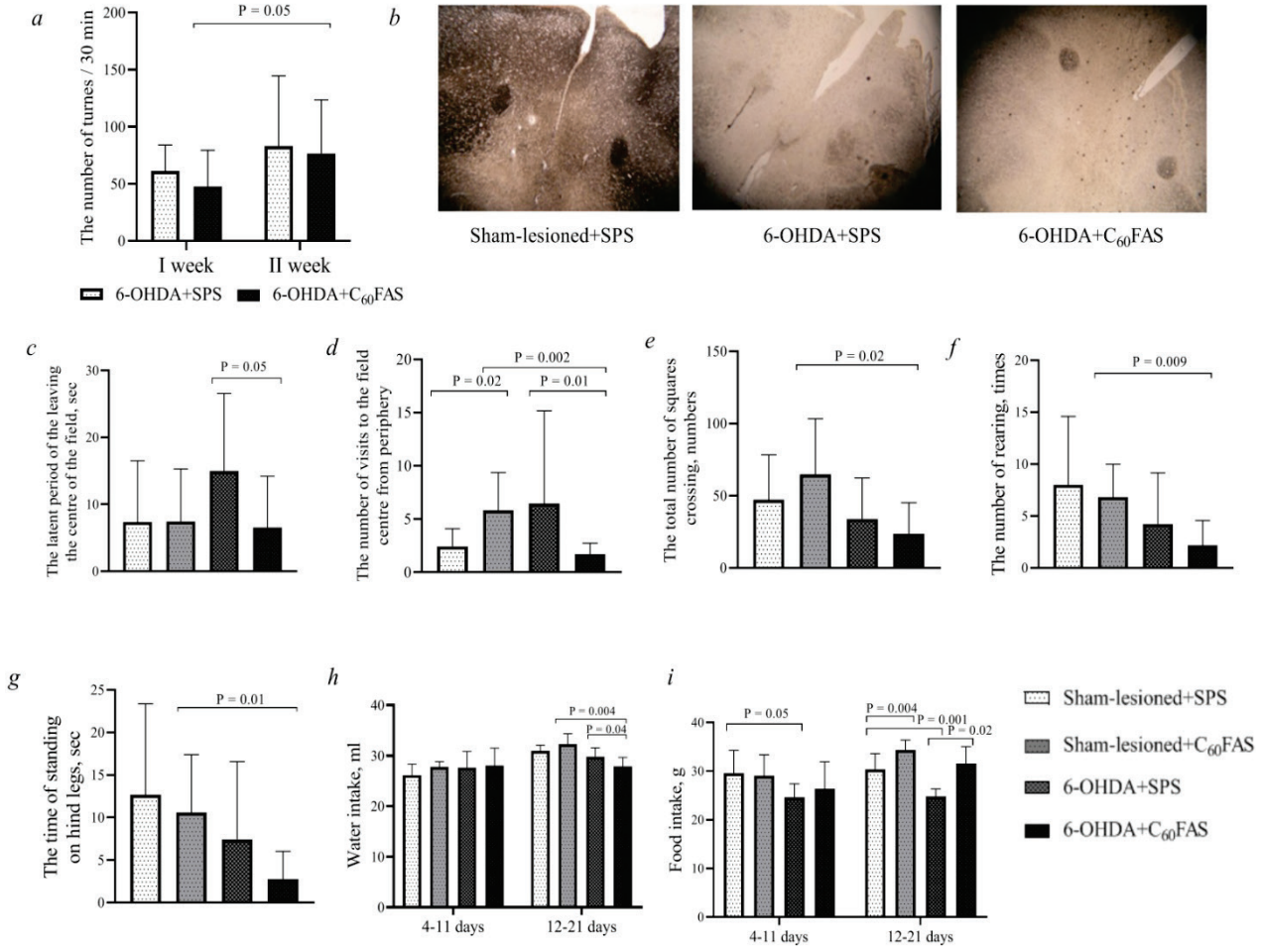

Fig. 2. Treatment with C_{60}FAS increased the central dopaminergic neurons surveillance and changed behavioural pattern in rats with 6-OHDA-PD: *a* – the apomorphine-induced rotational behaviour in rats in the 1th and the 2nd weeks after 6-OHDA-induced PD (n = 25), *b* – photomicrographs of TH-positive immunostaining (brown) of dopaminergic neurons in formalin-fixed, paraffin-embedded rat midbrain sections (5 μm) at the levels of SN (n = 15) (x10), *c* – the level of anxiety by the latent period of the leaving the center of the field (s), *d* – the number of visits to the center of the field from the periphery, *e* – the total number of square crossings (horizontal activity) during the test period in the Open Field test, *f* – the number of rearings (rises up on hind legs) in the Open Field test (times), *g* – the time of standing on hind legs in the Open Field test (s) (n = 43), *h* – the volume of water consumption by rats (n = 20), *i* – the amount of food consumption by rats (n = 20); the reliability of the differences between the samples was evaluated using the single factor dispersion analysis (ANOVA), using the Tukey test (x ± SD)

The apomorphine-rotation test revealed that in 5 out of 11 saline-treated 6-OHDA rats and in 4 out of 15 number the C_{60}FAS-treated 6-OHDA rats the number of turns between the 1st and the 2nd apomorphine test was decreased. In C_{60}FAS-treated 6-OHDA rats the percentage of reduction was 62.29 ± 14.83% vs. 23.07 ± 18.47% in saline-treated 6-OHDA rats.

Immunohistochemically, we observed that 50–75% neurons in midbrain were TH-positive with Q = 6.0 ± 0.0, while rats with 6-OHDA-induced PD had about 10–25% TH-positive cells with Q = 2.0 ± 1.4 (P < 0.001 vs. the sham-lesioned group). C_{60}FAS treatment increased the number of TH-positive cells with Q = 3.7 ± 0.5 vs. the sham-lesioned group (P < 0.05), although, this parameter did not return to control values (Fig. 2b).

Rats with 6-OHDA-induced PD were characterized by impaired emotionality vs. the sham-lesioned rats. We found 2-fold increase in the latent period of the leaving of the field center (Fig. 3c) and 2.5-fold in the number of visits to the center of the field from periphery vs. the sham-lesioned rats (Fig. 3d), but in both cases the difference did not reach significance. C_{60}FAS treatment decreased 4-fold the latent period of the leaving of the field center (P < 0.05) and the number of visits to the field center from the periphery (P < 0.01) vs. 6-OHDA-PD + SPS (Fig. 2c, d). Moreover, the total number of square crossings during the test, the number of rearings (rises up on hind legs) and the total duration of this form of behaviour were insignificantly decreased in 6-OHDA-PD vs. the sham-operated groups rats (Fig. 2e–g). This represents the slightly impaired explorative activity (vertical activity) and horizontal activity, which were further decreased by C_{60}FAS treatment.

