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The impact of single walled carbon nanotubes on the expression of microRNA in zebrafish (Danio rerio) embryos

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Objective. Single-walled carbon nanotubes (SWCNTs) are able to cross the blood-brain barrier, penetrate through the cell membrane, and accumulate in the cell nucleus, which purposefully allows their use in the health sciences as imaging probes and drug carriers in the cancer therapy. The aim of this study was to investigate the effect of low doses of SWCNTs on the expression of microRNAs associated with the cell proliferation and the brain development in zebrafish (*Danio rerio*) embryos.

Methods. The zebrafish embryos (72 h post fertilization) were exposed to low doses of SWCNTs (2 and 8 ng/ml of medium) for 24 or 72 h. The microRNAs (miR-19, miR-21, miR-96, miR-143, miR-145, miR-182, and miR-206) expression levels were measured by quantitative polymerase chain reaction analysis.

Results. It was found that low doses of SWCNTs elicited dysregulation in the expression of numerous cell proliferation and brain development-related microRNAs (miR-19, miR-21, miR-96, miR-143, miR-145, miR-182, and miR-206) in dose- (2 and 8 ng/ml of medium) as well as malformations in the zebrafish embryos brain development in a time-dependent (24 and 72 h) manner.

Conclusion. Taken together, the present data indicate that the low doses of SWCNTs disturbed the genome functions and reduced the miR-19, miR-21, miR-96, miR-143, miR-145, miR-182, and miR-206 expression levels in dose- and time-dependent manners and interrupted the brain development in the zebrafish embryos indicating for both the genotoxic and the neurotoxic interventions.

Key words: SWCNTs, microRNA expressions, zebrafish (Danio rerio) embryo, genotoxicity, neurotoxicity

Single-walled carbon nanotubes (SWCNTs) have extensive industrial applications due to their electronic, optical, mechanical, and thermodynamic properties, which increases their chance of human and environmental exposure to these nanoparticles. Carbon nanotubes have also been investigated for various biomedical applications, including therapeutic chemical compounds delivery in cancer therapy as well as imaging probes (Yan et al. 2019). At the same time, a potential risk has also been recognized, as the hazardous impact of various carbon nanotubes on the human health and the environment (Tejral et al. 2009; Toyokuni 2013; Francis and Devasena 2018; Kobayashi et al. 2017; Kavosi et al. 2018). The toxic potential of carbon nanotubes has been reported in numerous cell lines and animal models and their long-term toxicity has been attracted as an increasing concern (Moller and

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Jacobsen 2017; Eldawud et al. 2018; Yang and Zhang 2019).

It has also been shown that carbon nanotubes are able to penetrate via the blood-brain barrier as well as the cell membrane and accumulate in the nucleus, which permits their use in health sciences as carriers of chemical compounds in cancer therapy and imaging probes. However, SWCNTs are not biodegradable, which consequently restricts their use in medicine and biology raising a concern about their chronic genome toxicity (Ahmadi et al. 2017; Yang and Zhang 2019; Jiang et al. 2020). It has been shown that these carbon nanotubes may interfere with the immune response, because they strongly suppress the expressions of cell surface glycoproteins, such as HLA-DRA (major histocompatibility complex, class II, DR alpha) and HLA-DRB1 as well as dysregulate the level of microRNAs, such as miR-190b and miR-7, which possibly contribute to the down-regulation of both HLA-DRA and HLA-DRB1 mRNA expression (Minchenko et al. 2018). Besides, SWCNTs deregulate the expression of LMNB1 (lamin B1), which participate in the control of the genome stability. Many other genes also indicate for genotoxic effect of these nanoparticles (Minchenko et al. 2018).

Recently, we have shown that SWCNTs may affect the expression of genes associated with the endoplasmic reticulum stress, cell proliferation, cell survival, and carcinogenesis in normal human astrocytes (Rudnytska et al. 2021). The changes in the expression level of these mRNAs introduced by SWCNTs in astrocytes were dissimilar in magnitude and direction, showing a dysregulation in the genome stability. It is possible that alterations in the gene expression encoding DNAJB9, IGFBP3, IGFBP6, CLU, ZNF395, KRT18, GJA1, HILPDA, and MEST are mediated via the endoplasmic reticulum stress evoked by these nanoparticles. Moreover, the detected changes in the expression profile of these genes are possibly realized via both the transcriptional and the posttranscriptional mechanisms of regulation. Most studies have indicated that there are alterations in the expression of miRNAs with the binding sites at 3'-UTR level (Rudnytska et al. 2021).

