Genotoxicity of Titanium Dioxide Nanoparticles using the Mouse Bone Marrow Micronucleus and Sperm Morphology Assays

Bakare AA*, Udoakang AJ¹, Anifowoshe AT², Fadoju OM³, Ogunsuyi OI⁴, Alabi OA⁵, Alimba CG⁶ and Oyeyemi IT⁷

1Cell Biology and Genetics Unit, Department of Zoology, University of Ibadan, Ibadan, Nigeria
2Department of Zoology, University of Ilorin, Ilorin, Nigeria
3Department of Biology, Federal University of Technology, Akure, Nigeria

Abstract

Titanium dioxide nanoparticles (TiO₂-NPs) have recently been of public health and scientific concern due to their widespread use in industrial and household applications. However, there is limited information concerning its in vivo cytogenotoxicity. In this study, the cytogenotoxic effects of TiO₂-NPs on the somatic tissue using the mouse bone marrow micronucleus (MN) assay and on reproductive tissue using the mouse sperm morphology assay and testicular histopathology were investigated. Five concentrations of 9.38, 18.75, 37.50, 75.00 and 150.00 mg/kg bwt were administered intraperitoneally at 0.5 mL/mouse to mice for five and ten consecutive days in the MN assay; and for five consecutive days in the sperm morphology assay. Double distilled water and cyclophosphamide (20 mg/kg bwt) served as negative and positive controls, respectively. A significant (p<0.05) increase in MN was observed in bone marrow cells of treated mice at 37.50 mg/kg bwt concentration in the 5-day exposure and at all concentrations in the 10-day exposure. The sperm cells examined 5 and 10 weeks from the first day of exposure showed significant increase (p<0.05) in abnormal sperm cells at tested concentrations. Histopathologically, TiO₂-NPs disrupted the normal cellular architecture of testicular tissues in exposed mice; as it caused severe lesions such as congestion of the interstitium oedema, vacuolation and necrosis. These suggest that the bone marrow and testicular cells may be potential targets for TiO₂-NPs induced DNA damage and cytotoxicity in mice. This is of public health importance considering increasing exposure to TiO₂-NPs in consumer products.

Keywords: Titanium dioxide nanoparticles; DNA damage; Micronucleus; Histopathology; Mouse sperm morphology

Introduction

Nanotechnology as an emerging science in this millennium has led to the advancement in the production of nanoparticles [1]. It creates opportunities for engineers to manufacture superior and more durable devices and products [2], and boost scientific interest to ascertain their impact on the biotic and abiotic components of the ecosystem. The development and enlarging research interest in nanoparticles and nanomedicine have led to a huge potential for novel ways of rapid disease diagnosis, treatment and enhanced quality of life [2].

Nanoparticles (NPs) exist as naturally occurring nanoparticles (e.g. volcanic ash, ocean spray and storm dust) and engineered NPs (ENPs). ENPs include carbon based (e.g. fullerenes, carbon nanotubes), inorganic NPs such as metal (e.g. silver, iron, copper, manganese), and metal oxides (e.g. - titanium dioxide, zinc oxide, copper oxide, silicon oxide) and quantum dots (e.g. cadmium and selenium) [3]. They have a small size and large surface area to volume ratio with high reactivity potential; as a result of these unique properties, they have been massively produced by industries that use them on a large scale [4,5]. The increased production of these particles enhances the probability of exposure through inhalation, oral and dermal penetration, both in the occupational and environmental settings [6-8].

Among the available metal oxide NPs used in the manufacturing of consumer products are titanium dioxide nanoparticles (TiO₂-NPs), which are the earliest industrially produced nanomaterials [9] and one of the most highly manufactured in the world [10]. There are three different crystalline structures of TiO₂-NPs: anatase, rutile and brookite [11]. Anatase is more chemically reactive and capable of generating reactive oxygen species [12] while rutile is the most natural form of TiO, and is said to be chemically inert [11,12]. TiO₂-NPs account for over 70% of the total production volume of nanoparticles worldwide. It is a white pigment and mostly used because of its brightness and very high refractive index. It is used in diverse areas of application such as ointments, toothpaste, plastics, rubber, printing inks, floor coverings, automotive products, food colorants, catalysts, adsorbents, semi-conductors, mortar, ceramics, whitening and brightening of food, especially for confectionary and certain powdered foods. They are also used in the pharmaceutical industry as an opacity agent, and in environmental decontamination of air, soil, and water [13-15]. In some of these products, the amount of TiO₂-NPs is more than 10% by weight [16,17]. In spite of the increased application of TiO₂-NPs especially in consumer products, little is known about the potential toxicities and the underlying mechanisms, and this has generated major concerns among scientists especially toxicologists on their potential genotoxic and cytotoxic effects [18-20].

