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# Relationship between lymphocyte DNA fragmentation and dose of iron oxide ($Fe_2O_3$) and silicon oxide ($SiO_2$) nanoparticles.

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**ABSTRACT.** At present, the use of nanoparticles is a controversial topic, especially when analyzing their effects in human tissues. Nanoparticles (NPs) can cause oxidative stress by increasing membrane lipids peroxidation and reactive oxygen species, and decreasing intracellular glutathione. Oxidative stress plays an important role in cell signaling

and inflammatory responses. It can result in genotoxicity, affect cell proliferation, and induce DNA damage. The objective of this study is to evaluate the genotoxic potential of NPs in lymphocyte DNA. Wistar female rats (N = 45) were sorted in three randomized groups as follows: Group 1 (N = 20); Group 2 (N = 20) and a control group (N = 5). A single dose of iron oxide ($Fe_2O_3$) and silicon oxide ($SiO_2$) NPs dissolved in saline solution were administered orally to the rats. Cardiac puncture was performed to extract peripheral blood for genotoxic analysis. DNA fragmentation for lymphocytes was performed. Control rats showed a fragmentation percentage of 11.20 ± 2.16%. Rats exposed to $SiO_2$ and $Fe_2O_3$ NPs for 24 h showed statistically significant differences in DNA fragmentation percentages as compared with that of the control group. A lineal dose-response correlation between genotoxic damage and exposure to $SiO_2$ and $Fe_2O_3$ NPs was found ($r^2$ = 0.99 and 0.98 for $SiO_2$ and $Fe_2O_3$, respectively). In conclusion, we found that exposure to $Fe_2O_3$ and $SiO_2$ NPs can cause DNA fragmentation in lymphocytes in a dose-dependent manner.

**Key words:** Nanoparticles; DNA fragmentation; Lymphocytes; DNA damage; Iron oxide; Silicon oxide

## INTRODUCTION

Nanoparticles (NPs) have diverse therapeutic applications. Recently, there is great interest in their *in vivo* use, mainly for drug release, cancer therapy, implant coatings, contrast agents for diagnostic imaging, and cell therapy (Bruners et al., 2010; Di Bucchianico et al., 2013; Sadiq et al., 2015). One of NPs' characteristics is that the relationship between the number of superficial atoms and particle size is exponential (Volkovova et al., 2015). Because of this, characteristics associated with the particle's surface, such as electrical, mechanical, magnetic, optic, and chemical properties, differ from those of non-nanometric materials (Kumari et al., 2012, 2013). These properties play a very important role in particle toxicity. It is therefore of vital importance to understand these characteristics in order to predict and manage the potential risks that can occur in new biological studies (Magdolenova et al., 2015; Sanganeria et al., 2015). A major advantage of NPs is that they are capable of reaching a specific site using mainly the electric and magnetic fields on their surface. Once the magnetic or electric field is disrupted, magnetization disappears, and NPs can remain in the destined site for a period of time (Hong et al., 2011; Kumari et al., 2013; Sun et al., 2013). This is a property unique to NPs, and plays an important role in their diagnostic and therapeutic use. However, despite the fact that NPs have been commercialized in clinical applications, controversial research suggests that these nano-sized materials are associated with toxicity. In fact, there is much uncertainty on the rapid adoption of advanced biomedical nanotechnology, and comprehensive analysis still needs to be conducted on NP toxicity (Naqvi et al., 2010; Ahamed et al., 2013; Alarifi et al., 2014). Due to their small size and surface properties, NPs can pass through biological barriers and modify the physicochemical properties of matter. This causes increased interactions with tissues, and may lead to adverse biological effects in cells. Furthermore, in NPs that contain metal elements, solubility, temperature, and contact

