Consequences of subacute intratracheal exposure of rats to cadmium oxide nanoparticles: electrophysiological and toxicological effects

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Running head: nervous system and general effects of nanoparticulate cadmium

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Abstract

Cadmium is a metal used in various industrial applications, whereby exposure to Cd-containing fumes is likely. The submicron sized particles in the fumes represent an extra risk due to their high mobility within the organism and high surface area. Toxicity of Cd on the liver, kidney and bones is well known, but there are less data on its neurotoxicity. Here, male Wistar rats were treated for 3 and 6 weeks by intratracheal instillation of CdO_2 nanosuspension. The treated rats' body weight gain was significantly decreased, and in the high dose rats (0.4 mg/kg Cd daily) the weight of lungs and thymus was significantly increased. In this group, the spectrum of spontaneous cortical electrical activity was shifted to higher frequencies, the latency of sensory evoked potentials was lengthened, and the frequency following ability of the somatosensory evoked potential was impaired – even without detectable Cd deposition in the brain. The data support the role of the nano-sized Cd in the causation of nervous system damage and show the possibility of modeling human neurotoxic damage in rats.

Introduction

Environmental conditions constitute one of the four major determinants of human health, and the medium causing the most direct exposure to harmful substances is air. Airborne particulate matter can be classified as sedimenting dust (>10 μm), suspended or fine dust (100 nm-10 μm; often called PM10) and ultrafine dust or nanoparticles (NPs, <100 nm).
NPs as pollutants arise mainly from combustion and other high temperature processes (smeltimg, casting, welding of metals, etc.: Antonini et al., 2003). Another potential source of exposure to NPs today is nanotechnology. Manufactured nanomaterials are present in numerous consumers' goods and in technical applications (Oberdörster et al., 2005). Quantum dots, novel nanotechnological materials (with application, among others, in biomedical research) often contain cadmium (more precisely, CdTe) and show special toxicological properties (Rzigalinski and Strobl, 2009). Cd-containing metal dust and fumes, or paint spray, cause occupational airborne exposure in manufacturing and application of e.g. steel and other alloys, pigments and semiconductor materials (ATSDR, 2008).
Inhaled NPs are either deposited in the nasopharynx or get down to the alveoli (ICRP, 1994). Once deposited, NPs translocate readily to other body parts and reach target organs by different transfer routes and mechanisms, including transcytosis (by caveola formation) across epithelia of the respiratory tract into the interstitium (Oberdörster et al., 2005) and axonal transport along the olfactory fibers directly into the CNS (Calderon-Garciduenas et al., 2002). Due to their small size, high number concentration, and large specific surface area, NPs have greater biological activity per given mass than larger particles (Oberdörster et al., 2005), including oxidative stress induction (Li et al., 2003). In the target organs, the components of the NPs also exert their own toxic effects, after transport to these sites in whole or after being dissolved from the surface of the NPs (Lundborg et al. 1985). Cadmium, in airborne forms, is absorbed from the respiratory tract in 2-50%, depending primarily on particle size (Chaney et al., 2004). Its several target organs include the lungs, liver, kidney, testis, placenta, as well as the nervous system (ATSDR, 2008). Concerning the latter, the reported consequences of chronic Cd exposure include amyotrophic lateral sclerosis, optic nerve damage, striatal damage and peripheral polyneuropathy (Bar-Sela et al., 2001; Fern et al., 1996; O'Callaghan and Miller, 1986; Viaene et al., 1999). In children, a straight relationship between hair Cd and altered visual or auditory evoked potential parameters was found (Thatcher et al., 1984), and school behavioral problems were reported (Marlowe et al., 1985). Similar effects were observed in rats (Agar et al., 1999). In our previous works, several weeks oral application of Cd to rats resulted in altered electrocorticogram (ECoG) power spectrum, and in changes of cortical evoked potentials and peripheral nerve action potentials (Papp et al., 2003; Institóris et al., 2002). In the present work, a potentially more realistic way of exposure – intratracheal application of CdO_2 NPs – was chosen, and the general toxicological and electrophysiological measurements were supplemented with some biochemical ones.