In our study, we observed gradual increase in net water intake volume between the first 10 days (after surgery) and the second 10 days of the experiment in saline as well as C_{60}FAS-treated the sham-lesioned groups of rats (Fig. 2h). Opposite pattern occurred in 6-OHDA-PD rats. C_{60}FAS treatment did not improve water-intake behaviour of 6-OHDA-PD rats. Moreover, 6-OHDA-PD rats treated with C60FAS drank slightly less (12%) volume of water vs. saline-treated 6-OHDA-PD rats (P < 0.05).

The amount of food intake in 6-OHDA rats decreased by 1.2 times vs. the sham-lesioned groups of rats + SPS (P = 0.05) during 4–11 days of experiment (Fig. 2i). In control groups and 6-OHDA-PD rats with C_{60}FAS treatment no difference was observed during 4–11 days of the experiment. During 12–21 days of experiment we observed increased food consumption in rats treated by C_{60}FAS. The sham-lesioned groups of rats + C_{60}FAS showed increased values by 12% vs. the sham-lesioned groups of rats +SPS (P < 0.01). The values of food intake in 6-OHDA-PD rats treated with C_{60}FAS increased by 27% vs. saline-treated 6-OHDA-

PD rats ($P < 0.05$). In 6-OHDA rats + SPS the values decreased by 18% vs. the sham-lesioned groups of rats +SPS ($P < 0.001$).

We observed 20% delayed in GI transit time through the whole GI tract in 6-OHDA-PD rats ($P < 0.01$). Treatment with C_{60}FAS improved GI transit time in the 6-OHDA-PD rats almost to the level of the saline-treated sham-lesioned rats. Interestingly, sham-lesioned rats treated with C60FAS had delayed GI transit time at the same level as saline treated 6-OHDA-PD rats (Fig. 3c). In our study, the spontaneous colon motility was reduced by 22% of colon IMA (from 142.3 ± 49.8 to 100.97 ± 29.23 cm H_2O) in rats with 6-OHDA-induced hemiparkinsonism vs. the sham-operated rats, although these changes did not reach statistical significance. Treatment with C_{60}FAS insignificantly decreased the colon index of motor activity (IMA) in rats with 6-OHDA-PD vs. the placebo-treated 6-OHDA-PD rats by 10% (from 100.97 ± 29.23 to 99.14 ± 42.98 cm H_2O, Fig. 3b).

Stimulated motility which represents the digestion behaviour of the GI tract was modulated by i.p. injection of acetylcholine agonist carbachol (10 μg/kg). Carbachol increased 2-fold (from 142.3 ± 49.8 to 266.05 ± 48.57 cm H_2O) colon IMA in the sham-operated rats, while in 6-OHDA-PD rats carbachol-stimulated colon IMA was 2-fold ($P < 0.05$) less (131.05 ± 60.97 cm H_2O) vs. the sham-operated rats (266.05 ± 48.57 cm H_2O). The carbachol-stimulated motility responds to the phase III the intestine motor activity. Phase III is the strongest, and slow waves are accompanied by a spike that we can see from Figure 3a. C60FAS treatment completely restored the level of stimulated colon motor activity in 6-OHDA-PD rats (from 131.05 ± 60.97 to 256.02 ± 60.1 cm H_2O) vs. the sham-lesioned + SPS and returned to control value.

The sham-lesioned rats + SPS gained 35% of body weight between the 1st and last (the 21st) day of the experiment (from 183.9 ± 21.3 g to 248.0 ± 40.6 g). C_{60}FAS injection to the sham-lesioned rats did not affect the body weight gain, rats increased body weight 38% (from 206.4 ± 13.2 to 283.0 ± 20.2 g). The 6-OHDA-PD rats increased net body weight only 25% (from 187.3 ± 22.1 to 233.4 ± 34.8 g), which is 11% less vs. the sham-lesioned rats. C_{60}FAS treatment did not improve body weight gain in 6-OHDA-PD rats, they gained 24% body weight (from 190.1 ± 20.8 to 234.58 ± 28.5 g, Fig. 4a).

