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In vitro genotoxicity evaluation of tungsten (VI) oxide nanopowder using human lymphocytes.

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Abstract

Tungsten (VI) oxide (or tungsten trioxide) (WO_3, <100 nm particle size) nanoparticles (NPs) are used for many purposes including production of electrochromic windows, or smart windows, x-ray screen and gas sensors in everyday life. However, their carcinogenicity and genotoxicity have not been sufficiently evaluated. Therefore, the genotoxic potential of WO_3 nanoparticle was examined in cultured human lymphocytes by the use of the micronucleus (MN) test and the comet (SCGE) assay. Freshly isolated human lymphocytes were exposed to WO_3 nanoparticle at concentrations ranging from 0 to 500 μM for 72 hours at 37°C. Our results indicated that 400 and 500 μM of WO_3 nanoparticle treatment caused slight increases of the MN frequencies in cultured human lymphocytes. Likewise, WO_3 nanoparticle (at concentrations above 200 μM) led to increases of DNA damage (estimated with the comet assay) in human lymphocytes. The observed alterations in the MN and the comet assay parameters revealed that WO_3 nanoparticles have genotoxic potential and could pose environmental and human health risk.

Keywords: Genotoxicity, Health risk, Human lymphocytes, Nanoparticle, Tungsten trioxide.

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Introduction

Nanoparticles (NPs) are identified as particles with diameters under 100 nm, are unique in that their electronic, chemical, and physical properties enable many promising technical and medicinal applications [1,2]. Thanks to their unique features, NPs have been the focus of much research such as in industrial applications, environmental toxicity studies and human health impacts. Various industrial NPs are made from titanium oxide, silver, gold, cadmium selenite, other carbon NPs, cerium oxide and hydroxyapatite NPs [3-6]. Together with the fast development of nanotechnology today, NPs are used for various biomedical applications such as targeted delivery/ imaging, hyperthermia, cell therapy and stem cell tracking [7-14]. In recent years, many efforts were made to investigate the toxicity of micro sized natural and man-made mutagens to human life and the ability of therapeutic substances to reduce the toxicity of these chemicals [15-20]. But the toxic effects of NPs were not fully detailed except for some inorganic and organic NPs. In fact, the most recent report indicated that there was a lack of systematic assessment of the DNA damaging and carcinogenic potential of NPs in spite of their extensive use in nanotechnological applications. People are exposed to NPs from various sources and in many pathways, including inhalation, dermal absorption, eye contact and oral ingestion [21,22]. Therefore, the evaluation of NPs toxicity has become very important for public health and the environment [23-25].

Tungsten trioxide contains oxygen and the transition metal tungsten. It is gained as an intermediate in the recovery of tungsten from its minerals. To produce tungsten products tungsten ores are treated with alkalis. Tungsten trioxide can be prepared in several different ways. Scheelite ($CaWO_4$) is allowed to react with HCl to produce tungstic acid, which decomposes to WO_3 and water at high temperatures. Another

common way to synthesize WO_3 is by calcination of ammonium paratungstate (APT) under oxidizing conditions. There are many applications of the WO_3 in everyday life. It is used in industry to manufacture tungstates for fireproofing fabrics, for x-ray screen, in gas sensors, automobile glass and as a pigment in ceramics and paints because of its rich yellow color [26-30]. In recent years, WO_3 has been employed in biomedical applications as an endovascular coil, endovascular catheter and bone cement [31-33]. Although tungsten had been considered a relatively inert and toxicologically safe material, recent research findings have raised concerns about possible deleterious health effects after acute and chronic exposure to this metal [34, 35]. It was reported that soluble tungsten compounds were absorbed after oral exposure both in humans and in laboratory rats. It has been shown that the embedded tungsten alloy pellets caused metastatic tumors in rats. Tungsten was found to accumulate in several organs and/or tissues such as kidneys, liver, ovaries, prostate, pancreas, lung, heart, muscle, spleen and bone following a single oral dose [36]. In addition, a previous report indicated the potential for tungsten alloy-induced immunotoxicity [37]. The genotoxic potential of tungsten and tungsten compounds has not been extensively assessed [38]. Considering the latest information, the mutagenic potential of WO_3 nanopowder has not been accurately perused. Thus, in this paper, we thoroughly investigate the cytotoxic and genotoxic potentials of WO_3 nanoparticles in human lymphocytes culture by using the micronucleus (MN) test and the comet (SCGE) assay.