Materials and Methods

Animals and treatment
Adult male Wistar rats of 320-350 g body weight were obtained from the breeding centre of the university, and were housed under standard conditions (22 - 24ºC, 12-hour light/dark cycle with light on at 06:00) with free access to tap water and standard pellet. The rats were divided into 4 groups of 20 animals each at start.
Cadmium dioxide NPs were synthesized at the Department of Applied Chemistry by a dry process. Stochiometric amount of $CdCl_2$ and Na_2CO_3 were put, in NaCl matrix, in the drum of

a planetary ball mill and rotated with stainless steel 20 mill balls at 400 rpm for 4 hours (reaction 1: $CdCl_2 + Na_2CO_3 \rightarrow CdCO_3 + 2\ NaCl$). The mixture milled this way was then calcined at 480 °C for 4 hours in air (reaction 2: $CdCO_3 + ½\ O_2 \rightarrow CdO_2 + CO_2$). After calcination, the synthesis mixture was filtered (0.45 μm PTFE membrane filter) and washed with 80°C preheated water to remove any unreacted starting material and the soluble NaCl matrix. The precipitate was dried at 100 °C for 1 h and characterized with X-ray diffraction and transmission electron microscopy. The size distribution and electron micrograph of the CdO_2 NPs is shown in Fig. 1.

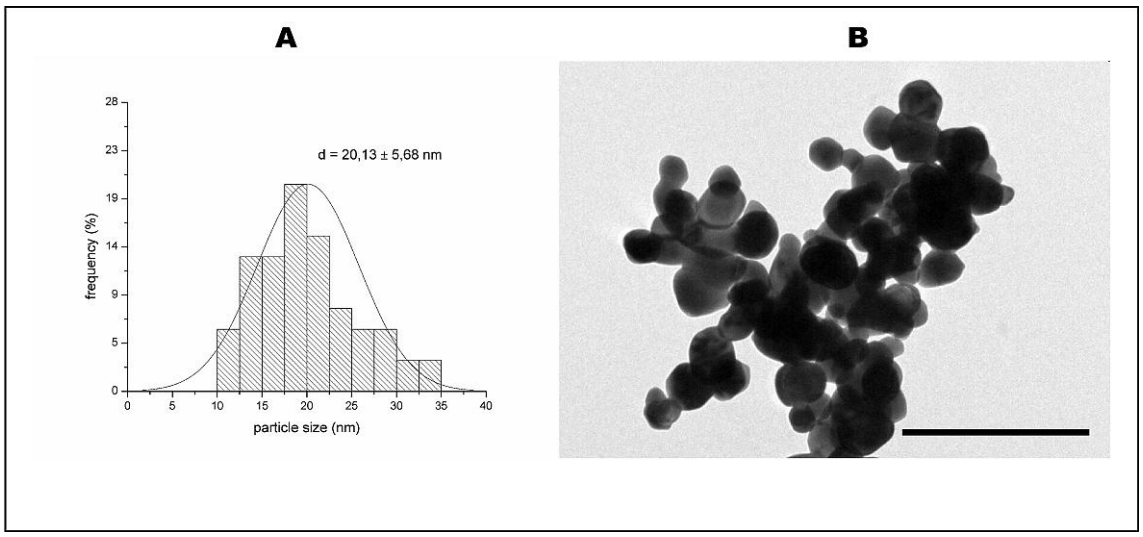

Figure 1
Size distribution histogram (A) and electron micrograph (B) of the CdO_2 nanoparticles. Scalebar: 100 nm.

The synthesized NPs were suspended in distilled water, and were instilled into the rats' trachea, in daily doses shown in Table 1, five days a week (Monday to Friday). The volume instilled was 1 ml/kg b.w. Treatment was continued for 3 and 6 weeks, whereby 10 rats from each group were sacrificed after 3 weeks treatment, and the remaining 10 after 6 weeks. There was an untreated control group (*Con*), and a vehicle control group (*W*). The choice of doses was influenced by literature data and by the technically possible concentration of the NPs in the distilled water medium.

