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DEVELOPMENTAL BIOLOGY

Cytogenetic Activity of Gold Nanoparticles in Germ and Somatic Cells of 129 Mice with a Nonsense Mutation in the DNA Polymerase Iota Gene

N. M. Mudzhiri[a, c], S. T. Zakhidov[a], V. M. Rudoy[b], O. V. Dement'eva[b], A. A. Makarov[d], I. V. Makarova[e], I. A. Zelenina[a], L. E. Andreeva[e], and T. L. Marshak[c, *]

[a] *Faculty of Biology, Moscow State University, Moscow, 119991 Russia*
[b] *Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, 119071 Russia*
[c] *Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, 119334 Russia*
[d] *Higher School of Economics National Research University, Moscow, 101000 Russia*
[e] *Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, 123182 Russia*
**e-mail: 4361.idb@bk.ru*

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Abstract—In this study, we investigated the cytogenetic effects of single and quadruple exposure of spermatogenic cells and hepatocytes of 129 mice, which have a mutation in the gene that encodes DNA polymerase iota, to ultrasmall gold nanoparticles (GNPs). The combined effects of GNPs and chemical mutagen dipin were evaluated. In all cases, except for the experiment with the quadruple GNP injection, we observed a slight, statistically nonsignificant increase in the frequency of round spermatids with micronuclei compared to the negative control (saline). It is established that, in the intact liver of 129 mice, in all variants of the experiment, GNPs behaved as a potentially cytotoxic agent, as evidenced by the decrease in the frequency of the micronucleated hepatocytes.

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INTRODUCTION

Earlier, using the model of male germ cells of CBA × C57Bl/6 hybrid mice, we first demonstrated (Zakhidov et al., 2017) that ultrasmall gold nanoparticles (GNPs) exhibited mutagenic, antimutagenic, or comutagenic properties depending on the experimental conditions. It was of interest to study the effects of separate and combined (with the alkylating mutagen dipin) exposure of spermatogenic and liver cells of 129 mice to GNPs. A characteristic feature of 129 mice is that they have a nonsense mutation in the second exon of the gene encoding the DNA polymerase iota. This leads to a lack of activity of the enzyme that plays an important role in providing genome stability (Makarova et al., 2008; Kazachenko et al., 2016). DNA polymerase iota is a specialized low-fidelity DNA polymerase involved in the replication of DNA containing lesions (Makarova and Kulbachinskiy, 2012). It should also be noted that 129 mice tend to form spontaneous teratomas in the germ primordia but are not susceptible to lymphomas induced by gamma rays (Newcomb et al., 1989) and, in general, exhibit an increased resistance to radiation (Roderick, 1963; Storer, 1966; Malashenko et al., 2003).

MATERIALS AND METHODS

The colloidal gold solution (hydrosol) used in this study was synthesized by the method of Duff et al. (1993), which is based on the reduction of $AuCl_4^-$ ions by tetrakis(hydroxymethyl)phosphonium chloride in an aqueous NaOH solution. According to the dynamic light scattering and high-resolution transmission electron microscopy data, the mean size of Au particles was ≈2.5 nm and their numerical concentration was $\sim 10^{15}$ mL^{-1}.

Experiments were performed on adult male mice of the 129/JY strain at two to three months of age (J, Jackson Laboratory, Bar Harbor, ME, United States; Y, animal breeding facility in Svetlye Gory, Russia). Experiments on animals were performed in accordance with the requirements of the Order of the Ministry of Health of the Russian Federation of June 19, 2003, no. 267 "On Approval of the Rules of Good Laboratory Practice and Ethical Standards," listed in the Rules of good laboratory practice (GLP) in the Declaration of Helsinki (2000).

Mice were divided into the control and experimental groups, each of which consisted of four males. The animals were housed under standard vivarium condi-

tions (temperature 18–22°C, 12-h daylight period) and received food and water ad libitum. The animals that were injected with 0.2 mL of saline for four days served as the negative control, and the animals that received single injections of the alkylated radiomimetic mutagen dipin in a genetically active dose of 30 mg/kg served as the positive control. Mice of the experimental groups received single or repeated (daily for four days) intraperitoneal injections of GNP sol (0.2 mL). One hour after the last GNP injection, some animals were injected with dipin at a genetically active dose of 30 mg/kg.

