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NEUROTOXIC RISK AND ADSORPTION PROPERTIES OF COARSE NON-FUNCTIONALIZED CARBON PARTICLES DERIVED FROM APPLE WASTE

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Aim. Carbon particles have been widely used in different technologies and have great potential for new biological application. Synthesis of carbon particles from agricultural waste using "green" principles is in the mainstream of biotechnology area and attract a great attention in biomedical application. Here, coarse carbon particles (CCPs) were synthesized using "green" principles from dry apple and used in the biological experiments without preliminary functionalization.

Methods. Neurotoxic features of CCPs were analysed in isolated presynaptic cortex nerve terminals (synaptosomes) monitoring the extracellular levels of excitatory neurotransmitter L-[^{14}C] glutamate and inhibitory one [^{3}H]GABA, as well as the membrane potential.

Results. Measuring the membrane potential of the nerve terminals, it was revealed an inadequate decrease in the fluorescence intensity of the potential-dependent dye rhodamine 6G in the presence of CCPs (1 mg/ml). This decrease was not due to membrane hyperpolarisation because CCPs did not change the extracellular synaptosomal levels of L-[^{14}C] glutamate and [^{3}H]GABA. CCP-induced decrease in the fluorescence intensity of the dye in nerve terminals can be due to its Interaction with CCPs. Indeed, the ability of CCPs to Interact with rhodamine 6G was shown in synaptosome-free incubation media.

Conclusions. Therefore, CCPs did not possess neurotoxic signs, and so are biocompatible. In both experiments, i.e. without bio object and in biological system, CCPs were able to to interact with fluorescent dye rhodamine 6G. In prospect, this feature of CCPs can be used in biotechnology after further investigation of dye Interaction conditions.

Key words: coarse non-functionalized carbon particles, apple, rhodamine, adsorption, neurotoxicity risk, glutamate, GABA, brain nerve terminals.

The conversion of biomass into useful products is one of the best solutions for waste managing. Synthesis of environmental friendly carbon materials and, in particular, carbon particles from agricultural waste using "green" principles is in the mainstream of technology area. Waste-derived carbon materials are very promising in numerous applications, e.g. environmental remediation, energy, catalysts, sensorics, and biomedical applications [1].

It is an increasing demand for porous carbon nanomaterials because adsorption is one of the most effective approaches for pollutant

management and detoxication. Carbon materials prepared from agricultural waste possesses porous structures, and contains functional groups, e.g. the carboxyl group and hydroxyl group. Agricultural waste gets the advantages of a wide range of sources, their low cost, and renewability [2]. In addition, important feature of the agricultural waste as the source of sorbent is the absence or very low content of toxic components (heavy metals, tar, carcinogenic polycondensed aromatic compounds, etc), ensuring, in many cases, formation of the materials which do not require further treatment for biomedical use. Biochar can be obtained by pyrolysis of biomass in different conditions. Porous carbons prepared by pyrolysis and hydrothermal pyrolysis are suitable for wastewater processing, such as adsorption of heavy metal ions [3–5], fluoride [6], ketamine [7], iodate [8], sulphide [9, 10]. For example, pomelo peel, rich in cellulose and lignin, has been broadly used for synthesis of porous carbon nanomaterials with large yield and numerous applications. Porous carbon nanomaterials derived from pomelo peel are widely applied as absorbers due to their loose and porous structure and stable chemical properties [11]. Agricultural biomass was shown to be effective in the remediation of environmental pollutant pesticides. The remediation effects of crop-derived waste, e.g cereal crops and cash crops, on pesticide pollution were shown [12].

Also, biochar attracts a great attention and has been intensively investigated due to its potential in biomedical application. Due to porous architecture of biochar, such materials are considered as suitable carriers for nanoparticles with other functional properties. The material, obtained by silver nanoparticles deposition on biochar, facilitated application and possessed antibacterial and anticancer properties. Such silver nanoparticle on biochar were suggested for treatment of bacteria and colorectal cancer cells [13]. The effects of rice straw biochar on cecal microbiome-related metabolic changes in rats was assessed. It was demonstrated that gut microbiome was altered and could be critical for good performance under rice straw biochar application through interacting with metabolism [14]. A magnetic solid-phase extraction materials on the basis of modified biochar were investigated to extract antiepileptic drugs from plasma. Biochar was derived from *Zizyphus jujuba* seed shells and was activated by phosphoric acid and magnetized via coprecipitation. It was shown that such material was appropriate for the assessment of antiepileptic drugs in plasma and expanded the usage of such biochar as the environmentally favorable matrix for the analysis of biological samples [15].

