Combined assessment of cyto-, geno-, and embryotoxicity of silica nanoparticles in experiments in vitro

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A complex of express methods for cyto-, geno-, and embryotoxicity assessment of nanoparticles (NPs), based on the simultaneous use of three models: the cultures of pre- and post-implantation embryos of mice (egg cleavage, blastocyst formation, 1−5 day of development) and rats (head fold−neurulation stages, 30 somite pairs, 9.5−11.5 day of development), as well as human peripheral blood lymphocytes, is developed. Pathogenic (dysmorphogenic) effects of SiO2 NPs in concentrations of 100 and 200 µg/mL on early embryo development processes were established. The cytogenetic methods used for cyto-, geno-, and mutagenicity assessment (micronuclear test under cytokinesis-block conditions, chromosomal aberrations test, and DNA comet assay) revealed no effects of SiO2 NPs in concentrations 200, 100, and 20 µg/mL.

Introduction

The broad-spectrum applications of SiO2 NPs in different fields, including medicine, food industry, optoelectronics, pharmacology, cosmetic industry, etc., make such NPs priority objects for detailed safety assessment [1, 2]. The toxic effects of SiO2 NPs are first of all associated with their ability to react with cell membranes and thus stimulate excess formation of reactive oxygen species (ROSs) [3, 4]. Some research on the effect of SiO2 NPs revealed damage of cell membranes, resulting in increased levels of reduced glutathione and malondialdehyde [5] on the background of disturbed cellular calcium homeostasis [6]. According to [7], SiO2 NPs enter the cell nucleus and cytoplasm, react with DNA, enhance topoisomerase I-mediated DNA cleavage, inhibiting replication, transcription, and proliferation processes. Yang et al. [6] suggested that the cytotoxic effect of SiO2 NPs can also be realized via inhibition of the mitochondrial function [6]. An important factor responsible for the potential impact of SiO2 NPs on human health is their size which is directly related to cytostatic activity. Napierska et al. [7] showed that 70-nm silica nanoparticles exhibit a stronger cytotoxic effect compared to their microsized analogs.

Thus, analysis of published data literature showed that amorphous SiO2 NPs have been insufficiently studied in terms of general toxicity and scarcely studied in terms of their effect on mammalian embryogenesis. Therefore, in the present work we set ourselves the aim to develop a complex approach to express in vitro cyto-, geno-, and embryotoxicity assessment of SiO2 NPs. Previously we developed a test system including rodent pre- and postimplantation embryo cultures and the human peripheral blood lymphocyte culture for complex assessment of the effect of chemical substances and unfavorable environmental factors on the genetic apparatus of the cell and human and animal embryogenesis. The complex assessment of the pathogenic impact on embryo development and human peripheral blood lymphocytes is based on an analysis of the embryotoxic, embryoletal, and teratogenic (dysmorphogenic) effects, dynamic morphofunctional disorders, as well as cytotoxic (cytostatic) and genotoxic (mutagenic) effects [8, 9, 10, 11]. The test system was many times applied in toxicological, environmental hygienic, and clinical research, and in the present work it was applied for the first time for the express toxicity assessment of silica nanoparticles.
Materials and methods

Silica nanoparticles 12 nm in diameter were purchased from Sigma Aldrich (Aldrich-718483). The stock aqueous suspension of SiO$_2$ NPs (2 mg/mL) was prepared by ultrasonication of a mixture of the NPs and water. To prevent agglomeration, following the protocol developed by Bihari et al. [12], the suspension was ultrasonicated and then BSA (15 mg/mL) was added as a stabilizer. The nanoparticles were introduced into the culture medium in concentrations of 20, 100, and 200 µg/mL. The size of SiO$_2$ NPs in the culture solutions was estimated by means of laser correlation spectroscopy (LCS) [13]. The LCS spectra were measured at an INTOX LCS-03 laser correlation spectrometer (INTOKS OOO, St. Petersburg, Russia). The processed LCS spectra of the prepared suspensions showed the Gaussian distributions of the hydrodynamic linear particle sizes peaking at $15 \pm 2$ nm for the concentrations 20 and 100 µg/mL and $16 \pm 2$ nm for the concentration 200 µg/mL.