Data regarding the genotoxicity studies of TiO₂-NPs are inconsistent, as their toxicities are complex and depend on the physico-chemical properties such as size, surface area, crystalline structure, surface properties, agglomeration and solubility [4,5,21,22]. There are studies on in vitro genotoxic effects of TiO₂-NPs in human lymphocytes [23,24], human hepatoma HepG2 cells [25,26], Chinese Hamster Ovary

*Corresponding author: Bakare AA, Cell Biology and Genetics Unit, Department of Zoology, University of Ibadan, Ibadan, Nigeria, Tel.: +23407032595419; E-mail: adekunle.bakare@ui.edu.ng
Received January 28, 2016; Accepted February 29, 2016; Published March 07, 2016

Citation: Bakare AA, Udoakang AJ, Anifowoshe AT, Fadoju OM, Ogunsuyi OI, et al. (2016) Genotoxicity of Titanium Dioxide Nanoparticles using the Mouse Bone Marrow Micronucleus and Sperm Morphology Assays. J Pollut Eff Cont 4: 156. doi:10.4172/2375-4397.1000156

Copyright: © 2016 Bakare AA, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Cells (CHO) [27,28], human bronchial epithelial cell line (BEAS 2B) [29], human epidermal cell line (A431) [30], human lung cancer cells (A549) [31], and human SHSY5Y neuronal cells [32]. Whether photo activated or not, TiO$_2$-NPs (anatase or rutile) have been found to induce DNA damages [33-36], micronuclei formation [26,28,37], cell necrosis and apoptosis through the formation of reactive oxygen species [21,38-40], impair cell function in human dermal fibroblasts and decrease cell area, cell proliferation, and cell mobility [41]. In vivo, TiO$_2$-NPs have been reported to cause inflammatory reaction [42,43], oxidative DNA damage [44-47], pulmonary fibrosis [48] and serious damage to the liver, kidneys and myocardium in mice [49]. Information on in vivo genotoxicity of titanium dioxide nanoparticles is limited. This may be of importance for bone marrow and germ cells, where adverse impacts may affect the potentials for self-renewal and differentiation. With the enormous applications of TiO$_2$-NPs in consumer products, they can be absorbed via inhalation, ingestion, and dermal penetration into the body systemic circulation and reach important viscera organs. Hence, in this study, we investigated the genotoxic effect of TiO$_2$-NPs in mice using induction of micronucleus and abnormal sperm morphology as the genetic end points. Additionally, the toxic effect on the histology of the testes of exposed mice was also examined.

Materials and Methods

Test substance and preparation of TiO$_2$-NPs stock solution

Titanium dioxide nanopowder [(TiO$_2$-)NPs, anatase, CAS number: 1317-70-0, product code - 637254], Purity: 99.7%, Average Particle Size: <25 nm, Specific Surface Area: 45 m$^2$/g, Color: white, Morphology: powder, Bulk density: 0.04-0.06 g/mL and relative density: 3.9 g/mL was obtained commercially from Sigma Aldrich Co. Germany. This NP was chosen because of its utilization in previous studies [30], and the physico-chemical characterization is as reported by Shukla et al. [30]. The TiO$_2$-NPs were suspended in double distilled water at a stock concentration of 150 mg/kg body weight (adapted from IP LD$_{50}$ in mice) [49] and ultrasonicated (BANDELIN Sonorex digitec Germany - DT 52H, 230 V – 50/60 Hz; 0.9 A; 60/240 W; 35 kHz) for 1h (3 min pulse on and 30 sec pulse off) at 60W. Freshly prepared working solutions were made by serial dilution from the stock solution; after vortex for 5-10 min and 10 weeks exposure periods respectively. Blood and body fluids were rinsed from the testes using normal saline and were fixed in Bouin’s fluid and 10 weeks from the first exposure. Four mice per dosage group were sacrificed by cervical dislocation. The femurs were removed and bone marrow flushed with Fetal Bovine Serum (FBS) (PAA Laboratories GmbH, PAA-Strasse 1, Pasching, Austria). Cells were centrifuged at 2000 rpm for 5 min and slides were stained with May-Grunwald and Giemsa stains. At least 2000 erythrocytes per mouse were scored at ×1000 for MN in polychromatic erythrocytes (MNPCE) and normochromic erythrocytes (MNCE). The same volume but of cyclophosphamide (20 mg/kg) and double distilled water was administered to mice in the positive and negative control groups respectively. Sperm cells were sampled from the cauda epididymes at 5 and 10 weeks from the first exposure. Four mice per dosage group were sacrificed by cervical dislocation and their caudal epididymes removed; sperm suspensions were then prepared from the cauda of each testis by mincing the cauda in normal saline and 1% eosin Y stain. The slides were air-dried and coded for subsequent microscopic examination at ×1000. For each mouse, 1000 sperm cells were assessed for morphological abnormalities according to the criteria of Wyrobel and Bruce [54].

