FUNCTIONAL NEUROTOXICITY AND TISSUE METAL LEVELS IN RATS EXPOSED SUBACUTELY TO TITANIUM DIOXIDE NANOPARTICLES VIA THE AIRWAYS

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Introduction and aims – Nanoparticles of titanium dioxide are suspected neurotoxic agents and have numerous applications possibly resulting in human exposure by several ways including inhalation. In the present work, rats were exposed to spherical TiO_2 nanoparticles of two different sizes by the intratracheal route. It was investigated how the neuro-functional alterations, detected by electrophysiological and behavioral methods, were related to the concentration of Ti in the tissue samples and what the influence of the size of the NPs was.
Materials and methods – Rats (young adult Wistar males, 10/group) were exposed to TiO_2 nanoparticles of ca. 10 and 100 nm diameter (suspension medium: neutral PBS with 1% hydroxyethyl cellulose) by intratracheal instillation in 5 and 18 mg/kg b.w. dose; 5 days per week for 6 weeks. Controls were instilled with saline, and vehicle controls, with the suspension medium. To see general toxicity, body weight was checked daily, and organ weights were measured at the end of experiment. Grip strength test, to assess motor function damage, was done before and after the 6-week treatment. Finally, the rats were anesthetized with urethane, spontaneous cortical activity and sensory evoked potentials were recorded, then the rats were dissected and tissue samples were taken for Ti level measurement.
Results – Body weight gain indicated no general toxicity, and no significant change in the relative organ weights, except that of the lungs, was seen. However, change of time-to-fall in the grip strength test, and latency of cortical evoked potentials, were altered in the treated groups, indicating functional damage. Correlation of these alterations with the cortical Ti level was dissimilar for the two sizes of nanoparticles.

FUNKCIONÁLIS NEUROTOXICITÁS ÉS SZÖVETI FÉMSZINTEK PATKÁNYBAN TITÁN-DIOXID-NANORÉSZECSKÉK SZUBAKUT LÉGÚTI ADAGOLÁSÁT KÖVETŐEN

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Bevezetés és célkitűzés – A feltételezetten neurotoxikus hatású titán-dioxid-nanorészecskék kiterjedt alkalmazása emberi expozícióval járhat, ami többféle módon, többek közt belégzés útján mehet végbe. A jelen munkában kétféle méretű gömbszerű TiO_2-nanorészecskével kezeltünk patkányokat, intratrachealis adagolással. Megvizsgáltuk, hogyan függ össze az elektrofiziológiai és magatartási módszerekkel kimutatott idegrendszeri funkcióváltozás a szöveti Ti-szintekkel és hogyan hat erre a részecskék mérete.
Anyagok és módszerek – Fiatal felnőtt hím Wistar-patkányokat (hat csoport, 10-10 állat) kezeltünk heti öt napon, hat héten át, 10 és 100 nm körüli átmérőjű TiO_2-nanorészecskékkel, 1% hidroxietil-cellulózt tartalmazó foszfátpufferelt fiziológiás oldatban szuszpendálva, 5 és 18 mg/ttkg dózisban. A vivőanyagos kontrollcsoportot a szuszpendáló közeggel, míg a kezeletlen kontrollokat fiziológiás sóoldattal instilláltuk. Az általános toxicitást a testtömeg naponta történt mérésével, illetve a kísérlet végén a szervtömegek meghatározásával mutattuk ki. A motoros funkciók károsodását a hathetes kezelés előtt és annak végén végzett kapaszkodási próbával vizsgáltuk. Végül uretános altatásban kérgi alapaktivitást és szenzoros kiváltott potenciálokat vezettünk el, majd az állatokat felboncoltuk és szövetmintákat vettünk fémszint-meghatározásra.
Eredmények – A testtömeg-gyarapodás nem utalt általános toxicitásra, és relatív szervtömegre tett hatás csak a tüdőnél látszott. A kezelt állatokban azonban funkcionális károsodásra utaló változás volt a kapaszkodási próbánál a leesési időben, és a kérgi kiváltott potenciálok latenciájában. Ezen változások és a kérgi Ti-szint korrelációja a kétféle méretű nanorészecske esetében eltérő volt.