Mice from the control and experimental groups were killed by cervical dislocation on day 14 after the last injection. In this period, an increase in the frequency of round spermatids with micronuclei is the result of the impact of chemical or physical agents on preleptotene and leptotene spermatocytes at the stage of active premeiotic DNA synthesis. Hepatocyte populations were also analyzed 14 days after the last injection.

Cytogenetic analysis was performed on squashed samples of isolated seminiferous tubules and intact liver fragments. The preparation of samples, their fixation, and staining were performed as described previously (Zakhidov et al., 2017).

The frequency of genetically abnormal cells (round spermatids and hepatocytes) was determined by the standard method of counting micronuclear aberrations. For each animal, 2000 to 5000 round spermatids and 2000 to 3000 hepatocytes were counted. The frequency of the cells with micronuclei was expressed in per mille. In the quantitative cytogenetic analysis, cells with micronuclei both attached to the nuclei and located separately from them were taken into account.

The data obtained was processed using the SPSS statistical package. To determine the significance of differences of the mean values and variances in different mouse groups, the parametric Student t test for two independent samples with the assumption of equal and unequal variations in groups was used. To determine the significant differences in variation in groups, Levene's test for equality of variances was used (Turin and Makarov, 1998).

RESULTS AND DISCUSSION

Spermatogenic cells. Figure 1 shows microphotographs of round spermatids with morphologically diverse micronuclei, found in the testes of the control and experimental 129 mice. It can be seen that some micronuclei retain contact with the major nuclei.

The results of estimating the frequency of the micronucleated round spermatids, which are summarized in Fig. 2, show that the treatment with dipin (positive control) does not increase the frequency of the micronucleated spermiogenic cells compared to the negative control: the difference between the mean values was statistically nonsignificant at $p = 0.312$. The highly efficient alkylating mutagen dipin, which has exhibited mutagenic effect in many studies performed on different mouse strains and Wistar rats (Zakhidov et al., 1994, 2008), had no marked damaging effect on the chromosomes of early primary spermatocytes in the mutant 129 mice. Apparently, this may be due to either the peculiarities of repair processes in the testes of these mice or the presence in their genome of some yet unidentified mutations affecting the sensitivity of chromosomes to mutagens (in particular, dipin).

In the experimental variant with a single injection of GNPs, we detected a statistically nonsignificant ($p = 0.165$) increase in the frequency of micronucleated round spermatids compared to the negative control. In contrast, after the quadruple injection of GNPs, the frequency of formation of aberrant spermiogenic cells was significantly ($p = 0.012$) higher than in the negative control group.

A single injection of GNPs in combination with dipin increased the frequency of the micronucleated round spermatids by 44% compared to the negative control. However, this did not lead to significant differences in its mean values ($p = 0.091$). It cannot be ruled out, however, that the absence of significant differences between the mean values may be associated with a high variance of this trait in the animals of these experimental groups. In addition, we found no statistically significant differences ($p = 0.182$) in the mean frequency of formation of the mutant spermiogenic cells when comparing the results of the negative control and the experiment with the quadruple exposure to GNPs and then to dipin (Fig. 2).

Hepatocytes. In the intact liver of the control and experimental 129 mice, a relatively large number of hepatocytes with micronuclei and residual chromosomal bridges was found (Fig. 3). As can be seen in Fig. 4, the frequency of micronucleated hepatocytes in the animals of the negative control group averaged $7.8 \pm 3.9‰$, which was 8.7 times higher than the spontaneous frequency of the micronucleated cells in the intact liver in hybrid CBA × C57Bl/6 mice ($0.9 \pm 0.9‰$, our unpublished data). Such a large proportion of the genetically abnormal hepatocytes in 129 mice testifies to the high genetic instability of these cells and may indicate specific patterns of growth, development, and functioning of the liver of mutant animals of this strain in the postnatal ontogeny. Earlier, a similar picture was observed in the intact liver of mutant senescence-accelerated mice of strains SAMP1 and SAMR1 (Zakhidov et al., 2002). Apparently, the absence of expression of the low-fidelity DNA polymerase iota, which plays an important role in maintaining the genome stability, in 129 mice may be the cause of such a sharp increase in the frequency of spontaneous chromosomal aberrations in the hepatocyte population. In the positive control, we detected a nearly fourfold increase in the number of micronucleated hepatocytes

