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Increasing the effectivity of the antimicrobial surface of carbon quantum dots-based nanocomposite by atmospheric pressure plasma

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Abstract

Preventing nosocomial infections is one of the most significant challenges in modern medicine. The disinfection of medical facilities and medical devices is crucial in order to prevent the uncontrolled spread of bacteria and viruses. Cost-effective, eco-friendly and fast-acting antibacterial coatings are being developed as the prevention of bacteria and viruses' multiplication on various surfaces. One of the possibilities to create such antimicrobial coatings can rely on a photoactive material, that produces singlet oxygen. However, a remote production of the singlet oxygen and disinfection of the desired surface is a time-consuming process. Hence, a coating material that would autonomously produce singlet oxygen employing ambient light will have a significant impact on the shortening of the disinfection time; leading into an increased number of patients that can be cured in one facility. In this work, an ultra-fast and eco-friendly method for decreasing the disinfection time of the photoactive surface is presented. The atmospheric pressure plasma surface treatment on the hydrophobic carbon quantum dots-polydimethylsiloxane nanocomposite is employed. The plasma-treated samples exhibited improved antibacterial properties compared to non-plasma
treated samples, with the best results obtained after only 30 seconds of plasma treatment. The short duration and the scalability potential of the here described method open new possibilities of how to improve the already existing antibacterial coatings.

**Keywords:** antibacterial polymers; carbon quantum dots; atmospheric pressure plasma; singlet oxygen

1. Introduction

Today, the importance of effective and widely accessible antimicrobial surfaces is found to be critical in the fight against the pandemic. Unquestionably, bacteria belong to the well-known sources of pandemics during history [1–3]. One of the crucial difference that makes bacteria even more dangerous is their prolonged half-life on surfaces [4]. Bacteria can persist on inanimate surfaces for an extremely long time. As an example, *Escherichia coli* (*E. coli*) can be detected on a surface even after 16 months [4]. Especially on plastics, bacteria tent to persists longer compared to other surfaces [4,5]. For example, *E. coli* can persist on the surface of polyurethane for fifteen days, while on cotton only one day. The same amount of *Serratia marcescens* can persist on the surface of the polyurethane for seven days, while on cotton only one day [5].

Antibacterial materials such as silver nanoparticles [6,7], TiO₂ [8,9], CuO [10–12], chitosan [13–15], curcumin [16] or quantum dots [17] are used to stop the uncontrolled spreading of the bacteria on the surfaces. In general, the antibacterial materials can be mixed with a polymer matrix, and the resulting composite will exhibit antibacterial properties as well [18–21]. The final antibacterial plastic will reduce the number of bacteria accumulated on its
surface and ideally will not allow the proliferation of bacteria at all. Such an artificial surface will significantly reduce the risk of nosocomial infections and substantially lower the expenses and time spent on the disinfection of medical facilities and devices and reduce the spreading of the disease. For the mass production of antibacterial materials, the antibacterial agent must be affordable, easy-to-prepare and biocompatible. One such candidate is carbon quantum dots (CQDs) [17]. CQDs owe their antimicrobial effect to the ability to produce singlet oxygen [20–23]. The generation of singlet oxygen is mediated by the light irradiation of photosensitizers (PSs). Different PSs have different absorption spectra given by their chemical structure. In our case, CQDs act as PSs, effectively absorbing the blue part of the ambient light spectrum. After the excitation of PS from the ground state to the 1st singlet excited state, the conversion via intersystem crossing into the triplet state is followed. Triplet state is well-known for its relatively longer lifetime (μs) compared to the singlet state lifetime (ns) [18]. Therefore, PS in this state is able to transfer energy to molecular oxygen (in the air or water) and produce singlet oxygen molecules. Singlet oxygen is one of the most reactive oxygen radicals and causes a biological response. After contact with cells, it inflicts cell membrane disruption and damage to the internal components of the cell. It can cause apoptosis via different biological pathways [18,19,24–29].

Low-temperature plasma is another source, well known for its antimicrobial effect [30–35]. The high concentration of reactive species such as OH-, H2O2, or O3 together with generated UV light makes plasma an extremely hostile environment. Plasma treatment of surfaces can provide fast and effective disinfection. On the other hand, the disinfection of a whole medical facility directly by plasma is technically not feasible.