Materials and Methods

Synthesis of tungsten trioxide nanoparticles

Metal oxide based semiconductors such as SnO_2, ZnO, TiO_2, CuO and WO_3 etc. have been used in many application areas [39]. Among these, WO_3 is one of the most valuable materials for electrochromic devices, information displays, smart windows and rechargeable lithium batteries [40]. WO_3 as transitional metal oxide not only has reversible electrochromism property and special catalysis property [41]; at one time because of its big surface area, WO_3 can be used excellent solar absorb material and contact material: but also WO_3 belonging to an n-type semiconductor has excellent gas sensing property [42]. In the recent years, nanopowder WO_3 materials have gained much attention due to its surface to volume ratio, which is much greater than that of coarse-grained materials [43]. There is a wide range of techniques for preparation of the powders such as the sol-gel process [44], the micro emulsion method [42], the inert gas condensation method and the chemical vapor condensation process [45]. Among these techniques, the sol-gel technique is attractive due to its easy manipulation of the samples, simplicity, safety, low cost [46], and easy control of chemical components [47]. The sol-gel method involves the dispersion of metallic salts in solutions. The sol is later 'solidified' through stages of stiffening and polymerization to give a gel (gelation) [48]. The gel so obtained is thoroughly washed with distilled water or alcohol, filtered, dried and finally heated to high temperatures to obtain the required material [49]. Therefore, in present study, WO_3 nanopowders were prepared via the sol-gel process. The experimental construction is shown in Figure 1. Firstly, nitric acid (HNO_3) solution was added drop by drop to sodium tungstate ($Na_2WO_4.2H_2O$), so tungstic acid deposit was formed. Oxalic acid ($H_2C_2O_4$) and citric acid ($C_6H_8O_7$) were used as complex forming agents in the sol solution. The precipitate obtained from this solution was washed several times with absolute ethyl alcohol and then dried at 50°C. In this manner yellow precipitates were formed and WO_3 powder was produced with calcination at 550°C for 3h.

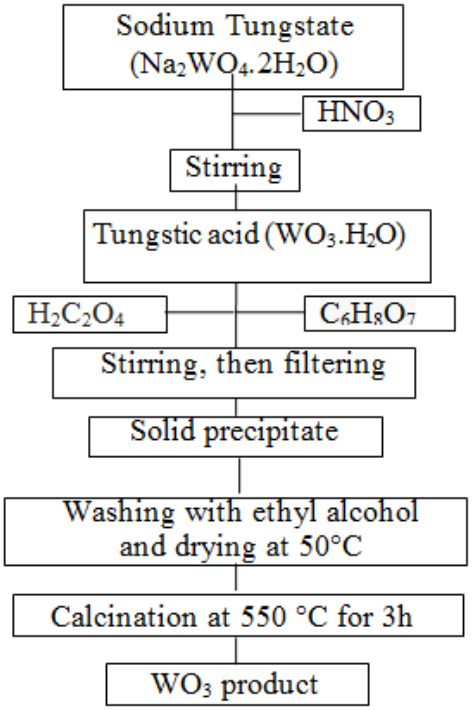

Figure 1: *Schematic diagrams of steps involved in obtaining WO_3 powders*

Lymphocyte cultures

Typically, two or three donors have been used in toxicity studies [18,19]. In this study, four donors were used to increase the statistical reliability. Human peripheral blood samples were drawn from healthy volunteers (age ≤ 30 years), by venipuncture in heparinized tubes. Leukocytes (lymphocytes +monocytes) were isolated on a Ficoll-Paque gradient, washed with *Phosphate Buffered Saline (*PBS) and resuspended in Ham's F10 medium containing 15% foetal calf serum (FCS). Lymphocytes were stimulated to divide by 2% phytohaemagglutinin (PHA). Cultures were set up at a concentration of 0.5×10^6 cells/ml in glass tubes and incubated at 37°C 24 h after PHA stimulation, WO_3 particles were dispersed in 1-ml cultures. All concentrations were prepared immediately prior to the application; H_2O (10 µl) was used as metal carrier.