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Conclusion – The results provided further support to the functional neurotoxicity of TiO_2 nanoparticles. The exact role of particle size, and the mechanisms involved, remain to be elucidated.

Keywords: *nanoparticles, titanium dioxide, neurotoxicity, tissue metal levels*

Következtetés – Az eredmények újabb érvet szolgáltatnak a TiO_2-nanorészecskék funkcionális neurotoxicitása mellett. A részecskeméret szerepe és a toxikus mechanizmus további vizsgálatokat igényel.

Kulcsszavak: *nanorészecskék, titán-dioxid, neurotoxicitás, szöveti fémszintek*

Recent advances in nanosciences and nanotechnology gave rise to numerous industrial processes and products based on nanoparticles (NPs) that is, particles with <100 nm typical diameter. By now, NPs have been used among others in health care, energy production, agriculture and environmental protection[1], which results in their release into the environment. In nanoparticulate state, substances show different physical and chemical properties than those seen in more conventional physical states, leading to biological, and hence, toxicological, interactions not seen with traditional materials, which also means novel health risks. When inhaled by humans, NPs are either deposited in the nasopharynx of or get down to the alveoli, and reach distant body parts by migration along fibres of the olfactory and other nerves, or by crossing the alveolar and capillary wall and entering systemic circulation[2]. Generation of reactive oxygen species (ROS) and other surface reactions known to be crucial in the biological effects of NPs, are promoted by their high number and large specific surface area[3]. Beyond that, NPs are known to migrate to mitochondria within the cells and directly interfere with oxidative phosphorylation, also resulting in ROS and finally in cell and tissue damage[2].

TiO_2 in NP form is a frequently applied engineered nanomaterial, present in coatings, and in skin care products like sunscreens, as radiation blocking agent[4]. Its anatase crystal form has photocatalytic properties in UV light which is utilized in antibacterial and anti-fouling applications, and in coatings and building materials that break down air pollutants[5]. This broad and growing range of application has raised questions about possible health risks[6], including nervous system damage[7].

Chemical safety is a primary requirement today[8], toxicological evaluation of novel materials should therefore take place not later than their introduction to everyday use. In occupational exposure to NPs, lung damage is the number one concern[9], but – regarding the known oxidative stress generating potency of TiO_2 NPs (directly and via mitochondrial damage[10]) and the especial sensitivity of neurons to oxidative stress (due to highly active mitochondrial energy production because of the high energy demand, to abundance of unsaturated structural lipids, and to low antioxidant defence capacity[11]) – nervous system effects are also expected. In humans exposed to airborne titanium-based pigment particles at the workplace, neurological symptoms were indeed found[12], but most results suggesting human nervous system affection came for in vitro investigations on cells[13, 14].

Rat-based models of nervous system effects of TiO_2 NPs are scarce in the literature[4]. Inhalation exposure of rats to 10 mg/m^3 nano-TiO_2 aerosol (daily 6 hours, 5 days a week for 4 weeks) resulted in increased level of inflammation markers and decreased expression of synaptophysin in the brain without relevant deposition of Ti[15]. In most other rat studies using airway application, pulmonary toxicity was investigated[16] and in some, the access of the NPs beyond the lungs was questioned[17]. On indirect application of TiO_2 NPs (young rats exposed during lactation via dam's milk who were treated orally) impaired synaptic plasticity in the dentate gyrus and significant Ti deposition in the hippocampus were found[18]. When adult rats received oral administration of TiO_2 NPs, decreased brain acetylcholinesterase activity, together with increased GFAP reactivity and interleukin-6 level, was observed[19]. In a less realistic model – using intraperitoneal (ip.) injection – TiO_2 NPs given for 20 days resulted in increased anxiety in young Wistar rats, with elevated Ti level in brain and other organs and signs of cellular damage[20].

Considering the above mentioned importance of airborne exposure and nervous system effects, in the present work rats were exposed to spherical TiO_2 NPs of two different sizes via the airways by intratracheal (it.) instillation. Internal Ti exposure was determined, and functional neurotoxicity was investigated by electrophysiological and behavioral methods. It was also investigated how the neurofunctional alterations induced were related to the level of Ti in tissues and how these were influenced by the size and dose of the NPs.

