EXPERIMENTAL MODELS AND INSTRUMENTAL SURVEYS FOR RISK ASSESSMENT IN HYGIENE AND EPIDEMIOLOGY

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RESEARCH ON ACUTE TOXICITY OF NANODISPERSE MANGANESE OXIDE AEROSOL FOR PREDICTING HEALTH HAZARDS FOR WORKERS AND POPULATION UNDER INHALATION EXPOSURE

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Our research object was nanodisperse manganese oxide synthesized at Mn^{2+} and MnO_4 ions interaction when nano-reactors, namely bromide cetyltrimethylammonium micelles, were present but they didn't become a part of an end product. We applied scanning electronic microscopy, X-ray phase analysis, dynamic laser light scattering, Brunauer, Emmeth, Taylor and Barret, and Joyner and Halenda techniques to confirm that the synthesized substance was a nanomaterial with particles sections having a needle form and being equal to mostly 13–29 nanometers (95.6 % of the total particles number).

Acute inhalation toxicity was assessed in conformity with the procedures stated in "OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects, Acute Inhalation Toxicity – Acute Toxic Class Method" (OECD, Test № 436:2008, IDT); it revealed that synthesized nanodisperse manganese oxide had acute toxicity when it was inhaled as an aerosol. CL_{50} under 4-hours exposure which male and female Wistar rats with body weight being equal to 190 ± 10 grams had to undergo was 120 mg/m^3. Acute intoxication had the following clinical picture: irritating and neurotoxic effects, and respiratory depression. As per CL_{50} criterion (>50–500) mg/m^3 the tested substance is of the 2nd hazard degree (in accordance with the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and of the 1st hazard degree (according to the State Standard 12.1.007.76. Classification and general safety requirements). The obtained inhalation toxicity parameters which nanodisperse manganese oxide has prove the substance can exert hazardous impacts on workers' health when they are exposed to it at the work places or on population health; they also call for safety precautions development.

Key words: *nanodisperse manganese oxide, aerosol, inhalation exposure, particles concentration, toxicity, health hazard.*

Nano-sized manganese oxide particles are a very promising nano-material for creating high technological components which are applied in up-to-date domestic industries such as nanoelectronics, nano-optics, and nanochemistry. In relation to that, manufacture and consumption of products with nano-disperse manganese oxide being one of their components have been growing quite rapidly over the last decade. Nano-disperse manganese oxide is widely used in manufacturing semi-conductor thermistors [1], solar batteries, various electrical appliances, cathode accelerators, nano-magnetic materials, and sorbents [2]. Sensory electrodes and biological sensors

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creation is another specific and very promising field where threadlike particles of manganese oxide can be applied [3].

Research results by Hussan, Stefanescu, and Frick [4–6] allow to assume that under specific conditions manganese oxide particles when introduced into a body via inhalation can cause adverse impacts on health, including toxic effects. Some data imply that catalytic generation of active oxygen forms (AOF) occurs in human alveolar epithelial cells and it enhances after 24-hour exposure [6]. Also, extracellular and intracellular oxidized glutathione form (GSSG) grows by 30 and 80% correspondingly in case of such exposure [4]. Elder et al. and Oberdorster describe certain conditions under which manganese oxide nanoparticles sized up to 30 nm are able to penetrate into PC-12 neuron-like brain cells via the olfactory bulb [7, 8], as well as accumulate in astrocytes and other brain cells [8, 9]. Researchers observed slight inhibition of mitochondrial activity in their experiments; it was combined with dose-dependent decrease in concentration of dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid and homovanilinic acid. All the detected phenomena were shown to be accompanied with a significant increase in AOF [5, 10], and neuro-degenerating disorders which occurred already after 2-3 weeks of exposure [7, 8], proteolytic breakdown activation mediated with caspase-3 and protein kinase Cδ (these enzymes take part in apoptosis, necrosis, and inflammatory processes). Phosphorylation cycle activation was also proved [10–13].

Crittenden and Filipov [14] described that when manganese nano-oxide particles concentration grew, it led to a linear increase in the level of p38 mutagen-active protein kinase. The latter, in its turn, stimulates apoptosis mechanism or early cells death. Elder et al. indicated there were facts proving that exposure to the substance in the olfactory bulb, frontal cortex, midbrain, and striate body, make gene expression of tumor necrosis factor-α double [7]. Long-term inhalation exposure to manganese oxide nano-particles is detected to cause transferrin activation in dopaminergic nervous cells. Researchers also revealed structural changes in Beclin 1 and LC3 proteins, which, in its turn, may indicate at potential autophagia process activation [5].

