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To cite this article: M P Sutunkova *et al* 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **918** 012119

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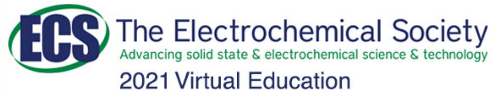

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IOP Conf. Series: Materials Science and Engineering **918** (2020) 012119 doi:10.1088/1757-899X/918/1/012119

Multisystemic damage to mitochondrial ultrastucture as an integral measure of the comparative *in vivo* cytotoxicity of metallic nanoparticles

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Abstract. Vehicles emissions of nanoparticles is a one of the major threat to humans in the modern conditions. Subchronic intoxication was induced in outbred male rats by repeated intraperitoneal injections of lead oxide, zinc oxide and copper oxide nanoparticles separately, or in three binary combinations, or in the full triple combination. Based on electron microscopy results, this paper considers the usefulness, feasibility and informativity of an approach based on a generalized semi-quantitative assessment of toxic damage to mitochondria in various organs using partial or complete destruction of cristae as an index of damage. The adequacy of such assessment is confirmed by its consistency with the previously published data on the relative and combined toxicity of nanoparticles of the above species and high protective efficacy of a complex of bioprotectors estimated by a great number of functional and optical-microscopy morphometric indices.

1. Introduction

Vehicles emissions of nanoparticles is a one of the major threat to humans in the modern conditions. (23rd ETH-Conference on Combustion Generated Nanoparticles, June 17th - 20th, 2019 at ETH Zurich, Switzerland). Mitochondria and mitochondrial DNA of cells in various organs and tissues are increasingly often considered to be a major subcellular target of exposure to the harmful effect of not only drugs but also various environmental toxicants (see reviews by [1-3]) though it is admitted that for some intoxications there could also be other primary sites of toxic action, and that damage to mitochondria may be a secondary effect. Examples from experimental research related to the toxicology of particulates including nanoparticles are [5-8], as well as such metals as cadmium and copper [9-12], manganese and lead [13-16], arsenic [17-19], and mercury [12, 16, 20].

It should be noted that researchers tend to focus on toxic damage, in vivo or in vitro, to some mitochondrial functions whereas visible damage to the ultrastructure (swelling, partial or full loss of cristae) revealed by transmission electron microscopy (TEM) is either ignored or is presented without any quantitative or, at least, semiquantitative ("score-based") assessment. In our studies, we aimed to identify an approach to such assessment that could render TEM characterization informative in comparative toxicological studies, and this paper presents our solution to this issue.

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IOP Conf. Series: Materials Science and Engineering **918** (2020) 012119 doi:10.1088/1757-899X/918/1/012119

For developing our approach, it was important to decide whether it would be appropriate to use a generalized analysis of data merged by one of the following two defining criteria.

Firstly, would it be admissible to combine in one quasi-homogeneous set data relating to cell mitochondria in different organs of an animal exposed to a certain toxic in vivo? There may be doubts concerning such merging of data because, as was stressed in one of the above-mentioned reviews [3], "mitochondria vary dramatically from tissue to tissue …, so it is not surprising that evidences from mitochondrial disease and mitochondrial drug toxicity indicate that different cell types are differentially susceptible to mitochondrial toxicants". So we had to check this statement in relation to the specific toxicants that we studied (metal oxide nanoparticles). Nevertheless, we assumed that, despite some differences between tissues, the mitochondria of all eukaryotes are of the same evolutionary origin and have the same set of important functions [21], their ultrastructure in the cells of all organs (within a concrete biological species) being largely similar, as well as disturbances to this ultrastructure. We thus believe that there are sufficient grounds to regard all mitochondria of the animal organism under various intoxications as one target of a specific toxic action in spite of the fact that their sensitivity to this toxic action in cells of different organs can be different.

The alternative criterion of experimental data merging that we assumed admissible a priori but that also had to be checked was the pooling of all estimated mitochondria of a certain organ irrespective of which specific toxicant the animal was exposed to in an experiment assuming that the mechanisms of the damaging action of toxicants are, in the first approximation, common to all of them. Mitotoxicity is quite likely to be one of these mechanisms in relation to the metal oxide nanoparticles that we study [2, 22].