Methods

ANIMALS

Young adult SPF Wistar rats were used, altogether 60, obtained from Toxi-Coop Ltd. (Budapest, Hungary). The animals (with 170±20 g body weight at start) were kept in polypropylene cages (3-4 rats/cage) under GLP-equivalent conditions. (12-12 hours light/dark cycle with light on at 06:00; temperature 22-24 °C; relative humidity 30-60%). After one week of acclimation, the rats were randomized to 6 treatment groups of 10 rats each, on the basis of their body weight. The groups and corresponding treatments are shown in **Table 1**.

PRODUCTION OF THE NANOPARTICLES

All chemicals for the synthesis of NPs were obtained from Sigma-Aldrich.

Spherical TiO_2 NPs of two sizes were synthesized, at the Department of Applied and Environmental Chemistry, Faculty of Science and Informatics, the following way:

To obtain NPs of ca. 10 nm diameter, titanium isopropoxide (TTIP, 7.32 g) was added to 50 mL ethanol (absolute) and stirred for ten minutes. Another 20 mL ethanol was mixed in 165.5 mL distilled water and stirred simultaneously for the same duration. The ethanol-distilled water mix was then added by slow dropping (one drop in 5 seconds) to the TTIP solution being continuously stirred at high speed (1200 rpm). After adding all the TTIP, stirring went on for half an hour. The TiO_2 NPs were collected from suspension by centrifuging and dried for 36 hours at 80 °C in air; their final diameter was 9.67±1.66 nm.

Larger NPs were prepared in a similar manner but other volumes and sequence was applied. 300 mL ethanol was added to 68.4 mL distilled water and stirred for 10 minutes. In this case the TTIP was given to the ethanol-water mix dropwise, at the same rate as mentioned above. These NPs had a diameter of 110±21.5 nm.

The medium for suspending the dried NPs was phosphate-buffered saline (PBS, pH 7.4) containing 1% hydroxyethyl cellulose (HEC), both obtained from the pharmacy of the Faculty of Medicine of the University of Szeged.

DOSES AND TREATMENT

Rats in the control group (*C,* see **Table 1**) received saline (NaCl 0.9%) and vehicle control (VC) rats received the medium described above (1% HEC in PBS) by it. instillation. Treated rats received TiO_2 NPs suspended in the medium; the two sizes of TiO_2 NPs were administered in two doses as shown in **Table 1**. Treatment was done every workday (i.e., 5 days per week) over a 6 weeks period, between 8:00 and 10:00 a.m. It. instillation was performed in light volatile anesthesia as described in[21]. Our Ti doses were ca. one order of magnitude higher than that applied in a similar dosing regime in[15] which – 10 mg/m^3 for 6 hours – corresponds to ca. 1.25 mg/kg on the basis of ventilation volume data published in[16], and is itself higher than the 0.3 mg/m^3 occupational limit[9].

INVESTIGATIONS

General toxicity

General toxicity of the instilled TiO_2 NPs was characterized by the rats' body weight gain. Body weight was measured before every treatment to calculate the exact doses. From these data, individual weight gain for every rat over the whole treatment period was obtained as the difference between body weight on the last and first treatment day.

After the electrophysiological recording described below, each rat was sacrificed by the twofold of the anesthetic dose of urethane. From the group *VC* and the metal-treated groups, 3 rats were randomly chosen for Ti level measurement. From these, a blood sample was taken from the left ventricle after opening the thorax, then the rat was transcardially perfused with 300 ml saline of 4 °C temperature to

Table 1. *Treatment groups, group coding, and treatments in the control and treated groups*

Treatment Groups and Codes	Vehicle and volume	NP dose*
Control (C)	saline, 1 ml/kg b.w.	–
Vehicle Control (VC)	HEC-PBS, 1 ml/kg b.w.	–
Small spherical NPs, low dose (S-LD)	HEC-PBS, 1 ml/kg b.w.	5 mg/kg b.w.
Small spherical NPs, high dose (S-HD)	HEC-PBS, 1 ml/kg b.w.	18 mg/kg b.w.
Large spherical NPs, low dose (L-LD)	HEC-PBS, 1 ml/kg b.w.	5 mg/kg b.w.
Large spherical NPs, high dose (L-HD)	HEC-PBS, 1 ml/kg b.w.	18 mg/kg b.w.