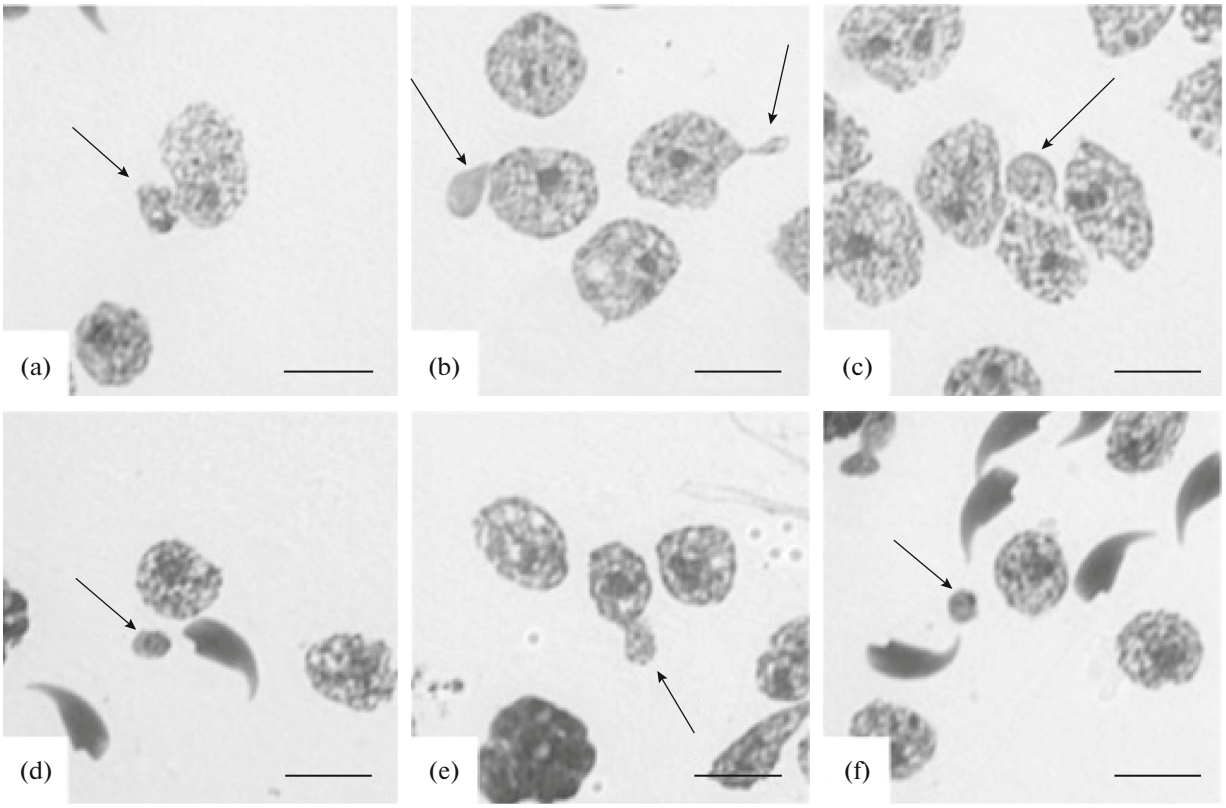

Fig. 1. Nuclei of round spermatids with attached and detached micronuclei (shown with arrows) of different sizes and morphology in the testes of (a–c) control and (d–f) experimental 129 mice. Here and in Fig. 3, scale bar, 10 μm; Feulgen stain.