A technique for addressing these challenges has been developed and tested on the basis of actual data from an experimental work the other results of which have been described in detail elsewhere [23].

2. Materials and methods

2.1. Nanoparticles

The suspensions of metal oxide nanoparticles (MeO-NPs) were produced by laser ablation of 99.99% pure metal (copper, lead or zinc) targets in deionized water. The details of this technique as well as the methods used for characterization of the obtained MeO-NPs were described by Minigalieva et al. (2017). In the present paper, we confine ourselves just to stating that their chemical composition was identified as CuO, PbO and ZnO; and that PbO-NPs and CuO-NPs were virtually spherical with a mean (±sd) diameter of 47±16 nm and 24.5±4.8 nm, respectively, while ZnO-NPs were rod-like with dimensions 83±20 x 30±11 nm.

The stability of all three nano-suspensions proved sufficient for carrying out the animal experiment described below without adding any chemical stabilizer.

2.2. The animal experiment.

The experiment was carried out on outbred male white rats from our breeding colony with an initial body weight of 150 to 220 g, a minimum of 12 animals in different exposed and control groups. The rats were housed in conventional conditions, breathed unfiltered air, and were fed standard balanced food. The experiments were planned and implemented in accordance with the "International guiding principles for biomedical research involving animals" developed by the Council for International Organizations of Medical Sciences (1985) and were approved by the Bio-Ethics Committee of the Ekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers.

As well as in other nanotoxicological studies published by our team [24-32 and others], the subchronic toxicity of the Me-NPs was investigated using repeated intraperitoneal (i.p.) injections of nano-suspensions. The experiment involved 8 groups of rats exposed in parallel during 6 weeks to 18 i.p. injections of:

•either CuO-NPs, or PbO-NPs, or ZnO -NPs in a dose of 0.5 mg in 1.0 mL of the

IOP Conf. Series: Materials Science and Engineering **918** (2020) 012119 doi:10.1088/1757-899X/918/1/012119

suspension plus 2.0 mL of deionized water;

•either [CuO-NPs+ PbO-NPs], or [ZnO-NPs + PbO-NPs], or [CuO-NPs+ ZnO-NPs] in a dose of 0.5 mg for each Me-NP species in 1.0 mL of a respective suspension plus 1.0 mL of deionized water;

•CuO-NPs+ PbO-NPs +ZnO-NPs in a dose of 0.5 mg for each Me-NP species in 1.0 mL of a respective suspension;

•3.0 mL of deionized water.

In the groups exposed to [CuO-NPs+ PbO-NPs +ZnO-NPs] together or to water without particles, 50% of the rats were orally administered during the same 6 weeks a bioprotective complex (BPC) comprising glutamate, glycine and cysteine (the latter in a highly active and metabolically well available form of N-acetylcysteine), vitamins A, E, and C, ω-3 polyunsaturated fatty acids; selenium, calcium, iron and iodine supplements, and pectin enterosorbent. The doses and methods of administration of these bio-protectors as well as theoretical premises for their inclusion into the BPC have been published by Minigalieva et al [23].

After the exposure period and registration of some functional indices for the organism's status, the rats were killed by decapitation, and their blood, collected by exsanguinations, was used for hematological and biochemical tests while the liver, spleen, kidneys, and brain – for histological examination with morphometry. All these indices and tests were described by us earlier [23], and they are not presented here because in this paper we do not describe the respective results. The intracellular accumulation of NPs by different organs and ultrastructural cell alterations were visualized by scanning transmission electron microscopy (STEM). To this end, pieces of an organ were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in a cacodylate buffer with 5% sucrose at pH 7.3, then post-fixed in 1% osmium tetroxide, contrasted with uranyl acetate en bloc and embedded in epoxy resin (Spurr). This sample preparation procedure was carried out in a microwave tissue processor, HISTOS REM (Milestone, Italy). Semi-thin (900 nm thick) sections of epoxy blocks were stained in toluidine blue with the addition of 1% borax and examined under the optical microscope for choosing a site for STEM. The 60 nm ultrathin sections of this site obtained using ultramicrotome (Power Tome, «RMC», USA) were contrasted with uranyl acetate and lead citrate. Grid-mounted sections were investigated by means of scanning electron microscope, Workstation AURIGA CrossBeam (Carl Zeiss NTS, Germany) in the STEM mode in the magnification range from 1200 to 200000.