*The doses were based on previous experience[24]; with increases to achieve more clear-cut effects.

remove blood from the organs. Samples of cerebral cortex and blood were stored at -20 °C. For measurement, the samples, after being dried to constant weight at 80 °C, were digested in 3 mL cc. HCl/g wet tissue for 90 min at 90 °C, then an equal volume of cc. HNO_3 was added for a further 90 min of digestion in order to fully dissolve all TiO_2 particles. The liquid obtained was filtered on 0.45 mikrom hydrophilic membrane filter and diluted to 100 mL final volume. Ti level was determined by inductively coupled plasma mass spectrometry at the Department of Inorganic and Analytical Chemistry, University of Szeged Faculty of Science and Informatics. All other rats were dissected, the organs brain, heart, kidneys, adrenals, liver, lungs, spleen and thymus were weighed, and relative organ weights (to 1/100 body weight) were calculated.

Functional neurotoxicity

The rats' motor function was assessed by the grip strength test[22, 23]. A wooden rod of ca. 8 mm diameter and rough surface was fixed horizontally in 60 cm height above a tray lined with a layer of wood chips litter. One by one, the rats were made grasp the rod with their front paws and let hang free. There were four trials in line for each rat and the length of time until falling was measured and averaged. Grip strength test was done before the treatment period (0^{th} week) and two days after the last treatment (6^{th} week). For each rat, the average time-to-fall on the 6^{th} week was divided by the corresponding data of the 0^{th} week, and this ratio was used to quantify the effect of nano-TiO_2.

Electrophysiological recording was done on the day following the 6^{th} week grip strength test. Preparation and recording was done in urethane anesthesia l (1000 mg/kg b.w. ip, the level of anesthesia was checked by the hind leg withdrawal reflex). After mechanically fixing the skull, head skin was opened by a mid-sagittal cut, soft tissues were removed, and the left hemisphere was exposed by removing the temporal bone along the inner circumference by means of a mini drill. Wounds were sprayed with 10% lidocaine, the exposed cortex was protected with a thin layer of petroleum jelly, and the animal was put aside for at least 30 min for recovery. For recording, the rat was placed into the stereotaxic frame of the electrophysiological apparatus. For sustaining normal body temperature, a thermostated (+36.5 °C) base plate was used to support the rat's underside during the recording procedure. Silver electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas. Spontaneous electrical activity was recorded from the sites for 6 min, and the relative spectral power of the frequency bands was determined by the software used for electrophysiological recording and analysis (Neurosys 1.1, Experimetria Ltd., Hungary). Then, sensory stimulation in trains of 50 was applied (SS: electric stimuli to the contralateral whisker pad; 3-4 V, 0.05 ms, 1, 2 and 10 Hz; VIS: flashes of a high-luminance LED to the contralateral eye, 0.2 ms, 1 Hz; AUD: clicks, 70 dB, 1 Hz to the contralateral ear through the hollow ear bar of the stereotaxic frame). The cortical evoked potentials (EPs) were recorded, averaged automatically, and onset latency was measured manually.

During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University of Szeged were strictly followed. The methods used in this work were licensed by the authority competent in animal welfare issues under No. XXI./151/2013.

Statistics

The sufficient number of animals in the groups was calculated by means of Power analysis ($p \geq 0.8$). From the data, group means were calculated and checked for normality by the Kolmogorov-Smirnov test. Depending on the result of that, the main statistical test used was parametric one-way ANOVA or non-parametric Kruskal-Wallis method. Post hoc

Table 2. *Body weight gain and relative organ weights (to 1/100 body weight) in the control and treated groups*