SPF animals: F1 female mice (C57BL x CBA) and outbred Wistar female rats, were obtained from the Pushchino Animal Breeding Facility (Moscow Region, Russia). The experimental animals were handled and used in accordance with the “Rules of Laboratory Practice (GLP) in the Russian Federation” (approved by the Order no. 267 of the Ministry of Health of the Russian Federation of 19.06.2003).

Culturing preimplantation mouse embryos. For dated pregnancy female mice were stimulated by injections of Puregon (10 ME) and Pregnyl (5 ME) (MSD Pharmaceuticals, The Netherlands) and placed together with male rats in a 2 : 1 proportion. The day of vaginal plug detection was designate as the gestation day 1 (GD 1). On GD 2 the female mice were sacrificed by cervical dislocation, after which 2-cell embryos were washed out from the oviducts using the EmbryoAssist medium (Origio, Denmark). Morphologically normal embryos were transferred to 40-µL droplets (15–20 embryos per a droplet) of the culture medium preloaded with SiO$_2$ NPs, and the cultured under paraffin oil in Petri dishes (35 mm, Denmark) by the Brinster method. For the control we used embryos cultured in a straight EmbryoAssist medium. Culturing was performed in a CO$_2$/O$_2$/N$_2$ incubator (Sanyo MCO-18M, Japan) for 72 h at 37 ºC under a humid air (5% CO$_2$, 5% O$_2$, and 90% N$_2$). After culturing had been complete, live, dead, and abnormal embryos were counted, and the development stages were determined. Embryos were fixed in a methanol–acetic acid mixture (3:1), and cytological preparations were prepared [14] to assess the cellular mass, proliferative activity, and cell death (as determined by pyknotic nuclei score).

Culturing early rat embryos. To obtain postimplantation rat embryos, pregnant female rats were sacrificed by cervical dislocation at GD 9.5, the embryos (2–4 somite pairs) were taken out from the uterus and transferred to the culture flasks containing 6–8 mL of inactivated rat blood serum (ca. 1 mL per 1 embryo) spiked with SiO$_2$ NPs at concentrations of 20, 100, and 200 µg/mL. Culturing was performed for 46–48 h in rollers (10–40 rpm, rolling diameter 15 cm) at 37ºC in a CO$_2$/O$_2$/N$_2$ incubator (Sanyo MCO-18M, Japan). after which the embryos were examined for the presence of heart beating and circulation in yolk sac vessels and chorioallantois to determine the number of live and dead embryos. In live embryos, a number of main development parameters which characterize the growth processes, degree of development of structures and organs, degree of establishment and completion of morphological processes were estimated, as well as the number of embryos with congenital abnormalities and the character of the pathologies.

Assessment of gene expression. Assessment of the expression of genes involved in embryonic neurulation was performed in rat embryos (E 11.5) in control (blank) cultural medium and the cultural medium spiked with a suspension of SiO$_2$ NPs (100 and 200 µg/mL). Quantitative PCR using RT2 SYBR Green I qPCR primer assay kit (Sabioscience, Germany) with normalization by the 18S rRNA gene was applied. Two- or higher-fold changes in gene expression, as calculated by delta-delta-Ct method, were considered significant. The markers were genes involved in primary neurulation in rat embryos: bone morphogenetic proteins 2 and 4 (BMP2 and BMP4), sonic hedgehog (SSH), tubby-like protein 3 (TULP3, SSH inhibitor, signaling regulator), and NEUROG1. Disorders in these gene expression lead to craniofacial abnormalities.
The cytotoxicity and genotoxicity (mutagenicity) of amorphous SiO\textsubscript{2} NPs in the human peripheral blood lymphocyte culture was assessed using (a) micronuclear test under cytokinesis-block conditions (score of binuclear cells with micronuclei, proliferative activity, frequency of pyknosis and apoptosis) [15]; (b) chromosome aberration assay [16]; and (c) alkaline DNA comet assay to reveal primary DNA damage in cell nuclei [17].