Genotoxicity testing

Micronucleus assay (MN): Cytochalasin B (6 µg/ml) was added after 44 h to block cytokinesis, and after a total of 72 h cells were spread on slides using a cytospin (Shandon, 5 min at 600 rpm). All slides were fixed in 100% methanol (20 min) and stained with 5% Giemsa in Sörensen buffer (pH 6.8). Duplicate cultures were analyzed for each dose tested: 1000 cytokinesis-blocked (binucleated) lymphocytes (CBs) were examined per culture for the presence of one, two or three MN. In addition, the percentages of binucleated cells (% CB), polynucleated cells (polyN), metaphases, and mononucleated cells with MN were recorded. As a measure for cell cycle delay and/or cytotoxicity, the relative division index (RDI) was used: RDI=[(CB+2polyN)/n treated sample]/[(CB+2polyN)/n control sample] × 100. All slides were coded and analyzed with a Zeiss microscope (1250 × magnification). Statistical differences between controls and treated samples were determined with the chi- square (χ^2) test. Because Mytomicin C (MMC) is more effective at lower doses [50], it was used at 10-7M as a positive control.

Comet assay (SCGE): Cell processing was performed as described by Singh et al., with some modifications [51]. Fully frosted slides (Richardson Supply, UK) were covered with 1% normal melting point (NMP) agarose and a coverslip. The agarose was allowed to solidify at room temperature and removed by scraping with a coverslip. Then the slides were covered with 300 µl NMP agarose (0.5%) and a coverslip, and placed on ice for 10 min to let agarose solidify. After removal of the coverslip, 5000-50,000 cells (in 10 µl incubation solution) were mixed with 90 µl of 0.6% low melting point (LMP) agarose and carefully layered on top, covered with a coverslip and put on ice to solidify. The coverslips were removed, and the slides put in cold, freshly made lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA disodium salt and 1% w/v N-lauroylsarcosine, pH 10, supplemented with 10% v/v DMSO and 1% v/v Triton X-100 before use) for at least 1 h at 4°C. For electrophoresis, the slides were placed in a horizontal electrophoresis box filled with freshly made alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >12) for 40 min at 18°C, to allow the DNA to unwind. Electrophoresis (300 mA, 0.7 V/cm) was performed in the same buffer for 20 min at 18°C. Slides were removed from the buffer, the excess alkali was neutralized with 0.4 mM Tris (Ph 7.5), and the slides stored with coverslip in a moist chamber at 4°C until analysis. Ethidium bromide (20 µg/ml) stained nuclei were analyzed by a computer-guided image analysis system. Images from a Zeiss fluorescence microscope (300X magnification) were captured with an air-cooled camera (Photonic Science) on a frame grabber type DT 2855. Depending on the quality of the slides, between 40 and 100 non-overlapping images per dose were selected randomly on the slides. Tail length (TL) of the comet was measured by defining manually the center and the leading edge of the nucleus, and the end of the tail. Besides TL, also tail moment (TM) (TM=TL × fraction of DNA content in the tail) and the percentage of DNA in the tail (%DNA tail) were assessed. All data were processed by a Macintosh (Performa 6200 PPC) computer resulting in, e.g. a box plot presentation to show the extent and distribution of DNA damage. Statistical differences between controls and treated samples were determined with the non-parametric Mann-Whitney U-test.

Results

The effects of WO_3 nanoparticle exposure on the frequency of MN formation are shown in Figure 2. No statistically significant difference was found between WO_3 nanoparticle applied samples and control group excluding 400 and 500 µM samples. The higher doses of WO_3 nanoparticle (400 and 500 µM) caused increases of MN rates. The results of comet assay are shown in Figure 3. Comet assay analyses did not show any statistically significant differences between control and the first five doses of WO_3 nanoparticle (from 10 to 150 µM). On the contrary, last three doses of WO_3 nanoparticle (200, 400 and 500 µM) caused increases of DNA damage.

