Evidence That Ultrafine Titanium Dioxide Induces Micronuclei and Apoptosis in Syrian Hamster Embryo Fibroblasts

Qamar Rahman,[1] Mohtashim Lohani,[1] Elke Dopp,[2] Heidemarie Pemsel,[2] Ludwig Jonas,[3] Dieter G. Weiss,[2] and Dietmar Schiffmann[2]*

[1]Industrial Toxicology Research Centre, Division of Fibre Toxicology, Lucknow, India; [2]Institute of Animal Physiology, Department of Biology, and [3]Electron Microscopy Division, Department of Pathology, University of Rostock, Rostock, Germany

Inhaled ultrafine titanium dioxide (UF-TiO_2) particles cause pronounced pulmonary inflammation, in contrast to fine TiO_2. Previous studies provide evidence for the production of reactive oxygen species by alveolar macrophages, after overloading with UF-TiO_2 particles and cytotoxicity of UF-TiO_2 in rat lung alveolar macrophages. UF-TiO_2 also causes pulmonary fibrosis and lung tumors in rats. UF-TiO_2 particles are photogenotoxic, but in general, information on the genotoxicity of UF-TiO_2 is still limited. We studied the potential of UF-TiO_2 (particle size ≤ 20 nm) and fine TiO_2 (particle size > 200 nm) to induce chromosomal changes, which can be monitored by the formation of micronuclei (MN) in Syrian hamster embryo (SHE) cells. We also analyzed UF-TiO_2–treated cells for apoptosis induction. The MN assay revealed a significant increase in MN induction ($p \leq 0.05$) in SHE cells after treatment with UF-TiO_2 (1.0 μg/cm^2) for 12 hr (mean, 24.5 MN/1,000 cells), 24 hr (mean, 31.13 MN/1,000 cells), 48 hr (mean, 30.8 MN/1,000 cells), 66 hr (mean, 31.2 MN/1,000 cells), and 72 hr (mean, 31.3 MN/1,000 cells). Bisbenzimide staining of the fixed cells revealed typical apoptotic structures (apoptotic bodies), and the apoptosis-specific "DNA ladder pattern" resulting from internucleosomal cleavage was identified by gel electrophoresis. Furthermore, transmission electron microscopy of the exposed cells revealed the typical chromatin compaction of apoptosis. *Key words:* apoptosis, genotoxicity, kinetochores, micronuclei, ultrafine titanium dioxide. *Environ Health Perspect* 110:797–800 (2002). [Online 21 June 2002]

http://ehpnet1.niehs.nih.gov/docs/2002/110p797-800rahman/abstract.html

Titanium dioxide (TiO_2) is a naturally occurring mineral found primarily as rutile, anatase, brookite, and ilmenite (*1*). It is widely used in the cosmetics, pharmaceutical, paint, and paper industries.

TiO_2 was previously classified as biologically inert, both in animals and in humans (*2,3*). However, recent investigations on the basis of both experimental studies on rats and epidemiologic surveys revealed the development of inflammation, pulmonary damage, fibrosis, and lung tumors after exposure to ultrafine TiO_2 (UF-TiO_2) (*4–9*). Dimensions of the TiO_2 particles are pivotal, as demonstrated by the fact that UF-TiO_2 causes more pronounced toxicity compared with fine TiO_2 particles (*10–12*).