period are key factors that affect their toxicity (Brunner et al., 2006; Franklin et al., 2007; Kahru et al., 2008; Frejo et al., 2011). The current knowledge regarding the effect of NP size on biological systems is still incomplete. However, NP properties associated with particle size are crucial factors that determine the biological safety of NPs, and can directly affect feasibility of NP in biomedical applications (Alarifi et al., 2014; Šebeková et al., 2014). The study of nanotoxicology has shown that NPs with the same composition but different magnetic surface charge can produce toxic damage (Kut et al., 2012; Ma et al., 2012). The production of reactive oxygen species (ROS) has been proposed as an underlying mechanism involved in genotoxicity of metallic oxide NPs, particularly, iron oxide NPs (Adams et al., 2006; Mesárošová et al., 2014; Yun et al., 2015; Rajiv et al., 2016). However, according to Lanone and Boczkowski (2006), the main mechanism behind NP toxicity is the induction of oxidative stress by free radicals. Recent studies have reported that exposure to NPs induces oxidative stress, as determined by an increase in ROS and cell membrane lipoperoxidation (Wang et al., 2010; Alarifi et al., 2014). These cellular changes have been attributed to the small size and large surface area of NPs (Xia et al., 2006). Oxidative stress is caused by the imbalance between ROS production and the ability of the biological system to quickly detoxify intermediate reactants or repair the resulting damage. Modifications of normal redox status can result in toxic effects through the production of peroxides and free radicals that damage cell components such as proteins, lipids and DNA (Xia et al., 2006; Pratt et al., 2008; Frejo et al., 2011). It is known that ROS cause oxidative stress by inducing superoxides ($O^{2-}$), hydrogen peroxides ($H_2O_2$), and hydroxyl radicals (OH) that cause DNA damage, and ultimately leads to cell apoptosis (Ott et al., 2007; Erdem et al., 2015; Rajiv et al., 2016). It is therefore possible that genotoxicity due to NP is an indirect result of ROS production. Genotoxicity leads to disruption of normal cell function, apoptosis, and even cancers (Alarifi et al., 2014). In fact, Stone and Donaldson (2006) suggested that screening strategies should be developed to discriminate between the adverse effects of different NPs at the cellular and molecular level (Xia et al., 2006; Pratt et al., 2008). The lack of information on the chemical composition, size, and shape of NPs, in addition to the scarcity of reports on NP exposure, has caused concerns regarding their impact on human health. As a result, regulation of NPs has been difficult. Both *in vitro* and *in vivo* studies in different animal species have been carried out to determine NP toxicity. Currently, more focus is placed on biological reactions of NPs composed of transition metals such as silicon, carbon, iron oxides, and other metallic agents that have been selected as potential vectors of pharmacological agents (Adams et al., 2006; Pratt et al., 2008; Yun et al., 2015; Rajiv et al., 2016). However, despite the growing number of industries and populations that utilize NPs, their toxic effects, as well as health surveillance systems and effective hygiene programs in the nanotechnology industrial sector is still lacking (Pratt et al., 2008). The comet assay is a very sensitive method for detecting DNA fragmentation induced by genotoxic agents in alkali-labile sites in individual cells (Singh et al., 1988). This technique can also be adapted for quantification of alkali-labile sites, DNA bases with oxidative damage, DNA-DNA or DNA-protein linkage, and abasic sites (Guillamet et al., 2004). The main objective of this study is to evaluate the genotoxic potential of NPs in peripheral blood lymphocytes as a cell and molecular biomarker.

## MATERIAL AND METHODS

### Animals

We used 45 homozygous Wistar pathogen-free female rats ranging between 7-9 weeks

of age, and weighing between 150-170 g. The experimental conditions were as follows: 12-h light/dark cycles, 25°-26°C, and relative humidity of 30-70%. Rats were given Nutri-cubos® (Agribrands Purina México, Irapuato, Guanajuato, Mexico) as food source, and water was given *ad libitum*. The use and care of the animals were performed according to technical specifications for the production, care, and use of laboratory animals (Diario Oficial de la Federación, 1999). Animal handling was performed by a certified veterinarian. The research was approved by the Bioethics Committee of the School of Medicine, Universidad Autónoma de Coahuila, Torreon Campus, Coahuila, Mexico (No. CONBIOETICA07CEI00320131015).

## Experimental groups

Animals (N = 45) was divided into three randomized groups as follows: group 1 (G1) (N = 20), group 2 (G2) (N = 20), and a control group (CG) (N = 5). Four subgroups consisting of five rats in each were formed from G1 and G2 (G1a, G1b, G1c, G1d and G2a, G2b, G2c, G2d). A single dose of iron oxide ($Fe_2O_3$) and silicon oxide ($SiO_2$) NPs dissolved in normal saline was administered orally to each subgroup of rats. The dose distribution is shown in Table 1. Following exposure to NPs, cardiac puncture was performed to extract peripheral blood for genotoxic analysis; the rats were later sacrificed by cervical dislocation at 24 h. NP doses were selected based on previous studies (Alarifi et al., 2014; Magdolenova et al., 2015; Sanganeria et al., 2015) to estimate the pharmacodynamics and genotoxic effects of NP.