Table 1 Treatment groups and doses.

Group	Code	Treatment and dose	Duration
Untreated control	*Con*	---	3 and 6 weeks[a]
Vehicle control	*W*	Distilled water 1 ml/kg b.w.	
Low dose	*LD*	CdO_2 nanosuspension, 0.04 mg Mn/kg b.w.; 1 ml/kg b.w.	
High dose	*HD*	CdO_2 nanosuspension, 0.4 mg Mn/kg b.w.; 1 ml/kg b.w	

[a] There were 20 rats in each group at start. Ten of them were processed and sacrificed after 3, and the another ten, after 6 weeks treatment.

Calculating with ca. 0.5 m^3/kg b.w. daily breathing volume for the rats (based on data by Strohl et al. 1997), our lower dose is comparable to that reported from industrial settings (ca.

30 μg/m^3, indoors in car body repair shops: Vitayavirasuk et al., 2005; or 1-19 μg/m^3, outdoors in bridge maintenance: Conroy et al., 1995), and the higher one, to the 550 μg/m^3 used by Takenaka et al. (2004) in a rat inhalation experiment. A more direct comparison is, however, impossible because the unknown retention fractions.
Intratracheal instillation was done in diethyl ether anesthesia, with the rat suspended on a 60° inclined board so that its upper incisors were held by a wire loop to keep the animal's mouth open. The trachea was illuminated transdermally. The tongue was pulled forward with a pair of non-traumatic forceps, and a custom-made laryngoscope was used to visualize the glottis. The nanosuspension (or distilled water for group W) was instilled into the trachea by means of a syringe and 1.2 mm OD plastic tubing, inserted between the vocal chords. The untreated control group (Con) had neither ether anesthesia nor instillation, while the water control (W) group was anesthetized and instilled with distilled water. The nanosuspension was vigorously sonicated before, and repeatedly during, administration to prevent agglomeration.

General toxicological and biochemical measurements
Body and organ weights were the endpoints for general toxic effect of the CdO_2 NPs. The rats' body weight was measured each workday during the treatment period, and the mean body weight of the groups was plotted against time to see the course of weight gain. Following electrophysiology (see below), the rats were sacrificed by an overdose of urethane, dissected, and the organ weight of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals was measured. Relative weights were calculated by relating organ weights to brain weight. To reduce costs, 5 of the 10 rats from each group were randomly assigned for chemical measurements. Of these, blood, brain, lung and liver samples were taken and stored at -22°C.
Metal level was determined from ca. 1 g of the samples, dried at 80°C to constant weight and digested in 5 ml 65 % HNO_3 at 90°C for 90 min. After filtration and dilution, metal level was determined by inductively coupled plasma mass spectrometry (at the laboratory of the MOL Hungarian Oil and Gas Company).
For biochemical measurements, another 1 g of the samples was homogenized with 4 ml saline and centrifuged under cooling for 10 min at 5000 rpm. The supernatant was centrifuged again for 20 minutes at 14,000 rpm.
From the supernatant, protein content was measured according to Lowry et al. (1951). As oxidative stress indicators, reduced glutathion (GSH) was measured by the method of Sedlak and Lindsey (1968), based on the reaction of non-protein bound SH groups with the Ellman reagent (DTNB), was used. Another oxidative stress parameter, superoxide dismutase (SOD) activity, was measured by the method of Misra and Fridovich (1972), modified by Matkovics et al. (1982), based on inhibition of the spontaneous adrenaline-adrenochrome transformation.