**Micronuclear test under under cytokinesis-block conditions.** For lymphocyte culturing, blood of healthy donors (0.5 mL) was taken from a finger with a micropipette filled with heparin and transferred to sterile tubes with the culture medium composed of 5 mL of RPMI-1640 (Sigma, USA) containing 15% of inactivated fetal bovine serum, a mixture of penicillin and streptomycin, and PHA-M (10 μg/mL). After 24-h culturing at 37°C, suspensions of SiO\textsubscript{2} NPs at concentrations of 100 and 200 μg/mL were added to the PHA-stimulated lymphocyte culture.

To inhibit cytokinesis and obtain polymuclear cells, cytochalasin B (6 μg/mL) was added to the medium 44 h after initiation of culturing, and in an additional 28 h the cells were separated by centrifugation (1000 rpm), resuspended in a cold (4°C) hypotonic solution (0.56% KCl) for 5 min, fixed in duplicate in a 4:1 methanol:glacial acetic acid, and air-dried using a cytocentrifuge. The preparations (20 object plates per concentration) were Giemsa stained and analyzed on an Axiostar plus microscope (Carl Zeiss, Germany); no less than 100 cells in each of 20 object plates for each concentration were analyzed [15, 18].

The cytotoxic effect was assessed using the entire cell pool, where the total scores of mono-, bi-, tri-, tetranuclear, and higher nuclear lymphocytes and their percentages were determined. The frequencies of pyknosis, apoptosis, and mitosis were calculated in per mille with respect to the absolute value, and the cytokinesis block proliferation index (CBPI) was calculated by the formula $\text{CBPI} = \frac{(M1+2M2+3(M3+M4))}{N}$, where M1–M4 are the scores of mono-, bi-, tri-, and tetranuclear cells, multiplied by the number of cell cycles that have passed and N is the total cell score (except for pyknotic cells) [19]. The mutagenic effect was assessed by the score of micronuclei per 1000 binuclear cells.

**Chromosome aberrations test.** The structural alterations in the human peripheral blood lymphocyte chromosomes were identified, as well as grouping of chromosome abnormalities was performed using the unstable chromosome aberration assay [20]. The blood samples obtained as described above were introduced into sterile tubes (0.6 mL per tube) with the culture medium composed of 6.0 mL of the RPMI 1640 medium, 1.0 mL of inactivated fetal bovine serum; 50 μL of a mixture of penicillin and streptomycin, and 0.2 mL of PHA-M. After 48-h culturing at 37°C, a suspension of SiO\textsubscript{2} NPs at a concentration of 200 μg/mL was added to the lymphocyte culture. The known mutagen Mitomycin-C (positive control) was introduced at a concentration of 0.1 μg/mL to the lymphocyte culture at the 48th hour of culturing. The control was the straight lymphocyte culture. To block mitosis at the metaphase stage, a solution of colcemid (Sigma, USA) was added to the culture medium 1 h before the end of culturing to the final concentration of 0.6 μg/mL. After culturing had been complete, the cells were precipitated by centrifuging at 1000 rpm for 10 min. The supernatant was discarded, and the residue was poured with a warm (37°C) hypotonic solution (0.75 M KCl), the mixture was thermostated at 37°C for 20 min, centrifuged under the above conditions, and the supernatant was discarded. A freshly prepared fixer (3:1 methanol–glacial acetic acid) was added to the precipitate. The fixing procedure was repeated three times. The cytological preparations were obtained immediately after the last fixing run. The cell suspension was applied by drops on cold object glasses prevetted with distilled water, and Giemsa stained. In each experimental group, 100 metaphase plates containing no less than 44 chromosomes having no less than 3 chromosome overlaps in one metaphase were analyzed. Chromosome-type (paired acentric fragments) and chromatid-type (single fragments) aberrations on metaphase plates were scored.

**Alkaline DNA comet assay.** Lymphocytes isolated from the whole blood of human donors by the Ficoll method were incubated for 3 h at 37°C in the L-glutamin-free RPMI-1640 medium spiked with a suspension of SiO\textsubscript{2} NPs (100 and 200 μg/mL) and then applied in an agarose gel (mp <42°C) on slides of agarose gel (mp < 65°C). Cell lysis, alkaline denaturation, electrophoresis, fixation, and
staining were performed. The resulting preparations were analyzed on an Axioskop 40 luminescent microscope equipped with a digital photo camera. Analysis of the resulting images was performed using the CometScore software (http://tritekcorp.com). The total level of DNA damage was estimated by the percentage of cells with tail DNAs.