UF-TiO_2 particles (≤ 20 nm) induce impairment of macrophage function, persistently high inflammatory reactions, and increased pulmonary retention compared to fine TiO_2 (particle size > 200 nm) (*6*). They also enter the epithelium faster and are translocated in greater proportion to the subepithelium space compared with fine particles (*13*). In situations such as "particle overload" after exposure to UF-TiO_2 particles, the activated alveolar macrophages produce excessive amounts of such mediators as oxygen radicals, proteases, and growth-regulating proteins (*14,15*). Furthermore, an increase in the level of mRNA specific for interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α in human alveolar macrophages has been found after exposure to UF-TiO_2 (*16*). Rahman et al. (*17*) also showed the production of reactive oxygen species (ROS) in human and rat alveolar macrophages after exposure to UF-TiO_2 using a luminol-dependent chemiluminescence assay. Nakagawa et al. (*18*) reported the photogenotoxic potential of TiO_2, using different *in vitro* genotoxicity assays and found that without ultraviolet (UV)/visible light it is weakly mutagenic, but combined with irradiation it is strongly mutagenic. Furthermore Lu et al. (*19*) reported the potential of TiO_2 to induce micronuclei (MN) sister chromatid exchanges in Chinese hamster ovary cells.

In this paper we report on the occurrence of mitotic disturbances, genetic damage, and oxidant-mediated DNA damage resulting in apoptosis (*20*) and induction of MN by UF-TiO_2 using Syrian hamster embryo (SHE) fibroblasts. Previous studies, by several groups as well as our own, have shown that this cell system is an efficient and reliable *in vitro* model to study the genetic damage induced by fibers or particles (*21*). Clastogenic and aneugenic effects have been monitored by the assessment of MN in combination with kinetochore analysis. We studied the induction of apoptosis using the DNA ladder assay and transmission electron microscopy (TEM).

Materials and Methods

Cell culture and treatment conditions. We isolated SHE fibroblasts from 13-day-old embryos and grew the cells in a humidified atmosphere with 12% CO_2 at 37°C. The culture medium was modified Dulbecco's Eagle's reinforced medium (Gibco, Karlsruhe, Germany), supplemented with 15% fetal calf serum (Flow Laboratories, Meckenheim, Germany), $NaHCO_3$ (7.5%), 1% glucose, 10,000 IU penicillin, and 10 mg/mL streptomycin according to the standard procedure described by Pienta (*22*).

UF-TiO_2 and fine TiO_2 were a gift from G. Oberdorster, University of Rochester (Rochester, New York, USA). The particle size was ≤ 20 nm for UF-TiO_2 and > 200 nm for TiO_2. Particles were sterilized by heating to 120°C for 2 hr and suspended in phosphate-buffered saline (PBS; 1 μg/μl). We treated cells with different concentrations of fibers (0.5, 1.0, 5, and 10 μg/cm^2) for different periods (12, 24, 48, 66, and 72 hr). We also treated cells separately with fine TiO_2 using similar concentrations and time periods.

MN assay. We grew SHE cells on coverslips after treatment with different doses of UF-TiO_2 and fixed them in cold fixative after different incubation periods. We then stored them at –20°C for at least 30 min. We carried out DNA staining using bisbenzimide (1 μg/mL; Hoechst 33258; Sigma, St. Louis, MO, USA) for 4 min; after washing in PBS, the slides were mounted for microscopy. We scored only MN smaller than one-third the diameter of the nucleus under a fluorescence microscope at 630 × magnification.

Kinetochore staining. For further MN analysis, we stained kinetochores by incubating the fixed cell preparations with CREST serum (Chemicon, Temecula, CA, USA) for 1 hr in a humidified chamber at 37°C. After

Address correspondence to Q. Rahman, Industrial Toxicology Research Centre, Division of Fibre Toxicology, Post Box 80, M.G. Marg, Lucknow 226 001 India. Telephone: 91-522-213786. Fax: 91-522-228227. E-mail: qamar@avadh.net

*Current address: Institute of Hygiene and Occupational Health, University of Essen, Essen, Germany.

We thank I. Poser and F. Schiffmann (University of Rostock) for excellent technical assistance and P.K. Seth (Director, Industrial Toxicology Research Centre) for his interest in the work. The work was done under the CSIR-DLR collaborative project.

Received 17 July 2001; accepted 30 January 2002.

rinsing with PBS, we incubated the cells with fluorescein isothiocyanate (FITC)-conjugated goat anti-human antibodies (Sigma, Heidelberg, Germany) before applying bisbenzimide 33258 (Hoechst). We examined at least 100 MN for the presence of kinetochores in each case.