**Table 1.** Determination of experimental groups.

| NP dose | Group and Subgroups | | | |
|---|---|---|---|---|
| | G1 (N = 20) | | | |
| | G1a | G1b | G1c | G1d |
| $SiO_2$ (mg/kg) | 3 | 7 | 10 | 13 |
| Subgroups | G2 (N = 20) | | | |
| | G2a | G2b | G2c | G2d |
| $Fe_2O_3$ (mg/kg) | 3 | 7 | 10 | 13 |
| Normal saline | CG (N = 5) | | | |
| | 1.5 mL | | | |

NPs, nanoparticles; G, group; CG, control group.

## Mass of $Fe_2O_3$ and $SiO_2$ NPs

The size of the $Fe_2O_3$ NPs used was 20-30 nm (CAS No. 1309-37-1, ≥ 98% purity), with a molecular weight of 231.53 g/mol; size of $SiO_2$ NP was 50-80 nm (CAS No. 7631-86-9, ≥98% purity), with a molecular weight of 60.2 g/mol. This was in accordance with the manufacturing report acquired from Nanostructured & Amorphous Materials, Inc., Houston, TX, USA.

## Lymphocyte sampling

Lymphocyte samples were obtained by cardiac puncture and stored in test tubes (BD Vacutanier®, Spain) with EDTA as the anticoagulant at 37°C for 30 min (with agitation) for DNA fragmentation analysis.

## DNA fragmentation analysis

DNA fragmentation of peripheral blood lymphocytes was performed using methods described by Singh et al. (1988) with modification by Guillamet et al. (2004). Samples were first lysed with cold alkaline lysis solution [2.5 M NaCl, 10 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA), 10% dimethyl sulfoxide, 1% Triton X-100], at a pH of 10. They were then placed on glass slides and incubated in cold electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 min, followed by electrophoresis at 25 V: 300 mA (1.25 V/cm) for 25 min. Finally, the slides were washed in neutralization buffer (0.4 M Tris, pH 7.5). The slides were stained with 35 µL GelGreen™ (Biotium, Hayward, CA, USA), and incubated at room temperature for 10 min in the dark. One hundred cells per slide were analyzed with a fluorescence microscope (LABOMED Lx 4000) at 100X magnification. Images were obtained with a LaboMed iVU 7000 16 Megapixel digital camera, and analyzed with TriTek CometScore™ Freeware v1.5. The Image J software V.1.8.0 was first used to remove background noise from the DNA images obtained. Automatic image processing software was used for analysis of the comet assay. The software was able to calculate the amount of DNA at specified location based on pixel intensity of images. DNA in the tail was computed as follows:

$$DNA = \text{total comet tail intensity} / \text{total comet intensity} \times 100$$

## Histopathological analysis

Histopathological analysis was performed in order to observe the effects of different doses of NPs in tissues. Rats in the experimental groups were sacrificed by cervical dislocation after NP treatments. The organs were harvested and fixed in 10% neutral formalin. Tissues were processed by standard histological techniques, and embedded in paraffin blocks. Tissue sections (5 µm) were prepared on a microtome, and were stained with hematoxylin and eosin. Stained sections were mounted, coverslipped, and sealed with synthetic resin prior to being observed via light microscopy.

## Statistical analysis

Statistical analysis was performed using the Minitab 17 software for Windows. Analyzed data are reported as means ± SD. For group comparisons, the Student *t*-test was used; for DNA fragmentation analysis, the Mann-Whitney U-test was used; Pearson's lineal correlation ($r^2$) was used to calculate correlations. Results with $P < 0.05$ were considered to be statistically significant.

# RESULTS

## Animals

No significant differences ($P < 0.05$) in body weight were found between all groups (Table 2).

**Table 2.** Body weight of all experimental groups (means ± SD).

| Group | Means ± SD |
|---|---|
| CG | 177.4 ± 15.27 |
| G1 exposed to $SiO_2$ | |
| Subgroup | |
| G1a | 162 ± 10.06 |
| G1b | 149.6 ± 13.58 |
| G1c | 164.4 ± 9.4 |
| G1d | 149.8 ± 8.76 |
| G2 exposed to $Fe_2O_3$ | |
| Subgroup | |
| G2a | 153.2 ± 20.5 |
| G2b | 149.6 ± 5.5 |
| G2c | 159.8 ± 6.5 |
| G2d | 163.1 ± 6.1 |

$P < 0.05$; statistically significant difference with Student *t*-test. G, group; CG, control group.