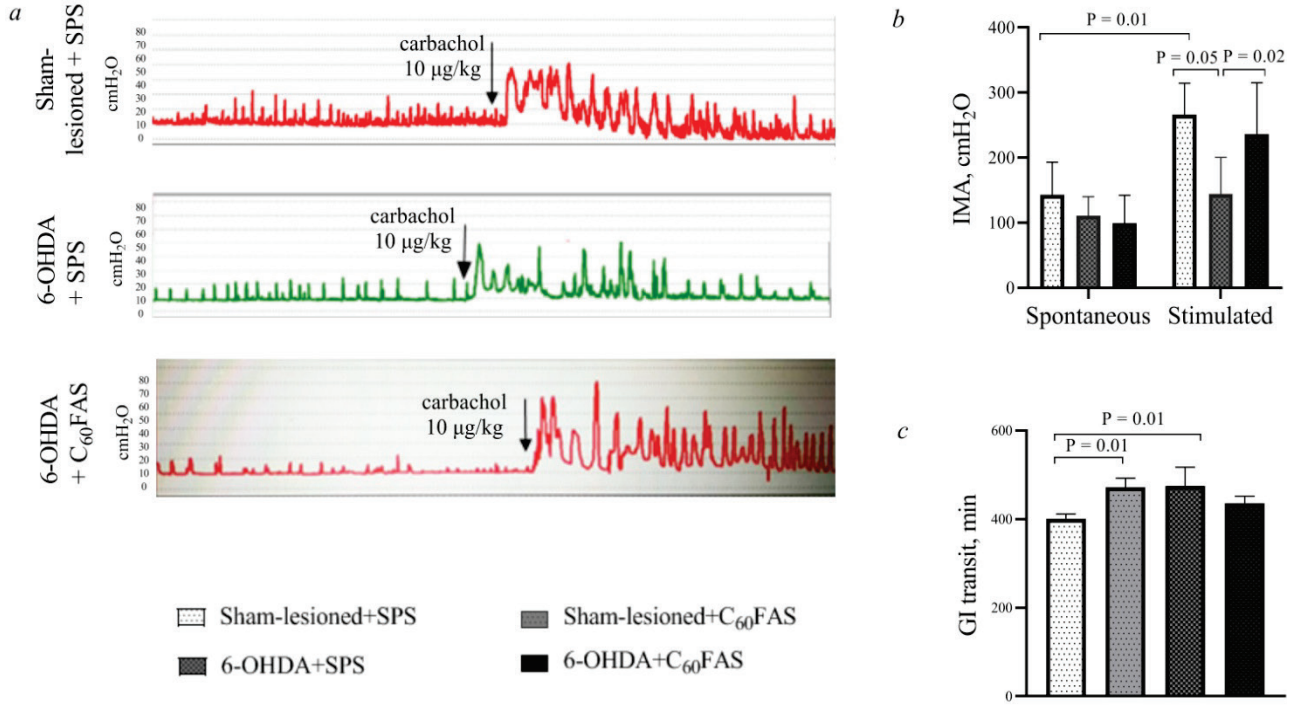

Fig. 3. C60FAS improved the motor activity and GI transit time of colon in rats with 6-OHDA-PD:
a – the typical record of spontaneous and stimulated motor activity of rat colon recorded by ballonographic method;
↓ – injection of carbachol (10 μg/kg, i.p.); *b* – the motor activity index (IMA) of spontaneous and stimulated colon motility in rats (n = 16),
c – the total GI transit time of gut (by 0.5 mL of 3% carmine red) (n = 18); the reliability of the differences between
the samples was evaluated using the single factor dispersion analysis (ANOVA), using the Tukey test (x ± SD)

The water content in faeces of rats with 6-OHDA-induced PD both saline and C_{60}FAS-treated were slightly increased on the 8th day of the experiment vs. the sham-lesioned groups ($P < 0.05$), which might be the result of overall toxic effect of 6-OHDA (Fig. 4b). In 2 weeks after modeling of the 6-OHDA-PD rats, the water content in faeces in the 6-OHDA-PD rats was significantly decreased vs. the sham-lesioned group ($P < 0.05$), which might the sign of delayed colonic motility. By the 21st day of the experiment, we observed tendency to increase in water content in faeces of C_{60}FAS-treated 6-OHDA-PD rats vs. saline-treated, that correlate with improvement of colonic motility and total GI transit time.

Macrophage activation is required to control inflammation and disease progression. So, in our research the percentage of spontaneous NADPH oxidase activity of peritoneal macrophages showed 1.5-fold decreased activity in sham-lesioned rats + C_{60}FAS and increased 1.3-fold in 6-OHDA-PD + SPS vs. sham-lesioned + SPS (Fig. 4c). C_{60}FAS treatment by 6-OHDA-induced PD rats showed 3.6-fold increase NADPH oxidase activity of peritoneal macrophages vs. sham-lesioned rats + C_{60}FAS ($P < 0.05$) and almost 2-fold vs. 6-OHDA-induced PD. Furthermore, we studied the effect of C_{60}FAS on oxidant-antioxidant system in the brain of rats with 6-OHDA-PD (Fig. 4d–i). C_{60}FAS treatment per se caused a significant 1-fold increase ($P = 0.05$) in activity of catalase and 3.6-fold ($P = 0.05$) in levels of GSH, while it did not affect significantly other markers of oxidative stress. The development of 6-OHDA-PD was associated with mild changes in markers of oxidative stress with only significant 1.4-fold ($P < 0.05$) upregulation of GP activity. C_{60}FAS treatment of 6-OHDA-PD rats significantly increased GSH levels ($P < 0.05$) and almost terminated the activity of GP ($P < 0.01$) vs. saline treated 6-OHDA-PD rats. Moreover, we observed 1.3-fold increase in catalase activity in rats after C_{60}FAS treatment vs. saline treated 6-OHDA-PD rats, but the difference did not reach statistical significance.

Discussion

In the present study, we reported for the first time on the toxicity effect of water-soluble pristine C_{60} fullerene-treatment on neurodegenerative changes and early non-motor symptoms in the rat model of 6-OHDA-induced PD *in vivo*. We used a model of partial lesion of the nigrostriatal system (unilateral injection of 6-OHDA) that mimics earlier stages of PD

where some dopaminergic cells and fibers should remain. A series of *in vitro* studies found that different C_{60} fullerene derivatives have neuroprotective effect through anti-inflammatory or antioxidant mechanisms. Ye et al. (2016) showed that pretreatment with hydroxylated C_{60} fullerenes inhibited the excessive production of inflammatory mediators (e.g. prostaglandin E_2, nitric oxide, tumour necrosis factors, IL-1β, and IL-6), blocked the expression of cyclooxygenase-2 and inducible nitric-oxide synthase on the model of the normal cellular prion protein-activated microglia; and prevented apoptotic neuronal cell death induced by culture media from activated microglia. These effects were nuclear factor erythroid 2-related factor 2 (Nrf2) dependent. C_{60} fullerene nanoparticles reduced β-amyloid (Aβ) 25–35-induced cytotoxicity towards Neuro-2A cells by increasing cell viability, the reduction of the intracellular reactive oxygen species accumulation (Lu et al., 2011). Pretreatment with the polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ increased cell viability, mitochondrial function (including mitochondrial membrane potential and activities of complex I and II), and decreased the levels of reactive oxygen species and oxidative damage to DNA and proteins in the 1-methyl-4-phenylpyridinium (MPP+)-induced acute cellular PD model in human neuroblastoma cells (Cai et al., 2008).