Statistical analysis was performed using the Bartlett's test with preliminary assessment of the homogeneity of variances by means of univariate dispersion analysis (ANOVA) with the subsequent adjustment of the level of difference between control and test groups by the Bonferroni t-test or Dannett q-test. In the absence of homogeneity, the group medians were compared using the Kruskal–Wallis test with Dunn's adjustment. Chi-squared test or Fisher's exact test [21, 22] were used for comparison of differences between discrete variables (embryolethal effect, pathological effects). The differences were considered significant at \( p < 0.05 \). Data processing was performed using the Prizm 5 software (GraphPad Software, USA).

Results and Discussion

For the assessment of cyto-, geno-, and embryotoxicity of silica nanoparticles in the present work we employed our developed combined approach based on a combined use of three models: pre-and post-implantation embryos of SiO2 NPs was performed using three models: in vitro cultured pre- and post-implantation embryos of laboratory animals (mice and rats) and human peripheral blood lymphocyte culture. Early embryos cultures were used to reveal the embryolethal and teratogenic effects (dysmorphogenesis), dynamic morphofunctional disorders, and gene expression disorders, caused by SiO2 NPs, and human peripheral blood lymphocyte cultures, to reveal their cytotoxic (cytostatic), genotoxic, and mutagenic effects.

The advantage of embryonic models is that embryonic development occurs at stages most sensitive to chemical hazards: the stage of embryo cleavage (from fertilization to formation of blastocyst capable for implantation) and initial organogenesis stages involving the most important morphogenetic processes (neurulation, body axis formation, somitogenesis, etc.), active initiation of organs (brain, vision and acoustic organs, limb rudiments, etc.), development of the embryo’s support system (establishment of trophic functions, change of the respiration modes, yolk sac and liver hematopoiesis, cardiac function activation, circulation initiation, etc.).

The advantage of human peripheral blood lymphocytes is their high proliferation rate in the culture, which makes them an appropriate model for direct human cytotoxicity and genotoxicity (mutagenicity) assessment.

In vitro assessment of the effect of SiO2 NPs on preimplantation mouse embryos at the stages cleavage and blastocyst hatching.

Silica NPs at concentrations of 200 and 100 \( \mu \text{g/mL} \) caused single embryo deaths (8.7–4.2 %) and decreased the rate of hatching, which resulted in a decrease of the total cell mass by the end of culturing by a factor of 1.3 compared to control \(( P < 0.01, P < 0.001)\). After 72-h culturing 14.3 and 17.4 % of developing embryos stayed at the early blastocyst stage and showed inhibited cavitation (Table 1, Fig. 1). As little as 85.7 and 82.6 % (control 100%) of embryos reached the formed blastocyst stage and had a well-defined internal cell mass (ICM). The number of hatching blastocysts (Table 1) was lower than in control \(( P \geq 0.05)\) with both concentrations of SiO2 NPs. Hatching (the process when an embryo frees itself from the enveloping pellucid zone) is quite an important stage of embryogenesis, which creates conditions for embryo implantation within uterine crypts. The changes in the in vitro cultured preimplantation mouse embryos in response to exposure to SiO2 NPs at concentrations of 200 and 100 \( \mu \text{g/mL} \) were similar both in character and in strength.

The nanoparticles at 20 \( \mu \text{g/mL} \) had no effect on the development of preimplantation mouse embryos. All explanted embryos reached the formed blastocyst stage by the end of culturing, and 38.1% had already hatched.
The cytological analysis of air-dried embryo preparations after exposure to SiO₂ NPs at 200, 100, and 20 µg/mL revealed no changes in the proliferative activity of blastomers (mitotic score) and cytotoxic effect (pyknotic score) with respect to control.

