MORPHOLOGICAL CHANGES IN RAT LIVER TISSUE DURING CHRONIC ORAL INTAKE OF MICRO- AND NANO-SIZED COPPER OXIDE

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Abstract. CuO in the form of micro- and nano-sized colloidal particles enters the human body from environment. The aim of study identification of morphological changes in rat liver tissues during chronic oral intake of micro- and nano-sized CuO by image analysis. The experiments were performed on 45 male Wistar rats (3 groups: experimental (nano-sized CuO); comparison (micro-sized CuO); control (water without CuO)). Suspensions CuO were administered to the rats orally once a day. Quantitative morphological parameters were determined by pathomorphological examination and image analysis using the method of constructing Voronoi diagrams (the ratio of cells of various shapes); average cell area; the average size of the cell perimeter and the number of cells per unit area. As a result of the analysis by the constructing Voronoi diagrams, was found that the main part of the model cells corresponding to hepatocytes has the shape of a hexagon. In the control group, their share is 35.89%, in the experience group – 29.09%, and in the comparison group – 30.59%. The density of cells' distribution in the comparison group is 7 times higher than in the control group; in the experimental group the same indicator is 4 times higher than in the control group. Collectively, the characteristics of morphological changes in liver tissue indicate greater toxicity of nano-sized copper oxide compared to its micro-sized analogue.

Keywords: nanotoxicology, copper II oxide, liver, image analysis, Voronoi diagram.

Introduction

Oxygen-containing copper compounds, including oxides, are used in reagents to prevent blooms in water bodies and as pesticides in agriculture. As a result of this use, copper compounds accumulate in food, water, and soil (Tsitsuashvili et al., 2017; Gladkova & Terekhova, 2013; Marcus et al., 2017). In addition, technological equipment and water supply pipelines can be sources of copper and its oxides in food and water (Kulakova et al., 2012). Studies (Hramov et al., 2019; Zaitseva et al., 2019) prove that oxygen-containing copper compounds accumulate in environmental objects, and therefore enter the human body in the form of both micro- and nano-sized colloidal particles. Assessment of human health risks associated with exposure to copper oxides is complicated by the fact that copper is an essential component of many enzymes and proteins that play an important role in redox processes. Copper ions are involved in the animal body's metabolism as a component of seven oxidoreductases, of which six enzymes are of great importance for humans. The effect of excessive amounts of copper (hypercuprosis) has been little studied in pathogenetic terms. It is known that copper excess is accompanied by a decrease in vital enzymes' activity and biosynthesis (Jing et al., 2015).

According to the WHO (WHO, 1996), the hepatotoxic effect is typical for chronic intoxication with copper compounds; it is most dangerous when exposed to children under the age of 1 year. Copper concentrations in drinking water above 2 mg/dm3 can lead to the development of hepatosplenomegaly in infants, an increase in the concentration of serum transaminases and bilirubin. In adults, centrilobular liver necrosis may develop at a copper content of 5 mg/dm3 in drinking water.

Data on chronic copper compounds toxicity are presented without taking into account the dimensionality, while studies (Sergievich et al.,

2013; Zemlyanova & Ignatova, 2019) proved that morphological changes in the tissues of the internal organs when exposed to a substance in nano-sized form quantitatively differ from the changes caused by exposure to the same substance in a micro-sized form. All of the above indicates the relevance of the problem of identifying reliable differences between morphological changes caused by exposure to copper oxide in nano- and micro-sized forms.

A number of studies (Zemlyanova & Ignatova, 2019; Zaitseva et al., 2018; Bekkers et al., 2018) have shown that quantitative indicators of morphological changes in the tissues of internal organs can be reliably determined by the method of image analysis. There are various techniques and algorithms applied to image analysis of structures of various biological tissues and objects. It has been proved (Serra-Picamal et al., 2015) that a technique based on the construction of Voronoi diagrams can be used to assess the morphological parameters of liver tissue since it reliably (with an accuracy of 90% to 98%) conveys the microanatomical structure of this organ (Barton et al., 2017).

The study aims to identify typical morphological changes in rat liver tissues during chronic oral intake of micro- and nano-sized copper oxide.

Materials and methods

To identify morphological changes in liver tissue during prolonged oral intake, experiments were performed on 45 male Wistar rats weighing 380–450 g.

The animals were freely divided into 3 groups of 15 individuals each: experimental group – exposure with nano-sized copper oxide; comparison group – exposure with micro-sized copper oxide; control group – exposure with bidistilled water without copper oxide.