Histopathological analysis of the testes

Testes were carefully excised from two mice randomly selected from each dosage group exposed to 9.38, 37.50 and 150.00 mg/kg bwt of TiO$_2$-NPs, and the negative and positive control groups at the end of 5 and 10 weeks exposure periods respectively. Blood and body fluids were rinsed from the testes using normal saline and were fixed in Bouin’s fluid for 48 hours. They were then dehydrated in ascending grades of ethanol (70%, 80%, 95%, and 100%), cleared using xylene and embedded in paraffin wax using Leica Histokinete tissue processor for 6 hours. Serial sections of 4 μm thickness were obtained on labeled glass slides using a rotatory microtome. The deparaffinized sections were stained routinely with haematoxylin and eosin (H & E) and mounted. The slides were scored randomly at 400X and photomicrographs taken accordingly.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 16.0 and Microsoft Excel 2007 were used for data analysis. Data obtained were expressed as percentage frequency and mean ± standard error. Significance at different concentration-level was tested using one-way ANOVA test and Duncan's New Multiple Range Test (DMRT). Correlation analysis was done to establish relationship between the frequency of induced MN and exposure period. Difference between the negative control-group and individual concentration-groups were analyzed at the 0.05 and 0.01 probability level.

Results

Micronucleus assay

Table 1 shows the frequency of MNPCE (Figure 1) observed in...
the bone marrow of mice exposed to the different concentrations of TiO$_2$-NPs for five and ten days. TiO$_2$-NPs increased the frequency of micronucleus in mice at all the tested concentrations compared to the negative control both in the 5 and 10 days exposure periods. At the respective tested concentrations, MNPC increased with a 3.2, 1.5, 4.8, 3.2, and 4.1 folds at the 5 days exposure period; and 6.5, 4.5, 14.3, 15.8 and 22.0 folds at the 10 days exposure period. Significant difference ($p<0.05$) was however observed at 37.50 mg/kg for the 5 days exposure and at all the tested concentrations ($p<0.01$) except 18.75 mg/kg for the 10 days exposure. A concentration dependent increase in the frequency of MNPC was observed in the 10 days exposure. There was a positive correlation between the frequency of MNPC and days of exposure ($r = 0.693$).

**Sperm morphology assay**

The effects of the different concentrations of TiO$_2$-NPs on the sperm morphology at the end of 5 and 10 weeks exposure periods are presented in Table 2. After 5 weeks of exposure, the percentage abnormal sperm cells were 16.44, 13.94, 15.22, 21.64 and 21.72% for 9.38, 18.75, 37.50, 75.00 and 150.00 mg/kg of TiO$_2$-NPs respectively, which were statistically significant ($p<0.05$) at all the tested concentrations compared to the negative control (8.40%). At the same concentrations for the 10 weeks exposure period, there were 11.65, 8.73, 15.98 and 15.05% abnormal sperm cells which were significant ($p<0.05$) at all concentrations (except at 18.75 mg/kg) compared to the negative control value of 8.20%. Generally, the mean of abnormal sperm cells induced in mice at each concentration after 5 weeks of exposure were greater than after 10 weeks of exposure. Abnormal sperm cells such as double hook, double heads, knobbed hook, double tails, pin head, banana shaped, amorphous head, folded and wrong tail attachment were observed in the mice exposed to TiO$_2$-NPs at the two exposure periods (Figure 2). Folded sperm cells were the most predominant while sperm cells with double hooks were the least.

**Histopathological assessment of the testes**

Microscopic examination of the testes in the negative control group showed a normal cellular architecture of the testicular tissues while the treated groups at the two exposure periods showed the disruption of the normal cellular architecture of the testicular tissues by TiO$_2$-NPs (Figure 3). There were severe histopathological lesions such as congestion of the interstitium oedema, congestion of the interstitial blood vessels, reduced height of germinal epithelium, numerous spermatocytes and elongate spermatids (Figure 3). The lesions were most severe in mice exposed to 37.50 mg/kg of TiO$_2$-NPs for 10 weeks as there were numerous variably-sized seminiferous tubules (ST) many of which were cystic and had irregular outlines, while the affected seminiferous tubules were severely depleted of spermatogenic cells. There were also marked necrosis and vacuolation of spermatogenic cells within the STs at this concentration.

**Discussion**

Nanotechnology has effectively improved a number of consumer products, through the manufacturing and use of Nanoparticles. Therefore, assessment of the toxicological effects of Nanoparticles on the human health and environment is inevitable as these nanoparticles through their small size, large surface area to volume ratio, and other physicochemical properties are able to disrupt the biochemical and physiological functions of the cell. In the present study, the potential genotoxic effect of TiO$_2$-NPs was evaluated in the somatic and germ tissues of mice using the mouse bone marrow micronucleus and sperm morphology assays. The data obtained in this study showed TiO$_2$-NPs to be genotoxic in mice.

The results of the MN assay showed that TiO$_2$-NPs are clastogenic and aneugenic. It caused chromosomal damage in dividing cells

---

**Table 2:** The frequency (%) and mean (± S.E) of morphologically abnormal sperm cells induced in mice exposed to different concentrations of TiO$_2$-NPs for 5 and 10 weeks.

| Concentrations (mg/kg) | % abnormalities | Mean ± S.E |
|------------------------|----------------|-----------|
|                        | 5 weeks        | 10 weeks   |
| Distilled water        | 8.4 ± 0.8      | 8.2 ± 0.6  |
| 9.375                  | 16.4 ± 1.1     | 16.4 ± 1.1 |
| 18.75                  | 13.9 ± 0.4     | 13.9 ± 0.4 |
| 37.5                   | 15.2 ± 0.6     | 15.2 ± 0.6 |
| 75                     | 21.6 ± 0.8     | 21.6 ± 0.8 |
| 150                    | 21.7 ± 0.8     | 21.7 ± 0.8 |
| Cyp                    | 11.6 ± 0.8     | 11.6 ± 0.8 |

*p* significant at $p<0.05$; Cyp- cyclophosphamide (20 mg/kg).