It has also been shown that the above-mentioned carbon nanotubes may affect the expression of genes encoding important regulatory factors and key enzymes, which control the cell proliferation and apoptosis, such as cyclin D2, parvin beta, dystrobrevin alpha (DTNA), 6-phosphofructo-2-kinase/fructose-2.6-bisphosphatases-3 (PFKFB3), and PFKFB4, in human U87 glioma cells (Minchenko et al. 2016). It is relevant to note that SWCNTs also disturb alternative splicing of pre-mRNA, which we have demonstrated for DTNA, PFKFB3, and PFKFB4 (Minchenko et al. 2016). At the same time, detailed mechanisms of this type of carbon nanotubes toxicity are still unidentified and it is possible that they may include signaling pathways of the endoplasmic reticulum stress. It has been shown that different nanoparticles may induce genotoxicity and that the endoplasmic reticulum stress signaling pathways may play an important role in mechanism of action and toxicity in variety of nanoparticles (Chen et al. 2014; Heim et al. 2015; Boran and Ulutas 2016; Agmal et al. 2017; Simon et al. 2017; Hou et al. 2019; Chen et al. 2020). There are data indicating that modulation of the endoplasmic reticulum stress by chemicals alters the toxicity of some nanoparticles (Barberet et al. 2017; Cao et al. 2017; Gu et al. 2017; Simon et al. 2017). The chemical compound 4-phenylbutyric acid repressed most effects of the nanoparticles, particularly of the titanium dioxide nanoparticles, ones by alleviation of some aspects of the endoplasmic reticulum stress signaling system (Hu et al. 2018). The toxicity of various nanoparticles has also been studied in the zebrafish larvae (Boran and Ulutas 2016; Hou et al. 2019; Zhu et al. 2019; Wang et al. 2020a). It is very important to note that the understanding of the nanoparticle molecular mechanism induced toxicity is significant for both the nanotoxicological studies and the nanomedicine.

MicroRNAs (miRNAs) are a class of small single-stranded non-coding, often phylogenetically conserved endogenous RNA molecules, which play a critical role in the gene expression control at the posttranscriptional level mainly via the interaction with the 3'-untranslated regions of the target messenger RNAs and a subsequent induction of their degradation or translational repression. MicroRNAs also play an important role in the regulation of all metabolic processes as well as the neurodevelopment (Krichevsky et al. 2003; Feng and Feng 2011; Xue et al. 2013; Petri et al. 2014; Horsham et al. 2015; Zou et al. 2015; Matamala et al. 2018; Ma et al. 2019; Mazzelli et al. 2020; Yapijakis 2020). The crucial regulatory role of miRNAs in the zebrafish development has also been shown (Mishima 2012; Bhattacharya et al. 2017). The zebrafish is an economically important freshwater fish species, globally considered to be as a good predictive model for human diseases and development studies (Agarwal et al. 2015). There are data indicating that the microRNAs dysregulation in the neural tissue may lead to brain development malformations as well as neurodegenerative diseases (Feng and Feng 2011; Gizak et al. 2020).

The attempt of this study was to examine the impact of low quantities of SWCNTs on the expression of microRNAs related to metabolism, cell proliferation, and neurogenesis in the zebrafish embryos. For this investigation, microRNAs: miR-19, miR-21, miR-96, miR-143, miR-145, miR-182, and miR-206 were selected, which play an important role in the control of cell proliferation and neurogenesis (Mishima 2012; Du et al. 2013; Wang et al. 2018; Ge et al. 2019; Cho et al. 2019; Wang et al. 2019, 2020b).

Materials and methods

Nanoparticles. The functionalized SWCNTs were received from Prof. Uwe Ritter, Institut für Chemie und Biotechnik, Technische Universität Ilmenau, Ilmenau, Germany and were suspended in a distilled water, sonicated on ice for 15 minutes, and filtered through membrane filter (pore size was 0.22 μm). These nanoparticles have partially been characterized previously using atomic force microscopy and transmission electron microscopy (Minchenko et al. 2018; Shapoval et al. 2020). The diameter of SWCNTs is 1–2 nm (atomic force microscopy study) and their length up to 210 nm (determined by pore size of membrane filter). The state of SWCNTs in solution was also characterized by dynamic light scattering (DLS) and zeta potential methods. The main fraction of light scattering particles had hydrodynamic diameters in the range of 183 nm and zeta potential –26 mV for 5 μg/ml SWCNTs in water suspension. Polydispersity index (PDI) of these nanoparticles was 0.273.

Zebrafish *(Danio rerio, Hamilton 1822).* The zebrafish AB wild strain was provided by Shandong Academy of Sciences (Jinan, Shandong, China). The condition of the culture was adopted from a zebrafish culture method of Zebrafish Book, as described previously (Liu et al. 2019). The Zebrafish was cultured under a condition of ventilation and circulating water. The light cycle was 14 h light – 10 h dark interchange, the water temperature was 28 °C, and the prawns and bait were fed twice a day. The zebrafish culture room was looked and managed by the staff of the Shandong Academy of Sciences.

The suitable female and male fish were paired before experiment according to a proportion of 2:2 or 1:2 for mating. The fertilized eggs were collected the next day. The embryos were washed for several times with 0.5% methyl blue aqueous solution, then the embryos were placed into incubators with the same illumination periods for light-controlled feeding at 28.5 °C. In this period, the dead embryos were timely removed. Into the experiment, matured embryo individuals were selected 72 h after the post fertilization (hpf). The zebrafish culture medium contained 5 mmol/L NaCl, 0.17 mmol/L KCl, 0.4 mmol/L $CaCl_2$, and 0.16 mmol/L $MgSO_4$ in deionized water purified in a purifying device.