Suspensions of nano- and microdispersed copper oxide II (CAS 1317-38-0) based on bidiistilled water (technical specification 6-09-2502-77) were administered to laboratory animals orally with an intragastric probe once a day. The powders used were: nanodispersed copper oxide with an average particle size of 45.86 nm and a particles' roundness (sphericity) coefficient of 0.59; microdispersed copper oxide with an average particle size of 13.9 microns and a sphericity coefficient of 0.46. The suspensions were preliminarily homogenized on a Sonopuls Hd 3200 ultrasonic unit (Bandelin, Germany) at room temperature for 2 minutes in a continuous pulsation mode with a frequency of 20 kHz.

The animals were kept and exposed in accordance with the requirements of Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS №123), sanitary and epidemiological rules of SR 2.2.1.3218-14 «Sanitary and epidemiological requirements for the design, equipment and maintenance of experimental biological clinics (vivariums) and the recommendations of the Ethical Committee of the Federal Budget Scientific Institution "Federal Scientific Center for Medical and Preventive Health Risk Management Technologies"».

The experiment on multiple oral exposure was carried out by the method of cumulation study according to Lim (Kevin H., 2013); the doses of the administered substance throughout the experiment are presented in Table 1. The total dose of copper oxide was 13187.5 mg/kg. The duration of the oral exposure was 20 days.

The surviving animals were removed from the experiment by the method of cervical dislocation one day after the last exposure, after which liver samples were obtained by the method of complete evisceration according to GV Shor. Afterwards, the whole liver was fixed in 10% aqueous neutral formalin solution. After fixation, it was dehydrated in alcohols of ascending concentration, then impregnated with chloroform and paraffin, then embedded in a homogenized paraffin medium "Histomix". Histosections 4 μm thick were obtained on a JUNG SM 2000R sled microtome (Leica, Germany), stained with Ehrlich's hematoxylin and eosin. Examination and obtaining of photomaterials was performed using an Axiostar light-optical microscope (Carl Zeiss, Germany).

Table 1

Dose of nano-sized copper oxide particles in a multiple oral exposure experiment

Experiment periods, days	LD_{50} portion	Predicted dose of substance, mg/kg of body weight per day
1-4	0.10 LD_{50}	250
5-8	0.15 LD_{50}	375
9-12	0.22 LD_{50}	575
13-15	0.34 LD_{50}	850
16-20	0.51 LD_{50}	1,275

The quantitative morphological parameters of liver tissues caused by exposure to nano- and micro-sized copper oxide were determined by image analysis using the universal software ImageJ-FiJi (open source software, developed by Wayne Rasband, National Institutes of Health, USA) using the Voronoi diagram technique. Additionally, we used the following software apps: BioVoxxel (developed by Jan Brocher) and EpiGraph (developed by Pablo Vicente-Munuera, Pedro Gomez-Galvez). Hepatocyte nuclei were used as centroids for constructing Voronoi diagrams, assuming that each cell of the diagram corresponds to one cell. As a result, the Voronoi diagrams, constructed on the basis of the liver tissue image, were its two-dimensional geometric model.

Further identification and comparison of tissue parameters' morphological characteristics were carried out according to the obtained two-dimensional models. The following parameters were calculated: the ratio of cells of various shapes (quadrangles, penta-, hexa-, hepta-, octagons); average cell area; the average size of the cell perimeter and the number of cells per unit area.

Statistical data processing. Differences between the group indicators corresponding to the normal distribution (checking the normality according to the Kolmogorov-Smirnov test) were carried out by the method of determining the Fisher's F-test at a given significance level of 0.05. BioStat 7.0 software (AnalystSoft Inc.) was used for statistical evaluation.

Results

Figure 1 shows examples of histological images of rat liver tissues obtained in experiments with oral exposure to copper oxide.

Figure 2 shows examples of Voronoi diagrams based on the results of processing samples of histological images of liver tissue. The diagram in Figure 3 reveals the ratio of cells of various shapes (quadrangles, penta-, hexa-, hepta-, octagons) in diagrams characterizing the tissue structure of rats of all experimental groups.

The results of determining the averaged value of the area and perimeter of the cells of Voronoi diagrams, characterizing the state of the liver tissue of rats from all experimental groups, as well as the number of cells per unit area and the results of data analysis to find significant differences between these parameters are presented in Table 2.