Plasma can be used not only as a primary antimicrobial agent but also as a secondary agent. Activation of the surfaces by plasma can facilitate the production of antibacterial nanocomposites and helps to prepare enduring composites with an enhanced antibacterial
activity [36–38]. On the other hand, the plasma treatment of the already existing antibacterial surface can lead to the improvement of its antibacterial potential [39–41]. Changing the surface roughness, chemical composition, and water contact angle alter the bactericidal potential of the material. To satisfy the requirements for the large-scale plasma treatment, the plasma source has to operate under the ambient pressure conditions and at a low temperature to prevent the thermal damage of the samples. The diffuse coplanar dielectric barrier discharge (DCSBD) [42] complies with the above-given requirements. DCSBD is non-thermal plasma generated at ambient conditions, which has a large power density in the order of 100 W/cm$^3$ [42]. This remarkably high power density gives the DCSBD plasma the potential to the high-speed surface treatment of temperature-sensible materials such as biodegradable polymers [43] or nonwoven fabrics [42].

Another possibility to enhance the antibacterial effect of antibacterial polymers is by using gamma irradiation [44]. However, the dosage needed for significant improvement of antibacterial properties of hydrophobic CQDs (hCQDs) filled polyurethane is extremely high. Such a dosage requires either irradiation time in order of weeks or powerful gamma source. Consequently, this discriminates its practical use in real-life applications.

In this work, the enhancement of the antibacterial properties of hCQDs/Polydimethylsiloxane (PDMS) nanocomposite by DCSBD plasma treatment is presented. The treatment with the atmospheric pressure non-thermal plasma generated at ambient air enhances the antibacterial potential of hCQDs/PDMS after only 30 seconds of treatment, which significantly reduces the time required for disinfection of its surface.
2. Experimental

The 1 mm thin PDMS foils were prepared by mixing two-component polymer Silpuran 6000/60A, and Silpuran 6000/60B obtained from Wacker Chemie AG in ratio 1:1. For polymer processing hydraulic press (Fontijne Holland SRA 100EC 225 x 320 NA) heated to 165 °C, with a force of 100 kN for 5 min. was used. Samples were left to cool-down in two steel plates for 2 min, and post-annealed in the oven for 4 h at 200 °C.

The preparation and characterization of hCQDs [45] and nanocomposite hCQDs/PDMS [46], as well as the evidence of inclusion of hCQDs into the PDMS, was reported previously in detail.

As a light source, we used a conventional LED lamp (purchased from LEDart s.r.o., Slovakia) with a wavelength of 470 nm, the power of 50 W, and the intensity of 700 μW/cm² on the sample surface placed at a distance of 50 cm from the LED source. At this particular distance, we obtained homogeneous light irradiation across the entire sample surface.

The plasma treatment was performed on a DCSBD plasma source operating in the air. The input power was set to 400 W, and the distance between the surface of the sample and the DCSBD reactor was adjusted to 0.3 mm. The treatment time was 10-60 s. The constant movement of the sample over the plasma was provided by a linear sample stage powered by an electric motor. A detailed scheme of surface modification with DCSBD plasma is shown in Ref. [41].

The amount of singlet oxygen generated by hCQDs/PDMS nanocomposites was measured by means of Electron Paramagnetic Resonance (EPR). The Varian E-line spectrometer (UK) and spin-trap 2,2,6,6-tetramethylpiperidine (TEMP; purchased from Sigma-Aldrich, USA) were used. All samples were measured in ethanol with p.a. purity (purchased from Mikrochem s.r.o., Slovakia) mixed with TEMP at room temperature, after
12 h of irradiation. The used frequency was 9.13 GHz, central field 3245 Gauss, received gain 500, power 1 mW, and a scan range of 100 Gauss.

The chemical changes of the surface of the plasma-treated PDMS were investigated by X-ray photoelectron spectroscopy (XPS) with Thermo Scientific K-Alpha XPS System using monochromatic Al Kα X-ray source.

Morphological changes of the PDMS surface were tracked by Atomic Force Microscopy (AFM). The ScanAsyst measurement mode on MultiMode 8 AFM (Bruker, USA), equipped with ScanAsyst-Air probes (Bruker, USA) was used. The RMS values were calculated as an average from three measurements using Gwyddion software [47].

