

# C<sub>60</sub> fullerenes increase the intensity of rotational movements in non-anesthetized hemiparkinsonic rats

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The effect of C<sub>60</sub> fullerene aqueous colloid solution (C<sub>60</sub>FAS) on the intensity of long-lasting (persisting for one hour) rotational movements in non-anesthetized rats was investigated. For this purpose, an experimental hemiparkinsonic animal model was used in the study. Rotational movements in hemiparkinsonic animals were initiated by the intraperitoneal administration of the dopamine receptor agonist apomorphine. It was shown that a preliminary injection of C<sub>60</sub>FAS (a substance with powerful antioxidant properties) in hemiparkinsonic rats induced distinct changes in animal motor behavior. It was revealed that fullerene-pretreated animals, in comparison with non-pretreated or vehicle-pretreated rats, rotated for 1 h at an approximately identical speed until the end of the experiment, whereas the rotation speed of control rats gradually decreased to 20–30% of the initial value. One can assume that the observed changes in the movement dynamics of the hemiparkinsonic rats after C<sub>60</sub>FAS pretreatment presumably can be induced by the influence of C<sub>60</sub>FAS on the dopaminergic system, although the isolated potentiation of the action of apomorphine C<sub>60</sub>FAS cannot be excluded. Nevertheless, earlier data on the action of C<sub>60</sub>FAS on muscle dynamics has suggested that C<sub>60</sub>FAS can activate a protective action of the antioxidant system in response to long-lasting muscular activity and that the antioxidant system in turn may directly decrease fatigue-related changes during long-lasting muscular activity.

Key words: C<sub>60</sub> fullerene nanoparticles, non-anesthetized animals, skeletal muscle fatigue, hemiparkinsonic animal model, dopamine, rat

## INTRODUCTION

Skeletal muscles have large energy reserves for long-term contractions, but excessive physical activity leads to reduced muscle force contractions and fatigue development. Long-lasting and intense muscle contraction associated with physical activity or work is often accompanied by muscular pain, posture impairment and motor control disruption (Gandevia, 2001;

Ervilha et al., 2005). In the process of muscle fatigue development, a violation of metabolism and the formation of products that result from the incomplete oxidation of oxygen, such as peroxide, free radicals, and oxygen ions, occur. The increased production of muscle-derived reactive oxygen species (ROS) is involved in the development of muscle fatigue and the mechanism of prolonged contractile activity-induced muscle damage (Pinheiro et al., 2012). The damage can include changes in protein structures, nitrogenous bases, and

the destruction of membranes (Powers et al., 2008). Cell protection during such injury is provided by the antioxidant system (Banerjee et al., 2003). Although the mechanisms of skeletal muscle fatigue have been described in detail (Boyas and Guével, 2011), the problem of preventing or correcting muscle fatigue remains unresolved. In studies of muscle fatigue, endogenous antioxidants, such as N-acetylcysteine and  $\beta$ -alanine, are widely used and speed up the muscle recovery process after fatigue (Reid et al., 1994; Harris and Sale, 2012). Recently, it was shown that bioactive soluble carbon nanostructures, such as pristine C<sub>60</sub> fullerenes, may be used as potential antioxidants (Gharbi et al., 2005). It is important that low and even high doses of such fullerenes do not present any acute or subacute toxicity in the animals; the maximum tolerated dose of C<sub>60</sub> fullerene for both oral and intraperitoneal (i.p.) administration in rats was found to be 5 g/kg (Gharbi et al., 2005). In our previous electrophysiological and biochemical study, we investigated the effect of pristine C<sub>60</sub> fullerene aqueous colloid solution (C<sub>60</sub>FAS) on fatigue of triceps surae (TS) muscles in rats induced by the intermittent high-frequency electrical stimulation of the tibial nerve. It was shown that the use of C<sub>60</sub>FAS led to a reduction in the recovery time of muscle contraction force and an increase in the time of active work in muscles during fatigue development in anesthetized Wistar rats (Prylutskyy et al. 2017; Vereshchaka et al. 2018). However, these studies were carried out on anesthetized animals and only on the TS. The effect of C<sub>60</sub>FAS on the development of general muscle fatigue in non-anesthetized animals has not yet been studied. It is known that animals with experimental hemiparkinsonism (EH) exhibit circular movements (Maisky et al., 2002; Talanov et al., 2017). We hypothesized that an EH animal model can be used to assess the development and modulation of skeletal muscle fatigue in non-anesthetized rats. That is, the i.p. injection of the dopamine (DA) receptor agonist apomorphine (AM), which induces long-lasting circular movements in hemiparkinsonic animals, can lead to skeletal muscle fatigue, and using C<sub>60</sub> fullerene as a powerful antioxidant will induce changes in the movement of the rats for 1 h. Thus, the aim of this study was to reveal the preventive effect of C<sub>60</sub>FAS on the development of muscle fatigue during long-lasting rotational movements induced by AM in non-anesthetized animals with EH.