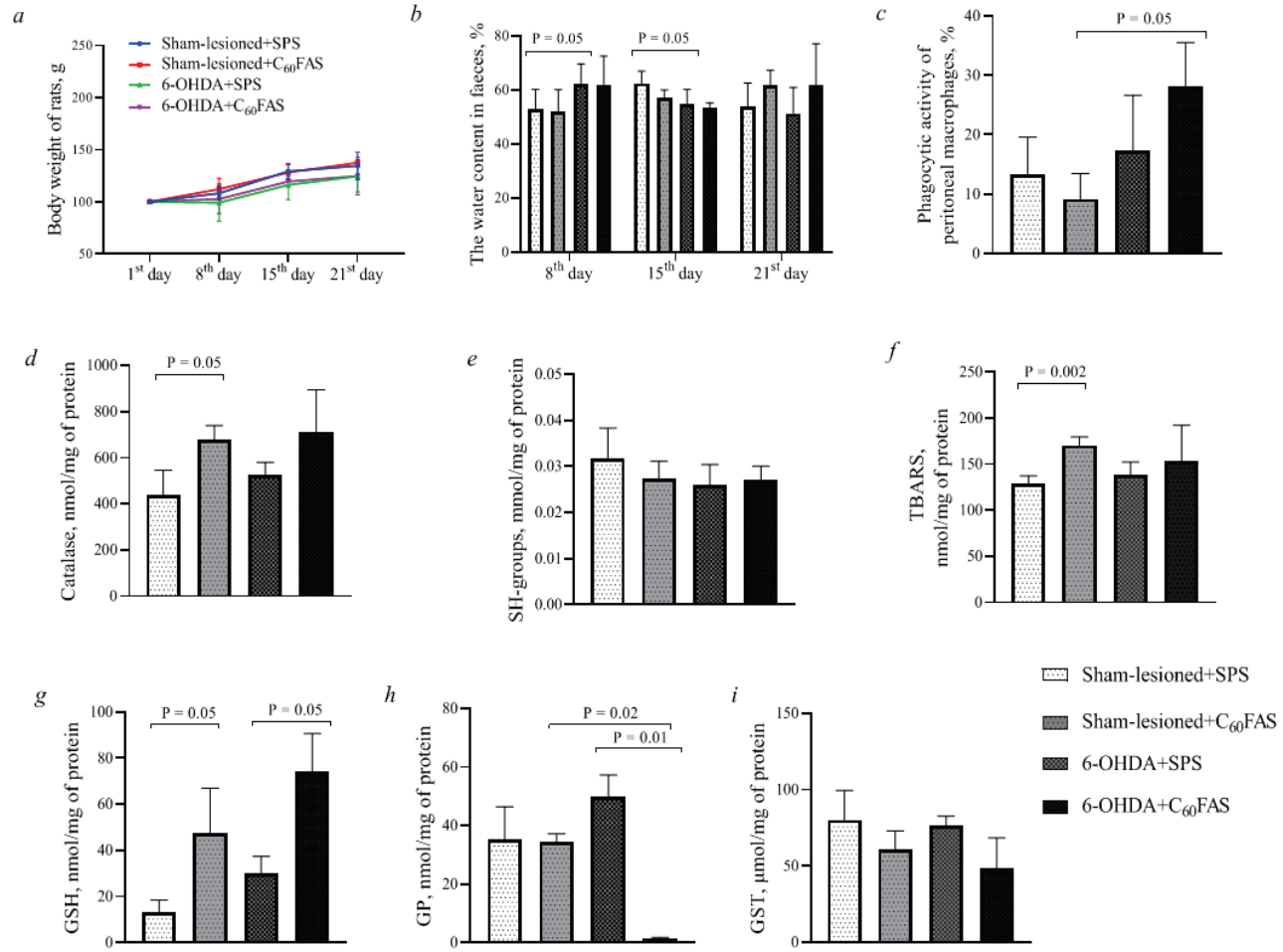

Fig. 4. C_{60}FAS treatment has systemic mild toxic effect in sham-lesioned and OHDA-PD rats:
a – the changes in dynamics of body weight in sham-lesioned and OHDA-PD rats after C60FAS treatment (n = 50); *b* – the water content in faeces of sham-lesioned and OHDA-PD rats in 8th, 15th and 21st days after C_{60}FAS treatment (n = 50); *c* – the phagocytic activity of peritoneal macrophages (%) in sham-lesioned and OHDA-PD rats on 21st day after C_{60}FAS treatment (n = 20); *d*–*i* – the changes in antioxidant system in brain of sham-lesioned and OHDA-PD rats after C_{60}FAS treatment (n = 20); *d* – catalase activity, nmoL/mg; *e* – content of protein thiol (SH) groups, mmoL/mg; *f* – the level of thiobarbituric acid reactive substances (TBARS), nmoL/mg; *g* – the level of reduced glutathione (GSH), nmoL/mg; *h* – the level of glutathione peroxidase (GP) activity, nmoL/mg; *i* – the total glutathione-S-transferase (GST) activity, μmoL/mg; the reliability of the differences between the samples was evaluated using the single factor dispersion analysis (ANOVA), using the Tukey test (x ± SD)

In our study, treatment with C_{60}FAS for 10 days prevented degeneration of the dopaminergic neurons in the midbrain in rats with 6-OHDA-induced PD, which was detected by the increased number of TH-positive neurons in the midbrain vs. the sham-lesioned rats (in 10 days after withdrawal from treatment). Interestingly, the apomorphine test showed effectiveness of C_{60}FAS only during treatment period (the 1st week after surgery). While in 3 days after C_{60}FAS withdrawal, there was no difference in the apomorphine test between C_{60}FAS and rats with 6-OHDA-PD + SPS. The apomorphine test is a indirect marker of degeneration of dopaminergic neurons, since positive correlation was found between the intensity of the apomorphine-induced circulatory movements to the side, contralateral to the toxin-treated hemisphere, and the percentage of destroyed neurons (Talanov et al., 2006). We assumed that increased number of turns in apomorphine test can be evoked by C_{60}FAS-induced neuronal plasticity and increased number of dopamine receptors. Vorobyov et al. (2015) showed on the rat model of Aβ1-42 Alzheimer's disease that introhipocampal preatretment with hydrated C_{60} fullerene (C_{60}HyFn) enhanced sensitivity to apomorphine in fullerene-versus saline-treated rats on cortical versus hippocampal interrelations, relative differences between cortical and hippocampal the electroencephalogram spectra.

The exposure of rats to a new environment leads to two opposing behavioural tendencies: the fear of the new environment and the desire to experience it. These two trends are characterized by different time course and different spatial preference. The locomotor activity and time spent in the center and at the periphery are the most significant indicators for assessing the level of anxiety in rats. Typically, rats prefer to walk close to the walls of the field (thigmotaxis) in order to escape an unknown, stressful environment. In the Open Field test, we found that rats with 6-OHDA-

PD had increased latent period of leaving the center of the field, the number of squares crossed in the center after revisiting from the periphery (markers of emotional stability), while the total number of squares crossed during the test period and the number and duration of rearings (rises up on hind legs) (markers of locomotor and explorative activity) were decreased. That confirmed the presence of changes in autonomic and cognitive functions of CNS, emotional instability and unbalancing of information processing and decreased locomotor activity in rats with modeled hemiparkinsonism. Treatment with C_{60}FAS improved the emotional state of the rats while it did not affect locomotor and explorative activity.