Discussion

According to the results of the pathomorphological examination, no pathologies were found in the control group. The experimental group animals were found to have acute active hepatitis; in some individuals, extensive centrilobular necrosis of the liver and hyaline-drop dystrophy of hepatocytes were observed. The comparison group animals were also found to have acute active hepatitis, while no other deviations were recorded.

As a result of the analysis of the liver tissue state of animals of all experimental groups by the method of constructing Voronoi diagrams,

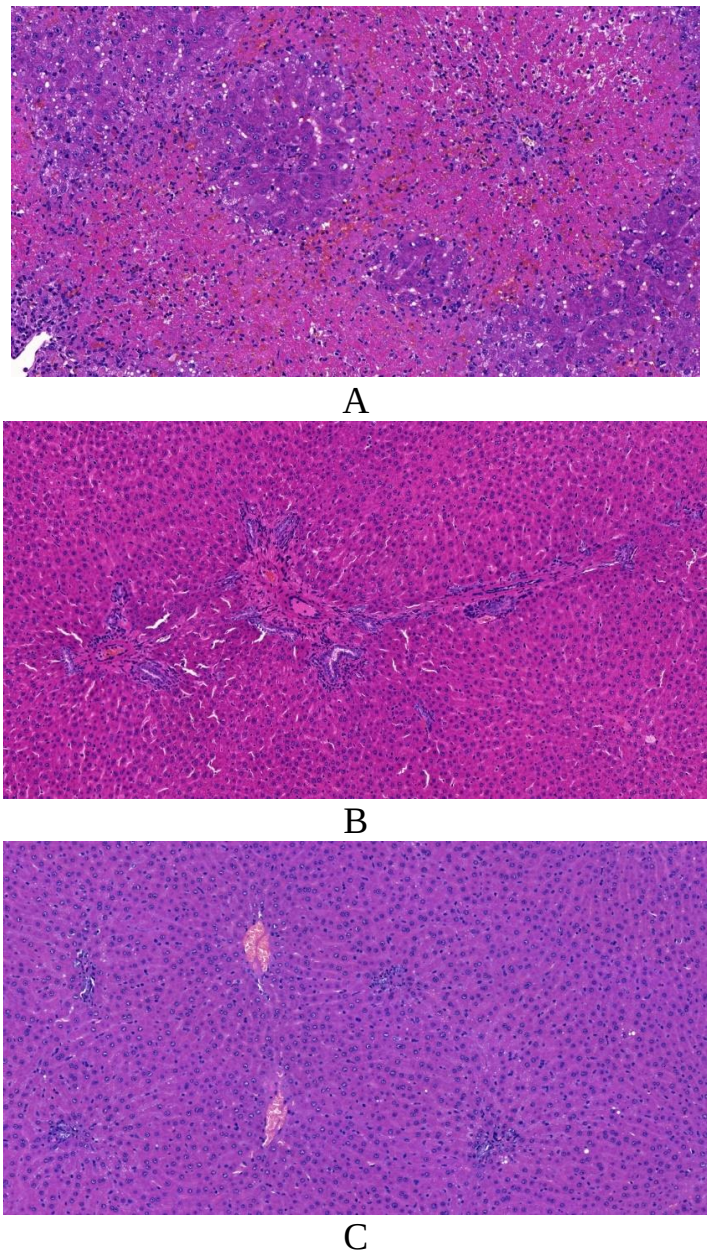
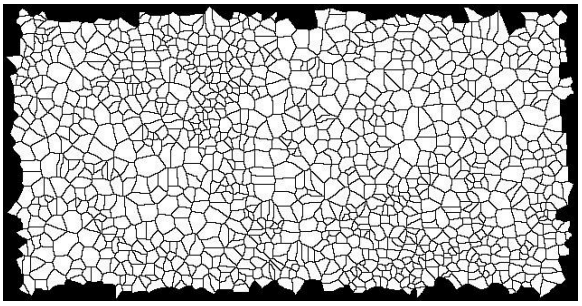


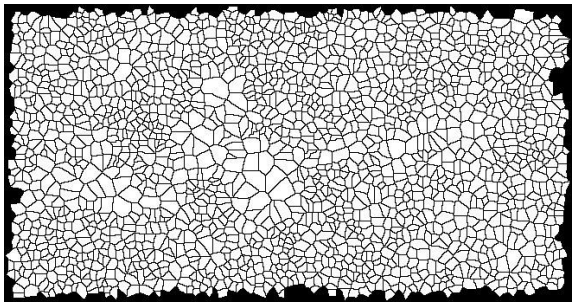
Fig. 1. Rats' liver tissues from an experiment to determine the nature of the impact of nano- and micro-sized particles of copper oxide, x200: A – experimental group; B – comparison group; C – control group

it was found that the main part of the model cells corresponding to hepatocytes has the shape of a hexagon.