Taking into account abovementioned facts on perspective biomedical application of carbon particles from agricultural biomass, the aims of the present study were (*) to synthesize non-functionalized **c**oarse **c**arbon **p**articles from dry apples (CCPs); (**) to analyse neurotoxic properties of CCPs in isolated presynaptic rat cortex nerve terminals (synaptosomes) analysing the extracellular levels of key excitatory and inhibitory neurotransmitters in the central nervous system, L-[^{14}C] glutamate and [^{3}H]GABA, respectively; and (***) to examine the membrane potential of CCPs using florescent dye rhodamine 6 G and their ability to interact with this dye.

Materials and Methods

Materials

HEPES, EGTA, EDTA, Ficoll 400, Sigma-Fluor® High Performance LSC Cocktail, salts of the analytical grade were obtained from Sigma, USA; L-[^{14}C] glutamate; [^{3}H] GABA– Perkin Elmer, Waltham, MA, USA.

Methods

Synthesis of CCPs from dry apples

CCPs from dry apples were synthesized using green synthesis principles according to [16]. In this study, coarse fraction of particles without preliminary separation into fractions by size and without any functionalisation was used in the biological experiments.

Scanning and transmission electron microscopy of CCPs

Transmission electron microscopy (TEM) images of the synthesized carbon particles were obtained using a microscope TEM125K (Selmi) with an accelerating voltage 100 kV. A suspension of the material in water was dropped on a Cu grid (300 mesh), covered by a film of amorphous carbon, and dried on air. Scanning electronic microscopy (SEM) images were obtained using th*e* TESCAN MIRA3 instrument operating at 10 kV. A suspension of the material was dropped on a conducting carbon film without additional treatment and dried on air.

Animals and Ethics

Wistar rats, 3 months' age males, were kept in a quiet and temperature-controlled vivarium room (22–23 °C) of the Palladin Institute of Biochemistry, NAS of Ukraine.

Animals were feed with dry food pellets and water *ad libitum*. All animal-involving procedures were performed in accordance with the guidelines of the European Community (2010/63/EU); "Scientific Requirements and Research Protocols"; "Research Ethics Committees" of Declaration of Helsinki; and "ARRIVE guidelines for reporting experiments involving animal" [17, 18]; and also local Ukrainian laws and policies. The experimental protocols were approved by the Animal Care and Use Committee of Palladin Institute of Biochemistry (Protocol # 1 from 10/01/2024). The total number of animals was 9.

Isolation of the synaptosomes from the rat cortex

The synaptosomes represented ~87% of the particles in preparations, and did not contain nerve cell bodies and functional glial fragments [19–21]. Presynaptic nerve terminals were isolated from the cortex regions of rat brains. Isolation procedures were conducted at + 4°C, the cortex regions were removed and homogenized in the following ice-cold solution: sucrose 0.32 M; HEPES-NaOH 5 mM, pH 7.4; EDTA 0.2 mM. One synaptosome preparation was obtained from one rat. The synaptosomes were isolated according to Cotman method with minor modifications [22–25] using differential and Ficoll-400 density gradient centrifugation. The synaptosomes were fitting for 2–4 hours after isolation. The standard saline solution contained: NaCl 126 mM; KCl 5 mM; $MgCl_2$ 2.0 mM; NaH_2PO_4 1.0 mM; HEPES 20 mM, pH 7.4; and D-glucose 10 mM. Protein concentrations were recorded according to Larson [26].