The antibacterial activity of here prepared nanocomposites was performed according to ISO standard 22196 “Measurement of antibacterial activity on plastics and other non-porous surfaces”. *Staphylococcus aureus* (CCM 4516), and *Escherichia coli* (CCM 4517) obtained from the Czech Collection of Microorganisms (CCM) was used as a model species of bacteria representing both, the G+ and G- bacteria. Before the experiment, all samples were sterilized by ethanol. The initial concentration of bacterial suspension was 1.1x10⁶ cfu/ml (*S. aureus*) and 5.3x10⁷ cfu/ml (*E. coli*). Plasma-treated samples were tested three months after the plasma treatment to simulate real-life applications. This is due to the fact, that the material from its production will be stored, transported and used for a long time. All samples were irradiated only 8 min. to observe the difference in the effect of the plasma and non-plasma treated samples. The aim of this study was not to kill all bacterial colonies as in our previous work [46] but to determine the effect of plasma. After 8 min. of irradiation, it is possible to compare samples with and without plasma treatment very precisely. Viable bacteria were calculated according to ISO standard mentioned above, for more detail please see our previous work [46].
3. Results and discussion

3.1. Measurement of singlet oxygen

The indirect EPR method was used to measure the production of singlet oxygen \([16,46,48]\). After the sample irradiation (12 h), TEMP reacted with light generated singlet oxygen \((^1\text{O}_2)\) and stable aggregates were created and measured (TEMPO). Figure 1 shows the same intensity for the reference sample – TEMPO and pure PDMS (with and without plasma), which validates no-production of \(^1\text{O}_2\). In the case of plasma-treated hCQDs/PDMS, there is an increase in the production of the \(^1\text{O}_2\). The increase of the production of singlet oxygen could be explained by mild oxidation of hCQDs' surface (as is discussed in detail in the XPS section). After prolonging the plasma treatment time over 30 s it was observed, that the production of the \(^1\text{O}_2\) was no longer improved. The resulting amount of \(^1\text{O}_2\) was even smaller than in the case of composite samples without plasma treatment. This can be caused by different factors. One of them could be degradation of polymer material itself, which happens during a longer time of plasma treatment \([41]\).

In the Supplementary information, the experiment was repeated using a standard radiofrequency plasma source. The results provide a positive evidence that DCSBD plasma is more efficient in the enhancement of \(^1\text{O}_2\) production.
Figure 1. Intensities of $^{1}$O$_{2}$-related peak in EPR spectra of pure PDMS and treated with and without plasma.

3.2. X-ray photoelectron spectroscopy

The XPS was employed to track the elemental composition changes at the surface before and after the 30 s plasma treatment. Table 1 shows the composition of all examined samples based on the deconvolution of the high-resolution C1s core-level spectra, in atomic % (at. %).

There is no principal difference between the pure PDMS and hCQDs/PDMS composite. After the addition of hCQDs, XPS detected an increase in C-C sp$^{3}$ and C-O signal.
The amount of hCQDs inside the polymer matrix is approximately 2 wt.% according to the swelling measurement. Such a small amount dispersed in the total sample volume is at the limit of detection of standard XPS measurements. We detected an increased amount of C-C bonds and C-O bonds after the addition of hCQDs.

