Original Article

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TANAFFOS

Toxicity of Carbon-Based Nanomaterials in the Human Lung: A Comparative In-Vitro Study

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Background: Carbon-based nanomaterials (CBNs) are the key elements in nanotechnology. The main challenge presented by CBNs is their relationship with the toxicity exposed in the biological systems, because of the incomplete information on their toxicity. This study is aimed to compare the cytotoxicity of graphite nanoparticles (GRNPs), graphene nanoparticles (GNPs), and multi-walled carbon nanotubes (MWCNTs) in A549 cells.

Materials and Methods: The physicochemical properties of nanomaterials were determined by instrumental techniques. CBNs were dispersed by the nongenotoxic standard procedure. After the cells were cultured, they were exposed to different concentrations of CBNs. Cellular viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Moreover, toxicological indicators were obtained using linear probit regression.

Results: The degree of cytotoxicity of CBNs in A549 cells was related to the time and, particularly, dose. At the concentrations of lower than 300 µg/mL, GNPs had stronger toxicity than MWCNTs, but the cytotoxic effects were reversed with the increase of the concentrations. The no-observed-adverse-effect concentration (NOAEC) of GRNPs, GNPs, and MWCNTs was 1.76, 0.06, and 0.65 µg/mL, respectively.

Conclusion: The results indicated that CBNs were toxic and GNPs had stronger toxicity than the others. The experimental results can be useful in increasing the knowledge about the toxicity and health risk management of CBNs.

Key words: Graphite nanoparticles; Graphene nanoparticles; Multi-walled carbon nanotubes; Cytotoxicity

INTRODUCTION

Carbon-based nanomaterials (CBNs) are the key elements in nanotechnology (1) with great potentials in nanomedicine (2, 3), food safety, agriculture, and industry (4). Among CBNs, multi-walled carbon nanotubes (MWCNTs) have been widely used in the industry (5). Because of their many applications, numerous individual workers can be potentially exposed to CBNs in environmental and occupational settings (6). Some investigations show that ecotoxicity can induce by bioaccumulation of CBNs in environment (7).

In 2013, National Institute for Occupational Safety and Health (NIOSH) published warnings about CBN effects, due to the similarities in their physical properties to particular materials in the workplace (8). Several studies have shown that individuals can be exposed to MWCNTs (9). For example, two studies conducted in an MWCNT primary manufacturing facility in Russia have suggested

that the concentration of exposure ranges from 3.5 to 17 µg/m^3 (10, 11). Previous studies have reported exposure to MWCNTs (12) and graphene nanoparticles (GNPs) in water resources(13) and also occupational settings (14).

Some CBNs may have the same carcinogenic effect as asbestos (15). Generally, investigations on graphite nanoparticles (GRNPs) have suggested their poor biological activity (16). Few studies have revealed that GRNP-exposed rats are able to induce programmed cell death and biological responses such as inflammation (17). The study by Sargent et al. was the first investigation which demonstrated that MWCNTs increase the growth of cells with DNA damage and lead to the development of tumors (18). MWCNTs are also known as nanomaterials with the potential for pulmonary, hematologic, and cardiovascular toxicity (15, 19). GNPs can possibly increase oxygen free radicals in live cells, damage to proteins and DNA (20) and apoptosis (21) and necrosis (22).

In view of NIOSH's recommended exposure limit (REL), OSHA's permissible exposure limits (PELs) have been reported for graphite: 5.0 mg/m^3, 8-h workdays, and 40-h workweek (23). In November 2013, NIOSH published the REL for CBNs at 1µg/m^3 (24). In 2017, MWCNT was categorized as a class 2B carcinogen, possibly carcinogenic to humans, by International Agency for Research on Cancer (IARC) (25). Currently, occupational exposure limits (OEL) for GNPs have not yet been defined; therefore, more data and information are required to obtain the real OEL (26, 27).

When the adequate dose–response data are accessible, systematic risk analysis can provide estimates for determining the appropriate exposure limits in the workplace (28) and assess potential ecological risks, too (29). Because of the variety of the physicochemical properties of nanomaterials including size, functionalized surface, bar, purity, agglomeration, corona effect, and type of sample preparation technique, there are different results concerning the in-vitro methods for nanomaterials (30, 31). Moreover, the toxicity information for determining the OELs for CBNs and the health risk assessment is incomplete (32).