Electrophysiological measurements
Electrophysiological recording was done 1-3 days after the last instillation. In urethane anesthesia the animal's head was fixed and the sensory areas of left hemisphere were exposed. The wounds were sprayed with 10% lidocaine, and a thin layer of petroleum jelly was applied on the dura to prevent drying. After 30 minutes recovery, silver electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas. Electrocorticogram (ECoG) was recorded from these areas for 6 minutes, and the relative spectral power of the frequency bands (delta, theta, alpha, beta1, beta2, gamma; standard human EEG bands) was determined. Then, sensory evoked potentials (EPs) were recorded by the same electrodes. For SS stimulation, 2 needles were inserted into the contralateral whiskery skin to deliver square electric pulses (3-4 V, 0.05 ms, 1-10 Hz). VIS stimulation was produced by a high-luminance white LED aimed directly at the rat's right eye, driven by 0.2 ms pulses at 1 Hz. The AUD

stimuli were clicks (1 Hz, 40 dB) guided from a miniature earphone into the animal's right ear via the hollow ear bar. Fifty stimuli of each modality per rat were applied and the EPs recorded. After averaging, latency and duration of the EPs was measured manually (for details, see Lukács and Szabó, 2007). The change of latency of the SS EP with increasing stimulation frequency was also investigated as a possible indicator of the action of the treatment on the state of the cortex. All electrophysiological recording and analysis was done by means of the Neurosys 1.11 software (Experimetria Ltd, Budapest, Hungary). The study was approved by the Ethical Committee for the Protection of Animals in Research of the University. During the whole procedure, the principles of the Committee (based on the EU-conform Hungarian law) were strictly followed.

Data processing

From the data, group means (±SD) were calculated. The results were tested for significance with one-way ANOVA and the post hoc analysis was done by Scheffe's test.

Results

Body and organ weights

Intratracheal exposure by the nanoparticulate CdO_2 had marked effect on the rats' body weight gain. As shown by Fig. 2, the untreated controls' (*Con*) weight gain was undisturbed. In the vehicle control (*W*) group (anesthesia and instillation but no CdO_2 NPs) the weight gain was lower, and with the advance of time became more and more similar to that seen in the treated rats. In the *HD* group, there was hardly any weight increase in the first weeks. Then, some compensation seemed to take effect and the weight gain was similar to that seen in the *LD* group and approached that of the vehicle control (*W*).

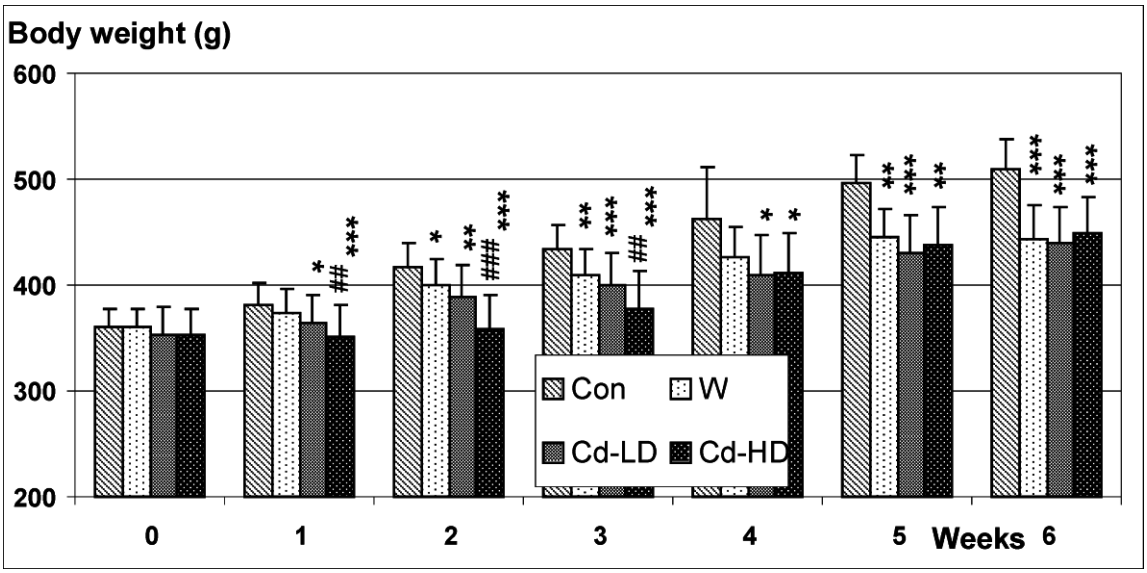

Figure 2

Body weight gain of the treated and control rats over the 6 weeks treatment period. Always the data from the first workday of the corresponding week are plotted.