## DNA fragmentation

Fragmentation percentage of CG was 11.20 ± 2.16%; which is considered normal, and is characteristic of DNA repair in the cell (Figure 1C). However, genotoxicity induced by $SiO_2$ NPs in G1 showed a dose-damage relationship; DNA fragmentation increased as dose of $SiO_2$ NPs increased, as shown in Table 3. G1 exposure to $SiO_2$ NPs for 24 h resulted in significantly higher DNA fragmentation in control group (CG). Figure 2A illustrates this lineal correlation, and shows that dose-damage association between NP exposure and genotoxic damage exhibited a positive linear relationship ($r^2 = 0.99$).

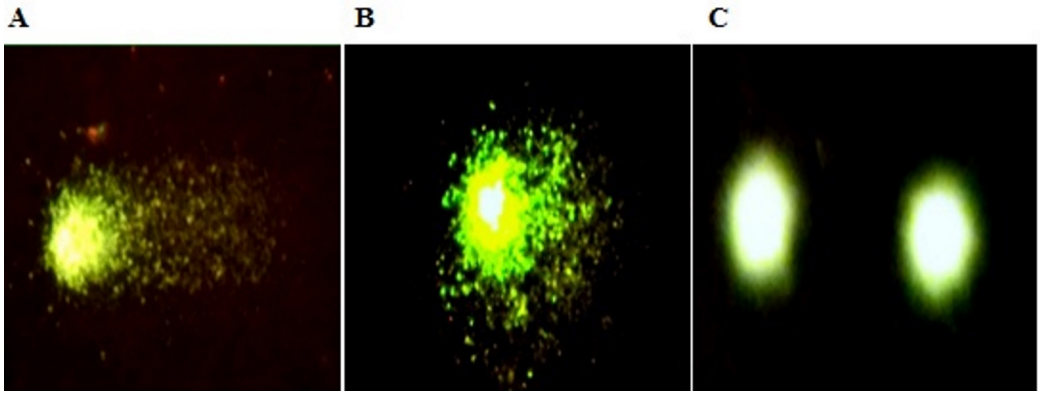


**Figure 1.** DNA fragmentation in lymphocytes from Wistar rats. DNA fragmentation in G1- exposed to $SiO_2$ NPs (**A**), G2- exposed to $Fe_2O_3$ NPs (**B**), and control (**C**). Images are taken at 100X magnification.

G2 exposure to $Fe_2O_3$ NPs exhibited similar pattern as that demonstrated by G1. A significant increase in CG DNA fragmentation was observed in G2 as compared with baseline damage. Table 4 shows DNA fragmentation induced by exposure to $Fe_2O_3$ NPs, which was similar in behavior as compared to that of $SiO_2$ NPs, but with reduced damage (Figures 1A and B). The $r^2$ value between $Fe_2O_3$ NP exposure and DNA damage was 0.98, as shown in Figure 2B.

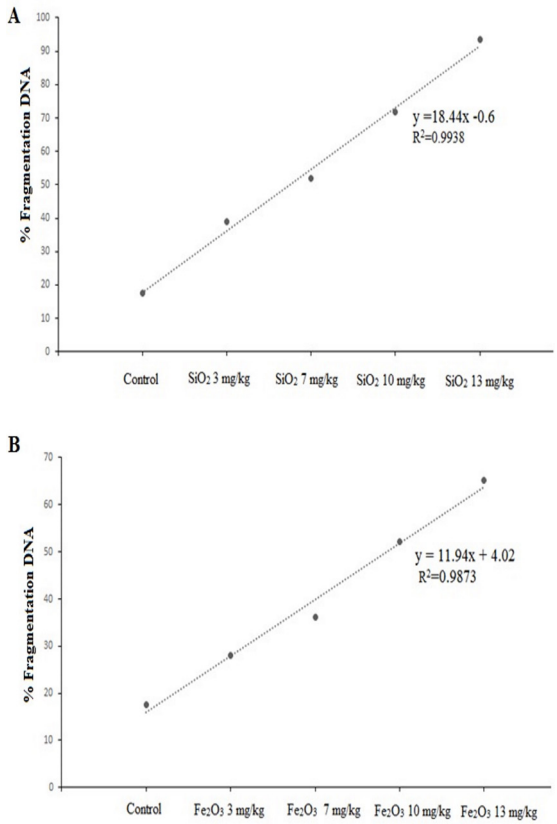


**Figure 2.** Linear correlation analysis for DNA fragmentation Association between DNA damage and exposure to $SiO_2$ NPs (**A**) and $Fe_2O_3$ NPs (**B**) in experimental groups.