TEM. We exposed SHE cells to 5 μg/cm^2 and 10 μg/cm^2 UF-TiO_2 or 100 μM cisplatin (positive control) for 48 and 66 hr and fixed them with 4% glutaraldehyde in 0.1 M phosphate buffer. The fixed samples were dehydrated, embedded, polymerized, and sectioned in the usual manner. After preparing ultrathin sections with an LKB-ultratom III (LKB Instrument, Rockville, MD, USA) and treating them with uranyl acetate and lead citrate, we examined samples using a TEM (EM 902A; Zeiss, Oberkochen, Germany).

Analysis of DNA fragmentation. We used agarose gel electrophoresis to analyze the fragmentation of DNA during apoptosis. SHE cells were treated with 5 μg/cm^2 or 10 μg/cm^2 UF-TiO_2 or 100 μM cisplatin for 48 hr and 66 hr, respectively, and then digested at 50°C overnight and incubated with RNAse (10 μg/mL). DNA was extracted with phenol/chloroform and precipitated in ethanol; the extracted DNA was then separated by electrophoresis in an agarose gel (1% agarose gel containing 0.4 μg/mL ethidium bromide) and visualized under UV light. We repeated the experiment twice with consistent results.

Statistical analysis. Each data point represents the mean of 2,000 nuclei counted from each of three treated separate cultures from one experiment. We carried out the experiments three times with consistent results. The chi-square test was used to compare the results of the MN assay and to compare kinetochore analysis of each treatment group with the control.

Results

We observed formation of MN and appearance of characteristic changes typical for apoptosis in SHE cells after treatment with different concentrations of UF-TiO_2 (0.5, 1.0, 5.0, and 10.0 μg/cm^2) for different exposure times (12, 24, 48, 66, and 72 hr). Figure 1 shows MN in SHE cells treated with 10.0 μg/cm UF-TiO_2. UF-TiO_2 induced MN, which significantly ($p \leq 0.05$) increased at concentrations between 0.5 and 5.0 μg/cm^2 (Figure 2), whereas fine TiO_2 did not induce significant alterations in the MN induction rate (data not shown). Cytotoxicity increased after exposure of cells to higher concentrations of UF-TiO_2 (> 10.0 μg/cm^2).

Kinetochore analysis of MN from all groups revealed only an insignificant increase in the kinetochore-positive MN compared with unexposed control cells (data not shown).

We observed induction of apoptosis after bisbenzimide DNA staining of the fixed cells showing apoptotic bodies after 24, 48, and 72 hr of UF-TiO_2 exposure (Figure 3).

Figure 4 shows the TEM results. The typical compaction and marginalization of chromatin toward the nuclear periphery is clearly visible in the exposed SHE cells. Both early and late stages can be recognized, whereas unexposed cells do not show these specific features.

Figure 5 shows the typical internucleosomal fragmentation of UF-TiO_2–treated DNA (concentration, 10.0 μg/cm^2; exposure times, 24 and 48 hr) and cisplatin-treated DNA (concentration, 100 μM; exposure time, 24 hr) after DNA agarose gel electrophoresis. In contrast, the DNA of untreated cells did not show this typical DNA ladder pattern in electrophoresis.

Discussion

We found UF-TiO_2 to induce MN; the number of MN was significantly increased ($p \leq 0.05$) at all concentrations between 0.5 and 5.0 μg/cm^2 (Figure 2). Cytotoxicity was clearly detectable after exposure of cells to higher concentrations (> 10.0 μg/cm^2). The time course of MN induction may reveal a saturation effect because the MN equency is identical at 24, 48, and 72 hr, whereas at 12 hr it was lower. In contrast, fine titanium dioxide did not induce MN to a significant extent (data not shown). MN induction results from very early genetic damage; therefore, linking these events directly to tumorigenesis would be too speculative. Nevertheless, this assay is one of the tests that are able to indicate the carcinogenic potential of chemicals and particles. Because the same effects would be expected in lung macrophages (no metabolism involved), these cells are also at risk.