The extracellular synaptosomal level of L-[^{14}C] glutamate

The synaptosomes were diluted up to a concentration of 2 mg of protein/ml, and then the synaptosomes were pre-incubated at 37 °C for 10 min, and loaded with L-[^{14}C] glutamate, 1 nmol per mg of protein, 238 mCi/mmol, at 37 °C for 10 min. The synaptosomes after loading were washed with 10 vol of the ice-cold standard saline solution, and then centrifuged (10,000×g, 20 s) at +4 °C; the pellets were re-suspended in the standard saline solution up to 1 mg protein/ml. The extracellular L-[^{14}C] glutamate level was assessed in the synaptosome aliquots (125 µl, 0.5 mg of protein/ml). The synaptosome aliquots were preincubated for 8 min in order to restore the ion gradients, and then CCPs were added to the synaptosomes, and this mixture were further incubated at 37 °C during 0 and 6 min; centrifuged at 10,000 × *g* for 20 s at room temperature. The values of L-[^{14}C] glutamate release were monitored in the supernatant aliquots (100 µl), and the pellets (preliminary treated with SDS, 100 µl of 10% SDS stock solution) by liquid scintillation counting using Sigma-Fluor® High Performance LSC Cocktail (1.5 ml) and liquid scintillation counter Hidex 600SL (Finland) [27]. The experimental data were collected from "n" independent experiments carried out in triplicate using different synaptosome preparations.

The extracellular synaptosomal level of [^{3}H] GABA

The synaptosomes were diluted up to 2 mg of protein/ml; and after pre-incubation at 37 °C for 10 min, the synaptosomes were pre-loaded with [^{3}H]GABA (50 nM, 4.7 µCi/ml) in the standard saline solution at 37 °C for 10 min. Aminooxyacetic acid (100 µM) was added to the incubation media throughout all [^{3}H] GABA experiments. After pre-loading, the suspension was washed with 10 volumes of the ice-cold standard saline solution. The pellets were re-suspended in the standard saline solution up to 1 mg of protein/ml. The synaptosome aliquots were pre-incubated for 8 min, and then CCPs were added and further incubated at 37 °C during 0 and 5 min; and then centrifuged at 10,000 × *g* for 20 s at room temperature [28]. [^{3}H] GABA content was measured in the supernatant aliquots (90 µl) by liquid scintillation counting with Sigma-Fluor® High Performance LSC Cocktail (1.5 ml) using liquid scintillation counter Hidex 600SL (Finland), and the extracellular level was expressed as the percentage of total accumulated [^{3}H] GABA. The data were collected from "n" independent experiments performed in triplicate with different synaptosome preparations.

The synaptosomal membrane potential

The membrane potential was monitored using 0.5 µM rhodamine 6G, the fluorescent potentiometric dye, based on potential-depended binding of the dye to the synaptosome membranes. The synaptosomes, 0.2 mg of protein/ml, were pre-incubated at 37 °C in a thermostated cuvette with continuous stirring for 10 min. The synaptosomes were equilibrated with the dye, and after that CCPs were applied. The ratio F, an index of the membrane potential, was calculated according to the following equation:

$$F = F_t/F_0,$$

where F_0 and F_t were the fluorescence intensity of rhodamin 6 G in the absence and presence of the synaptosomes, respectively. F_0 was assessed by the extrapolation of exponential decay function to t = 0. The fluorescence measurements were performed using a fluorescence spectrofluorimeter (QuantaMaster™ 40, PTI Inc., Canada), as well as Hitachi 650-10S at 528 nm excitation and 551 nm emission wavelengths.

Statistical analysis

The experimental data were expressed as the mean ± S.E.M. of *n* independent experiments. One-way ANOVA were applied; the accepted significance level was $P < 0.05$.

Results and Discussion

Electron microscopy of CCPs

According to SEM studies, the sample of CCPs contains particles with size from 1×1 to 20×20 μm (Fig. 1). All particles have irregular shape; the majority of particles have comparable dimensions in all directions and resemble bulky carboneous material, however several species looking like 2D sheets (implying that the size in one of the directions is significantly lower than the size in two other directions) can be distinguished. These results are consistent with images obtained by TEM. Several separate particles, which dimensions range from ca. 0.9×2.3 μm to 3.5×3.5 μm, are shown on Fig. 1. Notably, some of the particles on this TEM image have semi-transparent edges in contrast to non-transparent "main bodies" (these places are shown by arrows on Fig. 1, *b*). Different transparency of edge and center of the same particle can be indication of its non-uniform thickness, which precludes formation of thin graphene-like structure in this case. Thus, carbonization of dry apple resulted in formation of bulky carboneous material, which appearance is typical for disperse activated carbon.