For semi-quantitative estimation of the mitochondrial damage, we ranked each investigated organ of each rat by three scores: 0 – no mitochondria found with any visible damage; 1 - below 50%, and 2 – above 50% of examined mitochondria have explicit damage up to complete loss of cristae. Based on this scoring, we used 2 integrated estimates of mitochondria state in an organ of a group of rats, namely: (a) the weighted arithmetic mean score and (b) the proportion of mitochondria with any loss of cristae. Then, based on the assumptions described in the Introduction, we aggregated these estimates both for a given organ across combined groups of rats and for each group across all organs taken together.

The total number of estimated mitochondria in relation to which these indices were calculated is given in Table 1.

Table 1. Total number of mitochondria examined in different organs of control and Me-NP exposed rats.

Groups of rats	Organs							
	Brain	Heart	Kidney	Liver	Spleen	Testicle	Thymus	All together
Control	34	29	23	85	41	42	16	270
ZnO - exposed	50	80	64	68	92	76	84	514
PbO- exposed	44	85	113	32	46	93	91	504
CuO- exposed	36	37	40	50	38	111	24	336
(CuO+PbO) - exposed	82	36	36	34	91	121	16	416
(CuO+ZnO) - exposed	23	47	34	58	15	168	56	401

(PbO+ZnO) - exposed	26	82	88	103	73	142	92	606
(CuO+PbO+ZnO)	29	26	52	55	44	209	23	438
(CuO+PbO+ZnO) – exposed against the BPC background	44	2626	119	55	89	56	23	412
All together	368	448	569	540	529	1018	425	3897

3. Results and discussion

Scanning transmission electron microscopy (STEM) of different tissues revealed uniform ultrastructural changes, the most frequent being vacuolization of the cytoplasm and formation of concentric membranous bodies within it and especially damage to mitochondria with partial or complete loss of cristae (Figs. 1 - 2). In the STEM images of all organs one can see nano-sized electron-dense inclusions (Fig. 3), which are absent in the controls and are most likely to be the NPs that the rats were injected with. The above-mentioned pathological changes, however, were not always associated with the intracellular localization of the NPs: thus, we failed to detect any NPs in the testes while the mitochondrial damage was most pronounced just in the testicular tissue in the majority of the groups (see Tables 2 and 3).