Similar data were obtained in a double-blind, placebo-controlled study on MPTP-induced PD treated Macaque fascicularis monkeys. In this study, treatment for 2 months with water-soluble carboxyfullerene reduced striatal injury and improved behavioural motor function (Dugan et al., 2014).

Decreased food intake in sham-lesioned rats with C_{60}FAS during 12–21 days of experiment correlated with increased the level of anxiety by the latent period of leaving the center of the field and the number of visits to the field center from periphery.

Concomitant to behavioural changes in rats with the 6-OHDA-PD, we found decreased water intake by them. The amount of water intake correlates inversely with the severity of constipation and the depletion of water intake precedes the constipation in most cases. Both changes in water intake and constipation are common non-motor symptoms of PD. Interestingly, PD patients tended not to feel thirsty and thus they have no desire to drink water and this may also lead to constipation review (Ma et al., 2018)). In our research C_{60}FAS improved level of water intake and returned gastrointestinal transit time in 6-OHDA-PD rats to the control group. We found profoundly decreased carbachol-stimulated colon motility in rats with 6-OHDA-PD. Treatment with C60FAS restored it. Interestingly, C_{60}FAS treatment delay GI transit time in the sham-lesioned rats and one reason of this can be intestinal inflammation in rats with PD and accumulation C_{60} fullerene in the abdomen, because we injected C_{60}FAS intraperitoneally. Hendrickson et al. (2015) showed that after peroral administration C_{60} fullerene was detected mainly in the stomach and small intestines, liver, kidneys and spleen of animals and was excreted with faeces. In our research we observed accumulation of local high amount of C_{60}FAS in pancreas. The same results were observed by Kuznietsova et al. (2020). Beside this under chronic acetaminophen (at dose 1000 mg/kg) renal dysfunction also occurs, suggesting the possible cumulative effects of C_{60} fullerene.

The toxicity of nanoparticles on cells strongly depends on many physicochemical factors including size, shape, chemical composition, solubility, surface area, surface charge and experimental conditions (Aillon et al., 2009; Tolkachov et al., 2016; Emelyantsev et al., 2019). In our research we used the pristine (unmodified) C_{60} fullerene that was dissolved in water.

In review Kolosnjaj et al. (2007) say that pristine C_{60} fullerene has no acute or sub-acute toxicity in a large variety of living organisms, from bacteria and fungal to human leukocytes, and also in mice, rats and guinea pigs in contrast to chemically modified fullerenes. Moussa et al. (1996) showed for the first time that a highly concentrated aqueous suspension of micronized C_{60} (100 mg/mL) had neither lethal nor acute or sub-acute toxicity with respect to intraperitoneal injection to rodents (Moussa et al., 1996; Kolosnjaj et al., 2007).

Earlier it was shown (Prylutska et al., 2019) that the dose range of 75–150 mg/kg of pristine C_{60} fullerene had no toxic effect (LD_{50} value was 721 mg/kg). The toxic effect of C_{60} fullerene was observed at a concentration from 300 mg/kg. The changes were observed in mice behaviour disturbance, hematotoxicity and pathomorphological changes in spleen, hepatic and kidney tissues. In this study C_{60}FAS was injected to rats with PD intraperitoneally daily for 10 days (0.65 mg/kg per day). So, as indicated by previous research this dose is not toxic for the organism.

Recently, body weight changes have become important clinical marker of PD progression and exacerbation of the disease, which represents impairment of GI function and metabolic dysfunction (see for review Ma et al. (2018)). Moreover, Chen et al. (2003) observed that patients with PD had decreased body weight 10 years before disease onset. Halenova et al. (2018) showed that C_{60}FAS normalized the metabolic parameters and partially reduced body mass index in rats with diet-induced obesity. In present study we registered loss of body weight in the 6-OHDA-PD rats by 12% vs. the sham-lesioned animals and its increase after treatment with C_{60}FAS by 7% compared with the sham-lesioned.

Comorbidity of weight loss and malnutrition may impact Parkinson's disease progression, giving rise to dyskinesia, cognitive decline and orthostatic hypotension, and even resulting in disability and mortality (see for review Ma et al. (2018)). Since, the C_{60}FAS was effective in improving body weight, next we checked its effect on cognitive, locomotor state as well as function of GI tract.

Inflammation is characterized by a large infiltrate of macrophages, lymphocytes, eosinophils, and plasma cells. Lymphocytes are mobilized and stimulated by contact with antigen to produce lymphokines that activate macrophages (Munari et al., 2019). In our research C_{60} fullerene modulates phagocyte functions, such as stimulate phagocytic activity of peritoneal macrophages. Skivka et al. (2018) confirm that C_{60} fullerene can exert direct cytotoxic effect on phagocytes, more pronounced in the case of malignant cells. And this cytotoxic effect is associated with the vigorous induction of intracellular ROS generation. At low single therapeutic dose (5 mg/kg) C_{60}FAS can cause antitumour immune response, without direct cytotoxicity. Beside this, C_{60}FAS significantly decreases the number of tumours and total lesion area on the model of colorectal cancer in rats (Lynchak et al., 2017). Park et al. (2013) say "macrophages activated by the classical pathway show changes in gene expression, metabolic pathways, and their morphological and functional characteristics. These changes are responsible for increased phagocytic capacity, processing and presentation of antigens, lysis of tumour cells, reactive oxygen species, and lysosomal enzymes". If pristine C_{60} fullerene is absolutely nontoxic under dark conditions, this is not the same in the presence of O_2 where C_{60} fullerene solutions can be highly toxic through O_2 formation (Kolosnjaj et al., 2007; Scharff et al., 2008).

Research has shown that neuronal loss in the SN in patient with PD as a result of reactive oxygen species (ROS) is linked to the mitochondrial dysfunction of complex I associated with this disease (Mortiboys et al., 2007). The brain consumes a large amount of oxygen and is particularly sensitive to ROS-mediated damage. Catalase is antioxidant enzyme, which plays an active role in cells as a ROS destruction. Eom et al. (2015) showed that PEP-1-catalase transduced into SH-SY5Y cells protect them against MPP+-induced death by decreasing ROS and regulating cellular survival signals including Akt, Bax, Bcl-2, and p38. Immunohistochemical analysis showed that transduced PEP-1-catalase markedly protected against neuronal cell death in the SN in the PD animal model (Eom et al., 2015). In PD, the peroxidase and catalase activity was decreased in the SN, caudate, and putamen (Ambani et al., 1975). In our study the level of catalase activity did not change in 6-OHDA rats, but increased almost 2-fold in rats treated by C_{60}FAS, which confirms antioxidant function of C_{60}FAS. Oxidant-antioxidant balance disturbance can activate different redox-sensitive signaling pathways by oxidizing cysteine SH-groups of protein molecules. We detected an insignificant decrease in the level of reduced protein SH groups in all experimental groups. The TBARS increased only in sham-lesioned rats treated by C_{60}FAS but in PD rats there was no effect.