Since inhalation has been identified as the most common pathway for nanomaterials to enter the body and people may be occasionally exposed to nanomaterials in this way (33), this investigation was performed on A549 epithelial cells. A549 cells have been used in the lung cell biology (34). They have been applied in the cytotoxicity models of alveolar in the pulmonary epithelium, because of their characteristics including the production of lecithin, expression of cytochrome P450 enzymes, phospholipid biosynthesis, and secretory structures (35). Therefore, A549 cells were used for in-vitro investigations and evaluating the pattern of surfactant secretions (36).

In recent years, the use of CBNs (especially CNT, GRNPs, and GNPs) has increased and likely to keep increasing in the near future. The fascinations of their properties, particularly the possibility to enhance the composites performance using a tailor made methodology, have caused new materials, processes and products for highly demanding industrial applications. However, there are main challenge presented by these nanomaterials and the toxicity information for determining the OELs are far away from being understood and full of uncertainties. Due to our access to GRNPs, GNPs, and MWCNTs substances, we selected these materials as a priority for toxicity assessment. Thus, this study is aimed to compare the cytotoxicity of GRNPs, GNPs, and MWCNTs in A549 cells using toxicological indices.

MATERIALS AND METHODS

Characterizing used nanoparticles (CBNs):

The physicochemical properties of GNPs and MWCNTs were determined in the authors' previous works (37, 38).
Since the intensity of the nanoparticle dispersion had an important role in the cytotoxicity effects, dynamic light scattering (DLS) was performed on the solution containing GRNPs. Following the use of the DLS technique (Malvern Instruments Ltd., Zetasizer ver. 6.01), the suspension stability and hydrodynamic sizes of GRNPs were revealed.

Preparing stock solution:

The nongenotoxic standard procedure (39) was used to obtain good dispersion of CBNs. Separately, 15.36 mg of CBNs was weighed, then 30 µL of ethanol and 59.7 µL of distilled water containing 0.05% bovine serum albumin (BSA) were added to prepare the 2.56 mg/mL CBN solution. Finally, the mixture was sonicated for 16 min.

Cell culture and exposure of CBNs:

A549 cells were purchased from the cell bank of Pasteur Institute. Using Dulbecco's Modified Eagle's medium (DMEM) (BIO-IDEA, Iran) containing 10% fetal bovine serum (BIO-IDEA, Iran), 100 µg/mL penicillin, and 100µg/mL streptomycin, the cells were cultured in an incubator. Afterwards, the cell culture process was completed and the cells were added to a 96-well culture plate (1×10^4 cells/mL). During 24 hours, the cells were allowed to get adhered to the floor of the wells. The cells were exposed to ten different concentrations of CBNs (0.1, 1, 10, 50, 100, 200, 300, 500, 600, and 1000 µg/mL) for 24, 48, and 72 hours. In order to increase the accuracy and reduce the error, we have separately repeated the tests three times. Also, the cells containing DMEM without CBNs were selected as the control group (Samples size: 10 (concentration)×3(Control)×3(Time)×3(repeat)=270).

Cell morphology:

An optical microscope was used to observe cell morphology. After 24 hours of exposure to CBNs, A549 cells were observed by a microscope (Olympus 1x71, equipped with Olympus DP72 Camera 12.8 megapixel). Moreover, the cells containing DMEM without CBNs were selected as the control group.

Cell viability:

The MTT assay protocol, as a colorimetric method, was used to measure the cell viability of A549 cells (37). In live cells, MTT was converted into formazan and a pink color appeared; so the appearance of color is a valuable indicator of viable cells (40). In this study, phosphate-buffered saline (PBS) was used to wash the cells, because CBNs may interact with the MTT dye and create an invalid result. For assessing the cell viability, 150 µL of culture medium with 10 µL of MTT (5 mg/mL in PBS) were added to each well. After 3 hours of incubation, the surface culture medium was emptied and replaced with 150 µL of dimethyl sulfoxide (DMSO). The plates were placed in a shaker for 20 min. Finally, a microplate reader (ELX800, BioTek model, the US) was used to read the wavelengths absorbed at 570 nm.