## METHODS

Male Wistar rats weighing 260–330 g were used in the study. The experimental animals were housed in Plexiglas cages and kept in an air-filtered and tempera-

ture-controlled (21±1°C) room under 12-h light/12-h dark conditions. Rats received a standard pellet diet and water *ad libitum*. The use of the animals was approved by the Ethics Committee of the Institute and performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

## Stereotaxic surgery and behavioral assessment

Unilateral lesions of mesolimbic and nigrostriatal dopaminergic neurons were induced in rats by intracerebrally injecting the selective neurotoxin 6-hydroxydopamine (6-OHDA, 8  $\mu$ g, Sigma, USA) into the left medial forebrain bundle, and these lesions resulted in hemiparkinsonism (Maisky et al., 2002). The coordinates for the 6-OHDA injections were determined according to stereotaxic coordinates of the rat brain (Paxinos and Watson, 1997). The following coordinates were used: anteroposterior from bregma (AP)=-2.2 mm, mediolateral from the midline (ML)=+1.5 mm and dorsoventral from the *dura mater* (DV)=-8.0 mm. The tooth bar was located 4.5 mm above the interaural line. 6-OHDA was dissolved in 4  $\mu$ l of 0.9% ice-cold saline with 0.1% ascorbic acid to prevent the oxidation of the neurotoxin and injected using glass micropipettes (tip diameter of 80–100  $\mu$ m) attached to a microsyringe. Pargyline administration (40 mg/kg, i.p., Sigma, USA) was performed 30 min before neurotoxin injection to inhibit the metabolic transformation of the neurotoxin by monoamine oxidase. In addition, desipramine (25 mg/kg, i.p., Sigma, USA) was injected to block the uptake of 6-OHDA by noradrenergic neurons (Maisky et al., 2002). Stereotaxic surgery was performed under sodium pentobarbital (45 mg/kg, i.p., Nembutal, USA) anesthesia. Experimental animals were screened 7 days after 6-OHDA administration with an i.p. injection of AM (0.5 mg/kg, Sigma, USA) to verify the efficiency of the lesion. Application of the AM should induce contralateral rotational movements in animals with lesions in the nigrostriatal DA system (Kirik et al., 1998). Only animals that exhibited >180 rotations per 30 min period immediately following the injection of AM were used in the study. It indicates a decrease in the number of DAergic neurons in the pars compacta of the substantia nigra (SN) and ventral tegmental area (VTA) in the left hemisphere by 96.6% and 92.1%, respectively (Maisky et al., 2002).