Group code	Body weight gain (g)	Brain	Lungs	Liver	Right kidney
C	139.4±26.8	0.52±0.05	0.35±0.02	2.58±0.14	0.31±0.02
VC	154±17.1	0.49±0.05	0.50±0.05	2.85±0.39	0.32±0.05
S-LD	146.6±21.3	0.49±0.05	0.58±0.06	2.82±0.21	0.32±0.05
S-HD	163.7±19.9	0.50±0.04	0.58±0.07**##	2.86±0.45	0.34±0.07
L-LD	143.9±30	0.49±0.03	0.55±0.08	2.85±0.24	0.33±0.05
L-HD	133.9±42.6	0.48±0.06	0.57±0.06	2.87±0.30	0.32±0.05

Mean±SD, n=10. **: $p<0.01$ vs. C; ##: $p<0.01$ vs VC

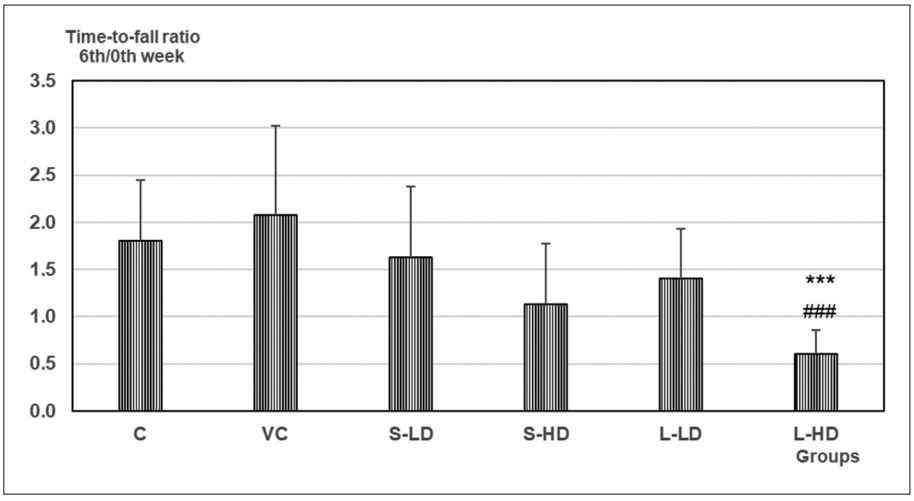

Figure 1. *Ratio of the grip strength test time-to-fall in the 6th and 0th week (see Methods)*

Mean+SD, n=10. *, **, ***: p<0.05, 0.01, 0.001 vs. C; #, ##, ###: p<0.05, 0.01, 0.001 vs. VC