Given all the above stated, if we want to implement promising production technologies which involve nano-sized manganese oxide application efficiently and on a large scale, it is necessary to provide safety for workers and population. And here it becomes truly vital to examine toxicity parameters of nanodisperse manganese oxide aerosol at inhalation introduction into a body [15].

Data and methods. We performed our experiment on water suspension of nanodisperse magnesium oxide which was synthesized via direct interaction between Mn2+ and MnO4 ions with micelles cetyltrimethylammonium bromide (CTAB, C16H33(CH3)3NBr) acting as nonreactors [16]. To do this, weighed CTAB was dissolved in alcohol at a room temperature under vigorous stirring for 30 minutes (CTAB/EtOH = 1:10). The alcohol CTAB solution was added with the water solution of 0.4M MnSO4·5H2O. The mixture was constantly stirred for 24 hours and a water solution KMnO4 of 0.05M was added into it slowly, drop-wise. This new mixture was also stirred continuously for another 24 hours to complete the reaction. The dark brown residue was washed with the distilled water. CTAB was removed by an extraction with ethanol, the extraction degree being not less than 98%. The residual concentra-

tion of cetyltrimethylammonium bromide after extraction was determined via chromato-mass spectrometry with tandem mass-spectrometric detector Agilent. The suspension matrix was the bidistilled water corresponding to the specifications TU 6-09-2502-77[1].

Size and shapes of manganese oxide particles in the water suspension were evaluated with dynamic laser light scattering performed with Horiba LB-550 analyzer ("Horiba", Japan) (The Department for Chemical and Analytical Research Techniques at the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies), via electronic microscopy performed with S-3400N ("HITACHI", Japan) high resolution scanning microscope (3-10 nm, maximum magnification equal to 300000X) with energy dispersive X-ray attachment for microanalysis ("Bruker", Germany) (Chemical Technology Department at Perm National Research Polytechnic University).

Textural parameters were determined via nitrogen sorption at 196 □C with ASAP 2020 (Micromeritics, США) but previously the examined material was degassed in vacuum during 3 hours. The samples specific surface area (SBET) was calculated as per a procedure developed by Brunauer, Emmett and Taylor [17]. The total pores volume (Vtot) was calculated from the amount of nitrogen adsorbed at a relative pressure p/p0≈0.99. Pores size distribution was determined by the desorption isotherms with a procedure developed by Barrett, Joyner and Halenda [18]. The structure of the sample was studied by X-ray diffraction (XRD) with XRD-7000 diffractometer (Shimadzu, Japan) under CuKα-radiation in the range of angles 2α=1–8°.

Mass concentration of manganese oxide in the suspension was determined via mass spectrometry with inductively coupled plasma (on Agilent 7500cx with octopole reactive/collision cell), with helium used as a reagent gas. Prior to any evaluations, the substance was dispersed with Sonopuls Hd 3200 ultrasound homogenizer by "Bandelin" in order to destroy any aggregates and agglomerates which could possibly occur due to the sample ageing. We achieved homogenous distribution of the particles in the volume via continuous pulsation during 2 minutes at a room temperature.

We assessed acute toxicity of nanodisperse manganese oxide at inhalation introduction as an aerosol according to the established procedure fixed in State Standard 32646-2014[2]. We performed our acute experiment on pubescent Wistar rats (bucks and does) with body weight equal to 190±10 grams. All the animals were kept in standards cages made of polypropylene, two rats in each, and they all before the experiment underwent 14-day quarantine. The cages were placed in a ventilated room, air temperature was 23.0±2.0 ^{o}C, air humidity was 60.0±5.0 %. The experimental animals were provided with semi-synthetic nutrition ration which fully satisfied their biological needs. They had free access to food and water. All the examinations and procedures strictly conformed to

[1] TU 6-09-2502-77. Highly purifiem water, OSCh trademark 27-5. technical conditions. 1978, 31 p.

[2] State Standard 32646-2014. Experimental techniques for assessing impacts exerted by chemicals on a human body. Acute inhalation toxicity – a procedure for acute toxicity class determination (ATC procedure). Moscow, Standardinform Publ., 2015. Available at: http://docs.cntd.ru/document/1200116047 (28.07.2017).

the principles and standards set forth by the European convention for the protection of vertebrate animals[3]. Experiments were controlled by the Ethical Committee of the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies.