## Experimental groups

After 7 days of 6-OHDA administration, all animals were randomly divided into 3 groups: non-pretreated

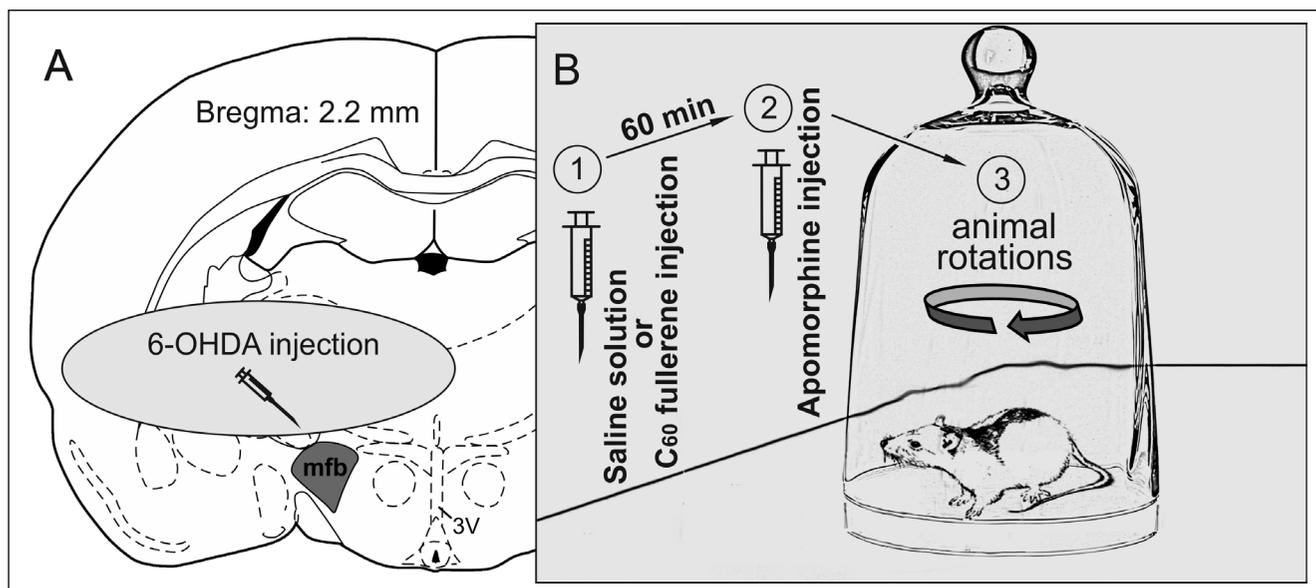


Fig. 1. Schematic representation of the experiment. The site of selective neurotoxin 6-hydroxydopamine (6-OHDA) administration (-2.2 mm caudal to bregma) at the level of brain structures (A) according to the stereotaxic coordinates of the rat brain (Paxinos and Watson, 1997). Experimental schedule: 7 days after 6-OHDA administration (B): 1 – preliminary i.p. injection of saline solution or  $C_{60}$  fullerene; 2 – i.p. injection of apomorphine; 3 – animal rotation counts. Structures: mfb – medial forebrain bundle; 3V – 3rd ventricle.

rats (control animals that only received AM (0.5 mg/kg, Sigma, USA,  $n=6$ ) i.p.; vehicle-pretreated rats (animals that received saline solution (0.3 ml per animal) i.p. 60 min prior to AM injection,  $n=6$ ); and fullerene-pretreated rats (animals that received  $C_{60}$ FAS (0.3 ml, 0.14 mg/kg) i.p. 60 min prior to AM injection,  $n=6$ ). To avoid AM addiction, the experimental procedure was carried out in each animal once a week. A general overview of the experiment is shown in Fig. 1A, B.

### Material preparation and characterization

A highly stable  $C_{60}$ FAS with a purity of more than 99.96% was prepared and characterized (Ritter et al., 2015) in the Institute of Chemistry and Biotechnology, Technical University of Ilmenau (Germany). The method is based on transferring  $C_{60}$  fullerenes from organic solution into the aqueous phase by ultrasonic treatment. The purity of the prepared  $C_{60}$ FAS (i.e., the presence/absence of any residual impurities such as carbon black and toluene phase) was determined by high-performance liquid chromatography (HPLC) and GC/MS analysis (Keykhosravi et al., 2019). The state of  $C_{60}$  fullerene in aqueous solution was monitored using atomic force microscopy (AFM “Solver Pro M” system, NT-MDT, Russia) as well as the dynamic light scattering (DLS) method (Zetasizer Nano-ZS90, Malvern, Worcestershire, UK) at room temperature. The AFM study of prepared  $C_{60}$ FAS revealed that the majority of  $C_{60}$  molecules were locat-

ed chaotically and separately along the surface (see the dotted objects with a height of  $\sim 0.7$  nm in Fig. 2), or in the form of bulk nanoclusters consisting of several molecules (objects with a height of 1.3–2 nm in Fig. 2). The