Effect of CCPs on the membrane potential of nerve terminals

The membrane potential of nerve terminals is a driving force for the transporter-mediated turnover (uptake/release) of neurotransmitters, and also reproduces the synaptosome membrane integrity [29]. As shown in Fig. 2, the steady state level of the dye fluorescence was achieved in 6 min. The addition of the dye to the synaptosomes resulted in the decreased fluorescence signal of the dye that reflected its accumulation in the negative charge area of the membranes of the nerve terminals.

In calculations, the membrane potential index, F_{st}, at the steady state level was used as 100%. The standard saline buffer applied to the incubation media of the synaptosomes showed weak insignificant depolarizing effect (appx 1.0%) that was considered as a control.

As shown in Fig. 2, the addition of CCPs to the nerve terminals resulted in further decrease in fluorescence of the dye by 10% that can reflect inadequate significant membrane hyperpolarisation.

A CCP-induced decrease in the dye fluorescence may result from both (*) CCP-induced changes in functional state of the nerve terminals; and (**) interaction of rhodamine 6G with CCPs that bring inaccuracy to the membrane potential measurements.

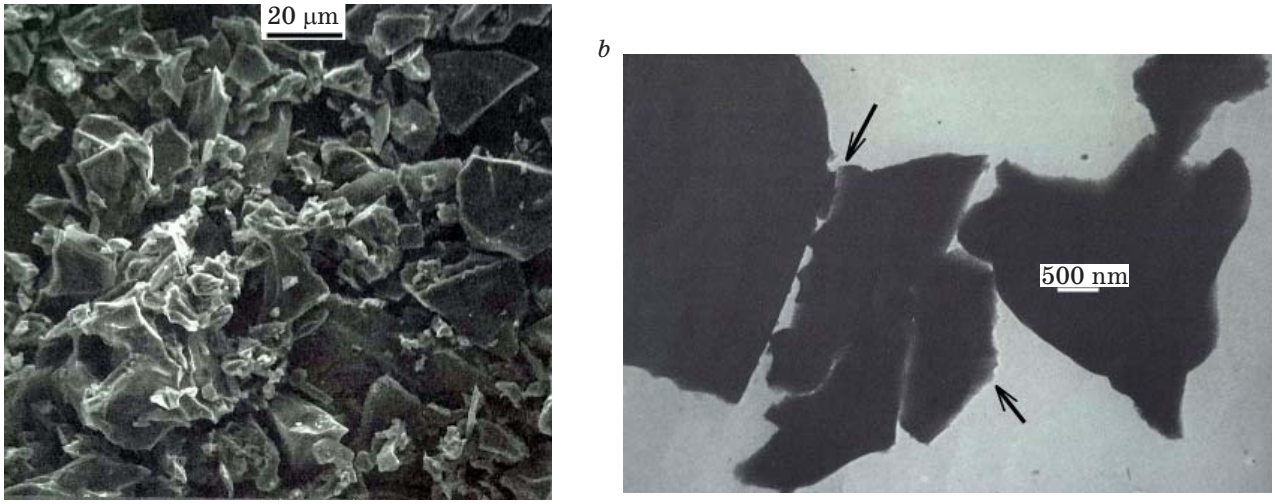

Fig. 1. **Images of CCPs obtained by scanning (*a*) and transmission (*b*) electron microscopy**