Mean+SD, n=10. Insert: group codes, see Table 1 for explanation.

*, **, ***: p<0.05, 0.01, 0.001 vs. *Con*; ##, ###: p<0.01, 0.001 vs. *W*.

The relative weight of the lungs was significantly higher in the *HD* group vs. *Con* after 6 weeks exposure (Table 2). In the *W* and *LD* groups, there was no noteworthy increase. There was also significant increase of the relative thymus weight in the *HD* group, and decrease of the relative spleen and liver weight in the treated groups. After only 3 weeks treatment (not shown) the trends were similar but less expressed.
Brain weight itself was little influenced by the Cd NP treatment (after 6 weeks exposure: *Con*, 1.278±0.054 g; *W*, 1.156±0.91 g; *Cd-LD*, 1.169±0.091 g; *Cd-HD*, 1.189±0.134 g) – so the relative organ weights were not biased.

Table 2 Relative organ weights after 6 weeks exposure to Cd nanoparticles.

Groups	***Con***	***W***	***Pb-LD***	***Pb-HD***
Organs				
Lungs	0.787±0.067	0.692±0.084	0.831±0.088	1.359±0.254***$^{\#\#\#}$
Liver	7.323±0.718	7.198±1.071	6.549±0.502*	6.198±0.595
Kidney	1.407±0.070	1.350±0.089	1.311±0.172	1.379±0.146
Heart	0.583±0.031	0.542±0.043	0.558±0.039	0.556±0.066
Spleen	0.468±0.062	0.358±0.040	0.334±0.056***	0.387±0.041**
Thymus	0.213±0.026	0.194±0.033	0.207±0.017	0.277±0.056**$^{\#\#}$
Adrenals	0.028±0.008	0.027±0.007	0.029±0.008	0.029±0.008

Mean±SD, n=10.
*, **, ***: p<0.05, 0.01, 0.001 vs. Con; $^{\#\#}$, $^{\#\#\#}$: p<0.01, 0.001 vs. W.

Cadmium levels and oxidative stress indicators
A shown by data in Table 3, most of the Cd content of the instilled NPs was located in the lungs but a significant amount was absorbed and deposited in the liver in a dose-dependent manner. In the brain (and blood) however, no Cd was detected.
SOD activity was affected neither in the brain nor in the lung and liver (in which organs Cd deposition was detected). The level of GSH was, on the contrary, dose-dependently influenced and the *HD* vs. *W* difference was significant in the lungs and the brain.

Table 3 Cd deposition and reduced glutathione level in tissue samples of rats after 6 weeks exposure by CdO_2 NPs.

	Treatment groups		
	W	***Cd-LD***	***Cd-HD***
Cd level (µg/kg)			
Brain	0	0	0
Liver	26±26	683±271**	9986±4171**$^{\#\#}$
Lung	1707±1391	43020±23904*	268399±199844*$^{\#}$
GSH (µM)			
Brain	0.0895±0.0037	0.0914±0.0075	0.1071±0.0035***$^{\#\#\#}$
Liver	0.0729±0.0040	0.0806±0.0065	0.1062±0.0020
Lung	0.2080±0.1780	0.2398±0.1335	0.1681±0.1620***$^{\#\#\#}$

Mean±SD, n=5. *, **, ***: p<0.05, 0.01, 0.001 vs. *W*; $^{\#}$, $^{\#\#}$, $^{\#\#\#}$: p<0.05, 0.01, 0.001 vs. *Cd-LD*.

Electrophysiological effects

The alterations of the spontaneous cortical activity (ECoG) were alike in all three cortical areas. There was a dose- and time-dependent shift from slower to faster waves which became significant in the *HD* group after 6 weeks exposure (Fig 3). After 3 weeks only, no significant changes were seen.