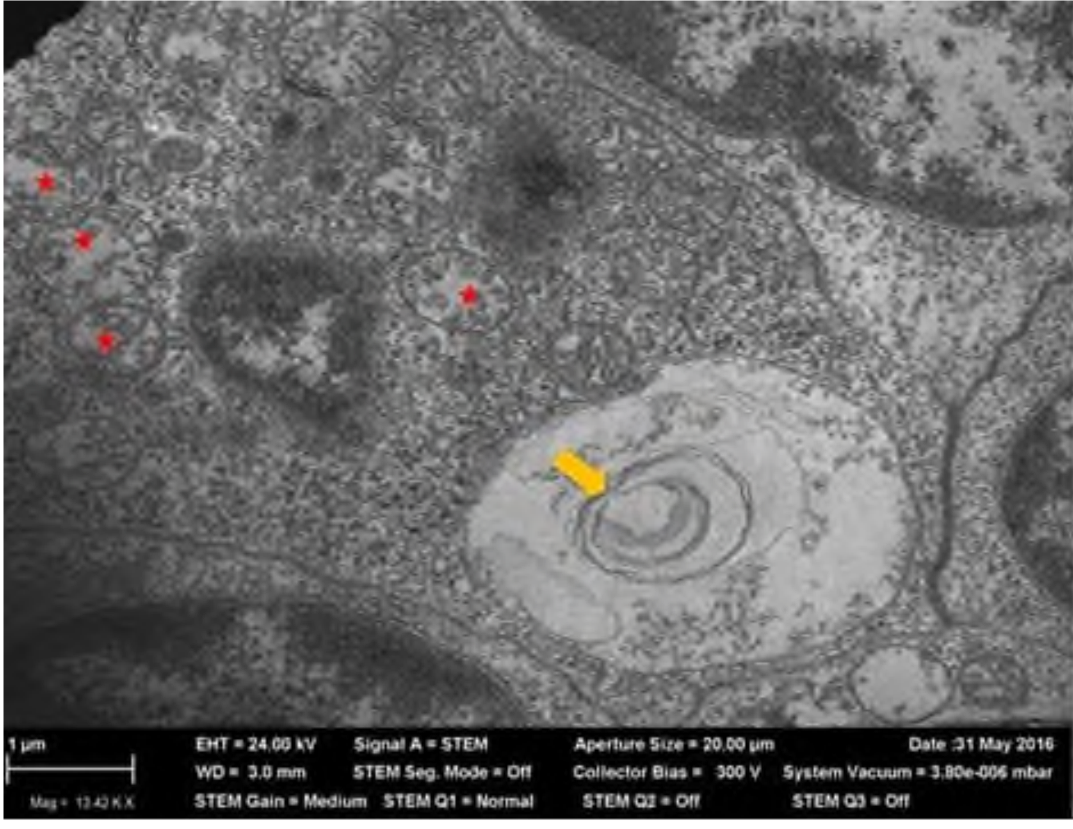

Figure 1. Concentric membranous formation and cytoplasmic vacuolization (arrow), and marked damage to mitochondria (asterisks) in a spleen cell from a rat exposed to ZnO-NPs. STEM, magnification *13420.

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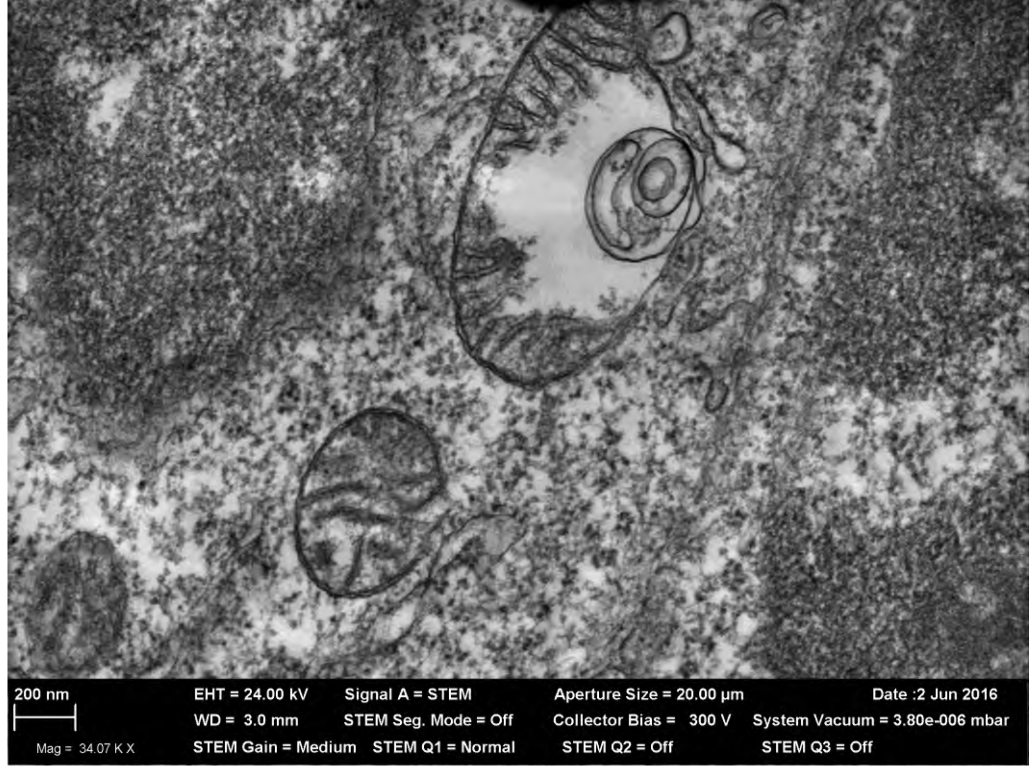

Figure 2. A partially destroyed mitochondrion (marked by asterisk) in a thymus cell of a rat exposed to PbO-NPs and ZnO-NPs . STEM, magnification *34070.