The brain contains only low to moderate superoxide dismutase, catalase and GP activity compared with kidney or liver. The best evidence of an altered glutathione metabolism as an important factor contributing to the pathogenesis of a neurodegenerative disease has been found in PD (Dringen et al., 2000).

The introduction of 6-OHDA can inhibit the development of protective endogenous mechanisms of cell in response, in particular the activation of the components of the antioxidant system in the form of GSH and glutathione-dependent enzymes antioxidant protection – GP and GST. Antioxidants such as GP can act as a compensatory mechanism in the fight against the toxic effect of 6-OHDA and prevent degeneration of DA-ergic cells (Talanov et al., 2006). Other research has shown decreases in GSH, increased iron content and activation of NFkB (Bharath et al., 2002). But in our research, GSH increased in rats treated by C_{60}FAS. So, the C_{60}FAS increased only the level of two enzymes – catalase and GSH, other level of enzymes decreased or did not change. The development of 6-OHDA-induced PD in rats did not affect significantly the markers of oxidant-antioxidant system in brain vs. sham-operated rats.

However, C_{60} fullerene treatment induced a mild shift in antioxidant-prooxidant system in the brain of 6-OHDA-PD rats vs. saline treated rats, with more profound negative effect on the glutathione system, which might be the sign of its toxic effect.

Kuznietsova et al. (2020) have shown that despite cumulative effects of C_{60}FAS in the kidneys and pancreas, it restores the liver, attenuates the dystrophic processes in hepatocytes and manifestations of apoptosis and necrosis and normalizes the values of most liver functional biochemical markers. This phenomenon has been explained by the therapeutic effects of C_{60} fullerene and its ability to scavenge free radicals (Prylutska et al., 2010) and affect the expression of EGFR. But in our research we observed heightened oxidative protection and a mildly toxic effect on the CNS and periphery in the gut, which was accompanied by inflammation in rats with PD.

Conclusions

C_{60}FAS increased the number of TH-positive cells in the midbrain, improved the colon motor activity and stimulated phagocytic activity of peritoneal macrophages compared with placebo-treated 6-OHDA-PD rats. C_{60}FAS treatment did not improve water intake behaviour and body weight changes in 6-OHDA-PD rats and induced a mild shift in the antioxidant-prooxidant system in the brain of 6-OHDA-PD rats vs. saline treated, with more profound negative effect on the glutathione system, which might be the sign of its toxic effect. Thus, the obtained results on the model of 6-OHDA-induced PD in rats can be explained by high antioxidant activity of the water-soluble pristine C_{60} fullerenes and neutralizing excess in ROS in the cells. Possibly, due to their aggressive antioxidant action, pristine C_{60} fullerenes cannot be used as an experimental basis for the treatment of early non-motor symptoms of 6-OHDA model of PD.

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References

Aillon, K. L., Xie, Y., El-Gendy, N., Berkland, C. J., & Forrest, M. L. (2009). Effects of nanomaterial physicochemical properties on *in vivo* toxicity. Advanced Drug Delivery Reviews, 61, 457–466.

Amani, H., Habibey, R., Hajmiresmail, S. J., Latifi, S., Pazoki-Toroudi, H., & Akhavan, O. (2017). Antioxidant nanomaterials in advanced diagnoses and treatments of ischemia reperfusion injuries. Journal of Materials Chemistry B, 48, 9452–9476.

Ambani, L. M., Van Woert, M. H., & Murphy, S. (1975). Brain peroxidase and catalase in Parkinson disease. Archives of Neurology, 32(2), 114–118.

Baldrighi, M., Trusel, M., Tonini, R., & Giordani, S. (2016). Carbon nanomaterials interfacing with neurons: An *in vivo* perspective. Frontiers in Neuroscience, 10, 1–27.

Bharath, S., Hsu, M., Kaur, D., Rajagopalan, S., & Andersen, J. K. (2002). Glutathione, iron and Parkinson's disease. Biochemical Pharmacology, 64(5–6), 1037–1048.

Braak, H., Del Tredici, K., Rüb, U., De Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiology of Aging, 24(2), 197–211.

Cai, X., Jia, H., Liu, Z., Hou, B., Luo, C., Feng, Z., Wenxin, L., & Liu, J. (2008). Polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ prevents mitochondrial dysfunction and oxidative damage in an MPP+-induced cellular model of Parkinson's disease. Journal of Neuroscience Research, 86(16), 3622–3634.

Chen, H., Zhang, S. M., Herna, M. A., Willett, W. C., & Ascherio, A. (2003). Weight loss in Parkinson's disease. Annals of Neurology, 53(5), 676–679.

Dragojevic-Simic, V., Jacevic, V., Dobric, S., Djordjevic, A., Bokonjic, D., Bajcetic, M., & Injac, R. (2011). Anti-inflammatory activity of fullerenol $C_{60}(OH)_{24}$ nano-particles in a model of acute inflammation in rats. Digest Journal of Nanomaterials and Biostructures, 6(2), 819–827.

Dringen, R., Gutterer, J. M., & Hirrlinger, J. (2000). Glutathione metabolism in brain: Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. European Journal of Biochemistry, 267, 4912–4916.

Dugan, L. L., Lovett, E. G., Quick, K. L., Lotharius, J., Lin, T. T., & O'Malley, K. L. (2001). Fullerene-based antioxidants and neurodegenerative disorders. Parkinsonism and Related Disorders, 7(3), 243–246.

Dugan, Laura L., Tian, L. L., Quick, K. L., Hardt, J. I., Karimi, M., Brown, C., Loftin, S., Flores, H., Moerlein, S., Polich, J., Tabbal, S. D., Mink, & Perlmutter, J. S. (2014). Carboxyfullerene neuroprotection postinjury in parkinsonian nonhuman primates. Annals of Neurology, 76(3), 393–402.

Ellman, G. L. (1959). Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82(1), 70–77.

Emelyantsev, S., Prazdnova, E., Chistyakov, V., & Alperovich, I. (2019). Biological effects of C_{60} fullerene revealed with bacterial biosensor-toxic or rather antioxidant? Biosensors, 9(2), 81.