Table 1: Effect of SiO₂ NPs on in vitro development of preimplantation mouse embryos (direct exposure)

| Concentration of SiO₂ NPs, µg/mL | Control | 200 | 100 | 20 |
|----------------------------------|---------|-----|-----|----|
| Total embryo score               |         |     |     |    |
| Dead embryo score, total/ %      | -       | 2/8.7 | 1/4.2 | - |
| Score of embryos formed by the end of culturing: |         |     |     |    |
| 9–16 blastomers total/ %         | -       | -   | -   | - |
| 17–32 blastomers total/ %        | -       | -   | 1/4.3 | - |
| 33–64 blastomers total/ %        | -       | 7/33.3 | 5/21.7 | 2/9.5 |
| 65 and more blastomers total/ %  |         | 14/66.7 | 17/74 | 19/90.5 |
| Score of embryos at the stage of morula total/ % | - | - | - | - |
| early blastocyst total/ %        | -       | 3/14.3 | 4/17.4 | - |
| blastocysts total/ %             |         | 17/100 | 18/85.7 | 19/82.6 | 21/100 |
| including hatched blastocysts    |         | 7/41.2 | 5/29.8 | 6/31.6 | 8/38.1 |
| Mean nuclei score total ± m      |         | 94.88 | 74.29** | 72.61*** | 91.90 |
| Mitotic score (per embryo) ± m   |         | 2.3   | 2.8   | 2.9   | 2.8 |
| Pyknotic score (per embryo) ± m  |         | 0.2   | 0.8   | 0.4   | 0.5 |
| Abnormal embryo score total/ %   | -       | -     | -     | -     |
| Net embryotoxic effect total/ %  | -       | -     | -     | -     |

(*, **, ***) Statistically significant result with P < 0.05, P < 0.01, and P < 0.001, respectively, relative to control.

Fig. 1. Culturing preimplantation embryos for 72 h: (A) control: all blastocysts are hatching and (B) exposure to SiO₂ NPs (200 µg/mL): the arrows point to early blastocysts (few-celled blastocysts with inhibited cavitation process). Magnification 100x.
Thus, amorphous SiO$_2$ NPs at concentrations of 200 and 100 $\mu$g/mL showed an embryotoxic effect on preimplantation mouse embryos developing in vitro, as evidenced by slowed down cleavage entailing a decrease in the total cell mass, appearance of few-celled blastocysts with inhibited cavitation process ($P < 0.01$, $P < 0.001$), and decrease in the hatching blastocyst score (by a factor of 1.4–1.3 compared to control). Silica nanoparticles at a concentration of 20 $\mu$g/mL had no effect on the preimplantation mouse embryo development.

**In vitro assessment of the effect of SiO$_2$ NPs on early rat organogenesis.**

Culturing postimplantation rate embryos (E 9.5–11.5) in the medium containing SiO$_2$ NPs at concentrations of 20, 100, and 200 $\mu$g/mL caused no embryolethality, but at the highest concentration some structural (neural tube dysgraphia, brain deformation, reduction of cerebral hemispheres, micro- and anophthalmia; disturbance of axial rotation) and dynamic (brain and pericardial edemas, brain and caudal artery hemorrhages) disorders were observed (Fig. 2, A–C). General morphogenesis inhibition, specifically retardation of somitogenesis, reduction of craniocaudal dimension, and reduction of total protein (Table 2) was observed. The yolk sac growth and vascularization rates did not differ from those in control.

Fig. 2. (A) Control: rat embryos in the course of culturing (the embryo is sheathed in the visceral wall of the yolk sac, containing blood vessels and abnormal embryos after 48-h after culturing with 200 $\mu$g/mL SiO$_2$ NPs: (B) disturbed neurulation at the head end and (C) reduction of brain hemispheres and brain deformation. Magnification 10x.

Sister chromatid exchange test revealed no genotoxicity in embryos growing in the medium containing 200 $\mu$g/mL SiO$_2$ NPs.