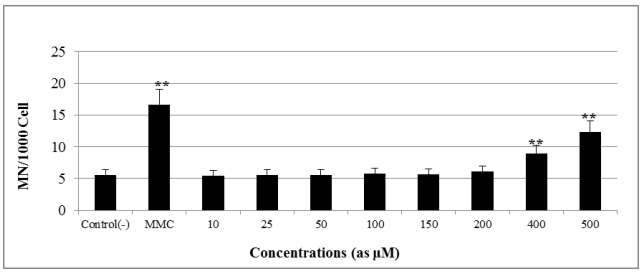

Figure 2: *The frequencies of MNs in human lymphocytes treated with different concentrations of Tungsten trioxide.*

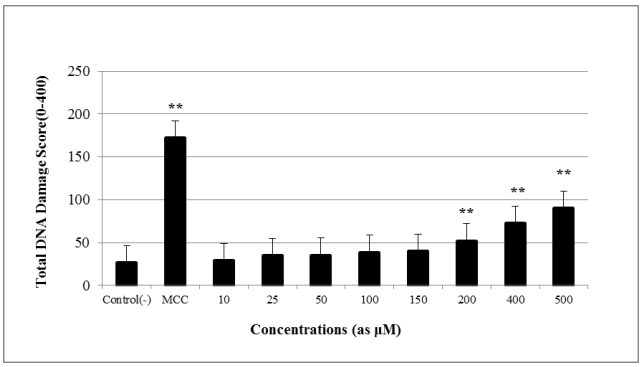

Figure 3: *The effects of Tungsten trioxide induced DNA damage determinated by SCGE assay in lymphocytes.*

Discussion

The aim of this laboratory study, was to evaluate the genotoxicity in the cultured human lymphocyte cells in response to different concentrations of WO_3 NPs. Present findings indicated that WO_3 NPs are a weak mutagen in human lymphocytes cultures. WO_3 nanoparticle induced insignificant increases of MN frequencies and DNA damage in human lymphocytes. In fact, the MN assay provides a measure of both chromosome breakage and chromosome loss or non-disjunction in clastogenic and aneugenic events, respectively [52]. Damaged DNA can lead to aneuploidy and/or chromosomal instability, which is believed to be a major

contributor to tumor progression [53, 54]. The SCGE assay is a rapid, simple, visual and sensitive technique for measuring DNA breakage in individual mammalian cells [55]. In line with recent findings, there are a few reports on genotoxicity of tungsten NPs in literature. Turkez et al., reported that WO_3 nanoparticle did not cause increase of the incidence of chromosome aberrations in rat bone marrow cells but led to increases of MN formation after chronic exposure for 30 days [56]. On the contrary, another study showed that WO_3 NPs did not induce MN frequency in cultured rat liver cells [6]. Again, in a study *in vitro*, WO_3 NPs showed positive mutagenic response in T A1537 and T A98 bacterial strains of Salmonella typhimurium by using Ames test [57]. Tungsten carbide NPs, which are used in hard metal industries for the production of wear resistant and hard tools, induced an increase in the rate of MN in human keratinocyte cells [58]. The probable genotoxic effect of NPs is rooted in several causes, namely their ability to penetrate into living cells and induce free radicals of oxygen and nitrogen [59], to reach the nuclei [60], to damage the cytoskeleton [61] and to interact with DNA [62]. The composition of some nanoclusters include elements that have a carcinogenic effect, such as tungsten [63]. Finally, the structures of some NPs are similar to asbestos fibers [64], which have well-known genotoxic and carcinogenic effects. In addition, a comparison of the genotoxic effects of nano- and microparticles of the same compounds verifies that NPs have higher activity [65].

As a summary, the present findings showed that tungsten oxide NPs have cytotoxic and a weak genotoxic potential and could pose human health risk. Further studies are warranted to investigate the mutagenicity or carcinogenicity of tungsten NPs in mammalian cells for offering certain cautions and assessing their risks on humans.

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