Inhalation introduction of manganese nano-oxide was provided via an inhalation system with a chamber for the whole body (TSE Systems GmbH) and integrated software. To prevent particles ingestion off hair we placed the experimental animals into individual cages. They were divided into two experimental groups, each made of 3 bucks and 3 does. The first experimental group underwent 4-hour inhalation exposure with manganese oxide concentration being 0.05 mg/dm3; the second experimental group also underwent 4-hour exposure but the admixture concentration in its case was equal to 0.5 mg/dm3. The speed of air flow in and out of the inhalation chamber was equal to 10 l/min. The suspension was fed into the aerosol generator at a speed being equal to 0.014 ml/min and 0.17 ml/min correspondingly. It allowed us to create homogenous substance circulation in the chamber. Air pressure in the chamber was maintained at -0.2±0.2 millibar, and air temperature, at 22–25°C. Oxygen level in the chamber was equal to about 19%; carbon dioxide concentration didn't exceed 1%.

Actual manganese oxide concentration in the inhalation chamber was estimated via mass spectrometry technique with inductively coupled plasma on Agilent 7500cx. Air sampling in the inhalation chamber was performed with the use of AFA-VG-10-1 filter (Russia) at the speed equal to 2 l/min during 5 minutes after 2 and 4 hours of exposure. Nanoparticles quantity in the chamber (concentrations) was performed with a diffuse aerosol spectrometry made by "AeroNanoTech" LLC (Russia).

The experimental animals didn't receive any food during exposure.

After exposure was over, the animals were under observation during 4 days allowing for any possible delayed toxicity effects. We determined hazard category as per average lethal concentration of a substance (CL50) according to animals deaths parameters, applying a procedure described in the State Standard 32646-2014[2], as well as inconformity with the State Standard 12.1.007.76[4].

Results. Examinations performed on the sample suspension in concentration equal to 36±2.3 mg/ml with CTAB residual in it being lower than the detection limit (0.00001 mg/ml) revealed that the particles distribution as per their cross section size is as follows: 13 nm (1.2 % out of the total particles number), 15-29 nm (94.3 % out of the total particles number), and 33-87 nm (4.1 % out of the total particles number). The greatest share in the suspension belongs to particles sized 19±4 nm (41.2 % out of the total number) (Table 1).

Scanning electron microscopy revealed that the particles being visualized (those exceeding 20 nm in size) were mostly threadlike (97.8% from the whole number of visible particles).

[3] The European convention for the protection of vertebrate animals used for experimental and other scientific purposes. Strasburg, 1986, 13 p.

[4] ГОСТ 12.1.007-76. System of Labor Safety Standards (SLSS). Hazardous substances. Classification and overall safety requirement (with Alterations No. 1 and 2). Available at: http://docs.cntd.ru/document/5200233 (28.07.2017).

Table 1

Manganese oxide nanoparticles dispersity in water suspension

Particles size in suspension, μm	Particles share, %	Particles size in suspension, μm	Particles share, %
0,0131	1,2	0,0387	0,38
0,0150	9,6	0,0443	0,64
0,0171	16,4	0,0507	0,90
0,0196	20,0	0,0581	0,73
0,0225	21,2	0,0666	0,42
0,0257	13,9	0,0762	0,39
0,0295	13,2	0,0873	0,15
0,0338	0,44		

The adsorption-desorption isotherm of nitrogen corresponds to the type IV (isotherm with a distinct capillary condensation). The shape of the hysteresis loop belongs to H3 type with the distinct area of mesopores filling within the range of relative pressures (p/p0) 0.7–1. Mesopores filling at higher relative pressures verifies the presence of large diameter mesopores (Figure 2). The specific surface area (SBET) of the nano-disperse manganese oxide particles amounted to 150.2 ± 2.6 m2/g; the total pore volume, to 0.676 cm3/g. The results of X-ray phase analysis showed the absence of the mesopores ordered structure (Figure 3).