In the control group, the proportion of hexagons is 35.89%, in the experimental group – 29.09%, and in the comparison group – 30.59%. It is noteworthy that the shape of the cells of the models is predominantly hexagonal, which corresponds to the shape of the Benard cells (Denisevich & Lyapcev, 2017). Based on this similarity, it can be suggested that the liver tissue has certain physical self-organization, which is disrupted during the development of the pathological process. The proportion of cells of quadrangular and pentagonal shapes increases as the proportion of hexagonal cells decreases, while the number of seven- and octagonal cells remains little changed, which can also be a sign of pathological changes in liver tissue.



A



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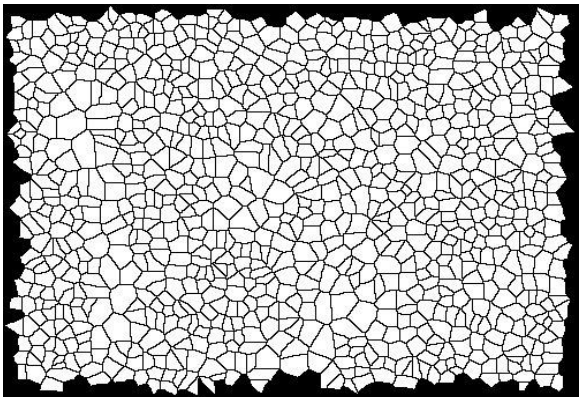

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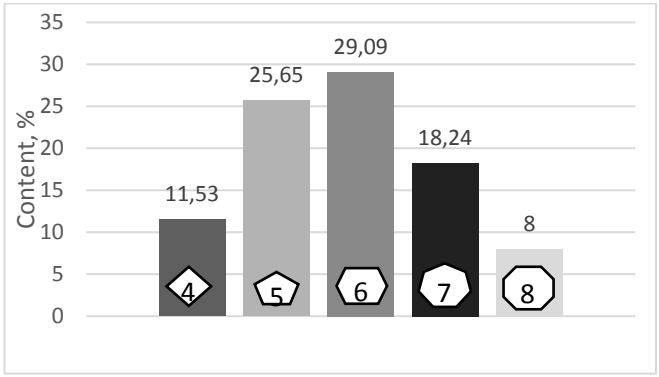
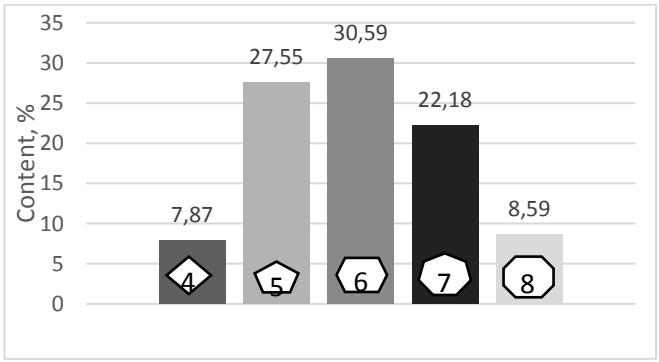
Fig. 2. Voronoi diagrams characterizing liver tissue: A – experimental group; B – comparison group; C – control group



A



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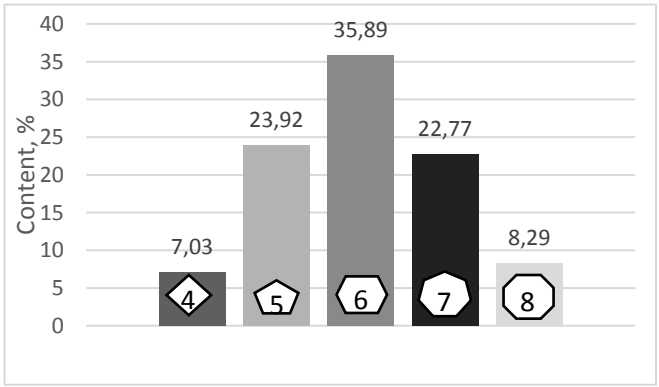