Eom, S. A., Kim, D. W., Shin, M. J., Ahn, E. H., Chung, S. Y., Sohn, E. J., Jo, H. S., Jeon, S. J., Kim, D. S., Kwon, H. Y., Cho, S. W., Han, K. H., Park, J., Eum, W. S., & Choi, S. Y. (2015). Protective effects of PEP-1-catalase on stress-induced cellular toxicity and MPTP-induced Parkinson's disease. BMB Reports, 48(7), 395–400.

Eswaran, S. V. (2018). Water soluble nanocarbon materials: A panacea for all? Current Science, 114(9), 1846–1850.

Faraji, B., Kang, H. K., & Valentine, J. L. (1987). Methods compared for determining glutathione peroxidase activity in blood. Clinical Chemistry, 33(4), 539–543.

Ferreira, C. A., Ni, D., Rosenkrans, Z. T., & Cai, W. (2018). Scavenging of reactive oxygen and nitrogen species with nanomaterials. Nano Research, 11(10), 4955–4984.

Gharbi, N., Pressac, M., Hadchouel, M., Szwarc, H., & Wilson, S. R. (2005). [60] Fullerene is an *in vivo* powerful antioxidant with no acute or sub-acute toxicity. Nano Letters, 5(12), 2578–2585.

Gonchar, O. O., Maznychenko, A. V., Bulgakova, N. V., Vereshchaka, I. V., Tomiak, T., Ritter, U., Prylutskyy, Y. I., Mankovska, I. M., & Kostyukov, A. I. (2018). C_{60} fullerene prevents restraint stress-induced oxidative disorders in rat tissues: Possible involvement of the Nrf2/ARE-antioxidant pathway. Oxidative Medicine and Cellular Longevity, 2018, 2518676.

Goodarzi, S., Da Ros, T., Conde, J., Sefat, F., & Mozafari, M. (2017). Fullerene: Biomedical engineers get to revisit an old friend. Materials Today, 20(8), 460–480.

Halenova, T. I., Vareniuk, I. M., Roslova, N. M., Dzerzhynsky, M. E., Savchuk, O. M., Ostapchenko, L. I., Prylutskyy, Y. I., Ritter, U., & Scharff, P. (2016). Hepatoprotective effect of orally applied water-soluble pristine C_{60} fullerene against CCl_4-induced acute liver injury in rats. RSC Advances, 6(102), 100046–100055.

Halenova, T., Raksha, N., Vovk, T., Savchuk, O., Ostapchenko, L., Prylutskyy, Y., Kyzuma, O., Ritter, U., & Scharff, P. (2018). Effect of C_{60} fullerene nanoparticles on the diet-induced obesity in rats. International Journal of Obesity, 42(12), 1987–1998.

Hendrickson, O. D., Morozova, O. V., Zherdev, A. V., Yaropolov, A. I., Klochkov, S. G., Bachurin, S. O., & Dzantiev, B. B. (2015). Study of distribution and biological effects of fullerene C_{60} after single and multiple intragastrical administrations to rats. Fullerenes Nanotubes and Carbon Nanostructures, 23(7), 658–668.

Kalia, L. V., & Lang, A. E. (2015). Parkinson's disease. The Lancet, 386(9996), 896–912.

Kolosnjaj, J., Szwarc, H., & Moussa, F. (2007). Toxicity studies of fullerenes and derivatives. Advances in Experimental Medicine and Biology, 620, 168–180.

Kroto, H. W., Heath, J. R., O'Brien, S. C., Curl, R. F., & Smalley, R. E. (1985). C_{60}: Buckminsterfullerene. Nature, 318(6042), 162–163.

Kuznietsova, H., Dziubenko, N., Hurmach, V., Chereschuk, I., Motuziuk, O., Oglobla, O., & Prylutskyy, Y. (2020). Water-soluble pristine C_{60} fullerenes inhibit liver fibrotic alteration and prevent liver cirrhosis in rats. Oxidative Medicine and Cellular Longevity, 2020, 1–14.

Lu, T. Y., Kao, P. F., Lee, C. M., Huang, S. T., & Lin, C. M. (2011). C_{60} fullerene nanoparticle prevents β-amyloid peptide induced cytotoxicity in neuro 2A cells. Journal of Food and Drug Analysis, 19(2), 151–158.

Ma, K., Xiong, N., Shen, Y., Han, C., Liu, L., Zhang, G., Wang, L., Guo, S., Guo, X., Xia, Y., Wan, F., Huang, J., Lin, Z., & Wang, T. (2018). Weight loss and malnutrition in patients with Parkinson's disease: Current knowledge and future prospects. Frontiers in Aging Neuroscience, 10, 1.

Mortiboys, H. J., Schaefer, J., Reichmann, H., & Jackson, S. (2007). Mitochondrial dysfunction in Parkinson's disease – revisited. Neurologia i Neurochirurgia Polska, 41, 150–159.

Moussa, F. (2018). [60] Fullerene and derivatives for biomedical applications. In: Narayan, R. (Ed.). Nanobiomaterials: Nanostructured materials for biomedical applications. Woodhead Publishing. Pp. 113–136.

Moussa, F., Trivin, F., Céolin, R., Hadchouel, M., Sizaret, P. Y., Greugny, V., Fabre, C., Rassat, A., & Szwarc, H. (1996). Early effects of C_{60} administration in swiss mice: A preliminary account for *in vivo* C_{60} toxicity. Fullerene Science and Technology, 4(1), 21–29.

Nilsson, L. O., Gustafsson, A., & Mannervik, B. (2000). Redesign of substrate-selectivity determining modules of glutathione transferase A1-1 installs high catalytic efficiency with toxic alkenal products of lipid peroxidation. Proceed-