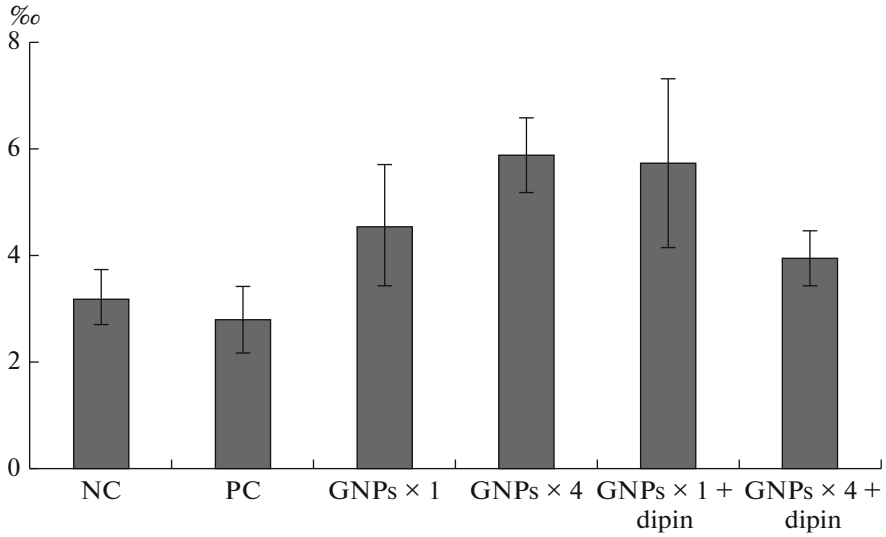

Fig. 2. Frequency of micronucleated round spermatids in the testes of control and experimental 129 mice. Here and in Fig. 4, NC, negative control; PC, positive control; GNPs × 1, GNPs × 4, single and quadruple injection of gold nanoparticles, respectively. The height of columns corresponds to the mean values, and vertical bars show the standard deviations.

compared to the negative control; the difference between the mean values was highly significant (p = 0.028) (Fig. 4).

In the experimental variant with a single injection of GNPs, the proportion of micronucleated hepatocytes was smaller by 59% than in the negative control; the difference between the mean values was significant (p = 0.046). After the quadruple injection of GNPs, the mean frequency of mutant hepatocytes decreased by only 27% compared to the negative control, and no