Kinetochore analysis revealed no significant increase in the kinetochore-positive MN compared with unexposed control cells (data not shown). This indicates that the MN mainly arise from clastogenic events.

The frequency of the induced MN reflects the extent of chromosomal changes induced (*23*). Furthermore, staining of kinetochores with anti-kinetochore serum (CREST) allows discrimination of clastogenic effects from aneuploidy. The observed formation of MN may be due to ROS and/or the physical presence of these particles around the mitotic apparatus, as reported earlier by Hesterberg et al. (*24*) for mineral fibers. Further investigations are required to identify the causative mechanisms.

Apoptosis is a unique type of programmed cell death, and oxidant-mediated

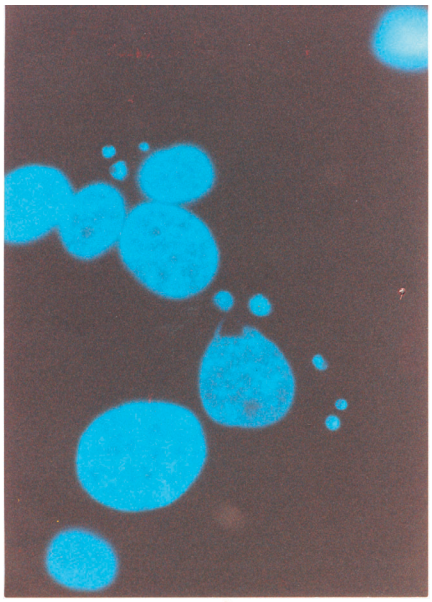


Figure 1. SHE cells exposed to UF-TiO_2 (10 μg/cm^2, 24 hr), showing induction of MN.

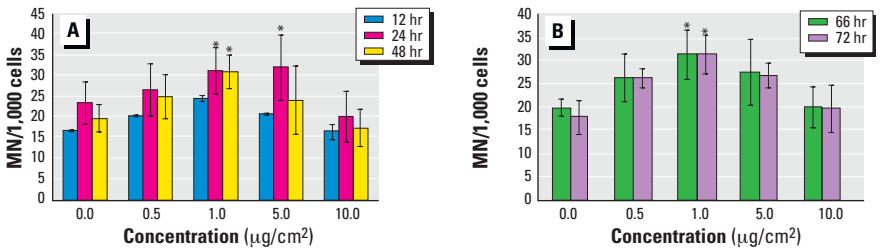


Figure 2. Formation of MN in SHE fibroblasts after treating cells with different concentrations of UF-TiO_2. (*A*) After 12, 24, and 48 hr. (*B*) After 66 and 72 hr.
*$p < 0.001$.

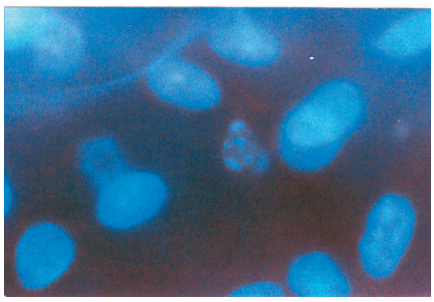


Figure 3. SHE cells exposed to UF-TiO_2 (10 μg/cm^2, 24 hr) showing formation of apoptotic bodies after staining with bisbenzimide.

DNA damage is one of the key factors for its induction. According to Jimenez et al. (*25*), it may be an important factor in both the induction and/or promotion of carcinogenesis and certain proliferative diseases.