The zebrafish embryos after 4–5 h of post-fertilization were collected and rinsed several times in culture medium to remove residues from the egg surfaces and maintained in a closed flow-through culture system at 28±0.5 °C. Then, the healthy embryos at the blastula stage were selected and transferred into the six-well cell culture plates (30 embryos in each well in a 5 ml of medium). Then, after 72 h post fertilization, they were exposed to 2 and 8 ng/ml of SWCNTs for 24 or 72 h. The development of the zebrafish larvae was detected using an inverse microscope equipped with a digital camera and documented photographically.

RNA extraction and cDNA synthesis. Total RNA was extracted from the zebrafish embryos using TRIZOL reagent as described previously (Auf et al. 2013; Rudnytska et al. 2021). The RNA pellets were washed with 75% ethanol and dissolved in the nuclease-free water. For an additional purification, the RNA samples were re-precipitated with 95% ethanol containing sodium acetate and the pellets were again dissolved in nuclease-free water. The RNA quantity and spectral characteristics were measured using NanoDrop One (Thermo Scientific). For polyadenylation and reverse transcription of miRNAs, Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan) was used.

Real-time quantitative polymerase chain reaction. The level of miRNAs (miR-19, miR-21, miR-96, miR-143, miR-145, miR-182, and miR-206)

Table 1
Forward primers used for quantitative real-time polymerase chain reaction of microRNAs

Gene	Specification	Primer
miR-19	microRNA-19-5p	5'- tgtgcaaatctatgcaaaactga
miR-21	microRNA-21-5p	5'- tagcttatcagactggtgttgg
miR-96	microRNA-96-5p	5'- tttggcactagcacatttttgct
miR-143	microRNA-143-3p	5'- tgagatgaagcactgtagctc
miR-145	microRNA-145-3p	5'- gtccagttttcccaggaatccct
miR-182	microRNA-182-5p	5'- tttggcaatggtagaactcaca
miR-206	microRNA-206	5'- tggaatgtaaggaagtgtgtgg
U6F	U6 forward	5'- cttgcttggggaggacatatac
U6R	U6 reverse	5'- aacgcttcacgaatttgcgtg

expression was measured by real-time qPCR using "QuantStudio 5 Real-Time PCR System" (Applied Biosystems) and Mir-X miRNA qRT-PCR SYBR Kit (Takara, Japan). Polymerase chain reaction was performed in triplicates. For analysis of microRNA expressions, a universal qPCR reverse primer and specific for each miRNA forward primers were used (Table 1).

The amplification products quality was analyzed by melting curves. An analysis of quantitative PCR was performed using special computer program "Differential Expression Calculator". The microRNA expressions values were normalized to the level of U6 RNA and represented as a percentage of control (100%).

Statistical analysis. All values are expressed as mean±SEM from triplicate measurements performed in 4 independent experiments. Statistical analysis was performed according to Student's t-test using Excel program as described previously (Rudnytska et al. 2021). A value of $p<0.05$ was considered to be statistically significant.

Results

We investigated the impact of SWCNTs on the genome function *in vivo* in zebrafish embryos by studying the numerous microRNAs expression levels, which have relation to the function of the nervous system, and the zebrafish embryonic development. As shown in Figures 1 and 2, the exposure of the zebrafish larvae, on the third day of the embryo development, to two different quantities of SWCNTs for 24 h led to variable changes in the microRNAs (miR-19, miR-21, miR-96, miR-143, miR-145, miR-182, and miR-206) expression levels. It was shown that the microRNAs miR-21 and miR-19 expression levels were significantly down-regulated in the zebrafish larvae by both SWCNT doses being more effective for miR-21 (Figure 1). Thus, smaller and bigger doses (2 and 8 ng/ml) of SWCNTs inhibited both miR-21 (–62 and –67%, correspondingly) and miR-19 (–26 and –43%, correspondingly), but the levels of miR-96 had significantly less sensitivity to these carbon nanotubes (Figure 1). A stronger dose-dependent suppressive influence was detected for the following microRNAs: miR-143, miR-145, miR-182, and miR-206 in the zebrafish embryos treated 24 h by SWCNTs (Figure 2). The expression levels of these microRNAs were significantly decreased in the zebrafish larvae treated by SWCNTs in doses 2 and 8 ng/ml: miR-143 (–32 and –75%, correspondingly), miR-145 (–13 and –73%, respectively), miR-182 (–15 and –51%, correspondingly), and miR-206 (–33 and –62%, respectively) (Figure 2).