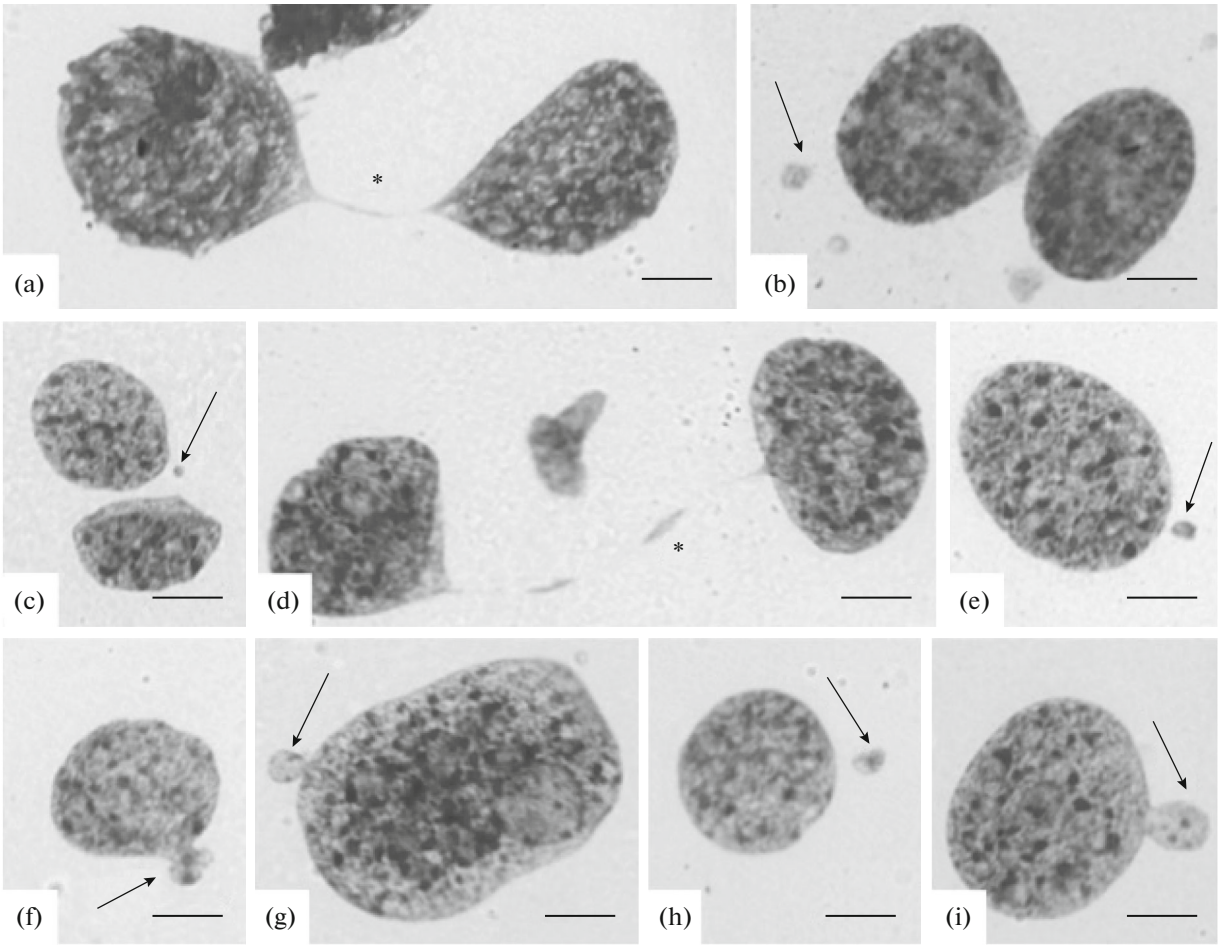

Fig. 3. Aberrant hepatocytes with micronuclei (arrows) and chromosomal bridges (asterisk) in the liver of (a–c) control and (d–i) experimental 129 mice.

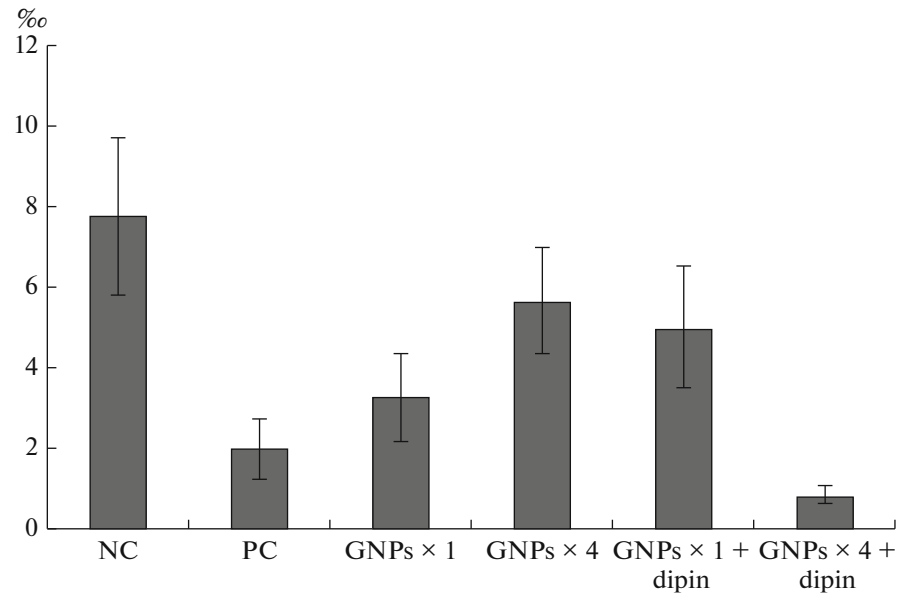

Fig. 4. Frequency of micronucleated hepatocytes in the intact liver of control and experimental 129 mice.

significant difference in this case was found ($p = 0.277$).