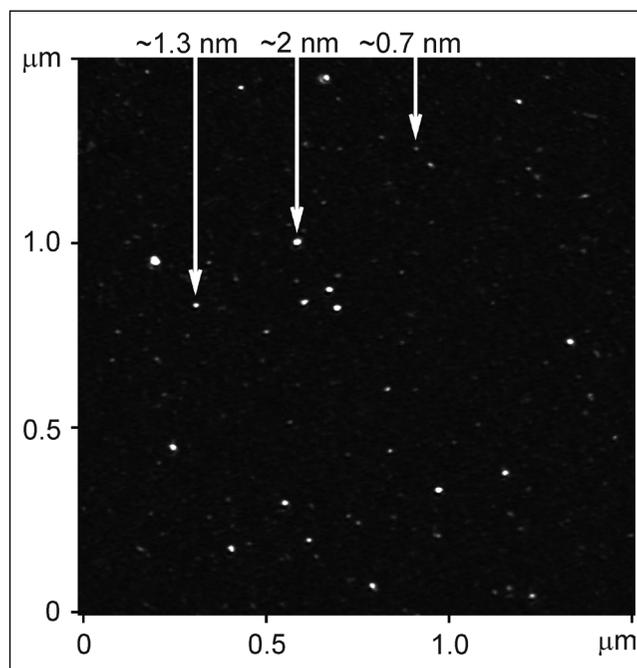


Fig. 2. AFM image of the single  $C_{60}$  fullerene ( $\sim 0.7$  nm) and its bulk nanoclusters (1.3–2 nm) on a freshly broken surface of mica (semicontact (tapping) mode).

formation of large C<sub>60</sub> fullerene nanoclusters in aqueous solution was confirmed by DLS measurements: the mean hydrodynamic diameter of light scattering nanoparticles equaled 82 nm, and the value of zeta potential was -23.9 mV, that indicates a high stability of the used C<sub>60</sub>FAS. The maximal concentration of C<sub>60</sub> fullerenes in water obtained by this method was 0.15 mg/ml.

### Statistical analysis

Six rats participated in 6 experiments, and the obtained data were averaged for each animal. The number of rotations induced by the injection of AM was also averaged every 6 min for an hour (1–6 min, 7–12 min, 13–18 min, 19–24 min, 25–30 min, 31–36 min, 37–42 min, 43–48 min, 49–54 and 55–60 min) and were normalized. The values are expressed as the mean ± standard error of the mean (S.E.M.) and were analyzed by one-way ANOVA. The factors of variation included two conditions: time and animal group. Values of  $P < 0.05$  were considered significant. Bonferroni *post hoc* analysis was used when a significant difference was detected.

## RESULTS

In the course of the research, it was found that in response to AM administration, the non-pretreated group of rats exhibited rotational movements for