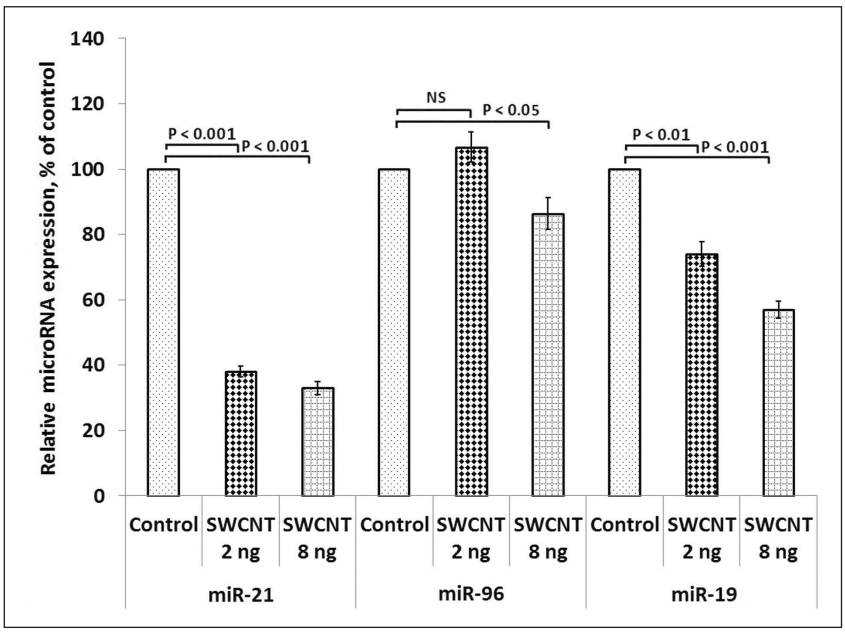

Figure 1. The impact of single-walled carbon nanotubes (SWCNTs; 2 and 8 ng/ml in water, 24 h) on the microRNAs (miR-21, miR-96, and miR-19) levels in comparison with the controls in the zebrafish embryo. Values of microRNAs levels were normalized to the U6 RNA level (n=3).

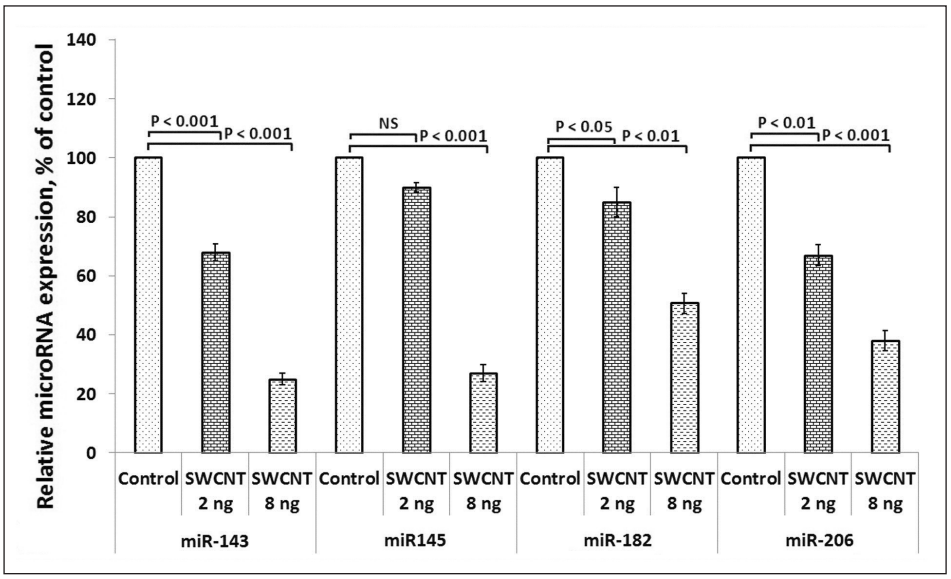

Figure 2. Dose-dependent effect of single-walled carbon nanotubes (SWCNTs; 2 and 8 ng/ml in water, 24 h) on the microRNAs (miR-143, miR-145, miR-182, and miR-206) levels in comparison with the controls in the zebrafish embryo. Values of microRNAs levels were normalized to the U6 RNA level (n=3).