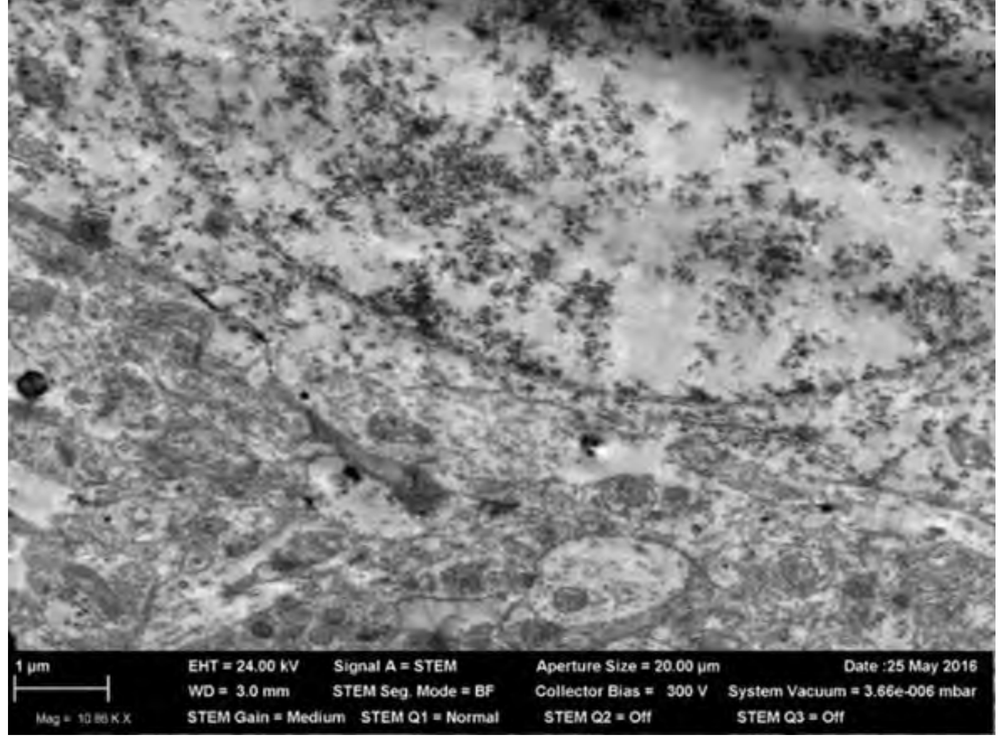

Figure 3. Numerous electron-dense nanosized granules in the cytoplasm and nucleus of a cell from the brain of a rat exposed to CuO-NPs + PbO-NPs + ZnO-NPs. STEM, magnification *10860.

Tables 2 and 3 present the results of mathematical processing as described in Section 2, applied to the visual assessments of mitochondria. In these Tables, we present together, for the purpose of more convenient comparability, the results of merging both by organ and by group. Such data representation does not, however, leave room for presenting each value together with its standard error, and we had to confine ourselves to denoting just the statistical significance of the differences between the indices pertaining to different organs or to different groups. Given a considerable number of such comparisons to be made, it was necessary to apply unconventional (but, hopefully, quite clear) notation.

Table 2. Weighted average score of damage to mitochondria by group and organ.