Apoptotic cells are characterized by a number of morphologic, molecular, and biochemical features, including shrinkage of cells, blebbing of cells and nuclear membranes, compaction and condensation of chromatin toward the nuclear periphery, and fragmentation of DNA into oligonucleosomes (*26,27*). Detection of more than one of these parameters is essential for a clear characterization of apoptotic cells. The present results confirm UF-TiO_2–mediated apoptosis in SHE cells through different end points such as DNA ladder formation (internucleosomal cleavage) and chromatin compaction as analyzed by TEM.

Dopp et al. (*28*) demonstrated that chrysotile asbestos induces apoptosis after 48 hr of exposure in SHE cells. Eastman (*29*) showed that other inducers of apoptosis such as cisplatin induce apoptosis after 18–24 hr, whereas camptothecin or teniposide induce apoptosis in rat thymocytes even after 6 hr of exposure. The induction of apoptosis appears to depend on the inducing agent, although apoptosis itself is a basic cellular inherent process (*29*). Because the manifestation of the genetic damage is one of the main prerequisites for apoptosis, the concentrations for the induction of this effect by UF-TiO_2 are considerably higher than those for MN induction.

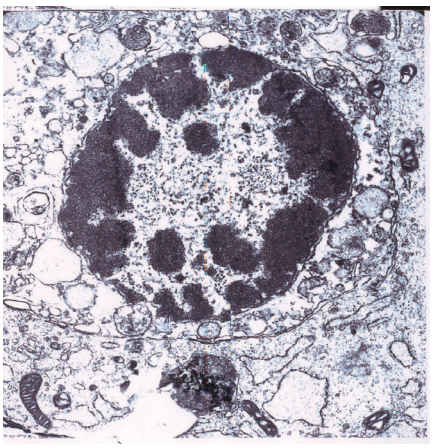
TEM revealed the typical compaction and marginalization of chromatin toward the nuclear periphery of exposed SHE cells, which is an important characteristic of the initial stages of apoptosis (Figure 4). We encountered both early and late stages, whereas unexposed cells did not show such specific features.

For the quantification of apoptosis, we also carried out dual staining of the nuclei of UF-TiO_2–exposed SHE cells with acridine orange and propidium iodide in a modified standard assay (*30*) (data not shown). According to Broaddus et al. (*31*), acridine orange enters the cells during the early stages of apoptosis and stains the nucleus bright green, but in later stages of apoptosis, due to loss of membrane integrity, both dyes enter the cell and stain the nucleus bright orange-red. We did not find this method appropriate because the cells showed staining in a number of shades between bright green and bright orange-red, making differential counting literally impossible.

Degradation of internucleosomal (linker) DNA segments as a consequence of activation of endogenous endonucleases is considered a characteristic end point of apoptosis and is analyzed using gel electrophoresis. DNA agarose gel electrophoresis depicted a typical internucleosomal fragmentation in DNA of UF-TiO_2–treated (concentrations, 5.0 and 10.0 μg/cm^2; exposure times, 24 and 48 hr) and cisplatin-treated (concentration, 100 μM; exposure time, 24 hr) cells. In contrast, the DNA of untreated cells did not show this typical DNA ladder pattern in electrophoresis. DNA fragmentation by UF-TiO_2 and cisplatin showed similar cleavage patterns. Because cisplatin is a very potent apoptosis inducer, these results indicate that UF-TiO_2 also has similar effects (Figure 5).

On the basis of the present findings and previous studies, we hypothesize that the size of UF-TiO_2 particles may stimulate phagocytosis. This evidence is supported by the results of the TEM analyses. The reaction of particles with cell membranes results in the generation of ROS, and the generated oxidative stress may cause a breakdown of membrane lipids, imbalance of intracellular calcium homeostasis, and alterations in metabolic pathways (*32–34*). The imbalance of calcium homeostasis finally results in calcium-dependent endonuclease activation, which is believed to initiate chromatin fragmentation, another key feature of apoptotic cells (*35,36*).