Table 2. Effect of SiO$_2$ NPs on in vitro development of postimplantation rat embryos

| Agent                        | Control | SiO$_2$  |
|------------------------------|---------|----------|
|                              |         | 20       | 100      | 200      |
| Concentration, $\mu$g/mL     |         | 20       | 100      | 200      |
| Total embryos score          | 42      | 30       | 22       | 28       |
| Dead embryos score, abs/\%   | 0/0     | 0/0      | 1/4.8 ±4.7 | 2/7.1 ± 4.1 |
| Live embryos score           | 42      | 30       | 21       | 26       |
| Yolk sac dimensions:         |         |          |          |          |
| longitudinal, mm             | 4.21 ± 0.06 | 4.51 ± 0.08 | 3.50 ± 0.18*** | 3.49 ± 0.11*** |
| transversal, mm              | 3.83 ± 0.05 | 3.92 ± 0.07 | 3.23 ± 0.17*** | 3.23 ± 0.09*** |
| vertical, mm                 | 3.68 ± 0.05 | 3.79 ± 0.06 | 3.18 ± 0.16*** | 3.23 ± 0.10*** |
| volume, mm$^3$               | 31.69 ± 1.26 | 35.78 ± 1.61 | 21.53 ±2.83*** | 20.03 ± 1.49*** |
| Total somite score           | 25.50 ± 0.24 | 25.97 ± 0.21 | 20.52 ±1.07*** | 18.54 ± 0.92*** |
| Craniocaudal size, mm        | 3.37 ± 0.04 | 3.63 ± 0.06 | 3.02 ± 0.11 **  | 2.46 ± 0.11*** |
Head size, mm

|   | a    | b    | c    | d    | e    |
|---|------|------|------|------|------|
|   | 1.70 ± 0.03 | 0.67 ± 0.01 | 0.89 ± 0.02 | 1.25 ± 0.03 | 0.94 ± 0.02 |
|   | 1.81 ±0.04 | 0.74 ± 0.02 | 0.91 ± 0.02 | 1.32 ± 0.03 | 1.00 ± 0.02 |
|   | 1.44 ±0.07*** | 0.58 ± 0.03** | 0.77 ± 0.04*** | 1.09 ± 0.05 ** | 0.83 ± 0.04** |
|   | 1.17 ± 0.05 *** | 0.47 ± 0.02*** | 0.60 ± 0.02*** | 0.88 ± 0.04*** | 0.71 ± 0.03*** |

Total protein, μg/embryo

|   | 375 ± 2.3 | 370 ± 6.6 | 316 ± 27** | 227 ± 4.7*** |

Dysmorphogenesis total

|   | 5   | 5   | 13  | 23  |
|---|-----|-----|-----|-----|
| % | 10.60 ± 5.56 | 18.18 ± 2.34 | 63.70±20.62** | 87.5 ± 7.97*** |

Dysmorphogenesis

|   | structural defects | dynamic disorders | retarded development |
|---|-------------------|-------------------|---------------------|
| % | 3   | 4   | 1   | 11  | 18  |

SCE frequency/cell

|   | 4.10 ± 0.22 | —   | —   | 3.83 ± 0.27 |

(***, ***) Statistically significant result with \( P < 0.05, P < 0.01, \) and \( P < 0.001, \) respectively, relative to control.

**Assessment of gene expression (quantitative PCR).**

Analysis of the expression of genes involved in neurulation showed that SiO\(_2\) NPs at concentrations of 100 and 200 μg/mL activated Neurog1 gene expression at the transcription level (6.77 and 5.1 times, respectively), and the expression levels of the other genes associated with neuronal differentiation and neurulation did not significantly differed from control (Table 3). The activation of neurogenin can be explained by either disorders in morphogenesis or neurulation processes.

**Table 3. Effect of SiO\(_2\) NPs on gene expression in rat embryos (E 11.5)**

| Gene   | Control | SiO\(_2\) (100 μg/mL) | SiO\(_2\) (200 μg/mL) | Effect       |
|--------|---------|-----------------------|----------------------|--------------|
| BMP2   | 1       | 1.33                  | 1.03                 | -            |
| BMP4   | 1       | 1.67                  | 1.20                 | -            |
| NEUROG1| 1       | 6.77                  | 5.10                 | Enhanced expression |
| TULP3  | 1       | 1.61                  | 1.26                 | -            |
| SHH    | 1       | 1.22                  | 1.30                 | -            |

Thus, high concentrations of SiO\(_2\) NPs exert dysmorphic effects at early embryogenesis stages (cleavage division, blastocyst formation, early organogenesis).