**Table 1:** Frequencies (Mean ± SE) of micronucleated polychromatic erythrocytes in bone marrow of mice exposed to Titanium dioxide nanoparticles.

| Concentration (mg/kg) | 5-days Mean ± SE | 10-days Mean ± SE |
|-----------------------|------------------|-------------------|
| DDW                   | 10.25 ± 2.17     | 9.50 ± 2.66       |
| 9.38                  | 33.25 ± 4.21     | 61.75 ± 8.42**   |
| 18.75                 | 15.25 ± 4.71     | 42.75 ± 9.69      |
| 37.5                  | 49.00 ± 7.49**   | 135.75 ± 12.04** |
| 75                    | 33.00 ± 9.53     | 149.75 ± 4.37**  |
| 150                   | 41.50 ± 9.43     | 208.75 ± 13.33** |
| Cyp                   | 36.50 ± 29.73    | 24.75 ± 1.97      |

*p* Significant at $p<0.05$; **Significant at $p<0.01$; DDW- double distilled water; Cyp- cyclophosphamide (20 mg/kg).

**Figure 1:** Micronucleus induced in mice exposed to TiO$_2$-NPs. (a) PCE: Polychromatic erythrocytes, NCE: Normochromatic erythrocyte (b) MNPC: micronucleated polychromatic erythrocyte. (c) Bi-MNPC: Bi-micronucleated polychromatic erythrocyte.
of exposed mice. The frequency of MN induction was directly proportional to the exposure periods; the longer the number of days of exposure; the higher the chromosomal damage induced by TiO$_2$-NPs. Mice exposed for 10 days had higher genomic damages in the form of micronuclei formation than those exposed for 5 days. This suggests that TiO$_2$-NPs might have accumulated in a dose-dependent manner, thus affecting the bone marrow PCE for a longer time [55]; it has also been reported that the retention halftime of TiO$_2$-NPs is long because of its difficult excretion [56]. TiO$_2$-NPs may have interacted (directly or indirectly) with the genetic material of the bone marrow cells producing primary and/or secondary genotoxicity resulting in accretic chromosome fragments or chromosome loss. Several factors may account for the calstogenic and aneugenic characteristics of TiO$_2$-NPs. A direct interaction between TiO$_2$-NPs and the genetic material is a possibility. Previous studies have shown TiO$_2$-NPs to have access to the cell membrane, without using a specific transporter or penetrate through the nuclear pore complex. They are able to produce titanium ion in the cell cytoplasm, having the potential of generating intracellular reactive oxygen species, of which the stable and diffusible forms such as hydrogen peroxide or lipid peroxidation intermediates could affect the nuclear DNA [57]. Another possible reason for the aneugenic effect of TiO$_2$-NPs may be explained by the physical interaction with the
components of the mitotic spindle during cell division or the interaction with proteins directly or indirectly involved in chromosome segregation [58]. They may physically interact with the mitochondrial membranes causing loss of the mitochondrial membrane potential, the opening of the permeability transition pores and ROS production [5,55].

Our result is in accordance with those of previous studies wherein mice [33,44,59], human peripheral blood lymphocytes [60,61] and cell lines [62] were used as test systems. However, it differs from those of Lindberg et al. [63], Sadiq et al. [64], Xu et al. [65] and Kim et al. [66] wherein TiO$_2$-NPs were reported to be non-clastogenic/aneugenic. This could be because of differences in exposure duration, degree of agglomeration, particle size, and chemical composition of the NPs.

The result of the sperm morphology assay showed the spermatotoxic effect of TiO$_2$-NPs. The mouse sperm morphology assay has potential in identifying chemicals that induce spermatogenic dysfunction and perhaps heritable mutations [52]. Sperm abnormalities have long been associated with male infertility and sterility in most species and the structure play a substantial role in both fertilization and pregnancy outcome [67]. Two sperm cells collection periods were considered: 5 and 10 weeks. The 5-week assessed sperm cells were exposed as differentiating and mitotically dividing spermatagonia, while the 10-week assessed sperm cells were exposed as mitotically dividing stem cells. Higher rate of sperm abnormalities was recorded for the 5-week assessed cells compared to the 10-week assessed cells. This could be an indication that differentiating and mitotically dividing spermatagonia are more susceptible to TiO$_2$-NPs damage than mitotically dividing stem cells.

The exact mechanism for the increase in the frequency of abnormal sperm is not clear and opinions on this subject differ. The induction of abnormal sperm is assumed to be as a result of an abnormal chromosome [68], minor alteration in testicular DNA [69], and point mutation [70]. According to several studies [71-74], small deletions, point mutations, and abnormal chromosomes are proposed as possible genetic causes of such alterations. Bruce and Heddle [75] attributed the occurrence of sperm head abnormalities to the chromosomal aberrations that occur during the packaging of genetic material in the sperm head or occurrence of point mutation in testicular DNA [75]. Sperm abnormalities may also arise as a consequence of mistakes in the spermatoozoa-differentiating process during spermatogenesis or by physiological, cytotoxic or genetic mechanisms or alterations in testicular DNA which in turn disrupts the process of differentiation of spermatooza [52].