The SS EP showed significant latency increase in the *HD* group vs. *Con* at each stimulation frequency (Fig. 4). The slight dependence of the latency on the frequency of stimulation, seen in *Con* and *W*, was more expressed in the treated groups, up to the significant difference between the latencies obtained with 1 and 10 Hz stimulation in the *HD* group. The latency of the VIS EP, and to a lesser extent of the AUD EP, also increased in the treated groups vs. *Con* (Fig. 5).

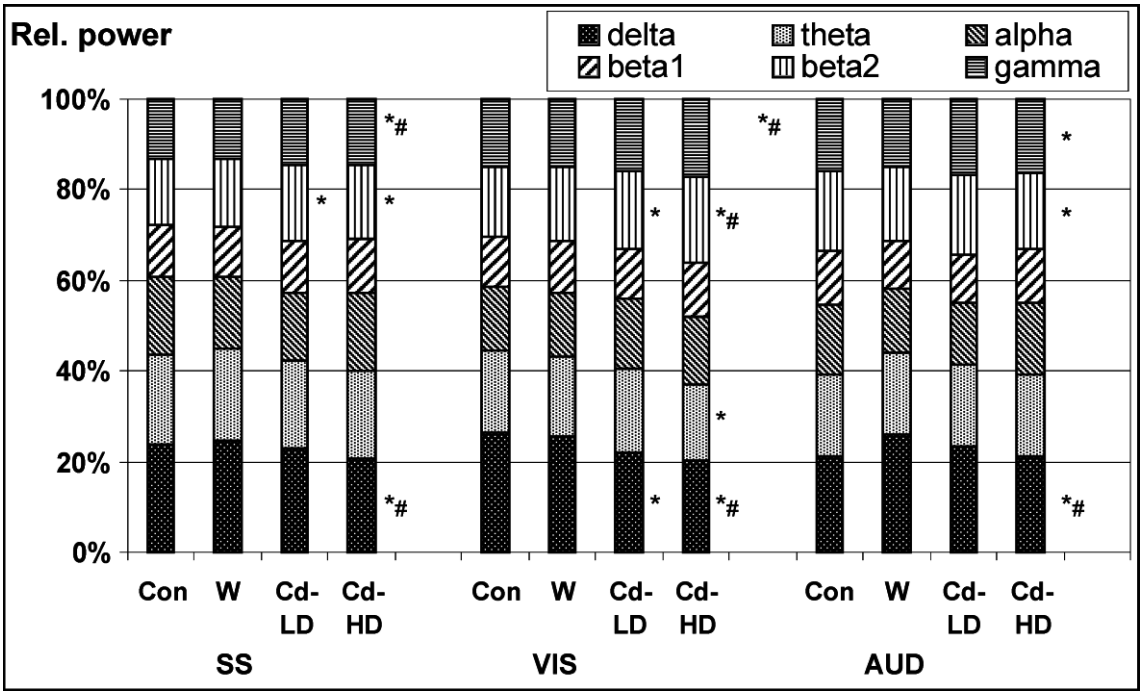

Figure 3

Band power spectrum (delta to gamma, see insert) of the rats' electrocorticogram after 6 weeks exposure.

Abscissa: group codes. SS, somatosensory area; VIS, visual area; AUD, auditory area.

*: $p<0.05$ vs. *Con*; $^{\#}$: $p<0.05$ vs. *W*.