Statistical analysis and determining toxicological indicators:

Using SPSS software (ver. 16), ANOVA test was used to determine the relationship between concentration/time and cell viability. Moreover, using Minitab software (ver. 18.1) and by obtaining the probit regression model, the toxicological indices including inhibitory concentration of 50% (IC_{50}) and non-observable-adverse-effect concentration (NOAEC) were calculated. NOAEC denotes the concentration of CBNs in the exposed cells when the dead cells reach the amount of 10%. IC_{50} denotes the concentration of CBNs in the exposed cells when the dead cells reach the amount of 50%.

RESULTS

The experimental results were categorized into several sections including characterization of CBNs, morphological changes of the cells, and toxicological indices.

Characteristics of materials:

According to the authors' previous studies, MWCNTs have fibril-shaped structures. The length of MWCNTs was in the range of 1 to 3 µm and the diameter was 10 nm. The average diameter of the GNPs was 13.28 nm (37). The average pore diameter of the GRNPs was 6.41 nm. The average hydrodynamic diameter of GRNPs, GNPs, and MWCNTs in the aqueous suspension was 96.77, 323.3, and 313.9 nm, respectively. Moreover, the polydispersity index (PDI) of GRNPs, GNPs, and MWCNTs was 0.653, 0.654, and 0.608, respectively. This means that there was moderate dispersion of CBNs.

Morphological changes of cells:

Following CBN exposure, cell morphology did not change. In the culture medium, both the CBN-exposed and the control cells adhered to the floor of the plate normally, without any difference in the spindle shape and structure of cells (Figure 1).

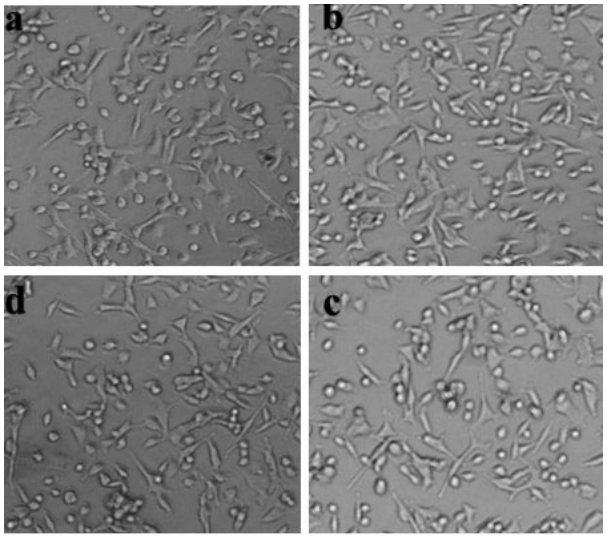

Figure 1. Representative microscopy images of A549 cells exposed to CBNs at hour 24. (a) Control, (b) GRNPs, (c) GNPs, (d) MWCNTs.

Cell viability:

At the high concentration of CBNs (1000µg/mL), GRNPs had higher cytotoxicity than the other CBNs. Similarly, cell viability for GRNPs was 19% after 24 hours. However, after 24 hours, cell viability was equal to 28.51 and 28.6% for MWCNTs and GNPs, respectively. After 48 hours, the obtained cell viability was 14.04 and 11.67% for MWCNTs and GNPs, respectively. Cell viability was also estimated at 8.6, 9.4, and 18.39% after 72-hour exposure to GRNPs, MWCNTs, and GNPs, respectively.

At the concentration of 500 µg/mL the cytotoxicity of CBNs was reversed. It means that GNPs had the highest cytotoxicity among the three CBNs. After 24 hours, cell viability was 37.67% for MWCNTs and GNPs similarly; but it was 46.09% for GRNPs. After 48 hours, the obtained cell viability was 14.28, 27.62, and 46.09% for GNPs, MWCNTs, and GRNPs, respectively.

Similar results were obtained for GNPs and MWCNTs at the low concentration of 300 µg/mL after 24 hours; but it was different after 48 and 72hours. Cell viability was 20.12, 41.51, and 58.44% for GNPs, MWCNTs, and GRNPs, respectively.

At the low concentration of CBNs (0.1 µg/mL), GNPs had higher cytotoxicity than the other CBNs. After 48 hours, cell viability was estimated at 83.02, 91.36, and 96.1% for GNPs, MWCNTs, and GRNPs, respectively. Interestingly, after 72 hours, GRNPs had higher cytotoxicity than MWCNTs and cell viability was 72.28, 96.8, and 91.51 for GNPs, MWCNTs, and GRNPs, respectively.