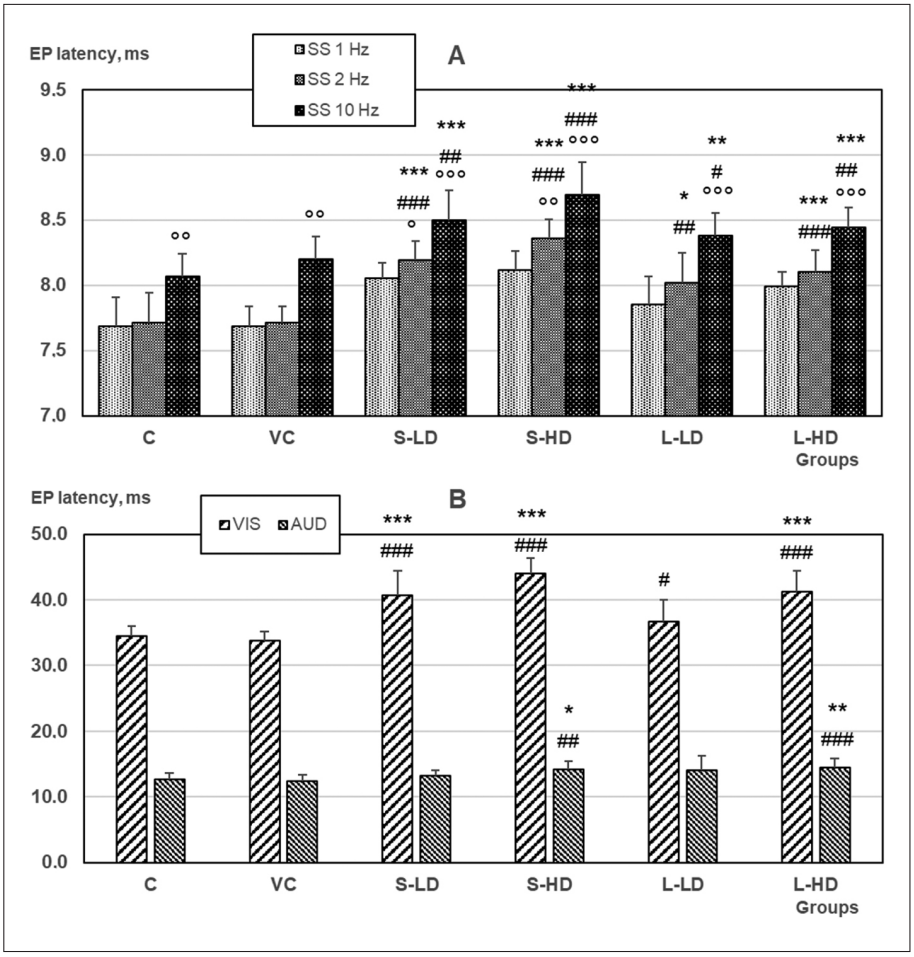

Figure 2. *Latency times of the averaged EPs in the somatosensory* **(A)** *and the visual and auditory* **(B)** *modalities in the control and treated rat groups*

Mean+SD, n=10. *, **, ***: p<0.05, 0.01, 0.001 vs. C; #, ##, ###: p<0.05, 0.01, 0.001 vs. VC; °°, °°°: p<0.01, 0.001 vs. 1 Hz stimulation in the same group

analysis of group differences was done by Tukey test and the Mann-Whitney U test. SPSS 24.0 (IBM Corporation, U.S.A.) was used.

Results

As judged from the group mean data of body weight gain, 6 weeks of TiO_2 NP exposure exerted no general toxicity in the treated rats **(Table 2)**. Also, among the organs, only the lungs appeared to be affected; their relative weight reflected the effect of the treatment procedure (group *VC* vs. *C*) and of the TiO_2 NP exposure.

In the grip strength test, the decrease of time-to-fall from the 0th to the 6th week (indicated by the 6th week / 0th week ratio; **Figure 1**) was stronger in the treated rats, with significant change in the *L-HD* group.

Among the electrophysiological phenomena recorded, spontaneous cortical activity was slightly shifted to higher frequencies in the treated rats (not shown). EP latency, however, was substantially lengthened on exposure to TiO_2 NPs, and in groups *S-HD* and *L-HD* the change was significant vs. *C* and *VC* **(Figure 2)**.

The causal relationship between TiO_2 NP exposure and the mentioned functional alterations, suggested by the group-by-group dose dependence of the changes, was further tested by examining the correlation of cortical Ti levels, and corresponding parameters of EP latency