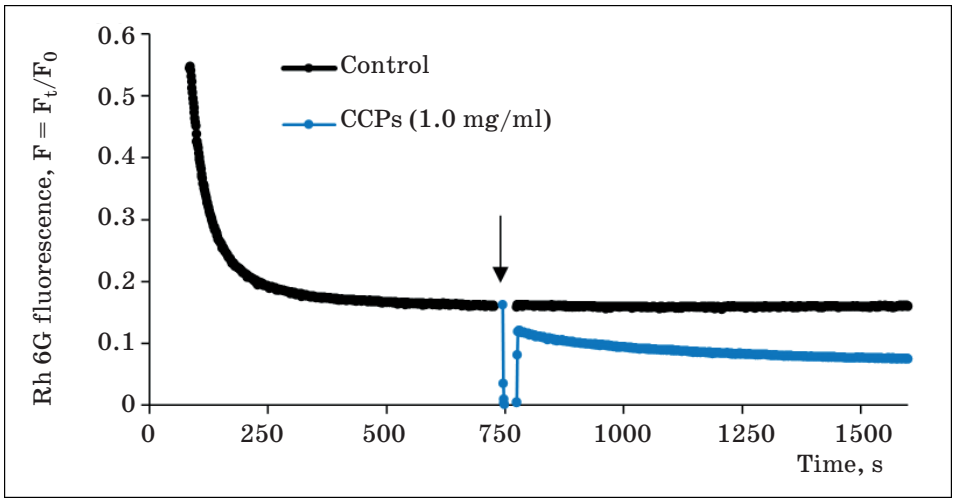

Fig. 2. **The membrane potential of synaptosomes and a decrease in the fluorescence of rhodamine 6 G after the addition of CCPs to nerve terminals**
Synaptosomes were equilibrated with rhodamine 6G (0.5 μM); when the steady level of the fluorescence was reached, the standard saline buffer (control) or CCPs were added to the synaptosomes (marked with arrow). The trace represents 9 experiments carried out using different preparations

In this context, the next series of the experiments were directed on accessment of the key synaptic characteristics, extracellular levels of excitatory neurotransmitter L-[^{14}C] glutamate and inhibitory one [^{3}H]GABA that in turn reflected the functional state of the nerve terminals.

Effect of CCPs on the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA in the nerve terminals

The extracellular levels of neurotransmitters glutamate and GABA are essential synaptic characteristics which show a balance of transporter-mediated uptake and unstimulated release of neurotransmitters [30, 31].

The effects of CCPs on the extracellular levels of neurotransmitters L-[^{14}C] glutamate and [^{3}H]GABA were assessed in the nerve terminals. It was revealed that CCPs did not affect the extracellular levels of both L-[^{14}C] glutamate and [^{3}H]GABA at a concentration of 1.0 mg/ml (Fig. 3).

Therefore, CCPs did not possess neurotoxic signs and was biocompatible at a concentration of 1.0 mg/ml. This results showed that a CCP-induced decrease in the dye fluorescence shown in the previous subsection (Fig. 2) was not a result of CCP-induced changes in functional state of the nerve terminals.

Effect of CCPs on the rhodamine 6G fluorescence

Interaction of rhodamine 6G with CCPs was investigated in the next set of the experiments. It was revealed that CCPs were able to decrease to zero the fluorescence signal of rhodamine 6G in the synaptosome-free incubation media (Fig. 4). This feature may result from interaction of the dye with CCPs in the synaptosome-free incubation media.

So, the changes in dye fluorescence during the membrane potential measurements (Fig. 2) were associated with interaction of the dye with CCPs but not with changes in the membrane potential value and membrane hyperpolarisation.

Here, we showed that CCPs did not affect the extracellular synaptosomal levels of L-[^{14}C] glutamate and [^{3}H]GABA at a concentration of 1.0 mg/ml. Therefore, CCPs can be applied in different neurotechnologies because they did not possess neurotoxic signs and is biocompatible at this concentration. Comparing changes in the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA in nerve terminals, CCPs were less neurotoxic as compared to carbon dots [32], nanodiamonds [27], fullerene C_{60} [33], plastic and wood smoke particulate matter [34–36].

In perspective, the ability of CCPs to ito interact with rhodamine 6G should be further investigated in details. Acquired knowledge on a interaction conditions of the dye, e.g. pH-dependence, ionic strength-dependence, can be useful for perspective usage of CCPs in bio sensing.