This report supports the chromosomal damage as possible genetic cause of such alterations, since the TiO$_2$-NPs were found to be clastogenic in the MN assay. The reproductive toxicity of TiO$_2$-NPs herein is in accordance with previous reports of other types of NPs. Yoshida et al. [76] showed adverse effects of carbon NPs on the male reproductive systems of adult mice. Also, Gromadzka-Ostrowska et al. [77] observed a decrease in sperm count, increased DNA damage and a change in testis seminiferous tubule morphology of male rats exposed to Ag NPs. Au NPs was also reported to cause a drop in sperm motility and increase sperm fragmentation [78].

The result of the histopathology of the exposed mice testes showed that TiO$_2$-NPs caused pathological changes to the testes. Administration of TiO$_2$-NPs caused severe histopathologic lesions such as congestion of the interstitium oedema, moderate/mild congestion of the interstitial blood vessels, reduced height of germinal epithelium, fairly numerous spermatocytes and few elongated spermatids. The degenerative changes observed in the seminiferous tubules such as necrosis, vacuolation and congestion of the interstitial/testicular blood vessels are evidence of the toxicity of TiO$_2$-NPs to the mouse male reproductive system. Degenerative changes in the seminiferous tubules indicate that TiO$_2$-NPs may directly interfere in the process of spermatogenesis [79]. This was probably a pre-requisite for the observed abnormal sperm morphology produced by the damaged testes. This result is consistent with previous reports on histopathological damage or lesions in the male testes of mouse/rat by TiO$_2$-NPs [80,81] but in contrast to the report of Guo et al. [82] who reported no obvious pathological changes in the testis of male mice exposed to TiO$_2$ NPs. That TiO$_2$-NPs induced abnormalities in mouse sperm cells and the testes suggests that the same could happen in other exposed male animal species especially mammals. DNA-damaged spermatooza may introduce damaged genome into the oocytes with dangerous drawbacks on fertilization, embryonic, foetal and post-natal development [83,84]. Although the clinical significance of sperm morphology is still a matter of debate, it has been recently recognized that an accurate definition of morphological anomalies plays a very important role in the determination of male fertility potential [85].

One of the possible mechanisms for TiO$_2$-NPs induced genotoxicity herein is oxidative stress [86] as TiO$_2$-NPs are photocatalytic and have been implicated to directly generate free radicals [87,88]. NPs are able to generate reactive oxygen species caused by secondary mechanical processes associated with inflammatory responses ultimately causing cell damage and eventually cell death [33,44]. Likewise, the small particle size and large surface area of TiO$_2$-NPs enables them to easily penetrate cells and cellular components thus interfering with several sub-cellular mechanisms and biomolecules causing lipid peroxidation, mitochondria disruption, immune reactivity and protein damage [5].

Our study has shown that TiO$_2$-NPs have the capacity to interact with mice genetic materials / machinery under the test condition. This is of public health importance considering industrial and household applications of TiO$_2$-NPs. Chemically induced genetic damage has been implicated in the etiology of many diseases; thus, there is need for stringent policies as regards the use of nanoparticles in human consumable and cosmetic products as well as their disposal into the environment.

Acknowledgements

We appreciate the support of Department of Zoology, University of Ibadan vote for postgraduate studies; and the technical assistance of Naomi Adeyemo.

Author Contributions

Bakare, Alimba and Alabi conceived and designed the experiments. Udoakang, Afnifowose, Fadoju, Ogunsuyi and Oyejemil performed the experiments and analyzed the data. Udoakang and Afnifowose drafted the manuscript; while Bakare, Alimba and Alabi critically revised it, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

References

1. Kim YR, Park JI, Lee EJ, Park SH, Seong NW, et al. (2014) Toxicity of 100 nm zinc oxide nanoparticles: a report of 90-day repeated oral administration in Sprague Dawley rats. Intern J Nanomed 9: 109-126.
2. Cheng-Teng N, Jasmine J, Boon-Huat B, Lin-Yue L (2010) Current studies into the genotoxic effects of nanomaterials. J Nucleic Acids 20: 1-9.
3. Nam D, Lee B, Eom I, Kip B, Yeo M (2014) Uptake and bioaccumulation of titanium and silver nanoparticles in aquatic ecosystems. Mol Cellular Toxicol 10: 9-17.
4. Zhang R, Bai B, Zhang B, Chenc L, Yan B (2012) The potential health risk of titania nanoparticles. J Haz Mat 211-212: 404-413.
5. Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V, et al. (2014)
Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8: 233-278.

6. Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Hlth Perspect 113: 823-839.

7. Handy RD, Owen R, Valsamis-Jones E (2008) The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicology 17: 315-325.

8. Wang J, Liu Y, Jiao F, Lao F, Li W, et al. (2008) Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO₂ nanoparticles. Toxicology 254: 82-90.

9. Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. (2006) Carcinogenicity of carbon black, titanium dioxide, and talc. Lancet Oncol 7: 295-296.

10. Liang G, Pu Y, Yin L (2009) Influence of different sizes of titanium dioxide nanoparticles on hepatic and renal functions in rats with correlation to oxidative stress. J Toxicol Environ Hlth H 72: 740-745.

11. Chen T, Yan J, Li Yan (2014) Genotoxicity of titanium dioxide nanoparticles. J Food Drug Anal 22: 95-104.

12. Shi H, Magaye R, Castranova V, Zhao J (2013) Titanium dioxide nanoparticles: a review of current toxicological data. Particle Fibre Toxicol 10: 15.

13. Cho M, Chung H, Choi W, Yoon J (2004) Linear correlation between inactivation of E. coli and OH radical concentration in TiO₂ photocatalytic disinfection. Water Res 38: 1069-1077.

14. Esterkin CR, Negro AC, Alfano OM, Cassano AE (2005) Air pollution remediation in a fixed bed photocatalytic reactor coated with TiO₂. Am Inst Chem Engin J 51: 2298-2310.

15. Choi H, Stathatos E, Dionysiou D (2006) Sol gel preparation of mesoporous photocatalytic TiO₂ films and TiO₂/Al₂O₃ composite membranes for environmental applications. Appl Catal B-Environ 63: 60-67.

16. Brumfitt G (2006) Consumer products leap aboard the nano bandwagon. Nature 440: 262.

17. Bangales M, Mitkare S, Gattani S, Sakarkar D (2012) Recent nanotechnological aspects in cosmetics and dermatological preparations. Intern J Pharm Pharmacol Sci 4: 88-97.

18. Oberdörster G, Finkelstein J, Johnson C, et al. (2000) Acute pulmonary effects of ultrafine particles in rats and mice. Res Rep Hlth Effects Instit Pharmacol Sci 4: 88-97.

19. Niel A, Xia T, Mäddler L, Li N (2006) Toxic potential of materials at the nanolevel. Science 311: 622-627.

20. Nowack B, Bucheli T (2007) “Occurrence, behaviour and effects of nanoparticles in the environment,” Environ Pol 150: 5-22.

21. Laura K, Braydich-Stolle, Schaebulin NM, Murdock RC, Jiang J, et al. (2009) Crystal structure mediates mode of cell death in TiO₂ nanotoxicity. J Nanoparticle Res 11: 1361-1374.

22. Stone V, Nowack B, Baun A (2010) Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci of the Total Environ 408: 1745-1754.

23. Ghosh M, Bandyopadhyay M, Mukherjee A (2010) Genotoxicity of titanium dioxide (TiO₂) nanoparticles at two trophic levels: plant and human lymphocytes. Chemosphere 81: 1253-1262.

24. Tavares AM, Louro H, Antunes S (2013) Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. Toxicol In vitro 28: 60-69.

25. Petkovic J, Zegura B, Stevanovic M (2011) DNA damage and alterations in expression of DNA damage response genes induced by TiO₂ nanoparticles in human hepatoma HepG2 cells. Nanotoxicology 5: 341-353.

26. Shukla RK, Kumar A, Gurbani D, Pandey AK, Singh S, et al. (2013) TiO₂ nanoparticles induce oxidative DNA damage and apoptosis in human liver cells. Nanotoxicology 7: 48-60.

27. Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, et al. (2007) Development of a base set of toxicity tests using ultrafine TiO₂ particles as a component of nanoparticle risk management. Toxicol Lett 171: 99-110.

28. Di Virgilio AL, Reigosa M, Arenal PM, de Mele MFL (2010) Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. J Haz Mat 177: 711-718.

29. Falck G, Lindberg H, Suohonens S (2009) Genotoxic effects of Nanosized and fine TiO₂. Human Exper Toxicol 28: 339-352.

30. Shukla RK, Sharma V, Pandey AK, Singh S, Sultana S, et al. (2011) ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicol in vitro 25: 231-241.

31. Srivastava RK, Rahman Q, Kashyap MP, Lohani M, Pant AB (2011) Ameliorative effects of dimethylthiourea and N-acetylcysteine on nanoparticles induced cyoto-
genotoxicity in human lung cancer cells-A549. PLoS One 6: e25767.

32. Valtidigesias V, Costa C, Sharma V (2013) Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. Food Chem Toxicol 57: 352-361.

33. Sycheva LP, Zhurkov VS, Iurchenko VV (2011) Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo. Mutat Res 726: 8-14.

34. Kermanizadeh A, Gaiser BK, Hutchison GR, Stone V (2012) An in vitro liver model: assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanomaterials. Particle Fibre Toxicol 9: 28.

35. Demir E, Burgucu D, Turna F, Aksakal S, Kaya B (2013) Determination of TiO₂, ZnO₂ and Al₂O₃ nanoparticles on genotoxic responses in human peripheral blood lymphocytes and cultured embryonic kidney cells. J Toxicol Environ Hlth Part A 76: 990-1002.

36. Botelho MC, Costa C, Silva S (2014) Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro. Biomed Pharmacoch 68: 59-64.