**Table 3.** Evaluation of DNA fragmentation in lymphocyte samples in groups treated with $SiO_2$ nanoparticles.

| G1 | % of DNA fragmentation |
|---|---|
| Control group | 11.20 ± 2.16 |
| G1a ($SiO_2$ 3 mg/kg) | 39.00 ± 2.45* |
| G1b ($SiO_2$ 7 mg/kg) | 51.80 ± 4.09* |
| G1c ($SiO_2$ 10 mg/kg) | 71.80 ± 4.32* |
| G1d ($SiO_2$ 13 mg/kg) | 93.40 ± 3.65* |

Results are reported as means ± SD. *P < 0.05, compared with the Mann-Whitney U test. G = group.

**Table 4.** Evaluation of DNA fragmentation in lymphocyte samples in groups treated with $Fe_2O_3$ nanoparticles.

| G2 | % of DNA fragmentation |
|---|---|
| Control group | 11.20 ± 2.16 |
| G2a ($Fe_2O_3$ 3 mg/kg) | 28.0 ± 2.55* |
| G2b ($Fe_2O_3$ 7 mg/kg) | 36.20 ± 1.78* |
| G2c ($Fe_2O_3$ 10 mg/kg) | 52.20 ± 3.83* |
| G2d ($Fe_2O_3$ 13 mg/kg) | 65.20± 2.39* |

Results are reported as means ± SD. *P < 0.05, compared with the U Mann-Whitney test. G = group.

## Histopathological findings

Liver histopathology of CG showed normal morphological characteristics with normal vascular congestion. In the groups exposed to $SiO_2$ and $Fe_2O_3$ NPs, there was evidence of vascular congestion, cell cytoplasm, and hyperchromatic hepatocyte nuclei (Figure 3). Similarly, kidney histopathological findings in the control group showed normal morphology with sparse vascular congestion. In groups exposed to $SiO_2$ and $Fe_2O_3$ NPs, moderate vascular congestion, vacuoles in the cytoplasm, and glomerular congestion were observed (Figure 4).

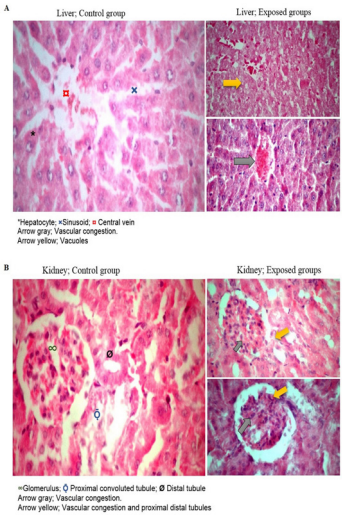


**Figure 3.** Histopathological analysis following $SiO_2$ NP exposure H&E staining of the liver (**A**) and the kidney (**B**) in Wistar rats exposed to $SiO_2$ NPs as compared to that in the control group.

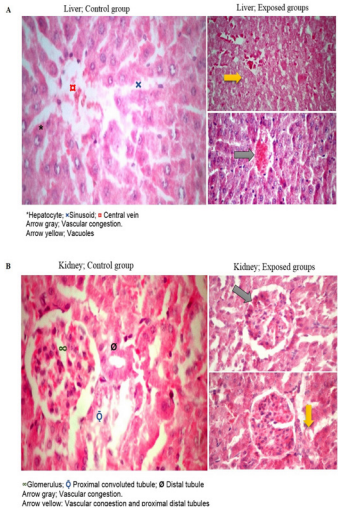


**Figure 4.** Histopathological analysis following $Fe_2O_3$ NP exposure Histopathology of the liver (**A**) and the kidney (**B**) in Wistar rats exposed to $Fe_2O_3$ NPs as compared to that in the control group.