60 min at different speeds and with a tendency to continuously decrease in speed. On average, the animals began to rotate at a speed of 10–18 rotations per minute (rpm), and the rotations gradually decreased to the end of the experiment to 2–5 rpm and even to a complete stop; that is, the average number of rats revolutions decreased to 20–30%. The rats in the vehicle-pretreated group demonstrated similar results. For example, after AM injection, one rat from group 1 start rotating at a speed of  $13.3 \pm 1.96$  rpm (average of 6 experiments) and finished an hour later at a speed of  $5.16 \pm 0.7$  rpm. One rat from group 2 start rotating at a speed of  $13.8 \pm 0.7$  and finished at a speed of  $5.9 \pm 2.8$  rpm (Fig. 3A). The total number of rotations (in one hour) for these rats was 557 and 585, respectively. It should be noted that there was no significant difference in the mean number of rotations performed by the rats from group 1 and the rats from group 2 (Fig. 3B). The animals from the third group, which were previously administered C<sub>60</sub>FAS, in response to the AM induction, began to rotate at a similar rate as the rats from groups 1 and 2. In the first 12 min, there was a 15–20% decrease in the mean number of rotations; after that, the intensity of the rotational movements gradually increased and reached the initial values or even exceeded them by 5–10% in some animals by the end of the experiment. For instance, one rat from the fullerene-pretreated group (after AM injection) start rotate at a speed of  $13.6 \pm 2.06$  rpm and finished an hour later at a speed of  $16.16 \pm 0.94$  rpm, exhibiting total of 801 rotations. One-way

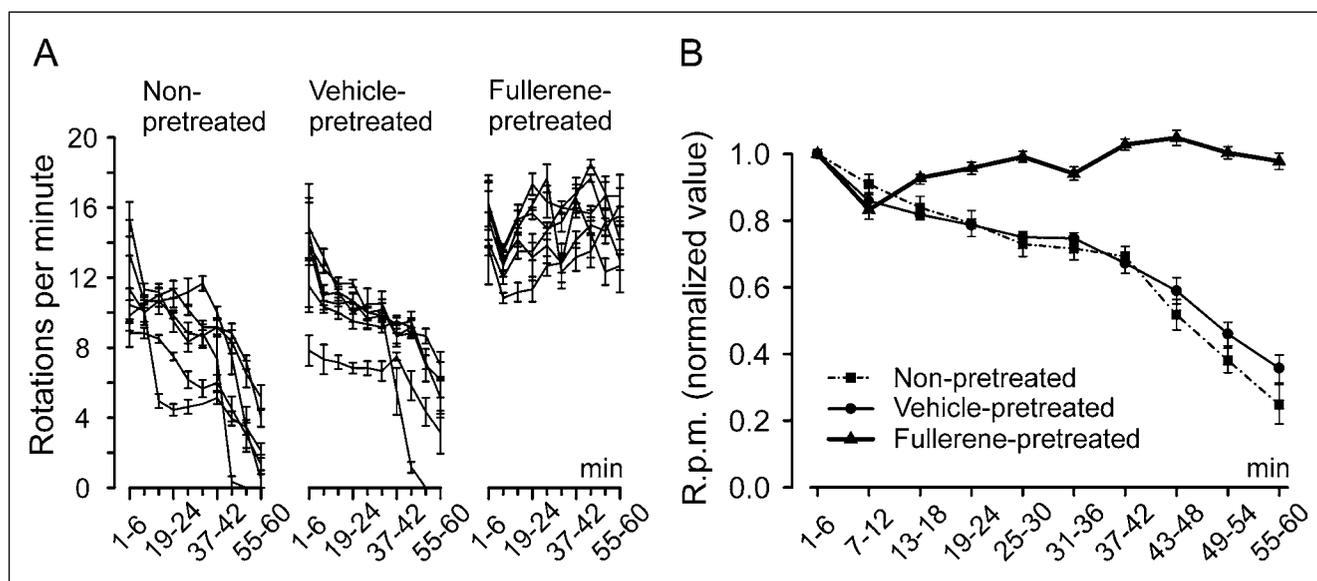


Fig. 3. Mean number ± S.E.M. of rotations per minute (rpm, averaged over 6 min) performed by non-pretreated, vehicle-pretreated and fullerene-pretreated rats (A). Each curve in the diagram corresponds to the values obtained from one animal and averaged over 6 experiments. Average group characteristics (mean ± S.E.M.) of normalized rpm values (with respect to the average rpm values in the first six min) in non-pretreated, vehicle-pretreated and fullerene-pretreated rats (B).