Several studies indicate that ROS and reactive nitrogen species are critical stimuli that induce apoptosis, either directly or via second messengers after exposure to various agents (*20*). Several groups have recently shown that fibers/particles induce apoptosis, the extent of which is reduced in the presence of various cellular antioxidants such as catalase and *N*-acetylcysteine (*37*). Further, antiapoptotic proteins such as Bcl-2 and baculovirus protein p35 are well known for their antioxidant properties (*38,39*). Broaddus et al. (*40*) showed a reduction of asbestos-induced apoptosis by catalase and deferoxamine, indicating an involvement of iron-catalyzed ROS. Further, a reduction of apoptosis under the influence of iron



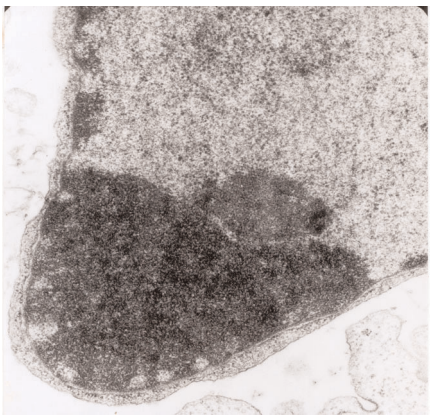


Figure 4. Electron micrographs of SHE cells exposed to UF-TiO_2 (5 μg/cm^2, 24 and 48 hr) showing an early stage of apoptosis (condensation and marginalization of chromatin toward the nuclear periphery. (*A*) Magnification 14,000×. (*B*) Magnification 32,000×.

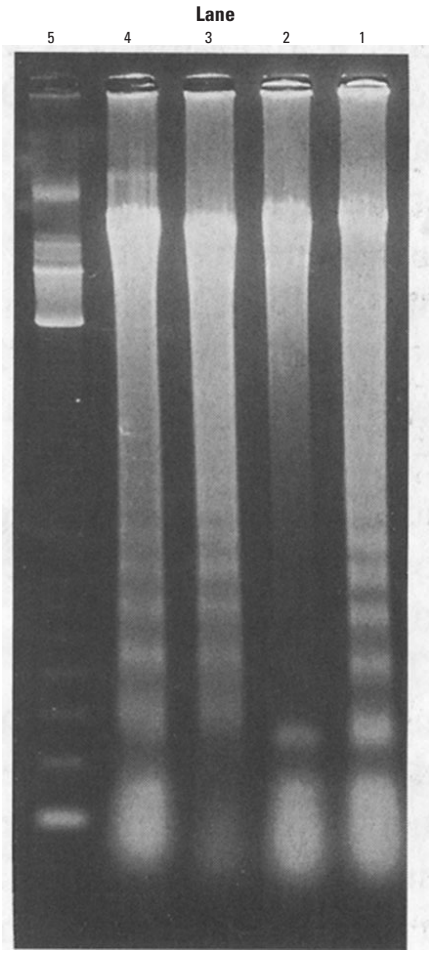


Figure 5. Agarose gel electrophoresis of DNA from SHE cells treated with UF-TiO_2 (10 μg/cm^2) for 24 hr (lane 1) and 48 hr (Lanes 1 and 2, respectively); purified DNA from untreated cells (control; Lane 3); SHE cells treated with cisplatin (100 μM, 24 hr; Lane 4) as a positive control; and the DNA marker (100 base pairs; Lane 5).

chelators and OH radical scavengers has also been demonstrated (*41*).

To our knowledge, this is the first study showing that UF-TiO_2 induces apoptosis. However, the precise mechanism of MN formation and induction of apoptosis by ultrafine particles and fibers is still unknown. Further insight is required into intracellular signaling and the role of oxygen radicals.