At the concentrations equal to or greater than 300 µg/mL for all the three CBNs, the decrease of cell viability was statistically significant in comparison with the control ($P<0.05$).

The cytotoxicity of GNPs at the concentrations of 50-300µg/mL was significantly higher than that of MWCNTs and GRNPs ($p<0.05$). Moreover, the mean cytotoxicity of GRNPs at the concentrations lower than 50 µg/mL was significantly higher than that of the other CBNs ($p<0.05$).

A significant relationship was obtained by ANOVA tests between cell viability and concentration of GRNPs ($p=0.001$), GNPs ($p=0.001$), and MWCNTs ($p=0.001$). Moreover, the time-dependent cytotoxicity of GRNPs (p-value=0.05), GNPs (p-value=0.011), and MWCNTs (p-value=0.026) was shown. Cell viability of A549 cells is displayed in Figure 2-4.

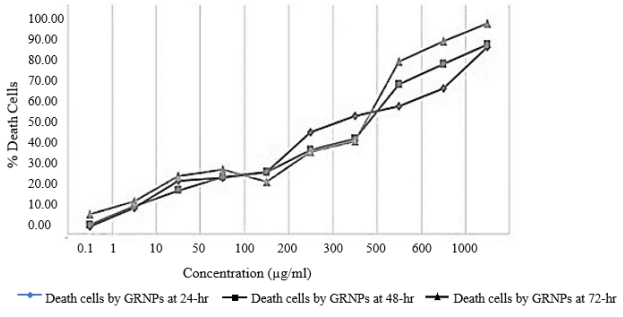

Figure 2. Cell viability of A549 cells was estimated after 24-, 48- and 72-hour exposure to GRNPs

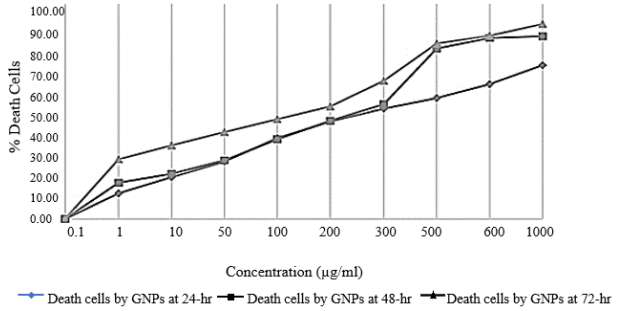

Figure 3. Cell viability of A549 cells was estimated after 24-, 48-, and 72-hour exposure to GNPs

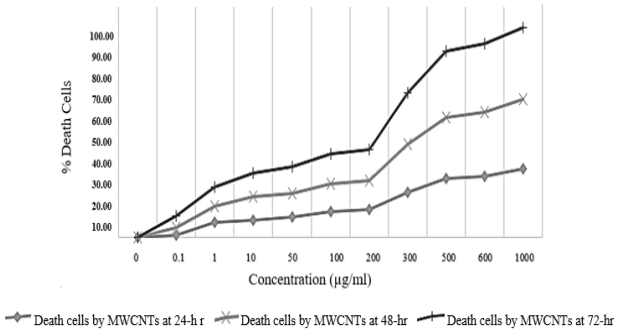

Figure 4. Cell viability of A549 cells was estimated after 24-, 48-, and 72-hour exposure to MWCNTs

Toxicological indices:

In comparison with the NOAEC estimated in the previous investigations about GNPs and MWCNTs, the toxicological indices of GRNPs were higher than those of the other CBNs at all three exposure times. After the 24-hour exposure, NOAEC was estimated at 2.35, 0.95, and 0.19 µg/mL for GRNPs, MWCTNs, and GNPs, respectively. However, after the 72-hour exposure to CBNs, the obtained NOAEC was 1.07, 0.49, and 0.03 µg/mL for GRNPs, MWCBNs, and GNPs, respectively.