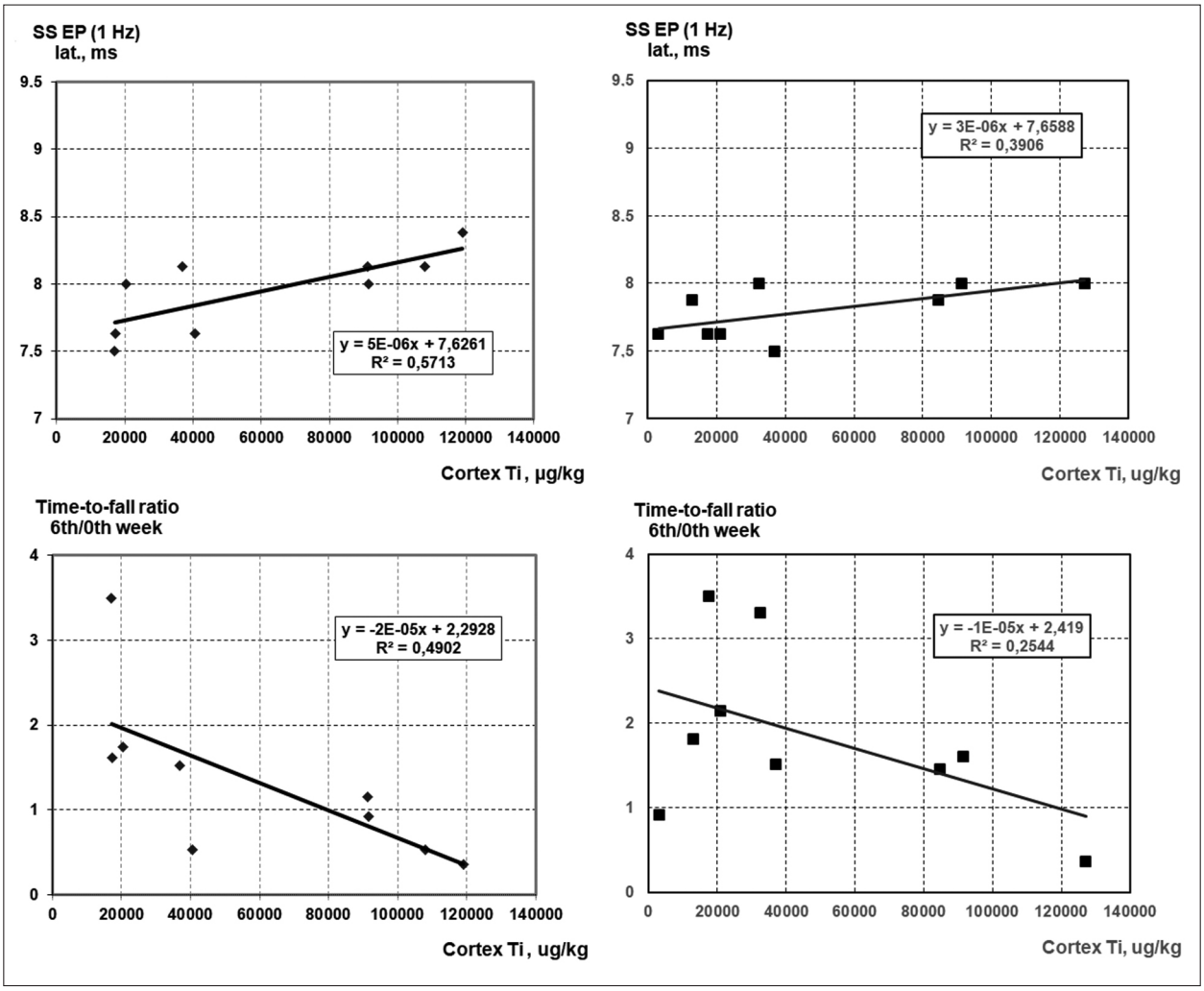

Figure 3. *Correlation diagrams of the somatosensory evoked potential latency to cortical Ti level in rats treated with the smaller (left column) and larger (right column) TiO_2 NPs*

(Figure 3) and grip strength test time-to-fall ratio **(Figure 4)**. Although Ti measurement was done only on 3 rats' samples per group, the diagrams show mostly fair correlations. The data show also that the effect of the smaller sized NPs was in most cases more clear-cut, resulting in relatively higher R^2 values.

Discussion

The neuro-functional alterations seen in the treated rats, and the correlation of these with the individual cortical Ti levels, pointed to the possible causative role of some TiO_2 NP-dependent processes. Most probably, mitochondrial damage and oxidative stress were involved, both known to be present in cells and tissues in NP exposure[2]. In rats undergoing artificial cerebral hypoxia-reperfusion, oxidative stress indicators increased (including lipid peroxidation, seen also in a previous work of us[24] with TiO_2 NPs) and muscle force, examined by grip strength test, decreased[25]. This is in parallel with our findings in nano-TiO_2 treated rats in the mentioned and the present study.

Latency lengthening of the EPs could result both from mitochondrial lesion and oxidative damage. The input-output relationship in[18] indicated impaired synaptic function in the nano-TiO_2 exposed rats. Decreased level of synaptophysin in rats after inhalation of nano-TiO_2, as described in[15] also suggests the role of impaired transmission in EP latency lengthening observed in our work. Beyond that, oxidative damage of membrane constituents, mainly lipids[11], impairs membrane-bound functions including synaptic transmission. In rats exposed the