References and Notes

1. Hedenborg M. Titanium dioxide induced chemiluminescence of human polymorphonuclear leucocytes. Int Arch Occup Environ Health 61:1–6 (1988).
2. Ophus EM, Rode LE, Gylseth B, Nicholson G, Saeed K. Analysis of titanium pigments in human lung tissue. Scand J Work Environ Health 5:290–296 (1979).
3. Lindenschmidt RC, Driscoll KE, Perkins MA, Higgins JM, Mourer JK, Belfiore KA. The comparison of a fibrogenic and two non-fibrogenic dusts by bronchoalveolar lavage. Toxicol Appl Pharmacol 102:268–281 (1990).
4. Oberdorster G, Ferin J, Gelein R, Soderholm SC, Finkelstein J. Role of the alveolar macrophages in lung injury: studies with ultrafine particles. Environ Health Perspect 97:193–199 (1992).
5. Driscoll KE, Maurer JK. Cytokine and growth factor release by alveolar macrophages: potential biomarkers of pulmonary toxicity. Toxicol Pathol 19:398–405 (1991).
6. Baggs RB, Fern J, Oberdorster G. Regression of pulmonary lesions produced by inhaled titanium dioxide in rats. Vet Pathol 34:592–597 (1997).
7. Afaq F, Abidi P, Matin R, Rahman Q. Cytotoxicity, prooxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultra-fine titanium dioxide. J Appl Toxicol 18:307–312 (1998).
8. Afaq F, Abidi P, Matin R, Rahman Q. Activation of alveolar macrophages and peripheral red blood cells in rats exposed to fibers/particles. Toxicol Lett 99:175–182 (1998).
9. Yamadori I, Ohsumi S, Taguchi K. Titanium dioxide deposition and adenocarcinoma of the lung. Acta Pathol Jpn 36:783–790 (1986).
10. Driscoll KE, Maurer JK, Lindenschmidt RC, Romberger D, Rennard SI, Crosby L. Respiratory tract responses to dust: relationships between dust burden, lung injury, alveolar macrophages, fibronectin release and the development of pulmonary fibrosis. Toxicol Appl Pharmacol 106:88–101 (1990).
11. Oberdörster G, Ferin J, Lehnert BE. Correlation between particle size, *in vivo* particle persistence, and lung injury. Environ Health Perspect 102(suppl 5):173–179 (1994).
12. Oberdorster G. Pulmonary effects of inhaled ultra-fine particles. Int Arch Occup Environ Health 74(1):1–8 (2000).
13. Churg A, Stevens B, Wright J. Comparison of the uptake of fine and ultrafine TiO_2 in a tracheal explant system. Am J Physiol 274:L81–L86 (1998).
14. Zhang Q, Kusaka Y, Sato K. Differences in the extent of inflammation caused by intratracheal exposure to three ultrafine metals: role of free radicals. J Toxicol Environ Health 53:423–438 (1998).
15. Churg A, Gilks B, Dai J, Induction of fibrogenic mediators by fine and ultra-fine titanium dioxide in rat tracheal explants. Am J Physiol 277:975–982 (1999).
16. Drumm K, Buhl R, Kienast K. Additional NO_2 exposure induces a decrease in cytokine specific mRNA expression and cytokine release of particle and fibre exposed human alveolar macrophages. Eur J Med Res 4(2):59–66 (1999).
17. Rahman Q, Narwood J, Hatch G. Evidence that exposure of particulate air pollutants to human and rat alveolar macrophages lead to different oxidative stress. Biochem Biophys Res Commun 240:669–672 (1997).
18. Nakagawa Y, Wakuri S, Sakamoto K, Tanaka N. The photogenotoxicity of titanium dioxide particles. Mutat Res 394:125–132 (1997).
19. Lu PJ, Ho IC, Lee TC. Induction of chromatid exchanges and micronuclei by titanium dioxide in Chinese hamster ovary-KI cells. Mutat Res 414(1–3):15–20 (1998).
20. Hampton MB, Orrenius S. Redox regulation of apoptotic cell death. Biofactors 8:1–5 (1998).