37. Ubould C, Urbán P, Gilliland D, Bajak E, Valsamis-Jones E, et al. (2016) Role of the crystalline form of titanium dioxide nanoparticles: Rulite, and not anatase, induces toxic effects in BAbt/3T3 mouse fibroblasts. Toxicol In Vitro 31: 137-145.

38. Rahman Q, Lohani M, Dopp E (2002) Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. Environ Hlth Perspect 110: 797-800.

39. Chen E, Rvulacaba M, Arajoo L, Chapman R, Chin W (2008) Ultrafine titanium dioxide nanoparticles induce cell death in human bronchial epithelial cells. J Exp Nanosci 3: 171-183.

40. Shi Y, Zhang J, Jiang M, Zhu L, Tan H, et al. (2010) Synergistic genotoxicity caused by low concentration of titanium dioxide nanoparticles and p,p'-DDT in human hepatocytes. Environ Mol Mut 51: 192-204.

41. Pan Z, Lee W, Slutsky L, Clark F, Perndedt N (2009) Adverse effects of titanium dioxide nanoparticles on human dermal fibroblasts and how to protect cells. Small 5: 511-520.

42. Chen HW, Su SF, Chien CT (2006) Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. FASEB J 20: 2393.

43. Ma L, Zhao J, Wang J (2009) The acute liver injury in mice caused by Nano- 

44. Trouiller B, Reliene R, Westbrook A, Solomani P, Schiestl R (2009) Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res 69: 8784-8789.

45. Zhang Y, Tao J, He P, Tang Y, Wang Y (2009) Bio-effects of nano-TiO₂ on lungs of mice. ShengWu Yi Xue Gong Cheng Xue Za Zhi 26: 803-806.

46. Liu HT, Ma LL, Liu J, Zhao JF, Yan JY, et al. (2010) Toxicity of nano anatase TiO₂ to mice: Liver injury, oxidative stress. Toxicol Environ Chem 92: 175-186.

47. Unnithan J, Rehrman MU, Ahmad FJ, Samim M (2011) Aquous synthesis and concentration-dependent dermal toxicity of TiO₂ nanoparticles in Wistar rats. Biol Trace Element Res 143: 1682-1694.

48. Kobayashi N, Naya M, Endoh S, Maru J, Yamamoto K, et al. (2009) Comparative pulmonary toxicity study of nano-TiO₂ particles of different sizes and agglomerations in rats: different short- and long-term post-institution results. Toxicology 264: 110-118.

49. Liu HT, Ma LL, Zhao JF (2009) Biochemical toxicity of nano-anatase TiO₂ particles in mice. Biol Trace Elem Res 129: 170-180.

50. Schmid W (1985) The micronucleus test. Mutat Res 31: 9-15.
51. Bakare A, Okunola A, Adetunji O, Jenni H (2009) Genotoxicity assessment of a pharmaceutical effluent using four bioassays. Gen Mol Biol 32: 373-381.

52. Wyrobek AJ, Gordon LA, Burkhart JG (1983) An evaluation of the mouse sperm morphometry test and cytology of Punica granatum L. (Punicaceae) whole fruit extracts. J Ethnopharmacol 298-308.

53. Bakare AA, Mosuro AA, Osibanjo O (2005) An in vivo evaluation of induction of abnormal sperm morphology in mice by land fill leachates. Mutat Res 582: 28-34.

54. Wyrobek AJ, Bruce WR (1975) Chemical induction of sperm abnormalities in mice. Proc Nat Acad Sci 72: 4425-4429.

55. Dobrzynska M, Gajowik A, Radzikowska J, Lankoff A, Dusinska M, et al. (2014) Genotoxicity of silver and titanium dioxide nanoparticles in bone marrow cells of rats in vivo. Toxicology 315: 86-91.

56. Zhang R, Niu Y, Li Y, Zhao C, Song B, et al. (2010) Acute toxicity study of the interaction between titanium dioxide nanoparticles and lead acetate in mice. Environ Toxicol Pharmacol 30: 52-60.

57. Patiolla A, Patra P, Flourent M, Tchounouv P (2015) Cytogenetic Evaluation of Functionalized Single-Walled Carbon Nanotube in Mice Bone Marrow Cells. Environ Toxicol 00: 000-000.

58. Muller J, Decorider I, Hoet P, Lamertabt N, Thomassen L, et al. (2008) Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. Carcinogenesis 29: 427-433.

59. Patiolla A, Hussain S, Schlager J, Patiolla S, Tchounouv P (2010) Comparative Study of the Cytotoxicity of Functionalized and Non-functionalized Multiwalled Carbon Nanotubes in Bone Marrow Cells of Swiss-Webster Mice. Environ Toxicol 25: 608-621.

60. Kang S, Kim B, Lee Y, Chung, H (2008) Titanium Dioxide Nanoparticles Trigger p53-Mediated Damage Response in Peripheral Blood Lymphocytes. Environ Mol Mutagen 49: 399-405.

61. Tavares A, Louro H, Antunes S, Quares S, Simar S, et al. (2014) Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. Toxicol In Vitro 28: 60-69.