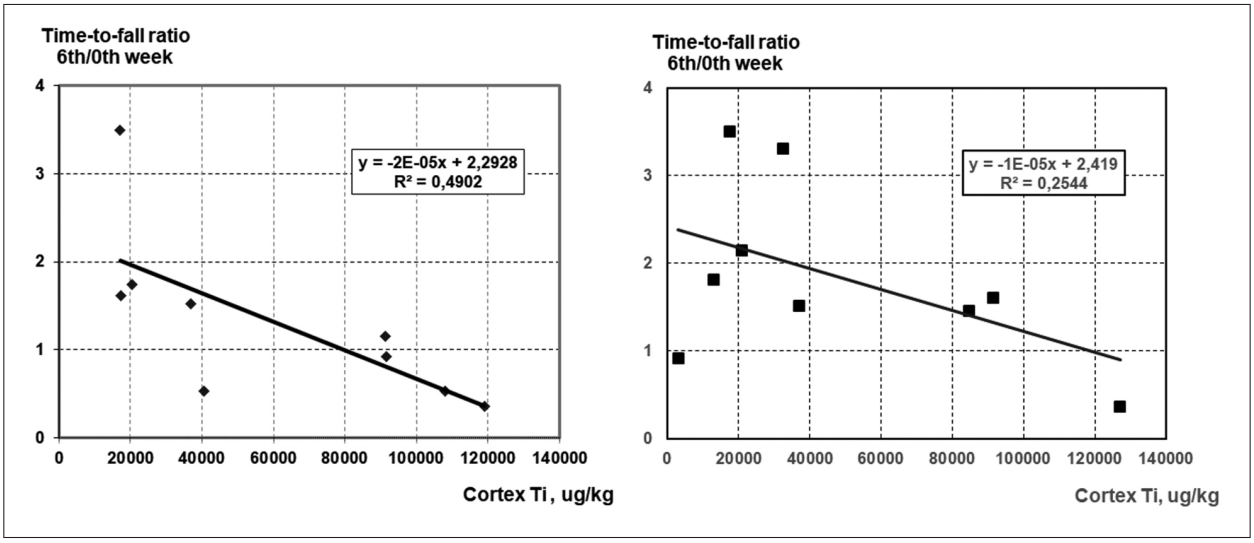

Figure 4. *Correlation diagrams of the time-to-fall ratio in the grip strength test to cortical Ti level in rats treated with the smaller (left) and larger (right) TiO_2 NPs*

same way with another metal oxide NPs, nano-MnO_2, lengthening of EP latency in the treated rats was reversed by antioxidant treatment[26] supporting the above described mechanism of NP-induced neuro-functional damage.

Mitochondrial damage leads to energy shortage in neuronal and glial cells. Glutamate is co-transported into the astrocytes with Na^+ along its concentration gradient which can be degraded if ion pumps do not operate properly due to energy shortage, ending up with excess perisynaptic glutamate and disturbed transmission[27]. Together with impaired axonal conduction this can result in depressed impulse propagation manifested in delayed cortical response to a peripheral stimulus.

The data in **Figure 1** and **2** suggest that at equal mass dose the smaller NPs had a stronger effect on cortical EPs, and the larger ones, on grip strength. One can suppose that 10 nm TiO_2 NPs had higher chance to reach the brain and 100 nm NPs stayed more in the peripheral circulation affecting nerves and muscles. With iridium NPs inhaled by rats, ca. 5 times less translocation of 75 nm size NPs than of 10 nm ones from the lungs to liver and kidneys was reported[28], underlining the effect of NP size on crossing barriers. Likewise, breakdown of blood-brain barrier and neuronal damage caused by metal NPs, given ip., was more severe with 20-30 nm than with 56-60 nm or >100 nm particles[29]. Blood-brain barrier damage was also observed in rats that inhaled nano-Ti[15].

The present study left a few questions open. It remains to be clarified, by electron microscopy, whether and to what extent the chemically measured Ti content of the organ (first of all brain) samples indicates the presence of TiO_2 NPs. Likewise, the actual role of the putative toxic mechanisms, especially in the periphery, awaits further investigations.

Conclusion

The results provided further support to the functional neurotoxicity of TiO_2 nanoparticles, including central and peripheral actions. The exact role of particle size, and the mechanisms involved, remains to be elucidated.

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