## DISCUSSION

According to results shown in this study, $Fe_2O_3$ and $SiO_2$ NPs produce genotoxic effects in Wistar rat lymphocytes at varying doses. $Fe_2O_3$ and $SiO_2$ NPs have important industrial impacts due to their characteristics and properties. However, NP exposure has potential effects on worker's health. Experimental studies using cell lines and animal models showed that NP exposure could have genotoxic and cytotoxic effects. Our study demonstrated that exposure dose is an important factor for generation of genotoxic damage, as NPs can accumulate in the organism. This was similar to the study by Alarifi et al. (2014), which suggested that genotoxicity and cytotoxicity of $Fe_2O_3$ NPs arise mainly due to accumulation in the body. On the other hand, it has been shown that accumulation of $Fe_2O_3$ NPs generate ROS, which induce radicals ($O^{2-}$, $H_2O_2$, and OH) that cause DNA damage in cell lines and animal models. Furthermore, it has been established that DNA fragmentation is the main mechanism by which cell apoptosis occurs (Ott et al., 2007; Alarifi et al., 2014). Our results are consistent with other studies which demonstrated that NPs have great potential for inducing DNA damage (Eom and Choi, 2009; Naqvi et al., 2010; Alarifi et al., 2014; Magdolenova et al., 2015; Sanganeria et al., 2015). Another way by which NPs can cause genotoxic damage is through their surface charge and time of exposure. Our results are consistent with that of Klien and Godnić-Cvar (2012), which showed that 1-2-day exposure is sufficient to cause DNA strand breaks. However, differences in exposure methods must be considered. In studies with short exposures, genotoxicity is evaluated by the capacity to repair DNA, while in long-term exposure studies, irreversible chromosomal and histological damage is determined. Magdolenova et al. (2015) reported that $Fe_2O_3$ NPs specifically cause dose-dependent cytotoxic damage in all exposed cell lines due to electrical surface characteristics of NPs. However, in other studies, it was suggested that iron oxide NPs do not cause genotoxicity and cytotoxicity in bacteria such as *Escherichia coli* (Gram negative) and *Bacillus subitilis* (Gram positive). We also detected biomarkers of ROS in kidney and liver tissues, including glutathione, malondialdehyde, DNA-protein cross-linking, and 8-hydroxy-2'-deoxyguanosine. Tissue lesions and molecular oxidative damage in cells were found. The recommended dose according to the results of this study is 5 mg/kg, and we feel that this is the upper limit to balance the benefits and risks of sub-long term exposure to $Fe_2O_3$ NPs (Ma et al., 2012). However, Singh et al. (2013) showed that a 30 nm $Fe_2O_3$ particle and bulk $Fe_2O_3$ did not cause significant damage in % tail DNA, micronuclei formation, and chromosomal aberration at all tested doses and intervals. Similar *in vivo* genotoxicity studies with $Fe_2O_3$, using the comet assay, have not been reported. A549 cells treated with $Fe_2O_3$ and micrometric-sized particles showed low toxicity and no significant differences between different sized particles Hong et al. (2011) observed that NPs affect cell viability and DNA stability of L-929 fibroblastic cells in a dose-dependent manner. In addition, supermagnetic NPs did not seem cytotoxic or genotoxic to fibroblastic cells at concentrations below 500 ppm. However, it was noted that small modification to nanoparticles induced subtle variations in cell internalization and endocytosis. Furthermore, the notable differences in genotoxicity of different NPs, observed at low doses, were possibly due to variations in size and charges. Porter and Jänicke (1999) reported that $Fe_2O_3$ and $SiO_2$ NP exposure leads to caspase-3 activation, which can cause chromosomal condensation and DNA fragmentation. Chen and von Mikecz (2005) reported that $SiO_2$ nanoparticles, due to their small size, are capable of reaching the nucleus and interacting with DNA. Martinez et al. (2003) stated that ROS are involved in DNA damage of purine and pyrimidine bases.

In conclusion, these controversial results regarding genotoxicity and cytotoxicity of NPs show that evaluation of NP risk pose a great challenge. Current *in vivo* studies are limited because they are not comparable; interpretation of NP toxicological results are controversial as an infinite number of NPs that differ in size, nanomaterial, surface, electrical charge, shape, molecular weight, stability, and coating can be produced. These properties have different impacts on live tissues and cells, which influences the degree of cytotoxicity and DNA damage. *In vivo* and *in vitro* experiments also do not necessarily mimic real mechanisms of interactions in live organisms when there is spontaneous contact due to the variability of NPs. Comparison of different toxicological studies of NPs exposure is questionable due to the lack of standardization of units of measure ($mg/m^3$, mg/kg, and type of exposure). However, despite these limitations, we believe that results obtained in different studies can be useful in evaluating the genotoxic potential of NPs, especially as they are increasingly used in human health and the environment. In conclusion, our study demonstrated that exposure to $Fe_2O_3$ and $SiO_2$ NPs induced lymphocyte DNA fragmentation in a dose-dependent manner.

## Conflicts of interest

The authors declare no conflict of interest.

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