CCP-induced decrease in the rhodamine 6G fluorescence may be due to the changes in the fluorescent properties of the dye in the presence of CCPs but not due to adsorption of the dye by the particles.

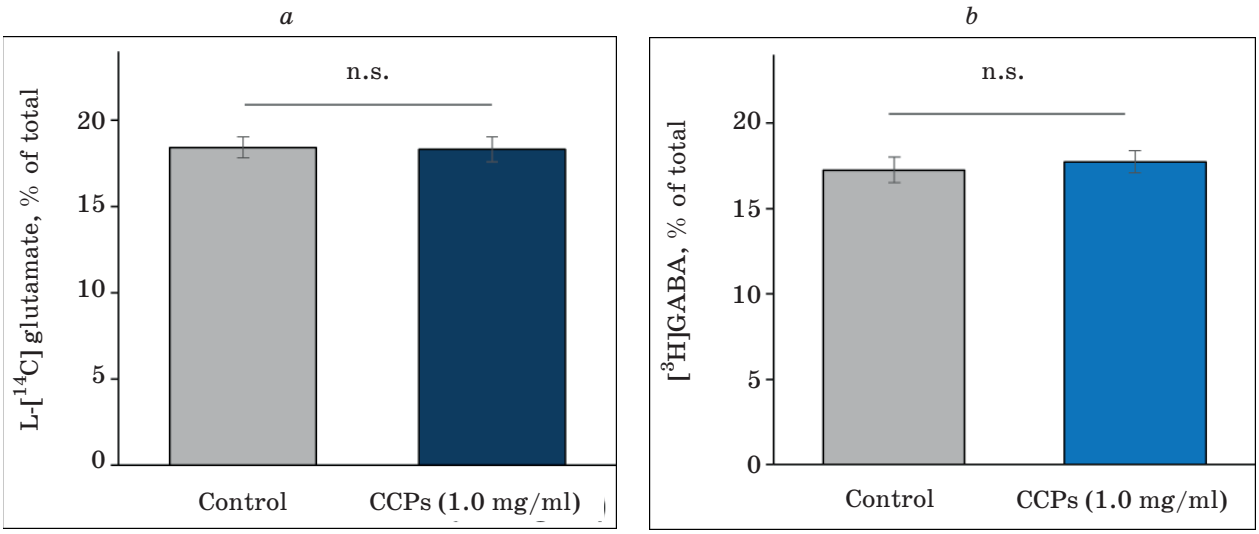

Fig. 3. **The extracellular levels of neurotransmitters L-[^{14}C] glutamate (*a*) and [^{3}H]GABA (*b*) in the nerve terminals in the presence of CCPs (1.0 mg/ml)**
Data are the mean ± SEM. n.s., no significant differences as compared to the appropriate control, $n = 9$

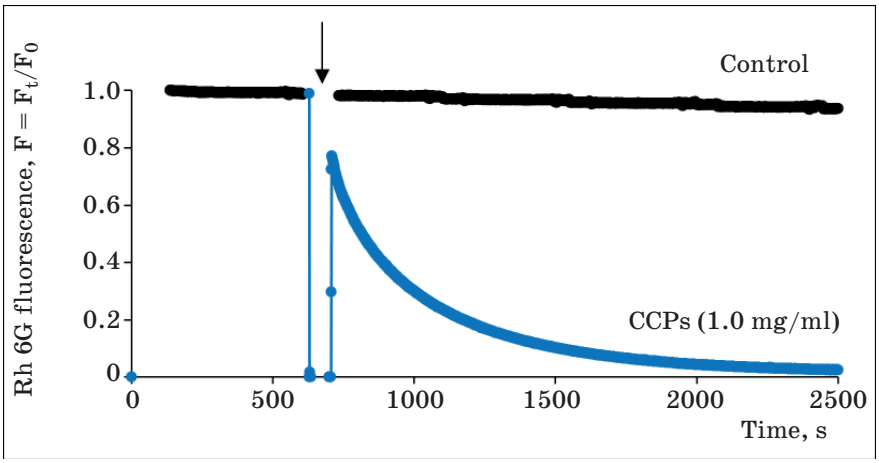

Fig. 4. **A complete elimination of rhodamin 6G fluorescence by CCPs**
When the steady level of the rhodamine 6G (0.5 μM) fluorescence was reached, the standard saline buffer (control) or CCPs were added to the incubation media (marked with arrow).
The trace represents 9 experiments carried out using different preparations