**Cytotoxicity and genotoxicity (mutagenicity) assessment of SiO\(_2\) NPs in the human peripheral blood lymphocyte culture.**

**Micronuclear test.** In the micronuclear test under cytokinesis-block conditions, the addition of SiO\(_2\) NPs to the lymphocyte culture (200 and 100 μg/mL) caused cell clustering but without signs of aggregation. The scores of pyknotic and apoptotic cells did not change (Table 4). Slightly more expressed mitotic activity of lymphocytes was noted \( (P \geq 0.05) \) but statistically significant differences were not obtained. Changes were observed in the scores of polynuclear cells and their proliferative activity. The data in Table 4 show that exposure to SiO\(_2\) NPs at concentrations of 200 and 100 μg/mL increase the scores of bi-, tri-, and tetranuclear cells with respect to control \( (P<0.05, P<0.01, \) and \( P<0.001, \) respectively). The cytokinesis block proliferation index (CBPI), too, revealed a statistically significant increase in the cell proliferation rate \( (P<0.05, P<0.01). \)

The micronuclear test for mutagenicity revealed no increase in the number of micronuclei in binuclear cells in the cultures containing SiO\(_2\) NPs in all the three studied concentrations.
Table 4. Cyto- and genotoxicity (mutagenicity) assessment of SiO₂ NPs in the human peripheral blood lymphocyte culture

| Agent                        | Control with BSA | SiO₂  |
|------------------------------|------------------|-------|
|                              |                  | 100 μg/mL | 200 μg/mL |
| Concentration                |                  |         |         |
| Total cell score, abs        |                  | 2373   | 2541   |
| ●Mean per 100 cells n ± m   | 113.0±2.5        | 121.0±3.1 | 124.6±4.9* |
| Mononuclear cells            |                  | 1160/48.9 | 1163/45.8 | 956/42.6 |
| ●Mean per 100 cells n ± m   | 55.2±1.6         | 55.4±2.0 | 53.1±3.0 |
| Binuclear cells              |                  | 1018/42.9 | 1050/41.3 | 1025/45.7 |
| ●Mean per 100 cells n ± m   | 48.5±1.2         | 50.0±1.4 | 56.9±2.3** |
| Trinuclear cells             |                  | 107/4.5 | 164/6.5 | 121/5.4 |
| ●Mean per 100 cells n ± m   | 5.1±0.4          | 7.8±0.4*** | 6.7±0.6* |
| Tetranuclear cells           |                  | 88/3.7 | 164/6.5 | 140/6.2 |
| ●Mean per 100 cells n ± m   | 4.2±0.5          | 7.8±0.5*** | 7.8±0.6*** |
| CBPI                         | n ± m            | 1.6±0.02 | 1.7±0.02* | 1.7±0.02* |
| #Pyknotic score total %     |                  | 12      | 12      | 10      |
| #Apoptotic score total %    |                  | 9       | 6       | 3       |
| # Mitotic score total %     |                  | 49      | 63      | 57      |
| Micronuclear test            |                  |         |         |
| Binuclear cells (BC) without micronucleus (MN) | 1013 | 1045 | 1021 |
| BC with 1 MN                 |                  | 4       | 4       | 3       |
| BC with 2 MN                 |                  | 1       | 1       | 1       |
| BC with 3 MN                 |                  | 0       | 0       | 0       |
| ##Micronuclei total         |                  | 6       | 6       | 5       |
| BC with MN total             |                  | 5       | 5       | 4       |

(●) calculated per total cell score; (●) calculated per total cell score; (●) averaged over 20 object plates, with no less than 100 cells scored at each plate; (*, ***, **) statistically significant result with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, relative to control.

Thus, the mutagenicity assessment of SiO₂ NPs using the micronuclear test revealed no increased scores of micronuclei in binuclear cells. The addition of SiO₂ NPs at concentrations of 200 and 100 μg/mL to the lymphocyte culture produced no other effect than proliferation activation as evidenced by an increased score of polynuclear cells, which can potentially lead to tumor cell transformation. This suggestion agrees with the data in [23, 24] and calls for further research.