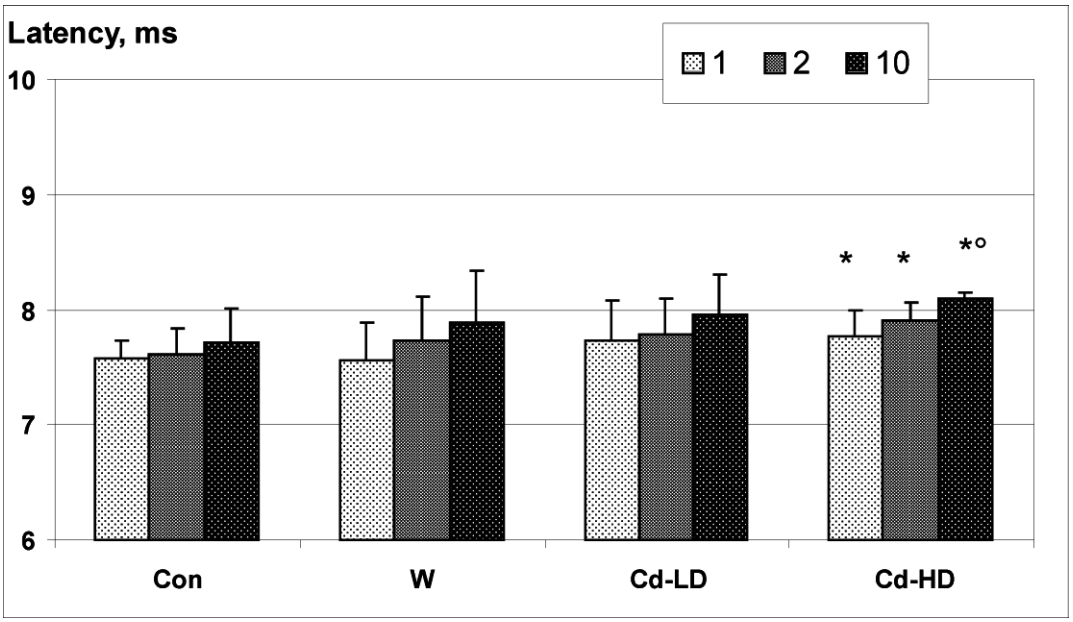

Figure 4
Latency of the somatosensory evoked potential after 6 weeks exposure.
Abscissa: group codes. Mean+SD, n=10. Insert: stimulation frequency.
*: p<0.05 vs. *Con*; °: p<0.05 vs. 1 Hz stimulation within the same treatment group.

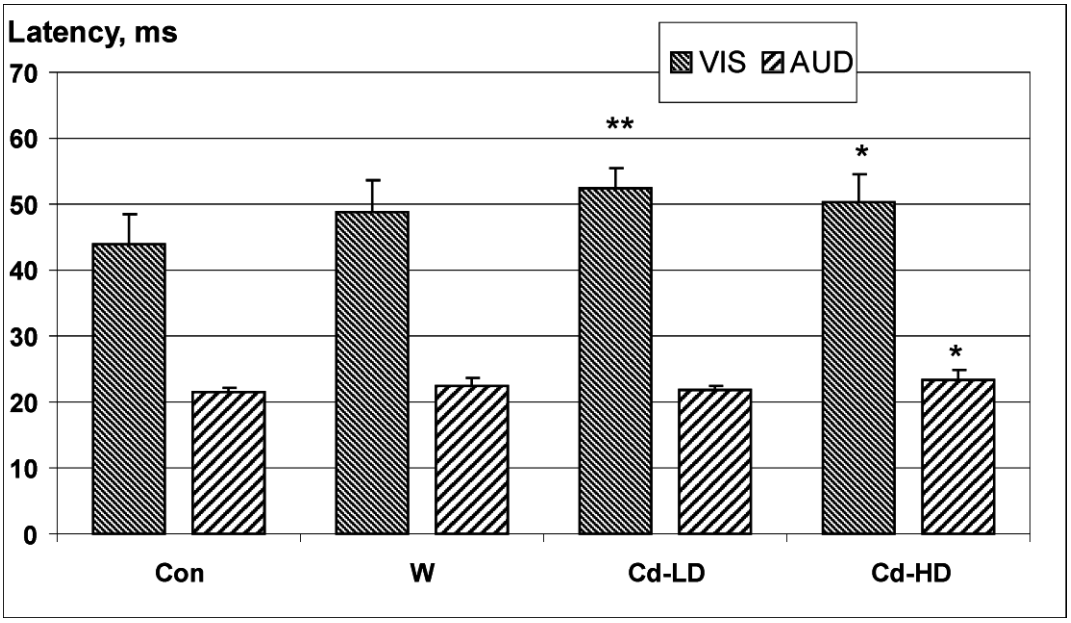

Figure 5
Latency of the visual and auditory (see insert) evoked potential after 6 weeks exposure.
Abscissa: group codes. Mean+SD, n=10.
*, **: p<0.05, 0.01 vs. *Con*.

