Experimental and Toxicologic Pathology 63 (2011) 453–457

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

The role of ascorbic acid on titanium dioxide-induced genetic damage assessed by the comet assay and cytogenetic tests

Hasan Turkez *

Department of Biology, Faculty of Sciences, Atatürk University, 25240 Erzurum, Turkey

ARTICLE INFO

Article history:
Received 12 November 2009
Accepted 11 March 2010

Keywords:
Ascorbic acid
Comet assay
Genotoxicity
Micronucleus
Titanium dioxide
Sister chromatid exchange

ABSTRACT

Titanium dioxide (TiO_2) is used in several commercial products such as cosmetics, sunscreen, toothpaste and pharmaceuticals. However, some recent investigations have revealed that titanium particles generate potential harmful effects on the environment and humans. Because of its strong antioxidant activity, ascorbic acid (AA) is admitted to act as an anti-mutagenic agent. The present study was undertaken to investigate the protective effect of AA against TiO_2-induced genotoxicity. Sister chromatid exchange (SCE), micronucleus (MN) and the comet assays were used to assess TiO_2-induced genotoxicity and to establish the protective effects of AA. There were significant increases ($P < 0.05$) in both SCE and MN frequencies of cultures treated with TiO_2 as compared to controls. However, co-application of AA (4.87 and 9.73 μM) and TiO_2 resulted in decreases of SCE and MN rates as compared to the group treated with titanium alone. Besides, significant reductions of primary DNA damage (comet assay) were determined when the AA was added to the cell culture medium simultaneously with TiO_2. In conclusion, the preventive role of AA in alleviating TiO_2-induced DNA damage was indicated for the first time in the present study.

Introduction

TiO_2 is used in many white or colored products like food, cosmetics, skin protection products, ceramics, fibers, paints, inks, paper and numerous plastic and rubber products. TiO_2 is also used for a colorant in pesticide formulations and sterilization of waste water (EPA, 2005; Wamer et al., 1997). Although the uses of TiO_2 have become so widespread, there is insufficient knowledge concerning its toxicity (Gheshlaghi et al., 2008). TiO_2 was reported to be non-toxic in a few investigations (Moore, 1997; Owolade et al., 2008). But an apprehension has arisen from animal and cultured mammalian cell studies and it has pointed to TiO_2 as carcinogen and mutagen. It was found that TiO_2 caused cystic and keratinizing squamous lesions (Carlton, 1994) and tumor formations (Hesterberg et al., 2005) in rats. Moreover, neurotoxicity (Long et al., 2006), hepatotoxicity, nephrotoxicity and myocardial damage have occurred in experimental animals (Jiangxue et al., 2006). Although the genotoxicity of TiO_2 has been investigated with a variety of genetic endpoints in animals and cultured mammalian cells (Nakagawa et al., 1997; Turkez and Geyikoglu, 2007; Xu et al., 2009), it remained controversial (Lu et al., 1998).

The free radical generation can be central for TiO_2 pathogenicity (Donaldson et al., 1996). In fact, oxidative stress increased after exposure to TiO_2 in human and rat alveolar macrophages (Rahman et al., 1997) and mouse microglia (Long et al., 2006). On the other hand, antioxidant vitamins can inactivate highly reactive molecules, such as free radicals, that are generated during various biochemical processes in the cells (Singh et al., 2008). The efforts are being made to examine therapeutic agents, which are capable of minimising the genotoxicity of various natural and man-made mutagens in human life. These include vitamins, sulfhydryl substances and plant products (Edenharder et al., 1999; Rao et al., 2001). AA (vitamin C) is known to be a strong antioxidant reported to reduce the mutagenic activity of many chemicals (Hisama et al., 2008); however, there is no information regarding its effect against titanium-induced genotoxicity. Therefore, in the present study, the toxicity induced by TiO_2 and the possible beneficial effect of AA against TiO_2-induced cytotoxicity (proliferation index, PI) and genotoxicity using comet, SCE and MN assays, which are rapid and sensitive methods for measuring the genetic damage, were evaluated.

Material and methods

The heparinized blood samples from three healthy non-smoking donors with no history of exposure to any genotoxic agent were used. Questionnaires were given to each blood donor to evaluate

* Corresponding author at: Atatürk Üniversitesi, Fen Fakültesi, Biyoloji Bölümü, 25240 Erzurum, Turkey. Tel.: +90 442 231 43 33; fax: +90 442 236 09 48.
E-mail addresses: hasanturkez@yahoo.com, hasanturkez@gmail.com.