62. Demir E, Akpa H, Turan F, Aksakal S, Burgucu D, et al. (2015) Genotoxic and cell-transforming effects of titanium dioxide nanoparticles. Environmental Research 136: 300-308.

63. Lindberg HK, Falcik GC, Catalan J (2012) Genotoxicity of inhaled nanosized TiO2 in mice. Mutat Res 745: 58-64.

64. Sadig R, Bhalia JA, Yan J (2012) Genotoxicity of TiO2 anatase nanoparticle in B6C3F1 male mice evaluated using Pig-a and flow cytometric micronucleus assays. Mutat Res 745: 65-72.

65. Xu J, Shi H, Ruth M (2013) Acute toxicity of intravenously administered titanium dioxide nanoparticles in mice. PLoS One 8: e70618.

66. Kim Y, Kim J, Cho H, Rha D, Kim J, et al. (2008) Twenty-Eight-Day Oral Toxicity, Genotoxicity, and Gender-Related Tissue Distribution of Silver Nanoparticles in Sprague-Dawley Rats. Inhal Toxicol 20: 575-583.

67. Saacke RG (2001) What is a BSE–SFT standards: the relative importance of sperm morphology: an opinion. Proc Soc Theriogenol 81-87.

68. Bruce WR, Furrer R, Wyrobek AJ (1974) Abnormalities in the shape of murine sperm after acute testicular x-irradiation. Mutat Res 23: 381-386.

69. Giri S, Prasad SB, Giri A, Sharma GD (2002) Genotoxic effects of malathion: an organophosphorus insecticide, using three mammalian bioassays in vivo. Mutat Res 514: 223-231.

70. Narayana K, D’Souza UJ, Seetharama Rao KP (2002) Ribavin-induced sperm shape abnormalities in Wistar rat. Mutat Res 513: 193-196.

71. Mendoza-Lujambio I, Burfeind P, Dööksen C (2002) The Hook1 gene is non-functional in the abnormal spermatозoon head shape (az) mutant mouse. Human Mol Genet 11: 1847-1857.

72. Pyle A, Handel MA (2003) Meiosis in male PJJU mice: a genetic model for gametic aneuploidy. Mol Reprod Dev 64: 471-481.

73. Escalier D, Bai XY, Sivus D, Xu PX, Xu X (2003) Spermatid nuclear and sperm periaxenomal anomalies in the Mouse Ube2b null mutant. Mol Reprod Dev 65: 298-308.

74. Lamara AS, Fonseca G, Fuentes JL (2008) Assessment of the genotoxic risk of Punica granatum L. (Punicaceae) whole fruit extracts. J Ethnopharmacol 115: 416-422.

75. Bruce W, Heddie J (1979) The mutagenicity of 61 agents as determined by the micronucleus, Salmonella and sperm abnormality assays. Can J Cytol Genet 21: 318-334.

76. Yoshida S, Hyoshi K, Ichinose T (2008) Effect of nanoparticles on the male reproductive system of mice. Intern J Androl 32: 337-342.

77. Gromadzka-Ostrowska J, Dziendziolowska K, Lankoff A (2012) Silver nanoparticles affects on epididymal sperm in rats. Toxicol Lett 214: 251-258.

78. Wiwanitkit V, Sereemaspun A, Rojanathanes R (2009) Effect of gold nanoparticles on spermatozoa: the first world report. Fertil Steril 91: e7-eb.

79. Thakur M, Gupta H, Singh D (2014) Histopathological and ultra-structural effects of nanoparticles on rat testis following 90 days (chronic study) of repeated oral administration. J Nanobiotechnol 12: 42.

80. Komatsu T, Tabata M, Kubo-Irie M (2008) The effects of nanoparticles on mouse testis Leydig cells in vitro. Toxicol In Vitro 22: 1825-1831.

81. EL- Sharkawy NL, Hamza SM, Abou-Zeid EH (2010) Toxic impact of titanium dioxide (TiO2) in male albino rats with special reference to its effect on reproductive system. J Am Sci 6.

82. Guo LL, Liu XH, Qin DX (2009) Effects of nanosized titanium dioxide on the reproductive system of male mice. National J Andrology (Zhonghua Nan Ke Xue) 15: 517-522.

83. Lewis SE, Aitken RJ (2005) DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 322: 33-41.

84. Zink A (2011) Are sperm chromatim and DNA defects relevant in the clinic? Syst Biol Reprod Med 67: 78-85.

85. Tosti E, Menezo Y (2012) IMSI, Useful, Useless or Harmful? J IVF Reprod Med 23: 1508-1511.

86. Wiwanitkit V, Sereemaspun A, Rojanathanes R (2009) Effect of gold nanoparticles on spermatozoa: the first world report. Fertil Steril 91: e7-eb.

87. Zhang Q, Kusaka Y, Sato K, Nakakuki K, Kohyama N, et al. (1998) Differences in the levels of DNA damage detected by the comet assay in blood cells from mice exposed to diesel exhaust. Toxicol Appl Pharmacol 153: 157-163.

88. Bhattacharya K, Davoren M, Boertz J, Schins R, Hoffmann E, et al. (2009) Genotoxicity of poorly soluble particles. Inhal Toxicol 21: 6-17.