Conclusions

CCPs were synthesized using "green" principles from non-toxic agricultural waste, and so they are environmentally friendly throughout the synthesis process. CCPs did not influence the extracellular levels of L-[^{14}C] glutamate and [^{3}H] GABA in the nerve terminals. Therefore, CCPs did not possess neurotoxic signs and is neurocompatible.

CCPs were able to adsorb or change the fluorescent properties of the dye rhodamine 6G in both biological system and without biological objects, and this feature of CCPs can be used in biotechnology in prospect after further investigation.

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Competing interests

The authors declare no financial and non-financial competing interests exist.

Authors contributions

CCPs synthesis and characterisation — OP, DM, AT; synaptosome preparations were obtained by RS, MD, L-[^{14}C] glutamate experiments — NK, AB; [^{3}H] GABA experiments — NP, MD; data analysis and figure preparation — OP, VK, YK, NP, AB, NK, TB, SK; funding acquisitions, project leading, data analysis and paper writing — NK, TB, SK.

Ethical Approval

Animal-involved experiments were performed according to the "Scientific Requirements and Research Protocols" & "Research Ethics Committees" of Declaration of Helsinki; the "ARRIVE guidelines for reporting experiments involving animal" [17, 18], and were preliminary approved by Institutional Animal Care and Use Committee (Protocol # 1 from 10/01/2024).

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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НЕЙРОТОКСИЧНИЙ РИЗИК І АДСОРБЦІЙНІ ВЛАСТИВОСТІ НЕОБРОБЛЕНИХ НЕФУНКЦІОНАЛІЗОВАНИХ ВУГЛЕЦЕВИХ ЧАСТИНОК З ЯБЛУК

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Мета. Вуглецеві частинки широко використовуютья у різних технологіях і мають великий потенціал для нових біологічних застосувань. Синтез вуглецевих частинок із відходів сільського господарства з використанням «зелених» принципів є популярним і привертає велику увагу в галузі біотехнологій. Грубі вуглецеві частинки (ССР) були синтезовані з використанням «зелених» принципів із сухих яблук та використані в біологічних експериментах без попередньої функціоналізації.

Методи. Нейротоксичні властивості ССР аналізували в ізольованих пресинаптичних нервових закінченнях кори головного мозку (синаптосомах), контролюючи позаклітинні рівні збудливого нейромедіатора L-[^{14}C] глутамату та інгібіторного [^{3}H]ГАМК, а також мембранний потенціал.

Результати. Під час вимірювання мембранного потенціалу нервових закінчень виявлено неадекватне зниження інтенсивності флуоресценції потенціалзалежного барвника родаміну 6G у присутності ССР (1 мг/мл). Це зниження не було зумовлено гіперполяризацією мембрани, оскільки ССР не змінювали позаклітинні синаптосомальні рівні L-[^{14}C] глутамату та [^{3}H]ГАМК. ССР-індуковане зниження інтенсивності флуоресценції барвника в нервових закінченнях може бути наслідком його прямої адсорбції ССР. Дійсно, здатність ССР адсорбувати родамін 6G була показана в інкубаційному середовищі без синаптосом.

Висновки. ССР не мали нейротоксичних ознак, тому є біосумісними. В обох схемах, тобто без біооб'єкта і в біологічній системі, ССР змогли зв'язати флуоресцентний барвник родамін 6G. У перспективі ця властивість ССР може бути використана в біотехнології та екологічних технологіях після подальшого дослідження умов абсорбції/десорбції барвника.

Ключові слова: необроблені нефункціоналізовані вуглецеві частинки, яблуко, родамін, адсорбція, ризик нейротоксичності, глутамат, ГАМК, нервові закінчення мозку.