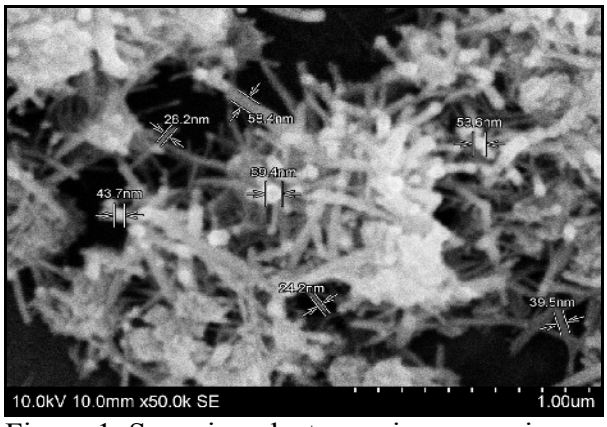

Figure 1. Scanning electron microscopy image of nanodisperse manganese oxide particles

Data on physical parameters of manganese oxide nanoparticles obtained via template synthesis corresponded to properties needed for sensory electrodes creation [3] and correct acute inhalation exposure modeling.

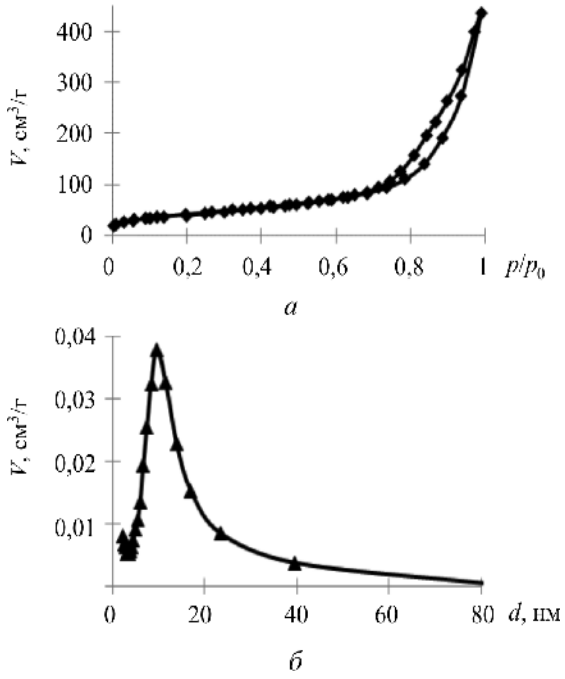

Figure 2. Nitrogen absorption-desorption isotherm (A) and pores distribution as per size d(нм) (B) for nanodisperse manganese oxide

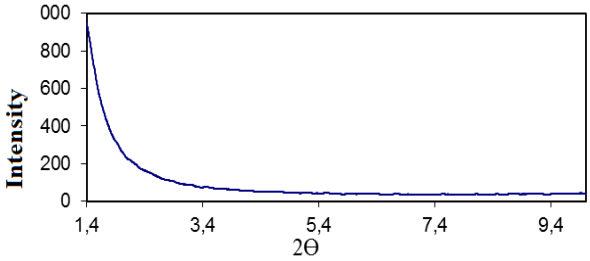

Figure 3. X-ray pattern of the nanodisperse magnesium oxide

Data obtained due to analytical measuring proved that if preset manganese oxide concentration was equal to 0.05 mg/l, then actual concentration in the inhalation chamber amounted to 0.029±0.001 mg/dm3; and with preset concentration equal to 0.6 mg/l, the actual one was 0.472±0.005 mg/l. Estimation of nanoparticles quantity in the air in the chamber revealed that when nanodisperse fraction was fed into it and transformed into aerosol, it didn't agglomerate up to micro-meter sizes (Table 2). The greatest number of particles at the examined actual concentrations didn't exceed 100 nm in size after 2- and 4-hour exposure.

Table 2

Examinations of nanodisperse manganese oxide particles concentrations and sizes in the inhalation chamber air

Parameters	Initial level	After 2-hour exposure	After 4-hour exposure
Manganese oxide concentration, mg/dm3	0,002	0,456	0,472
Particles concentration, sized 0-20 nm, un./dm3	63	10421	9980
Particles concentration, sized 20-40 nm, un./dm3	48	35 930	31 207
Particles concentration, sized 40-60 nm, un./dm3	42	35 602	36 783
Particles concentration, sized 60-80 nm, un./dm3	22	26 370	25 809
Particles concentration, sized 80-100 nm, un./dm3	8	17 783	14 320

Table 3

Dynamics of the experimental animals deaths during research on acute inhalation toxicity which nanodisperse manganese oxide had when introduced as water suspension aerosol

Animals group	Actual concentration, mg/l	Number of animals in group, abs.	Observation duration in days (hours)				Number of dead animals	
			1 (1)	2 (2)	3 (3)	4 (6)	abs.	%
No. 1	0,029 ± 0,001	6	0/6	0/6	0/6	0/6	0	0
No. 2	0,472 ± 0,005	6	0/6	0/6	5/6	6/6	6	100