21. Fritzenschaf H, Kohlpoth M, Rusche B, Schiffmann D. Testing of known carcinogens in the Syrian hamster embryo (SHE) micronucleus test in vitro; correlations with in vivo micronucleus formation and cell transformation. Mutat Res 319:47–53 (1993).
22. Pienta RJ. Transformation of Syrian hamster embryo cells by diverse chemicals and correlation with their reported carcinogenic and mutagenic activities. Chem Mutagen 6:175–202 (1980).
23. Heddle JA, Hite M, Krikhart B, Mavournin K, MacGregor JT, Newell GW, Salamone MF. The induction of micronuclei as a measure of genotoxicity. Mutat Res 123:61–118 (1983).
24. Hesterberg TW, Butterick CJ, Oshimura M, Brody AR, Barrett JC. Role of phagocytosis in Syrian hamster cell transformation and cytogenetic effects induced by asbestos and short and long glass fibers. Cancer Res 46(11):5795–5802 (1986).
25. Jimenez LA, Zenela C, Fung H, Janseen YMW, Vacek P, Charland C, Goldberg J, Mossman BT. Role of extracellular signal-regulated protein kinases in apoptosis by asbestos and H_2O_2. Am J Physiol 273:1029–1035 (1997).
26. Darzynkiewicz Z, Bruno S, DelBine G, Gorczyca H, Hotz MA, Lassota P, Traganos F. Features of apoptotic cells measured by flow cytometry. Cytometry 13:795–780 (1992).
27. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide range applications in tissue kinetics. Br J Cancer 26:239–257 (1972).
28. Dopp E, Nebe B, Hahnel C, Papp T, Alonso B, Simko M, Schiffmann D. Mineral fibres induce apoptosis in Syrian hamster embryo fibroblasts. Pathobiology 63:213–221 (1995).
29. Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells 2:275–280 (1990).
30. Duke RC, Witter RZ, Nash PB, Young JD-E, Ojcius DM. Cytolysis mediated by ionophores and pore-forming agents: role of intracellular calcium in apoptosis. FASEB J 8:237–246 (1994).
31. Broaddus VC, Yang L, Scavo LM, Ernst JD, Boylan AM. Crocidolite asbestos induces apoptosis of pleural mesothelial cells: Role of reactive oxygen species and poly(ADP-ribosyl)polymerase. Environ Health Perspect 105(suppl 5):1147–1152 (1997).
32. Clutton S. The importance of oxidative stress in apoptosis. Br Med Bull 53:662–668 (1997).
33. Petruska JM, Leslie KO, Mossmann BT. Enhanced lipid peroxidation in lung lavage of rats after inhalation of asbestos. Free Rad Biol Med 11:425–432 (1991).
34. Slater TFS. Free radicals fact or fiction? Br J Cancer 55:5–10 (1987).
35. Wolf M, Cautrecasas P, Sahyoun N. Interaction of protein kinase C with membranes is regulated by Ca^{+2}, phorbol esters and ATP. J Biol Chem 260:15718–15722 (1985).
36. Orrenius S, McCabe MJ, Nicotera P. Ca^{+}-dependent mechanisms of cytotoxicity and programmed cell death. Toxicol Lett 64/65:357–364 (1992).
37. Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. Free Rad Biol Med 29 (3/4):323–333 (2000).
38. Jacobson M. Reactive oxygen species and programmed cell death. Trends Biochem Sci 21:83–86 (1996).
39. Sah NK, Taneja TK, Pathak N, Begum R, Athar M, Hasnain SE. The baculovirus antiapoptotic p53 gene also functions via an oxidant-dependent pathway. Proc Natl Acad Sci USA 96:4838–4843 (1999).
40. Broaddus VC, Yang L, Scavo LM, Ernst JD, Boylan AM. Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species. J Clin Invest 98(9):2050–2059 (1996).
41. Kamp DW, Aljandali A, Pollack N. Asbestos induces apoptosis in cultured alveolar epithelial cells. Am J Respir Crit Care Med 157:385–394 (1998).