Moreover, GRNPs had the highest IC50 than the IC50 estimated for GNPs and MWCNTs in previous studies. After the 24-hour exposure, IC50 was calculated as 273.55, 134.8, and 148.72 µg/mL for GRNPs, MWCTNs, and GNPs, respectively. This index was also reduced with the increase of time. After the 72-hour exposure, IC50 was 124.92, 21.51, and 71.41 µg/mL for GRNPs, MWCTNs, and GNPs, respectively. Details of the other toxicological indices of CBNs are summarized in Table 1.

Table 1. IC50 and NOAEC indicators for CBNs

Time exposure (hr.)	Toxicology indicators (µg/mL)					
	MWCNTs		GNPs		GRNPs	
	IC_{50}	NAOEC	IC_{50}	NAOEC	IC_{50}	NAOEC
24	148.72	0.95	134.8	0.19	273.55	2.35
48	105.72	0.68	41.19	0.06	234.13	2.02
72	71.41	0.46	21.51	0.03	124.92	1.07

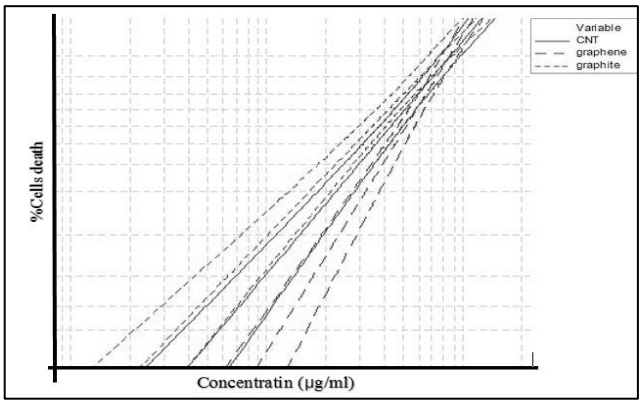

Figure 5. Comparing A549 cell viability for CBNs after 24, 48, and 72 hours using probit regression model

DISCUSSION

Potential applications of CBNs in industrial and biomedical sectors have increased human exposure and concerns about the possible adverse health effects. There are many studies on various toxicology profiles of CBNs (41, 42). Some of the investigations have indicated that CBNs have toxic effects on A549 cells (43), HeLa cells (44), bronchial epithelial BEAS-2B cells (43), and PC12 cells (45, 46). Similarly, in the current study, CBNs decreased the viability of A549 cells.

No changes occurred in cell morphology of the CBN-exposed A549 cells. Several studies have reported that the GO-treated A549 cells had the normal spindle shape and their cell morphology did not change (47, 48). However, it changed in stem cells (49) and H9c2 cells (50). In the study by Zhang et al., the morphology of the PC12 pheochromocytoma cells did not change by GNPs and the cell membrane appeared to be without damage; but,

single-walled carbon nanotubes (SWCNTs) caused cell membrane damage (45). Therefore, cell morphology can be affected by the type of cells. In addition, this might be due to the sharp edges of some of the CBN sheets, which can damage cell membranes.

Results of the present study revealed that GNPs had higher toxicity than GRNPs and MWCNTs-COOH. Zhang et al. demonstrated that, at low concentrations, GNPs had stronger toxicity than carbon nanotubes (CNTs); but, the cytotoxic effects were reversed with the increase of concentrations (42, 45). Similarly, the present study indicated that GNPs were more toxic than MWCNT-COOH although the cytotoxic effects were reversed at higher concentrations (more than 600 µg/mL). Therefore, toxicity may be related to the shape of these CBNs and their interactions in the biological system.

Surface functionalization and purity are important issues affecting toxicity. In many investigations, the cytotoxicity of functionalized CBNs has been reduced (51-53). The toxicity of carboxyl-functionalized GNP and amine-functionalized GNP leads to less DNA damage than that of pristine GNPs (54, 55). Figarol et al. suggested that the functionalization of GRNPs and CNTs triggered weaker cytotoxicity than that of pristine CBNs (42). In line with the present results, Chatterjee et al. reported the toxicity of MWCNTs (56). In the present work, GRNPs and GNPs did not have functionalized surfaces; but, MWCNTs had carboxylic groupings. Comparison shows that the findings of the present study are in agreement with the results of different studies. In contrast, some investigations have indicated that the functionalized MWCNTs are more toxic than pristine MWCNTs (56, 57). Hence, surface functionalization of CBNs may lead to a different toxic effect. Therefore, the best approach is to always keep exposure as low as possible.