Clinical picture at an initial stage of acute intoxication with nanodisperse manganese oxide aerosol in actual concentration being equal to 0.029±0.001 mg/l included the following effects: all the animals started to sneeze and cough, they had colorless discharge from their noses which evidenced inflammation evolvement in the respiratory tracts. Starting from the 3rd hour of exposure and up to the 4th one, animals had tachypnea and dyspnea. And here we registered that animals' accessory muscles had to participate in their respiration; we also detected forced postures (backs bent, heads tilted, etc.). When the 4th hour of exposure started, the animals became depressed and sluggish, they had no reaction to sound stimuli, and their movement coordination was disordered. During 24 hours after exposure we detected body trembling, motor activity absence, and extremely weak reaction to sound stimuli in the experimental animals; they also refused to eat or drink. 72 hours after exposure there were no signs of depression, and the animals ate their food. However, we detected that motor activity and reactions to sound stimuli were rather weak. We didn't register any animals death in this experiment over 96 hours of observation.

Clinical picture of animals intoxication under exposure to nanodisperse manganese oxide in actual concentration equal to 0.472±0.005 mg/l was rather different. Thus, respiratory failure was detected already starting from the 30th minute of exposure. After 3 hours of exposure we registered respiratory depression and animals deaths. We fixed that the animals were sluggish before they took the lateral position; motor activity and any reactions to sound stimuli were completely absent. 83% of the exposed animals died within 150-190 minutes after the experiment started (Table 3).

According to acute inhalation toxicity assessment, CL_{50} of the examined nanodisperse manganese oxide was assumed to be equal to 120 mg/m^3. This concentration is within 50-500 mg/m^3 range, which allows us to rank the examined substance, nanosized manganese oxide, among the sub-

stances with the 2nd hazard class as per The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and among the substances with the 1st hazard class according to the State Standard 12.1.007.76[3]

Discussion. Threadlike particles of nanodisperse manganese oxide are applied as an active matrix in sensory electrodes and storage batteries manufacture; it is seen as a most promising technology. Application of the substance causes its probable occurrence in the working area air and its further spread over territories located in the vicinity of production plants. And it leads to hazards of possible inhalation exposure for workers employed at such productions and for population living on territories close to them.

The results which we obtained in our research prove that the examined nanosized manganese oxide particles are able to cause negative effects: irritating and neurotoxic ones, as well as respiratory depression; their combination probably caused the experimental animals deaths.

Toxic impacts exerted by the substance on the nervous system cells and negative neuropsychological effects occurring under exposure even to low doses have been proved both for manganese oxide nanoparticles and its micro-disperse analogue [10, 12, 19]. Neurotoxic mechanism can be based on functional disorders in neuron membranes which result from membrane lipids peroxidation caused by direct cytotoxic effects exerted by nanoparticles; these effects were detected for dopaminergic neurons [10, 12, 20]. These effects can be much more apparent when they are caused by nanodisperse particles in comparison with their microdisperse analogue; it is due to the fact that nanodisperse particles have greater specific surface area. Clinical picture of acute intoxication proves toxic effects exerted by nanodisperse manganese oxide particles which have been described in the previous research works [21]. Respiratory failure occurrence can be related to a potential ability of the examined nanoparticles to cause inflammatory changes with the consequent alveolar epithelial cells apoptosis. And given the fact that nano-sized manganese oxide particles are more resistant mucociliary clearance, they contact the respiratory tract cells much longer than their microdisperse analogue [22]. The CL50 value for the substance is equal to 120 mg/m3; therefore, nanodisperse manganese oxide belongs to substances having the 2nd hazard class according to the international classification for chemicals' hazards [23] and to substances having the 1st hazard class according to the State Standard 12.1.007.763.

Conclusions. Nanodisperse manganese oxide with its threadlike particles having their cross section sizes mostly equal to 13-29 nm had acute toxicity at inhalation introduction as an aerosol. CL50 for Wistar rats amounts to 120 mg/m3 under 4-hour exposure. Clinical picture of acute inhalation exposure involves the following: irritating and neurotoxic effects as well as respiratory depression. Neurotoxic effects remained in the animals who survived the experiment even 92 hours after the exposure. So, the substance has the 2nd hazard class (according to CL50 criterion) as per the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and the 1st hazard class as per the State Standard 12.1.007.763[3].

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