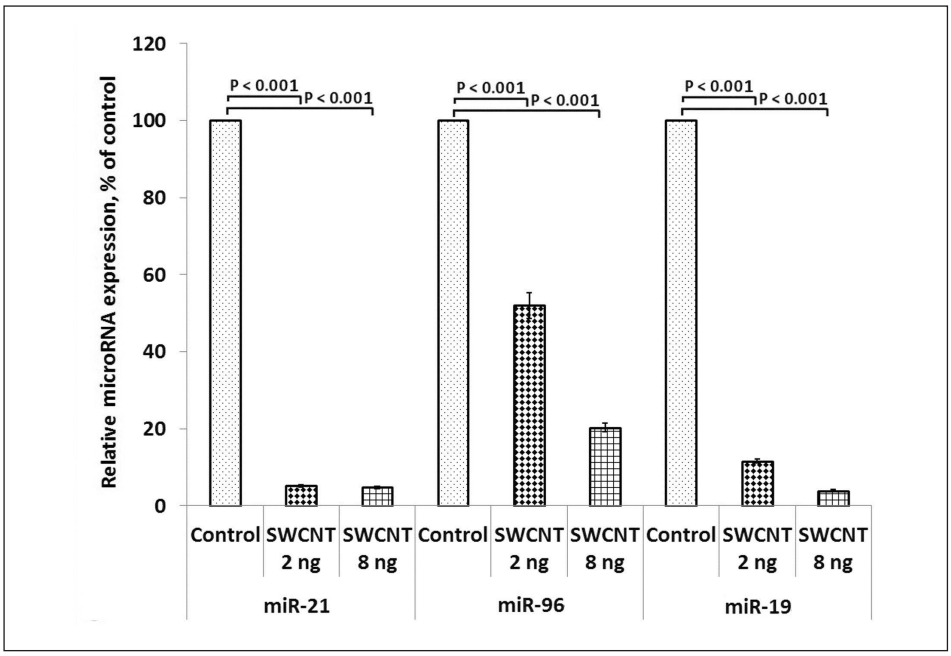

Figure 3. The impact of single-walled carbon nanotubes (SWCNTs; 2 and 8 ng/ml in water, 72 h) on the microRNAs (miR-21, miR-96, and miR-19) levels in comparison with the controls in the zebrafish embryo. Values of microRNA levels were normalized to the U6 RNA level (n=3).

At the same time, more significant and mostly dose-dependent changes were observed in the zebrafish larvae treated with low quantities of SWCNTs for longer period (72 h). As shown in Figure 3, the exposure of the zebrafish embryos to SWCNTs (2 and 8 ng/ml) for 72 h led to a robust down-regulation in the miR-21 expression levels (–95% independent of nanotubes quantities), miR-96 (–48 and –80%,

correspondingly), and miR-19 (–88 and –96%, respectively). Significant and markedly dose-dependent changes in the microRNA expression levels were detected in miR-145 (–18 and –80%, correspondingly), miR-182 (–70 and –95%, respectively), and miR-206 (–66 and –79%, correspondingly) (Figure 4). Even a stronger suppression was found for miR-143 (–98 and –99%), correspondingly for smaller and bigger doses of SWCNTs), as compared to the control zebrafish embryos (Figure 4).

The exposure of the zebrafish larvae to SWCNTs in doses 2 and 8 ng/ml for shorter (24 h) or longer period (72 h) significantly affected the expression of different microRNAs mostly in dose-dependent and time-dependent manners.

We have also studied the impact of SWCNTs (2 and 8 ng/ml of water for 72 h) on the zebrafish embryonic development in an inverse microscope equipped with a digital camera. Results are presented in Figure 5. The analysis of the photomicrographs of the zebrafish embryos treated with SWCNTs (72 h) revealed significant and dose-dependent malformation in the brain revealing the neurotoxic effect of these nanotubes on the early embryonic development stages of the central nervous system.

The SWCNTs at very low quantities dysregulated the microRNAs expression related to cell proliferation and neurogenesis in the zebrafish embryo leading to the significant and dose-dependent malformations in the area of the brain showing genotoxic and neurotoxic effects in the early stages of the zebrafish embryonic development, particularly on the development of the central nervous system.

Discussion

The results of this study clearly demonstrate that the carbon nanotubes used have a significant toxic effect on the important regulatory mechanisms via dysregulation of the genome function, which lead to a disruption of the normal development of the zebrafish embryos and particularly, the development of the brain.

For this study, the zebrafish was selected, since it is an economically important freshwater fish species globally accepted as a good predictive model for studying human diseases and development. It has been shown that very low quantities of SWCNTs may significantly disturb the expression of microRNAs, which participate in the post-transcriptional regulation of most mRNAs and regulate variable physiological and pathophysiological processes in different cells and tissues, including brain tissue (Schratt et al. 2006; Agarwal et al. 2015; Matamala et al. 2018; Wang et al. 2018; Goldewski et al. 2019; Krichevsky et al. 2019; Ma et al. 2019; Brennan and Henshall 2020).