The plasma treatment of the surface of the pure PDMS without hCQDs filler exhibited slight oxidation. The DCSBD plasma generated in the atmospheric air has a large concentration of reactive oxygen species [49–51]. Hence, the measured increase in C-O (C1s signal at ~ 286.4 eV), C=O (C1s signal at ~ 287.4 eV) and O-C=O (C1s signal at ~ 288.9 eV) was detected. The oxidation of the surface was in the order of percent. The atmospheric air plasma treatment also leads to the incorporation of nitrogen atoms. Besides some amidic NC=O group (N1s signal at ~ 399.9 eV (not shown here) and C1s signal at ~ 288.2 eV (Table 1)) also some NOx structures were adsorbed on the surface (N1s signal at ~ 406.8 eV (not shown here)). Nitrogen content was below 1 at. % and quite noisy, so difficult to properly quantified (not shown here). However, the plasma treatment of the hCQDs/PDMS nanocomposite leads to a different result. The oxidation of the surface was more profound compared to the pure PDMS. In Table 1 we can see the increase of all types of carbon-oxygen bonds as hydroxyls (C-O increase, 4.8 → 8.5 at. %), carbonyls (C=O increase, 0.2 → 2.0 at. %), amides (NC=O increase, 0.7 → 2.5 at. %), carboxyls (OC=O increase, 0.5 → 3.3 at. %) on the nanocomposite surface after the plasma treatment. It can be explained as the oxidation of hCQDs. The oxidation of the PDMS after plasma treatment is increased by only 3.7%, and such an oxidation level must be due to the interaction of plasma with hCQDs. Therefore, after the plasma treatment, the pure PDMS polymer is only mildly oxidized, but the majority of oxygen is bonded within hCQDs.
The extra oxidation of hCQDs can explain an increased production of the singlet oxygen. Wang et al. [51] reported enhanced production of the singlet oxygen by oxidation of graphitic carbon nitride. The incorporation of carbonyl groups helps to facilitate the energy transfer from carbon nitride to molecular oxygen. In our case, after the plasma treatment of hCQDs/PDMS nanocomposite, only 2 at. % of carbonyl groups were detected. Even in the case of different antibacterial mechanisms than the generation of singlet oxygen, the plasma treatment was shown to be enhancing the antibacterial effects. Espana-Sanchez et al. [52] reported the increased antibacterial activity of Ag and Cu nanoparticles after plasma treatment. They claimed that the plasma generated C-N and C-O bonds are responsible for the enhanced antibacterial activity. Zhang et al. [53] detected increased antibacterial properties after nitrogen functionalization and the creation of C-N and C=N bonds.

The decrease of the singlet oxygen production after the prolonged plasma treatment can be caused by the saturation of the hCQDs surface, decreased reactivity or due to the oxidative disruption of the nanomaterial.

Table 1. Carbon bonds in samples before and after 30 s plasma treatment.

<table>
<thead>
<tr>
<th>Binding energy (eV)</th>
<th>C-Si (atomic %)</th>
<th>sp³ (atomic %)</th>
<th>C-O (atomic %)</th>
<th>C=O (atomic %)</th>
<th>O=C=O (atomic %)</th>
<th>N=C=O (atomic %)</th>
<th>sp² (atomic %)</th>
<th>π-π* (atomic %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS pure</td>
<td>92.8</td>
<td>4.7</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hCQD pure</td>
<td>0</td>
<td>20.4</td>
<td>12.0</td>
<td>6.1</td>
<td>2.1</td>
<td>0</td>
<td>58.3</td>
<td>1.0</td>
</tr>
<tr>
<td>hCQD/PDMS</td>
<td>88.6</td>
<td>8.1</td>
<td>3.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDMS plasma</td>
<td>84.8</td>
<td>9.0</td>
<td>4.8</td>
<td>0.2</td>
<td>0.5</td>
<td>0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hCQD/PDMS plasma</td>
<td>56.5</td>
<td>27.2</td>
<td>8.5</td>
<td>2.0</td>
<td>3.3</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3. Atomic force microscopy
PDMS samples and their morphological changes after the plasma treatment were studied by using AFM. The DCSBD plasma has the ability to smooth surfaces, as it is evident in Figure 2 and Figure 3. The statistical roughness values were calculated from 5×5 µm² area scans. The RMS roughness of approximately 100 nm of the pure PDMS before plasma exposure was more than halved to some 42 nm after 30 s of plasma treatment. Similarly, the RMS roughness value for nanocomposite hCQDs/PDMS dropped from the initial 103 nm to 64 nm after 30 s exposure to DCSBD plasma. The small size of hCQDs (~ 6 nm) and their amount inside the polymer matrix do not significantly affect the resulting structure and roughness of the polymer. The decrease of the roughness was accompanied by the formation of round objects. We assume the plasma processes induces the formation of such objects on the polymer surface (etching, crosslinking, etc.). The increase of the sample’s temperature could as well cause the creation of such objects. The DCSBD plasma is highly non-equilibrium plasma with the rotational temperature of neutral gas around 100°C [42,54].