DNA comet assay was used to assess DNA damage in peripheral human blood lymphocytes, caused by exposure to SiO₂ NPs. The DNA damage was measured in terms of the percentage of DNA in the comet tail. After 3-h incubation of the human peripheral blood lymphocyte culture containing 200 and 100 μg/mL silica NPs, no statistically significant difference with control was found (Table 5).
Table 5. Levels of DNA damage in human peripheral blood lymphocytes after exposure to SiO₂ NPs

| Method                        | Alkaline DNA comet assay |
|-------------------------------|-------------------------|
| Agent (concentration)         | Control with BSA | SiO₂ (100 μg/mL) | SiO₂ (100 μg/mL) |
| Total comet score             | 136 | 85 | 176 |
| Tail DNA % (mean ±m)          | 5.7±0.3 | 7.1±0.5 | 6.5±0.3 |
| Fractions of cells with damaged (tail) DNA |
| 0–5%                          | total mean ±m | 59 | 31 | 73 |
|                                | mean ±m      | 1.9±0.2 | 2.4±0.3 | 2.3±0.2 |
| 5–10%                         | total mean ±m | 55 | 33 | 62 |
|                                | mean ±m      | 7.1±0.2 | 7.2±0.3 | 7.6±0.2 |
| 10–15%                        | total mean ±m | 20 | 13 | 39 |
|                                | mean ±m      | 12.0±0.3 | 11.5±0.3 | 12.2±0.2 |
| 15–20%                        | total mean ±m | 2 | 7 | 2 |
|                                | mean ±m      | 15.5±0.3 | 16.8±0.6 | 15.9±0.6 |
| > 20%                         | total mean ±m | 0 | 1 | 0 |

Chromosome aberrations test. The cytogenetic analysis of the human peripheral blood lymphocytes exposed to SiO₂ NPs at a concentration of 200 μg/mL revealed no statistically significant increase in the frequency of unstable chromosome aberrations, and the numbers of double and single fragments did not differ from control (Table 6).

Table 6. Frequency of metaphase chromosome aberrations in the human peripheral blood lymphocyte culture on direct exposure to SiO₂ NPs

| Agent (concentration) | Negative control | Mitomycin-C (0.1 μg/mL) | SiO₂ (200 μg/mL) |
|-----------------------|------------------|--------------------------|------------------|
| Number of analyzed metaphase plates | 400 | 400 | 400 |
| Total number of aberrations, abs/\% of them: | 9/2.25 | 71/17.75*** | 11/2.75 |
| single fragments | 8/2.0 | 57/14.25 | 9/2.25 |
| double fragments | 1/0.25 | 14/3.5 | 2/0.5 |
| Number of aberrations per one metaphase plate | 0.02±0.07 | 0.2±0.01*** | 0.03±0.008 |

(*, **, *** ) Statistically significant result with P < 0.05, P < 0.01, and P < 0.001, respectively, relative to control.

Thus, the cyto- and genotoxicity (mutagenicity) assessment of amorphous SiO₂ NPs in the human peripheral blood lymphocyte culture by three cytogenetic methods (chromosome aberration assay, DNA comet assay, and micronuclear test) did not reveal increased scores of chromatid- and chromosome-type aberrations, single- and double-strand DNA breaks, and micronuclei in binuclear cells.

Conclusions
The developed combined approach to the express assessment of the effect of amorphous SiO$_2$ nanoparticles using three models (pre- and post-implantation rodent embryos, and human peripheral blood lymphocyte culture) allowed us to show that SiO2 NPs at concentrations of 200 and 100 μg/mL impairs early embryogenesis (cleavage division, blastocyst formation, early organogenesis). The cytogenetic tests in the human peripheral lymphocyte culture (micronuclear test under cytokinesis-block conditions, chromosomal aberrations assay, and alkaline DNA comet assay) revealed no cytotoxic, genotoxic, and mutagenic effects, except that SiO2 NPs at concentrations of 200 and 100 μg/mL caused more active proliferation as evidenced by the increased score of polynuclear cells, which may result in the subsequent tumorigenic cell transformation.

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