The intensity of the dispersion of nanoparticles may influence the cytotoxicity effects. Dispersion can affect the agglomeration of nanoparticles and the entry of the nanomaterials into the cell (58). The agglomeration of GRNPs appeared after they were dispersed in ultrapure water (59). When GNPs were dispersed in double distilled water, agglomeration also occurred (54). Wang et al. stated that MWCNTs had better dispersion in the mixture of the serum containing dipalmitoyl phosphatidylcholine than the pure serum (60). In the present work, distilled water containing ethanol and BSA was used as the dispersing agent for CBNs and moderate agglomeration of CBNs was achieved.

After the 48-hour exposure, IC_{50} of GRNPs, GNPs, and MWCNTs-COOH was estimated to be 234.13, 41.19, and 105.77 µg/mL, respectively. Zhou et al. reported that the IC_{50} of MWCNTs-COOH (external diameter: 13–18 nm; length: 1–12µm) was at the concentration of 1 mg/mL or above it (61). This result is in contrast with the present findings. Although the IC_{50} of GRNPs (41, 42) and GNPs (22, 45) has not been clearly defined or reported, the percentage of cell viability/death has been reported in these studies. In some works, several factors such as laboratory conditions (62) and cell types (63) have been stated, which can influence various values of IC_{50}.

NOAEC of GRNPs, GNPs, and MWCNTs-COOH was 1.76, 0.06, and 0.65 µg/mL, respectively. One study stated that at the concentration of 0.01 µg/mL, GNPs could reduce the number of surviving PC12 cells. The GNP concentrations lower than 0.01 µg/mL could probably be introduced as the values of NOAEC (45). For MWCNTs-COOH, NOAEC was determined to be 0.1 mg/m^3 in a 13-weeks inhalation study on Wistar rats by Baytubes (64). In 2013, NIOSH proposed an REL for CNTs (1µg/m^3) based on the limit of quantification, which was derived from NOAEC (65). These results are not in agreement with the results of the present study. It was justified that the adverse effects of CBNs on the respiratory system can be created below these estimated levels. Therefore, attempts should be made to decrease the concentrations of these CBNs as low as possible.

The cytotoxicity of CBNs depends on the exposure period. Roberts et al. confirmed that the pulmonary and systemic toxicity of GRNPs was dependent on the dose and period of exposure (66). Several studies have

confirmed this phenomenon (37, 40, 67, 68). The cytotoxicity effects after 24 and 48 hours of exposure to CBNs were similar, but they were different from the results of 72 hours of exposure. It was similar to that determined by the precision of the results. The reason for this was probably the high activation of some toxicity mechanisms including lactate dehydrogenase (LDH) and the apoptotic mechanism after 48 hours of exposure to cells.

The MTT assay was unable to evaluate LDH, necrosis, apoptosis, and other mechanisms. Therefore, the results of the cytotoxicity assays require extra deliberation and evaluation. On the other hand, the limitations and challenges of the CBN toxicity still remain and even OEL is not yet reported for GRNPs and GNPs. Therefore, well-designed cell studies are required to diagnose the dangerous characteristics of CBNs.

CONCLUSION

According to the findings of the current study, while the concentration of the CBNs and the exposure period increased, the number of A549 cells significantly decreased in the culture medium. In general, the degree of cytotoxicity of CBNs in A549 cells was related to the time and, particularly, dose. At the concentrations of lower than 300 µg/mL, GNPs had stronger toxicity than MWCNTs. However, the cytotoxic effects were reversed with the increase of the concentrations. The NOAEC toxicological indices of GRNPs, GNPs, and MWCNTs were 1.76, 0.05, and 0.65 µg/mL, respectively. In addition, NOAEC can be derived from repeated toxicity experiments. Moreover, many factors including laboratory sample preparation and various kinds of cells can result in various values of NOAEC. Therefore, further investigations are required.

The experimental results can be useful in increasing the knowledge about the CBN-induced toxicity and health risk management in occupational and environmental settings. Nevertheless, the results of the cytotoxicity assays require more deliberation and evaluation.

Fund/supporter

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Conflict of Interest

Authors of this manuscript declare that there is no funding or conflict of interest for this work.

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