		Organs							
		a	b	c	d	e	f	g	
N	Groups of rats	Brain	Heart	Kidney	Liver	Spleen	Testicle	Thymus	All together
1	Control	0 .176	^{a}0 .000	0 .000	^{a}0 .000	0 .049	$^{b.c.d.e}$0 .214	0 .000	0.063
2	ZnO - exposed	0 .200	0 .275^{1}	0 .234^{1}	0 .162^{1}	$^{a.b.c.d}$0 .663^{1}	$^{a.b.c.d}$0 .553^{1}	$^{a.b.c.d}$0 .571^{1}	0.407^{1}
3	PbO- exposed	0 .409$^{1.2}$	^{a}0 .071^{2}	^{b}0 .478$^{1.2}$	^{b}0 .344^{1}	$^{b.c}$0 .239$^{1.2}$	$^{b.e}$0 .452^{1}	^{b}0 .429^{1}	0.359^{1}
4	CuO- exposed	0 .139^{3}	0 .108	$^{a.b}$0 .400^{1}	^{c}0 .080$^{1.3}$	$^{a.b.d}$0 .421^{1}	$^{a.b.d}$0 .423^{1}	0 .250$^{1.2}$	0.292^{12}
5	(CuO+PbO) - exposed	0 .390$^{1.2.4}$	^{a}0 .028^{2}	^{b}0 .222$^{1.3}$	^{a}0 .176^{1}	$^{b.c.d}$0 .538$^{1.3}$	$^{b.c.d}$0 .438^{1}	$^{a.e.f}$0 .063$^{1.2.3}$	0.361^{4}
6	(CuO+ZnO) - exposed	0 .217	0 .128	0 .176^{3}	$^{a.b.c}$0 .000$^{2.3.5}$	0 .000$^{2.4.5}$	$^{a.b.c.d.e}$0 .714$^{3.4.5}$	$^{b.d.e.f}$0 .375^{5}	0.394^{14}
7	(PbO+ZnO) - exposed	0 .115$^{3.5}$	0 .061^{2}	$^{a.b}$0 .341^{1}	$^{a.c}$0 .010$^{1.2.3.4.5}$	$^{a.b.d}$0 .479$^{3.6}$	$^{a.b.c.d.e}$0 .690$^{3.4.5}$	$^{a.b.c.d}$0 .565$^{1.4.5}$	0.370^{14}
8	(CuO+PbO+ZnO)	0 .172	0 .038^{2}	^{b}0 .308^{1}	^{c}0 .109$^{1.3.6.7}$	0 .227$^{1.2.5.7}$	$^{a.b.c.d.e}$0 .861$^{1.2.3.4.5.6.7}$	^{f}0 .130$^{1.2.3.7}$	0.505^{1234567}
9	(CuO+PbO+ZnO) – exposed against the BPC background	0 .091$^{3.5}$	0 .000^{2}	$^{a.b}$0 .261$^{1.3}$	^{c}0 .036$^{1.2.3.5}$	^{c}0 .022$^{2.3.4.5.7.8}$	$^{a.b.c.d.e}$0 .696$^{3.4.5}$	$^{b.d.e.f}$0 .217$^{2.7}$	0.201^{12345678}
	All together	0.239	^{a}0.100	^{ab}0.309	^{ac}0.076	^{abd}0.352	^{abcde}0.619	^{abcdf}0.412	

Note: The right-hand superscripts indicate the ordinal numbers of groups located above a given one from which a group is statistically significantly different ($p < 0.05$) in the value of the weighted score within a given column. Similarly, the left-hand alphabetical superscripts indicate the presence of a statistically significant ($p < 0.05$) difference from the weighted score for a given organ located to the left of a given one within the same row. The statistical significance of the differences between the weighted scores was estimated by computing Newcombe confidence intervals after transformation weighted score into binomial probability [33].

Table 3. Proportions of damaged mitochondria in groups of rats and in organs under study.

		Organs							
N	Groups of rats	Brain	Heart	Kidney	Liver	Spleen	Testicle	Thymus	All together
		a	b	c	d	e	f	g	
1	Control	0.176	$^{a}0.000$	$^{a}0.000$	$^{a}0.000$	0.049	$^{bcd}0.167$	0.000	0.056
2	ZnO - exposed	0.200	0.225^{1}	0.234^{1}	0.162^{1}	$^{d}0.348^{1}$	$^{abcd}0.395^{1}$	$^{abcd}0.429^{1}$	0.296^{1}
3	PbO- exposed	0.273	$^{a}0.071^{2}$	$^{b}0.283^{1}$	$^{b}0.313^{1}$	$0.174^{1.2}$	$^{be}0.355^{1}$	$^{b}0.330$	0.260^{1}
4	CuO- exposed	0.139	0.108	0.275^{1}	$^{c}0.080^{1.3}$	0.211^{1}	$^{abd}0.360^{1}$	$0.167^{1.2}$	0.226^{12}
5	(CuO+PbO) - exposed	$0.390^{1.2.3.4}$	$^{a}0.028^{2}$	$^{a}0.139$	$^{ab}0.176$	$^{bc}0.330^{1}$	$^{bcd}0.388^{1}$	$^{aef}0.063^{1.2.3}$	0.293^{14}
6	(CuO+ZnO) - exposed	0.217	0.128	0.118	$^{abc}0.000^{2.3.4.5}$	$0.000^{2.5}$	$^{abcde}0.494^{3.4}$	$^{def}0.268$	0.282^{1}
7	(PbO+ZnO) - exposed	0.115^{5}	0.061^{2}	$^{b}0.182^{1}$	$^{ac}0.010^{1.2.3.4.5}$	$^{abcd}0.329^{6}$	$^{abcd}0.444$	$^{abcd}0.446^{1.4.5.6}$	0.252^{1}
8	(CuO+PbO+ZnO)	0.172^{5}	0.038^{2}	0.173^{1}	$0.109^{1.3.6.7}$	$0.136^{1.2.5.7}$	$^{abcde}0.464^{1}$	$^{f}0.130^{2.7}$	0.290^{14}
9	(CuO+PbO+ZnO) – exposed against the BPC background	$0.091^{3.5}$	0.000^{2}	$0.134^{3.4}$	$^{c}0.018^{2.3.5}$	$^{c}0.022^{2.3.4.5.7.8}$	$^{abcde}0.375$	$^{bde}0.174^{2.7}$	$0.117^{12345678}$
	All together	0.223	$^{a}0.092$	$^{b}0.190$	$^{ac}0.072$	$^{b\,d}0.212$	$^{abcde}0.414$	$^{abcdef}0.315$	