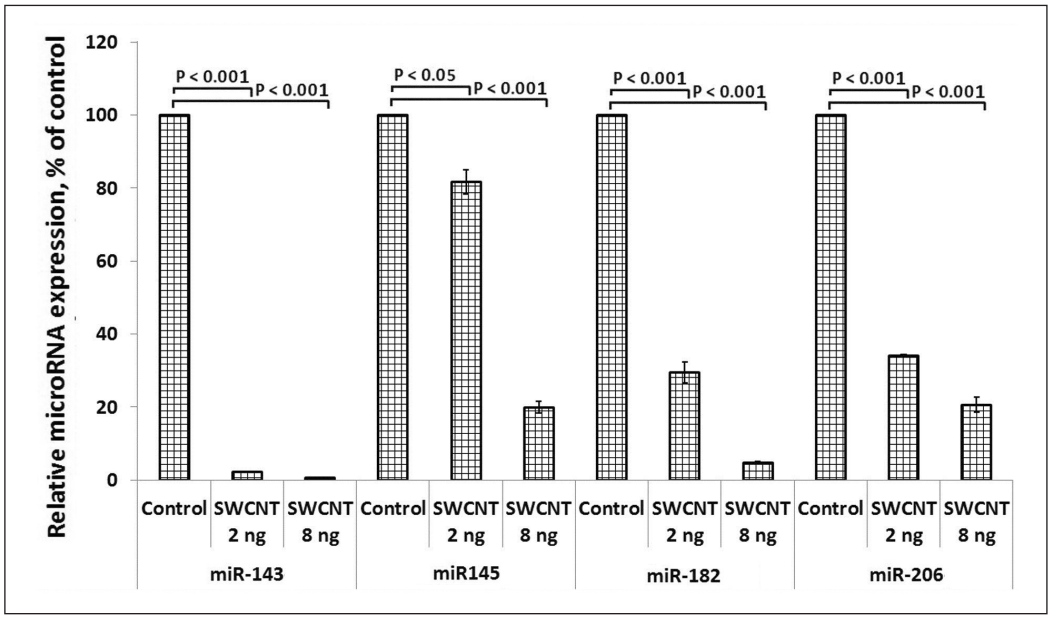

Figure 4. Dose-dependent effect of single-walled carbon nanotubes (SWCNTs; 2 and 8 ng/ml in water, 72 h) on the microRNAs (miR-143, miR-145, miR-182, and miR-206) levels in comparison with the controls in the zebrafish embryo. Values of microRNA levels were normalized to the U6 RNA level (n=3).

Previously we have shown that SWCNTs affect the expression of genes encoding enzymes and factors related to immune response, cell proliferation, and cell cycle control as well as some microRNAs in U87 glioma cells and normal human astrocytes (Minchenko et al. 2016, 2018; Rudnytska et al. 2021). The present study indicates that SWCNTs at very low quantities significantly down-regulate the expression of numerous microRNAs in the zebrafish embryos in dose- and time-dependent manners. The results clearly demonstrating this impact are presented in Figures 6A, B, and C. The dose-dependent changes in microRNAs expression in the zebrafish embryos introduced by SWCNTs after 24 h of exposure are schematically depicted in Figure 6A. Similar results were shown in Figure 6B, where the exposure time of the zebrafish embryos to SWCNTs was 72 h. The Figure 6C displays data of time-dependent changes in studied microRNAs expression levels in the zebrafish embryos induced by SWCNTs after 24 and 72 h. All studied microRNAs (miR-19, miR-21, miR-96,

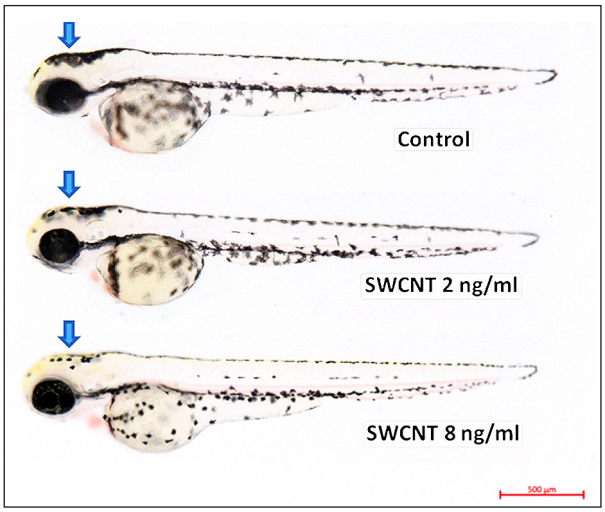

Figure 5. Dose-dependent impact of single-walled carbon nanotubes (SWCNTs, 2 and 8 ng/ml in water, 72 h) on the formation of the central nervous system in comparison with the controls in the zebrafish embryo. Arrows show brain developmental malformations in the SWCNTs (2 and 8 ng/ml water) and the control embryos.