After a single injection of GNPs in combination with dipin, the mean frequency of micronucleated cells did not differ significantly from that in the negative control ($p = 0.155$). In contrast, a quadruple injection of GNPs combined with subsequent injection of dipin led to an almost tenfold decrease in the frequency of micronucleated hepatocytes in the intact liver of the experimental mice; the difference between the mean values compared to the negative control was highly significant ($p = 0.019$).

In recent years, the problem of mutagenicity of GNPs has been studied actively. For instance, Balansky et al. (2013) found that intraperitoneal injection of pregnant female mice with GNPs of 100, but not 40 nm in size resulted in a dramatic increase in the frequency of micronucleated polychromatic erythrocytes in the peripheral blood and liver of fetuses. In other experiments on cultures of human peripheral blood lymphocytes and Raw264.7 strain (mouse macrophages), exposure to 5- and 15-nm GNPs caused a marked dose-dependent increase in the number of micronucleated cells (Di Bucchianico et al., 2014). In both types of cells, micronuclei emerged as a result of disturbance of segregation of chromosomes during cell division but not as a result of their damage. That is, the GNPs used by these authors functioned, more likely, as efficient aneugens rather than clastogens. In experiments on *Drosophila,* "nanomutants" were obtained for the first time in the world: various changes in the structure of the eye, wings, and thorax were identified in the offspring of *Drosophila* that received food containing 15-nm GNPs at all stages of ontogeny (Vecchio et al., 2012).

Experiments performed on healthy and cancer cell models (Tsoli et al., 2005), as well as with the use of particles of the cholesteric liquid-crystal dispersion (CLCD) of DNA (Yevdokimov, 2015a, 2015b), showed that ultrasmall GNPs are able to interact with the double-strand DNA by incorporating into the major groove of the double helix (Liu et al., 2003) or between DNA molecules in quasi-nematic CLCD layers. By disrupting the spatial organization of DNA and, therefore, presumably the structural and functional integrity of genes, GNPs in this case may act as a potentially strong genotoxic agent. GNPs conjugated with surface active glycolipids (so-called OA-SL-AuNPs) showed a weak genotoxic activity against HepG2 cells (human hepatocellular carcinoma epithelial cells). According to the results obtained by Singh et al. (2010) using the DNA comet assay, such GNPs at low concentrations (5.14, 0.51, and 0.0005 mg/mL) did not cause a significant increase in the number of lesions in the DNA structure. In the samples of HepG2 cells studied, the proportion of the so-called DNA tails was 6.29, 6.12, and 6.72 units, respectively, compared to 6.08 units in the control (Singh et al., 2010).

It was also shown (Schulz et al., 2012) that GNPs of 2, 20, and 200 nm in size did not cause an increase in the number of micronucleated polychromatic erythrocytes in the bone marrow of rats as well as DNA lesions in the lung cells after a single instillation of these particles into the trachea. Using the analysis of metaphase chromosomes and the micronucleus test, George et al. (2017) showed that 14- and 20-nm GNPs at concentrations of 6.2, 12.5, 25, and 50 mg/mL did not have a genotoxic effect on Chinese hamster ovary cells (CHO). The frequencies of occurrence of CHO cells with different structural chromosomal aberrations or micronuclei were minimal and did not differ significantly from those recorded in the negative control. According to Mytych et al. (2015), a 96-h exposure of the cell culture to GNPs at concentrations of 1.1×10^{11} and 5.5×10^{11} mL^{-1} did not cause an increase in the number of micronuclei in the population of astrocytes. Conversely, the frequency of occurrence of binucleate cells with micronuclei was significantly reduced compared to the negative control. GNPs also showed no mutagenic properties in the Ames bacterial test system, which was due to the inability of GNPs to penetrate the bacterium *Salmonella typhimurium* (Wang et al., 2011; George et al., 2017).