Mošovská et al. [32] measured the temperature of black pepper seeds after the plasma treatment. According to their work, the pepper heated up to ~35°C after 60 seconds of plasma treatment. On top of that, the black pepper is directly in contact with the DCSBD reactor. Our samples were positioned 0.3 mm above its surface and were continually moving. This causes additional cooling of our samples, and they were even cooler than the above-mentioned black pepper seeds.

The PDMS is commonly prepared by a moulding method at a temperature of 165 °C and dried (crosslinked) in the oven for 4 h at 200 °C [46]. The thermal degradation (depolymerization) of PDMS, according to Camino et al., is in the range around 400 – 650°C [54]. Consequently, plasma treatment can not cause any thermal damage to our samples even if the sample would be heated up to 50°C (the temperature of black pepper after 5 min plasma
treatment). However, the increase of the temperature can cause polymer chains re-arrangement of PDMS, which can result in observed changes in the surface's morphology.

The smoothening of the surface can be in the case of some bacteria strains undesirable because extremely high roughness can act as a passive antibacterial surface. However, if the material should be antibacterial only because of its surface, extremely large/low roughness is required [55,56]. In our case, neither extremely high nor extremely low roughness is achieved. Therefore, the detected changes in the surface roughness will not significantly affect antibacterial activity. Decreasing the roughness of a photoactive material can lead to a drop in the generation of singlet oxygen [46] due to a lower specific surface. Accordingly, high specific surface materials are desired for photodynamic therapy. On the other hand, if the material has a lower surface roughness, bacteria cannot easily adhere to its surface. In addition to surface smoothening, the plasma treatment in our study has a number of different effects on the nanocomposite. For this reason, a reduction in surface roughness does not necessarily affect the resulting improvement in singlet oxygen generation.

Figure 2. The AFM images of pure PDMS before (left) and after 30 s of plasma treatment (right).
3.4. Antibacterial activity

The hCQDs/PDMS polymer nanocomposite can eradicate all bacterial colonies on the surface after 15 minutes of blue light irradiation [46]. According to our previous study, the nanocomposite not exposed to irradiation did not exhibit an antibacterial effect [48]. The antibacterial effect, however, depends on various factors, e.g. microorganism species, time of irradiation or composite composition. The mechanism of antibacterial activity is mainly connected to the production of singlet oxygen, its diffusion outside the polymer matrix and effective disruption of the bacterial plasma membrane, eventually even interaction with inner biomacromolecules. Here, the antibacterial activity was conducted on two different bacterial model species *S. aureus* and *E. coli*. Both species are commonly used for these purposes and the results are therefore easily comparable to the results of other authors. Differences between plasma-treated samples and non-plasma-treated samples are presented in Table 2. As previously mentioned, it is known that irradiation of here tested nanocomposites can effectively eliminate all viable bacteria from the surfaces. On the other hand, time can be critical in terms of pandemic situations when a huge amount of material must be sterilized.
within a short period. The aim of the here presented study was not, therefore, to show that nanocomposites with hCQDs can kill bacteria cells, but that plasma treatment can accelerate this process. The results clearly show, that even 8 minutes of irradiation of *S. aureus* by blue light decreases the number of bacteria for one order of magnitude in the case of plasma-treated hCQDs/PDMS nanocomposite compare to untreated hCQDs/PDMS nanocomposite. In the case of *E. coli*, the plasma treatment resulted in only a minor decrease, but the tendency also shows the effectiveness of combined plasma-treated surfaces with subsequent irradiation. The difference in the efficiency of the photodynamic therapy between *S. aureus* and *E. coli* is because of the structure of their cell membrane [52].