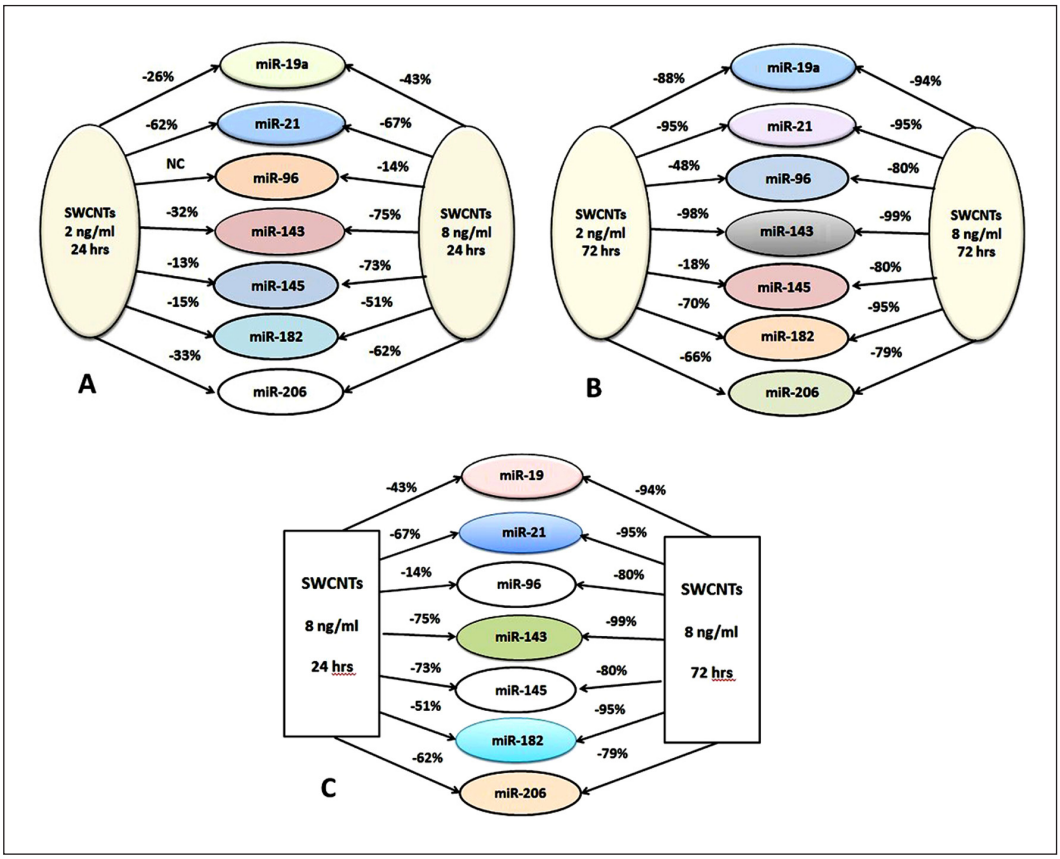

Figure 6. Schematic representation of the dose-dependent (A and B) and the time-dependent (C) impacts on a single-walled carbon nanotubes (SWCNTs, 2 and 8 ng/ml in water, 24 and 72 h) on the microRNAs levels in the zebrafish embryo.