Note: The right-hand superscripts indicate the ordinal numbers of groups located above a given one from which a group is statistically significantly different ($p < 0.05$) in the proportion of damage to mitochondria within a given column. Similarly, the left-hand alphabetical superscripts indicate the presence of a statistically significant ($p < 0.05$) difference from the proportion of damage to mitochondria for a given organ located to the left of a given one within the same row. The statistical significance of the differences was estimated by computing Newcombe confidence intervals for difference of binomial probabilities [33].

4. Discussion

When performing quantitative or semi-quantitative morphometric estimation of histopathological changes in the tissues of a laboratory animal revealed by optical microscopy, the researcher often obtains values related to a certain clearly identified type of cells (either a set of them or an individual cell of this type). A similarly cell-oriented morphometry is extremely difficult to perform in relation to ultrastructural changes revealed by electron microscopy since more often than not these changes are reliably visualized only under a magnification which does not enable one to see the cell general structure and boundaries within which they are found. A simple way out of this deadlock as proposed by us is described in the Materials and Methods and the theoretical grounds supporting the admissibility of such simplification are provided in the Introduction. We believe that the mathematical treatment and the logical analysis of experimental data performed with the help of this approach confirm convincingly (albeit indirectly) this admissibility.

As is seen from Tables 2 and 3, the degree of damage to the mitochondria in both controls and NP-exposed rats shows little intergroup differences for some organs, being, as we expected it (see the Introduction), noticeably different for others. Nevertheless, although the ranking of organs by this feature does not demonstrate the same pattern in different experimental groups, this ranking tends to

be similar in the majority of them. Thus, in terms of weighted average score, testicles rank first in 6 groups out of 9 (including the controls), spleen in 2 groups, and kidneys in one group only. The organ ranking last, also in 6 groups out of 9, is the heart, sharing this place in the control group with kidneys and liver and yielding it to liver or brain in two exposed groups only (Table 2). A similar pattern is displayed by the proportion of damaged mitochondria, for which testicles rank first in 5 groups out of 9, giving the first place to thymus or brain in the other four groups, though only by a very small margin over testicles (Table 3).

In addition to the theoretical premises mentioned in the Introduction, such close agreement between the actual results enabled us to be guided, in ranking the organs by degree of damage to mitochondria, by the data merged across all groups of rats taken together, which rendered the ranking in many respects much more obvious and statistically significant. Thus, in this case, the weighted average score decreases in the following sequence: testicles > thymus > spleen > kidneys > brain > heart > liver. If we consider the difference between neighboring ranks only, it proves statistically significant between ranks 1 and 2, ranks 4 and 5, and ranks 5 and 6, while the difference between more distanced ranks is statistically significant for even a greater number of organs. Thus, for example, the second place differs significantly not only from the first place but from all the others as well.

The ranking of organs by proportion of damaged mitochondria, as follows from Table 3, is not essentially different from the just considered ranking by weighted average score, being: testicles > thymus > brain > lien > kidneys > heart > liver.