Discussion

The metal levels, as well as the electrophysiological and biochemical changes, indicated that cadmium instilled into the rats' trachea in form of CdO_2 NPs was in fact absorbed from the airways and unfolded its toxicity. The time trend of body weights and the increase of alterations after 6 vs. 3 weeks suggested a gradual build-up of Cd. Absorption of the metal via the airways has been described repeatedly. Takenaka et al. (2004) detected Cd in the blood, liver and kidney of rats after inhalation cadmium oxide NPs – in an experiment with much shorter duration, and faster tissue sampling after exposure than it was in our work, which may explain the main difference viz. the absence of detectable Cd level in our treated rats' blood. In bulk, Cd oxide is hardly water soluble but its absorption from the lung is rather good (Oberdörster, 1979). No detectable blood Cd level in our work probably meant that the absorbed amount was promptly sequestered in the liver where it was detected in fact. In the study by Dill et al. (1994) the blood Cd level after ca. 3 months inhalation exposure by CdO particles of about 1 μm diameter was 10^3 times lower than in the kidney, the other organ known to accumulate Cd in the organism. The absence of noteworthy amounts of Cd in the blood was, logically, the reason for not detecting Cd in our brain samples. Beyond that, Cd is known to have low permeability across the blood-brain barrier (ATSDR, 2008) and no transneuronal movement from the periphery to the brain (Tjälve et al., 1996).

In spite of the latter, neurotoxicity of Cd in humans has been reported repeatedly (see Introduction). In exposed workers, elevated urine Cd level was associated with reduced visuomotor performance and difficulties of concentration and stance (Viaene et al., 2000), and with peripheral neuropathy (Viaene et al., 1999). In children, the exposure indicator was hair Cd, and the outcomes, cortical EPs (Thatcher et al., 1984) and behavior (Marlowe et al., 1985). The significant change in the latency of cortical EPs in our work, without detectable Cd deposition in the CNS, was probably due to secondary effects. Along the peripheral part of the afferent pathways, Cd^{2+} ions, if present, could interfere with ion channels (primarily Ca-channels: Viarengo and Nicotera, 1991), and with mitochondrial energy production (López et al., 2006), resulting in delayed arrival of the excitation to the subcortical and cortical centers, and so to lengthened cortical EP latency. Cd-induced liver damage could affect the substrate supply for synthesis of monoamine transmitters (Yourdaydin et al., 1990), the abnormal activity of which is known to alter cortical electrical activity (Sebban et al., 1999). The ECoG shift in the present study was similar to that found with oral application of dissolved Cd for 12 weeks (Papp et al., 2003).

The oxidative stress inducing effect of Cd is indirect; due, among others, to depletion of GSH (Valko et al., 2005) and to mitochondrial damage (López et al., 2006). In our treated rats' lungs (the organ having the highest Cd load) GSH level was in fact significantly reduced. The increase in the brain samples was possibly of compensatory nature. Others, e.g. Tandon et al., (2003) found depletion of GSH in Cd-exposed rats but in that experiment Cd was applied per os in dissolved form, in higher dose (1.5 mg/kg b.w.) and was detected in brain samples after 5 days treatment.

In spite of some disagreements with others' findings it can be stated that the data, presented above, emphasize the role of the nano-sized fraction of Cd-containing industrial fumes in the causation of nervous system damage, and show that it is possible to model the human neurotoxic damage caused by inhalational Cd exposure in rats.

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Conflict of Interest Statement

None declared.

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