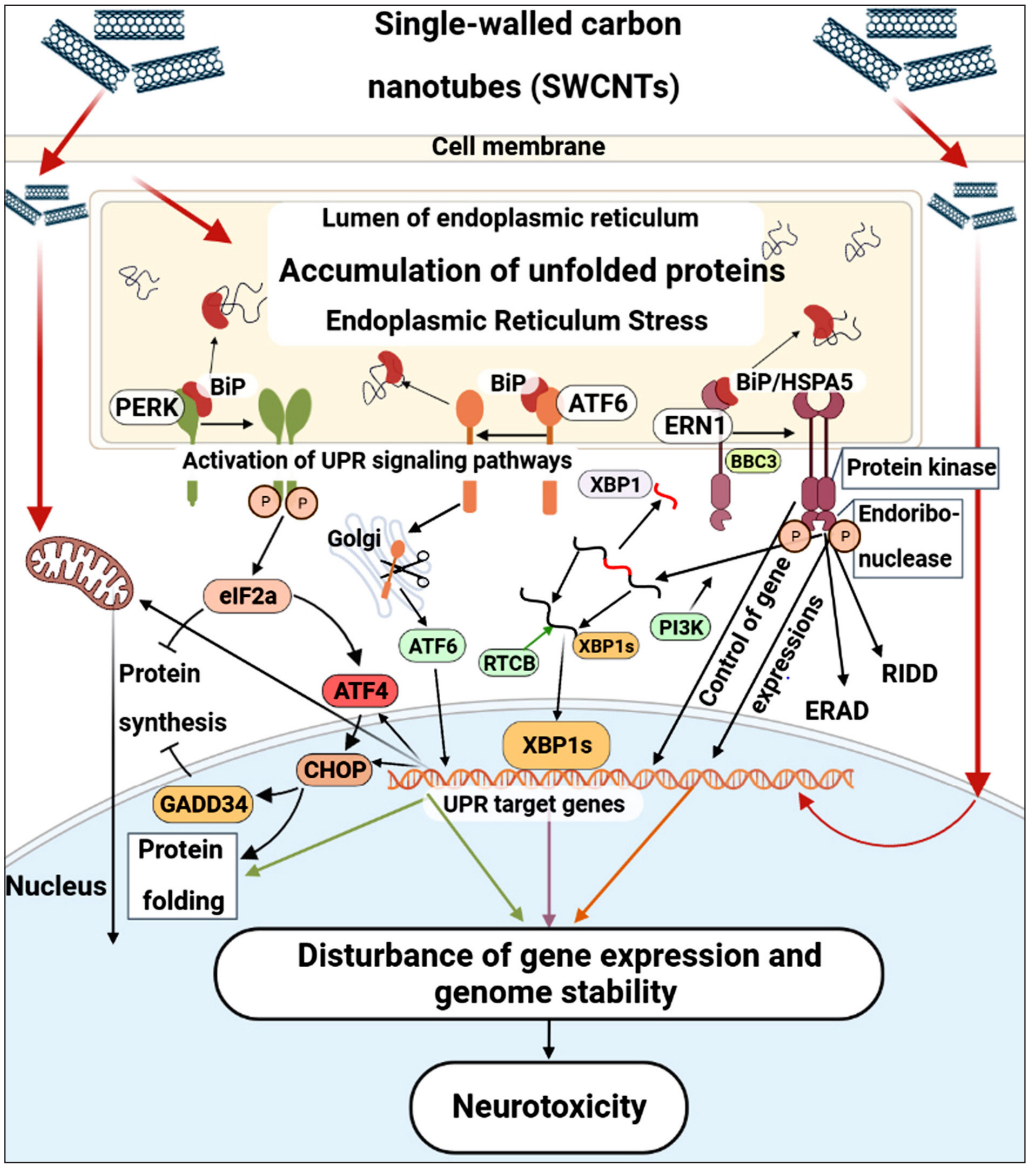

Figure 7. Schematic representation of possible molecular mechanisms of genotoxic and neurotoxic impacts of single-walled carbon nanotubes (SWCNTs on the cells via induction of the endoplasmic reticulum stress and the genome functional integrity and stability disturbances.

miR-143, miR-145, miR-182, and miR-206) play an important role in the control of cell proliferation and neurogenesis as well as variable metabolic processes (Mishima 2012; Du et al. 2013; Wang et al. 2018; Cho et al. 2019; Ge et al. 2019; Wang et al. 2019, 2020b). These results agree well with data of Krichevsky et al. (2003), Ma et al. (2019), and Cho et al. (2019) regarding the important role of microRNA in the development of brain and variable cerebrovascular pathophysiology. Furthermore, Ge et al. (2019) have shown that long-term downregulation of miR-19 level is responsible for the neurodevelopmental pathways alterations. Results of our investigation also demonstrate that the level of miR-96 is associated with malformations in the brain. These data agree well with the results of Du et al. (2013) who have demonstrated that transcription factor PAX6 (paired box 6) is a target of miR-96 and plays an essential role in the embryonic neural stem cells induction.

As consequences of such important changes in microRNA expressions induced by SWCNTs in zebrafish embryos, dose-dependent malformations

in the brain were present reflecting the genotoxic and neurotoxic effects of SWCNTs on the zebrafish embryonic development early stages. This correlates well with the dysregulation of microRNAs.

Many diverse consequences of carbon nanotubes toxicity have been shown including DNA damage, membrane damage, oxidative stress, changes in mitochondrial activities, altered intracellular metabolism including dysregulation of numerous gene expressions, and disturbances in the genome function (Minchenko et al. 2016, 2018; Kobayashi et al. 2017; Francis and Devasena 2018; Jiang et al. 2020; Rudnytska et al. 2021). It is possible that most of these changes are the results of the genome reprogramming function induced by an endoplasmic reticulum stress. The miRNAs have also emerged as key regulators of the endoplasmic reticulum homeostasis and important players in the unfolded protein response-dependent signaling (Maurel and Chevet 2013). Moreover, the miRNAs biogenesis machinery appears to be also regulated upon the endoplasmic reticulum stress supporting the idea that endoplasmic reticulum stress is an important component of SWCNTs action similar to other nanoparticles (Maurel and Chevet 2013; Chen et al. 2014; Barberet et al. 2017; Simon et al. 2017; Chen et al. 2020).

Possible mechanisms of SWCNTs impact on astrocytes include direct action on the subcellular organelles and induction of the endoplasmic reticulum stress followed by the activation of its signaling pathways (PERK, ATF6, and ERN1). As shown in Figure 7, activation of these signaling pathways reprograms genome function, significantly modifies the proteostasis, and leads to a decreased immune response and cell apoptosis as well as increased cell proliferation and survival. However, SWCNTs are not biodegradable and the endoplasmic reticulum stress they cause is long lasting and induces disturbances in the cell genome functional integrity and malformations in the zebrafish embryos brain, which possibly may reflect the genotoxic and neurotoxic effects of this unique carbon nanocompound.

Conclusion

We have revealed that very small quantities of the functionalized SWCNTs exert dose-dependent genotoxicity and developmental neurotoxicity in the zebrafish embryos and disturb genome function inducing malformations in the development of the central nervous system. Therefore, malformations in zebrafish brain development induced by SWCNTs are associated with strong disturbances in the expression of microRNAs and may reflect both genotoxic and neurotoxic effects. Likewise in many other nanoparticles, very little is currently known about the genotoxicity and neurotoxicity of SWCNTs. Thus, to better understand the detailed molecular mechanisms of the carbon nanotubes action is important not only in terms of their promising therapeutic applications, but also the assessment of their potential exposure-related risks for the human health. Actually, these data suggest that carbon nanotubes biological and medical applications, like other nanoparticles, require more cautions.

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Conflict of interest: *The authors declare no conflict of interest.*

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