ings of the National Academy of Sciences of the United States of America, 97(17), 9408–9412.
Park, E. J., Roh, J., Kim, Y., & Park, K. (2013). Induction of inflammatory responses by carbon fullerene (C_{60}) in cultured RAW264.7 cells and in intraperitoneally injected mice. Toxicological Research, 26(4), 267–273.
Pedrosa Carrasco, A. J., Timmermann, L., & Pedrosa, D. J. (2018). Management of constipation in patients with Parkinson's disease. Npj Parkinson's Disease, 4(1), 6.
Pirtošek, Z., Bajenaru, O., Kovács, N., Milanov, I., Relja, M., & Skorvanek, M. (2020). Update on the management of Parkinson's disease for general neurologists. Parkinson's Disease, 2020, 9131474.
Podolski, I. Y., Podlubnaya, Z. A., Kosenko, E. A., Mugantseva, E. A., Makarova, E. G., Marsagishvili, L. G., Shpagina, M. D., Kaminsky, Y. G., Andrievsky, G. V., & Klochkov, V. K. (2007). Effects of hydrated forms of C_{60} fullerene on amyloid β-peptide fibrillization *in vitro* andperformance of the cognitive task. Journal of Nanoscience and Nanotechnology, 7(4–5), 1479–1485.
Prylutska, S. V., Burlaka, A. P., Klymenko, P. P., Grynyuk, I. I., Prylutskyy, Y. I., Schütze, C., & Ritter, U. (2011). Using water-soluble C_{60} fullerenes in anticancer therapy. Cancer Nanotechnology, 2(1–6), 105–110.
Prylutska, S. V., Grebinyk, A. G., Lynchak, O. V., Byelinska, I. V., Cherepanov, V. V., Tauscher, E., Matyshevska, O. P., Prylutskyy, Y. I., Rybalchenko, V. K., Ritter, U., & Frohme, M. (2019). *In vitro* and *in vivo* toxicity of pristine C_{60} fullerene aqueous colloid solution. Fullerenes Nanotubes and Carbon Nanostructures, 27, 715–728.
Prylutska, S., Panchuk, R., Gołuński, G., Skivka, L., Prylutskyy, Y., Hurmach, V., Skorohyd, N., Borowik, A., Woziwodzka, A., Piosik, J., Kyzyma, O., Haramus, V. M., Bulavin, L., Evstigneev, M., Buchelnikov, A., Stoika, R., Berger, W., Ritter, U., & Scharff, P. (2017). C_{60} fullerene enhances cisplatin anticancer activity and overcomes tumor cell drug resistance. Nano Research, 10(2), 652–671.
Ritter, U., Prylutskyy, Y. I., Evstigneev, M. P., Davidenko, N. A., Cherepanov, V. V., Senenko, A. I., Marchenko, O. A., & Naumovets, A. G. (2015). Structural features of highly stable reproducible C_{60} fullerene aqueous colloid solution probed by various techniques. Fullerenes Nanotubes and Carbon Nanostructures, 23(6), 530–534.
Siderowf, A., & Lang, A. E. (2012). Premotor Parkinson's disease: Concepts and definitions. Movement Disorders, 27(5), 608–616.
Skivka, L. M., Prylutska, S. V., Rudyk, M. P., Khranovska, N. M., Opeida, I. V., Hurmach, V. V., Prylutskyy, Y. I., Suchodub, O. F., & Ritter, U. (2018). C_{60} fullerene and its nanocomplexes with anticancer drugs modulate circulating phagocyte functions and dramatically increase ROS generation in transformed monocytes. Cancer Nanotechnology, 9(1), 1–22.
Stocchi, F., & Torti, M. (2017). Constipation in Parkinson's disease. International Review of Neurobiology, 134, 811–826.
Sun, F., Deng, Y., Han, X., Liu, Q., Zhang, P., Manzoor, R., & Ma, H. (2019). A secret that underlies Parkinson's disease: The damaging cycle. Neurochemistry International, 129, 104484.
Talanov, S. A., Oleshko, N. N., Tkachenko, M. N., & Sagach, V. F. (2006). Pharmacoprotective influences on different links of the mechanism underlying 6-hydroxydopamine-induced degeneration of nigro-striatal dopaminergic neurons. Neurophysiology, 38(2), 128–133.
Tipple, T. E., & Rogers, L. K. (2012). Methods for the determination of plasma or tissue glutathione levels. Methods in Molecular Biology, 889, 315–324.
Turov, V. V., Chehun, V. F., Krupskaya, T. V., Barvinchenko, V. N., Chehun, S. V., Ugnivenko, A. P., Prylutskyy, Y. I., Scharff, P., & Ritter, U. (2010). Effect of small addition of C_{60} fullerenes on the hydrated properties of nanocomposites based on highly dispersed silica and DNA. Chemical Physics Letters, 496(1–3), 152–156.
Viola, A., Munari, F., Sánchez-Rodríguez, R., Scolaro, T., & Castegna, A. (2019). The metabolic signature of macrophage responses. Frontiers in Immunology, 10, 1–16.
Vorobyov, V., Kaptsov, V., Gordon, R., Makarova, E., Podolski, I., & Sengpiel, F. (2015). Neuroprotective effects of hydrated fullerene C_{60}: Cortical and hippocampal EEG interplay in an amyloid-infused rat model of Alzheimer's disease. Journal of Alzheimer's Disease, 45(1), 217–233.
Walsh, S., Finn, D. P., & Dowd, E. (2011). Time-course of nigrostriatal neurodegeneration and neuroinflammation in the 6-hydroxydopamine-induced axonal and terminal lesion models of Parkinson's disease in the rat. Neuroscience, 175, 251–261.
Wang, I. C., Tai, L. A., Lee, D. D., Kanakamma, P. P., Shen, C. K. F., Luh, T. Y., Cheng, C. H., & Hwang, K. C. (1999). C_{60} and water-soluble fullerene derivatives as antioxidants against radical-initiated lipid peroxidation. Journal of Medicinal Chemistry, 42(22), 4614–4620.
Williams-Gray, C. H. (2016). Parkinson's disease key points. Medicine, 44(9), 542–546.
Yano, J. M., Yu, K., Mazmanian, S. K., Correspondence, E. Y. H., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K., & Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis article indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell, 161, 264–276.
Ye, S., Zhou, T., Pan, D., Lai, Y., Yang, P., Chen, M., Wang, Y., Hou, Z., Ren, L., & Jiang, Y. (2016). Fullerene C_{60} derivatives attenuated microglia-mediated prion peptide neurotoxicity. Journal of Biomedical Nanotechnology, 12(9), 1820–1833.
Yudoh, K., Karasawa, R., Masuko, K., & Kato, T. (2009). Water-soluble fullerene (C_{60}) inhibits the development of arthritis in the rat model of arthritis. International Journal of Nanomedicine, 4, 217–225.
Zha, Y., Yang, B., Tang, M., Chen, J., Wen, L., & Wang, M. (2012). Concentration-dependent effects of fullerenol on cultured hippocampal neuron viability. International Journal of Nanomedicine, 7, 3099–3109.
Zhang, X., Cong, H., Yu, B., & Chen, Q. (2018). Recent advances of water-soluble fullerene derivatives in biomedical applications. Mini-Reviews in Organic Chemistry, 16(1), 92–99.