ANOVA was used to determine the effect of  $C_{60}$ FAS. Bonferroni *post hoc* analysis revealed a significant ( $P < 0.001$ ) increase in the mean number of rotations exhibited by the rats from group 3 in comparison with the animals from groups 1 and 2 beginning 13 min after the injection of AM (13–18 min ( $F_{2,105}=7.54$ ,  $P < 0.001$ ), 19–24 min ( $F_{2,105}=14.47$ ,  $P < 0.001$ ), 25–30 min ( $F_{2,105}=37.15$ ,  $P < 0.001$ ), 31–36 min ( $F_{2,105}=27.34$ ,  $P < 0.001$ ), 37–42 min ( $F_{2,105}=59.02$ ,  $P < 0.001$ ), 43–48 min ( $F_{2,105}=59.45$ ,  $P < 0.001$ ), 49–54 min ( $F_{2,105}=115.57$ ,  $P < 0.001$ ) and 55–60 min ( $F_{2,105}=185.46$ ,  $P < 0.001$ ) (Fig. 2).

## DISCUSSION

The obtained results showed a significant increase in the number of rotations exhibited by the animals from the fullerene-pretreated group in comparison with the non-pretreated and vehicle-pretreated rats. It is known that, after long-lasting muscle activity, metabolism is significantly increased in the muscles, and this increase leads to the accumulation of secondary oxidation products in muscle fibres and further fatigue development (Casey and Joyner, 2011). The flow of oxygen through muscle cells is greatly increased due to intense muscle activity. High levels of oxygen uptake can lead to excessive ROS generation and have been implicated in muscle soreness and myofibril disruption (Clanton et al., 1999). In our previous findings (Vereshchaka et al., 2018), a significant decrease in the force contraction of the TS muscle induced by the intermittent electrical stimulation of the tibial nerve was shown. However, animals previously treated with  $C_{60}$ FAS demonstrated an increase in the time of active work of muscles until fatigue development. In addition, our biochemical study revealed a significant increase in thiobarbituric acid reactive substances and hydrogen peroxide content in fatigue development that led to an increase in catalase (CAT) activity and reduced glutathione (GSH) content in the TS muscle fibers. After  $C_{60}$ FAS administration under fatigue development, GSH content and CAT activity were significantly reduced compared to those in the control. It is assumed that  $C_{60}$ FAS affects the content and activity of endogenous antioxidants and can, to a certain extent, prevent fatigue in actively contracting muscles, thereby maintaining their normal physiological states (Vereshchaka et al., 2018). It is known that the enhancement of free radical processes is the main pathogenic factor in the development of skeletal muscle fatigue (Lee et al., 2014). With considerable physical activity, there is a robust overproduction of free radicals in muscle tissue (Clarkson and Thompson, 2000). Using exogenous antioxidants of different natures

leads to a significant decrease in skeletal muscle fatigue during intense physical activity and increases the time of muscle fatigue onset during prolonged intense loads (Ferreira and Reid, 2008; Mach et al., 2010; Hong et al., 2015). These data demonstrate the feasibility of using antioxidants for correcting the level of oxidative stress in muscle tissue during extreme conditions and increasing its efficiency. By comparing the behavioral performance of the rats from the three groups, it can be assumed that the decrease in the number of rotations exhibited by the animals from the non-pretreated and vehicle-pretreated groups was not due to the termination of the effect of apomorphine but rather the development of muscle fatigue during long-lasting rotational movements. At the same time, after the application of  $C_{60}$ FAS, a decrease in the average number of rotations was not observed in the rats from the third group. Presumably, this effect is related to the effect of  $C_{60}$ FAS on the dopaminergic system in the brain, although the enhancement of the effect of AM by  $C_{60}$ FAS cannot be excluded. However, based on our previous studies (Prylutskyy et al., 2017; Vereshchaka et al., 2018) we assume that the observed effect indicates the activation of the protective effect of the antioxidant system in response to long-lasting muscle activity, and  $C_{60}$ FAS can be considered a powerful activator of protective mechanisms aimed at reducing skeletal muscle fatigue.

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