The pilot experiments performed in this study on mutant 129 mice showed the ability of ultrasmall GNPs alone or in combination with the alkylating mutagen dipin to increase (though only slightly) the number of spontaneous chromosomal mutations (micronuclei) in the population of round spermatids. Recently, in a similar experiment on a model of male gametes of physiologically stable hybrid CBA × C57Bl/6 mice, we showed that GNPs exhibited a mutagenic, antimutagenic, or comutagenic effect depending on the experimental conditions (Zakhidov et al., 2017). The fact that GNPs affect the mutability of spermatogenic cells of 129 mice and hybrid CBA × C57Bl/6 mice differently can be explained by certain differences in the organization of their meiotic chromosomes or by the specificity of intracellular metabolic and reparative processes involved in the regulation of mutagenesis in mutant 129 mice. It should also be noted that the spontaneous (natural) frequency of micronucleated round spermatids in the gonads of 129 mice was four times higher than that in the gonads of CBA × C57Bl/6 hybrid mice (Zakhidov et al., 2017).

Diametrically opposite results were obtained in the quantitative cytogenetic assessment of the effects of GNPs on the hepatocyte population in 129 mice: in almost all experimental variants, exposure to GNPs significantly reduced the frequency of micronucleated hepatocytes compared to the negative control. The question of whether GNPs affect the neighboring genetically normal hepatocytes or if their cytotoxic (eliminating) effect is limited only to the aberrant liver cells remains unanswered. In this connection, it is worth mentioning that earlier, using the test for sperm

head shape abnormalities, we showed the ability of ultrasmall GNPs to cause elimination of a significant proportion of genetically defective gametes in gonads of CBA × C57Bl/6 hybrid mice (Zakhidov et al., 2012).

The analysis of the data obtained also showed that a combined single exposure to GNPs and dipin did not lead to a simple addition of their cytotoxic effects. Conversely, the number of micronucleated hepatocytes that survived in this case was higher than in the positive control (38%) and in the experimental group with a single exposure to GNPs (23%). The causes of the increased stability of a large number of micronucleated hepatocytes to a single exposure to GNPs in combination with dipin are obscure. Possibly, in such a short time interval, GNPs could have intensified the functioning of the enzyme systems involved in the "neutralization" of the highly reactive mutagenic dipin molecules, thus dramatically increasing the chances of survival of genetically unstable mutant liver cells. It can also be assumed that the interaction between GNPs and dipin molecules in the cytoplasm might have led to mutual attenuation of the cytotoxic potential of these structurally distant agents. In this connection, it is worth mentioning that Zhang et al. (2011) demonstrated that HeLa cells, which exhibited a high sensitivity to doxorubicin, showed a relatively low sensitivity to the conjugate of this antibiotic with ultrasmall GNPs.

After a quadruple exposure to GNPs in combination with dipin, the elimination of micronucleated hepatocytes reached a maximum value (76%), which is comparable with the level characteristic of the positive control. In our opinion, the key role in the cytotoxic effect in this case was played by the molecular partner of GNPs, the mutagen dipin, rather than GNPs themselves. However, it cannot be ruled out that, in the case of repeated (long-term) exposure, GNPs could create "favorable" conditions for the manifestation of the cytotoxic effect of dipin.

Thus, in 129 mice, which have defects in the cell defense system against genotoxic factors, ultrasmall GNPs showed a weak mutagenic (cytogenetic) activity in spermatogenic cells and had a strong cytotoxic effect on the mutant genetically unstable hepatocytes under almost all experimental conditions studied. Therefore, cells belonging to self-renewal (spermatogenesis) and growing (liver) populations respond differently to such GNPs administered into the body.

Our preliminary data on the genotoxicity and cytotoxicity of GNPs indicate the importance of conducting additional, advanced research on mutant and/or genetically modified animals, the patterns of development of which may differ from those in animals with normal genotypes. Only such comprehensive research will elucidate the issue of the genetic and biological safety of these unique nanoparticles, which may be very promising for use in biotechnology and, probably, in practical medicine.

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