Here the observed impact of plasma treatment of surfaces on the antibacterial activity can be related to previously presented changes in material properties of nanocomposites. As visible from AFM scans, the plasma smoothens the surface of the nanocomposite. This alone can lead to the smaller adhesion of some bacterial species on the surfaces [16,52]. On the other side, plasma could make nanochannels, and singlet oxygen can diffuse much faster and in a higher quantity. Also, atmospheric plasma can create oxygen-containing function groups on the surface, and singlet oxygen is produced in a more significant amount. All these facts lead to the improvement of nanocomposite antibacterial properties in a very short time of plasma treatment. Barnes et al. [57] reported a 2-log reduction in bacterial counts after 50 h of cultivation on a PVC polymer surface with 0.15 – 0.25 µm thick silver film. In our study, we improved the antibacterial effect of hCQDs/PDMS after only 30 s of plasma treatment by 1-log (*S. aureus*). In comparison to the above reference, we achieved 2-logs reduction after 8 min. of BL irradiation of 30 s plasma-treated samples (*S. aureus*, *E. coli*). Such an improvement in the antibacterial effect can lead to a shortening of disinfection time. On top of that, treatment with atmospheric air, low-temperature plasmas is environmentally, and user-
friendly method, and therefore this material and procedure is very suitable for large-scale application.

Table 2. Antibacterial activity of pure and modified PDMS with and without plasma treatment after 8 minutes of blue light irradiation.

<table>
<thead>
<tr>
<th>Samples</th>
<th>S. aureus CCM 4516</th>
<th></th>
<th>E. coli CCM 4517</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (cfu/cm²)</td>
<td>R = U₁ - A₁</td>
<td>N (cfu/cm²)</td>
<td>R = U₁ - A₁</td>
</tr>
<tr>
<td>PDMS pure</td>
<td>4x10⁵</td>
<td>U₁ = 5.6</td>
<td>8x10⁵</td>
<td>U₁ = 6.9</td>
</tr>
<tr>
<td>PDMS pure/plasma</td>
<td>5.8x10⁵</td>
<td>≥ 5.6</td>
<td>1.5x10⁶</td>
<td>0.7</td>
</tr>
<tr>
<td>hCQDs/PDMS</td>
<td>5.6x10⁴</td>
<td>U₁ = 4.7</td>
<td>6.8x10⁴</td>
<td>U₁ = 4.8</td>
</tr>
<tr>
<td>hCQDs/PDMS/plasma</td>
<td>7.4x10³</td>
<td>0.8</td>
<td>3.1x10⁴</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Note: N means the number of viable bacteria recovered per cm² per test specimen; R = the antibacterial activity where U₁ is the average of the common logarithm of the number of viable bacteria (cells/cm²) recovered from the untreated test specimens, and A₁ means the average of the common logarithm of the number of viable bacteria (cells/cm²) recovered from the treated test specimens.

4. Conclusion

In summary, the surface treatment by the atmospheric pressure low-temperature plasma leads to an improvement of antibacterial effect of hCQDs/PDMS nanocomposite. The plasma generated in atmospheric air oxidizes the surface of hCQDs and therefore enhances the energy transfer between the hCQDs and molecular oxygen. On top of that, the plasma treatment smoothens the surface of the nanocomposite. The smoother surfaces are well known for their ability to suppress the adhesion and proliferation of bacteria.

Because of these reasons, the irradiation time required for the resulting antibacterial effect can be shortened. Comparing the results obtained by the DCSBD plasma and gamma irradiation treatment [44], the plasma-treated samples acquired their antibacterial properties in
much shorter treatment time. Furthermore, DCSBD plasma processing enables considerable energy savings and offers facile upscaling to large surfaces. Finally, and most importantly, employing this relatively simple, easily scalable, ecological, efficient and time and cost-saving method can significantly decrease the disinfection time in medical facilities, food industry or ultra-clean rooms.

**Supplementary information**

A control experiment using radiofrequency plasma, all EPR and XPS spectra, AFM scans of 10 and 60 s plasma treatment and dynamic mechanical analysis of untreated and 30 s plasma-treated samples are presented in the supplementary information.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Conflict of interest**

The author reports no conflicts of interest in this work.

**CRediT author statement**

Mária Kováčová - Writing - Original Draft, Writing - Review & Editing, Investigation
Michal Bodík - Writing - Review & Editing, Investigation
Matej Mičušik - Investigation
Petr Humpoliček - Investigation
Peter Šiffalovič - Supervision
Zdenko Špitálský - Project administration, Funding acquisition, Supervision

**Ethical statement.**

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2. The authors declare that they have no conflicts of interest.

3. This article does not contain any studies involving animals performed by any of the authors.

4. This article does not contain any studies involving human participants performed by any of the authors.

Financial Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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