As was stressed in the Results, these ranks do not match the differences between the organs established by visual estimation of NPs accumulated in them. Thus, whereas such accumulation was well visible in the brain (ranking only 5th by degree of damage to mitochondria), no metal NPs were observable in the testicles, which sustained greatest damage. This seems to be in agreement with the suggestion (see the Introduction) of a possibility of not only direct but also secondary damage to mitochondria caused by toxic agents. Probably, the blood-testis barrier, which is well-known to be one of the tightest blood-tissue barriers in the mammalian body [34], is almost impenetrable for nanoparticles but, nevertheless, does not fully preclude the penetration of metals in ionic form to the seminiferous epithelium, which appears to be also highly sensitive to their mitotoxic action. Note that the spermato-toxicity of various metal salts, and of lead in particular, has been demonstrated by various researchers [35]. On the other hand, the elevated circulation of lead, copper and zinc ions in rat blood and their elimination with urine under subchronic exposure to the metal oxide nanoparticles considered in the paper is beyond doubt [23]. As well as in the other experiments carried out by our team with various other metal nanoparticles, this may be explained by their solubility in biological milieus.

Going over to comparison of the various groups of rats by the same aggregated indices of damage to mitochondria, we again see some differences between the ranks of these groups for different organs. Thus, in terms of weighted average score of damage to mitochondria (Table 2), the PbO-exposed group ranks first for the brain, kidneys and liver; the ZnO-exposed group, for the heart, spleen and thymus; and the triple combined exposure group, for thymus. Similar (but, nevertheless, not the same), differences between the organs are observed for the proportion of damaged mitochondria as well (Table 3).

A considerably greater consistency between group ranks is observed for the indices merged for all organs. Now the mitotoxicity of NP species acting alone and estimated both by the average score and by the proportion of damaged mitochondria is not only doubtless when compared with the control values but also decreases in the sequence ZnO > PbO > CuO (with a statistically significant difference between the groups ranking first and third for both indices). Interestingly, cytological examination of the bronchoalveolar lavage fluid from rats after a single intratracheal instillation of the same NPs revealed that their decreasing pulmonary cytotoxicity as estimated by an increase both in the absolute number of neutrophil leukocytes and in the ratio of the latter to the number of alveolar macrophages [23]. As for the subchronic experiment considered in this paper, the majority of the few dozens of various functional and morphometric indices used in it also provide evidence of the lowest organ and

system toxicity of CuO nanoparticles. The effects of PbO-NPs and ZnO-NPs in relation to the same indices were more comparable, and where these effects were indeed different, they differed for various outcomes of intoxication with opposite signs [23].

The mitotoxic effect of the three binary NP-combinations was almost identical and was significantly higher compared only with CuO-NPs acting separately. The effect of the triple combination, although being in terms of the proportion of damaged mitochondria about the same as the effect of the binary combinations, proved to be the highest in terms of the average weighted score, being statistically significantly different from the effect of all the other NP-exposures.

What is most interesting is that, although the same triple intraperitoneal NP-exposure conducted along with peroral administration of a bio protective complex (BPC) also provided a proportion of damaged mitochondria and an weighted average score of damage to them that were significantly higher than the corresponding control values, they were noticeably and statistically significantly lower compared with the values for all other groups. We should again refer the reader to our previous publication [23] demonstrating, based on a lot of functional and morphometric indices at organ and system level, that the triple combination was especially toxic and that the BPC ensured a noticeable protective effect.

It should be noted that in this discussion we are persistently emphasizing the consistency of the main conclusions drawn previously from various indices of the systemic toxic effect of the NPs acting alone or in combination with the ones drawn from semiquantitative estimates of their mitotoxicity not so much for enhancing the validity of these conclusions as for highlighting that this consistency, in our opinion, confirms most convincingly the informativity of the approach proposed for assessing toxic damage to mitochondria (especially by generalized average score).

5. Conclusion

Based on an example of subchronic intoxication with nanoparticles of lead, zinc and copper oxides acting alone, or in three binary combinations, or in a full triple combination (the latter with and without a background administration of an effective bioprotective complex), we have demonstrated the feasibility and satisfactory informativity of a method of generalized semiquantitative assessment of electron microscopy results obtained for the totality of mitochondria of various organs based merely on partial or full loss of cristae as a single, most easily and unequivocally distinguishable index of mitochondrial damage.

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