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Fig. 3. The ratio of cells of different shapes in Voronoi diagrams: A – experimental group; B – comparison group; C – control group

The density of cells' distribution in the diagrams corresponding to the state of the liver tissue of animals exposed to nano- and micro-sized copper oxide increases compared to the indicators of animals from the control group. However, the density of cells' distribution in the comparison group is 7 times higher than in the control group and is 702.80 ± 34, units/mm2; in the experiment group, the same indicator is 4 times higher than in the control group and is 438.04 ± 28 units/mm2. This can be explained by the fact that the inflammatory reaction in the liver tissue when exposed to a nano-sized substance is accompanied by necrosis, which leads to a reduction in the number of cells. Therefore, the diagram reflecting the state

Table 2

Parameters of Voronoi diagrams characterizing the state of liver tissue in rats exposed to nano- and micro-sized copper oxide

Parameter	Experimental group	Comparison group	Control group
Average size of the cell perimeter, μm	154,59±6,29 * F = 1,75 ($F_{кр}$ =1,10; p =0,001) **F = 1,92 ($F_{кр}$ = 1,08; p = 0,001)	129,95±3,58 * F = 1,11 ($F_{кр}$ =1,10; p =0,080)	164,87±2,67
Average cell area, μm^2	1626,28±30,42 * F = 1,58 ($F_{кр}$ =1,10; p = 0,001) **F = 2,58 ($F_{кр}$ = 1,08; p = 0,005)	1125,59±30,42 * F = 1,63 ($F_{кр}$ =1,09; p =0,009)	1733,7±25,11
Density of distribution of cells, units/mm^2	438,04±28 * F = 13,32 ($F_{кр}$ =6,38; p = 0,0060) **F = 11,81 ($F_{кр}$ = 6,38; p = 0,0073)	702,80±34 * F = 7,65 ($F_{кр}$ = 6,38; p =0,0069)	108,21±15
* significant difference from the control group's indicator; ** significant difference from the comparison group			

of the tissue in this state will have a smaller number of cells. In case of inflammation without the development of necrosis, the number of cells increases, since in such tissue, there are more macrophage cells, and, consequently, the number of cells will be higher.

The data on the density of the cells' distribution correlate with the values of the area and perimeter of the cells of Voronoi diagrams. In the experimental group, the cell perimeter's value is 154.59±6.29 μm on average, which is 1.1 times less than in the control group; this difference is significant (p=0.001). The cell area size in the experimental group is 1,626.28±30.42 μm2 on average, which is 1.1 times less than in the control group; this difference is significant (p=0.001). In the comparison group, the cell perimeter's value is 129.95±3.58 μm on average, which is 1.3 times less than in the control group; this difference is significant (p=0.08). The cell area size in the comparison group is 1,125.59±30.42 μm2 on average, which is 1.5 times less than in the control group; this difference is significant (p = 0.009).

Taken together, the analysis of the morphometric parameters of Voronoi diagrams, which are flat geometric models of liver tissue, allows us to conclude that the toxic effect of nano-sized copper oxide is not only more pronounced than the toxic effect of a micro-sized substance, but also different by nature. Thus, when exposed to a micro-sized substance, inflammation is characterized more by a decrease in the size of the cells and an increase in the density of distribution than a violation of the ratio of cells in shape. In contrast, when exposed to a nano-sized substance, the size and distribution of cells change less than the ratio of cells in shape.

Conclusion

The study revealed that morphological changes in rat liver tissues during chronic oral intake of nano-sized copper oxide are characterized by the development of acute hepatitis with foci of necrosis; when exposed to micro-sized copper oxide, only acute hepatitis is revealed. The method of identifying and assessing the quantitative and qualitative char-

acteristics of morphological changes in liver tissue is the construction of a Voronoi diagram with centroid points in the places of hepatocyte nuclei. A quantitative and qualitative characteristic of morphological changes caused by exposure to nano-sized copper oxide is a reduction in the proportion of hexagonal cells. A quantitative characteristic of morphological changes caused by the exposure to micro-sized copper oxide is a reduction in the average cell size, rather than a qualitative change in the ratio of the proportion of cells of different shapes. Collectively, the characteristics of morphological changes in liver tissue indicate a greater toxicity of nano-sized copper oxide compared to its micro-sized analogue.

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