0940-2993/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.etp.2010.03.004

exposure history; and informed consent forms were signed by each of them. For all the volunteers, hematological and biochemical parameters were analyzed and no disease was detected. Human peripheral blood lymphocyte cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (1975). The heparinized blood (0.5 ml) was cultured in 5 ml of culture medium (Chromosome Medium B, Biochrom®, Leonorenstr. 2-6.D-12247, Berlin) with 5 μg ml^{-1} of phytohemagglutinin (Biochrom®). TiO_2 (< 100 nm, CAS No. 13463-67-7) (Sigma® Chemical Co., St. Louis, MO, USA) (in concentrations of 3, 5 and 10 μM) and AA (CAS No. 50-81-7, $C_6H_8O_6$) (Sigma®) were added to the cultures (in concentrations of 4.87 and 9.73 μM) just before incubation, separately and together. Thus, the experiments were performed on different groups as follows: TiO_2 alone, AA alone and TiO_2 plus AA. Experiments conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki). These investigations stem from the works of Lu et al. (1998) and Turkez (2008). Each individual lymphocyte culture without TiO_2 and AA was studied as a control group. For the studies, a stock solution of the TiO_2 was prepared with sterile dimethyl sulfoxide (DMSO).

Cytogenetic analysis

SCE assay and proliferation index

With the aim of providing a better visualization of SCEs, 5-bromo-2′-deoxyuridine (Sigma®, final concentration 20 μM) was added after culture initiation. The cultures were incubated in complete darkness for 72 h at 37 °C. Exactly 70 h and 30 min after beginning of the incubations, colcemid (Sigma®) was added to the cultures to achieve a final concentration of 0.5 μg l^{-1}. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation and resuspension. The cell suspension was dropped onto chilled and grease-free microscopic slides, air-dried, aged, and then differentially stained for inspection of the SCE rate according to the fluorescence plus Giemsa (FPG) procedure (Perry and Wolff, 1974). For each treatment condition, 25 well-spread second division metaphases were scored and the values obtained were calculated as SCEs per cell.

In addition to SCEs, cells were analyzed for the relative frequency of first-division metaphases (M1; identifiable by uniform staining of both sister chromatids), second-division metaphases (M2; identifiable by differential staining of the sister chromatids), and third- and subsequent division metaphases (M3; identifiable by nonuniform pattern of staining). PI is the average number of replications completed by metaphase cells and is calculated as follows: $PI = 1 \times (\%\,M1) + 2 \times (\%\,M2) + 3 \times (\%\,M3)/100$.

MN Assay

The MN test was performed by adding cytochalasin B (Sigma®; final concentration of 6 μg ml^{-1}) after 44 h of culture. At the end of the 72-h incubation period, the lymphocytes were fixed with ice-cold methanol:acetic acid (1:1). The fixed cells were put directly on slides using a cytospin, and stained with May Grünwald–Giemsa. All slides were coded before scoring. The criteria for scoring micronuclei were as described by Fenech (1993). At least 2000 binucleated lymphocytes were examined per concentration (two cultures per concentration) for the presence of one, two or more micronuclei.

Comet assay

The comet assay also known as single cell gel electrophoresis (SCGE) was performed and scored according to Singh et al. (1988), Kizilian et al. (1999), Tice et al. (2000), Saleha Banu et al., (2001) and Das et al. (2006). The cultures were set up by incubating lymphocytes for 72 h with TiO_2 and AA. The control cultures were set up by incubating lymphocytes with the solvent DMSO (at a final concentration of 1%). Ten milliliters of the 100 μl aliquots of the lymphocytes treated as above along with untreated samples were mixed with 120 μl of 0.5% low melting agarose and layered on the surface on glass slides previously coated with 140 μl of 1% normal melting agarose. After the application of coverslips, the slides were allowed to gel at 4 °C for 20 min. After carefully removing the coverslips, a second layer of 0.5% low melting agarose was pipetted onto the slides and allowed to gel for a further 20 min at 4 °C. The slides were immersed in freshly prepared cold lysing solution and refrigerated overnight followed by alkali treatment, electrophoresis and neutralization. The dried slides were then stained using silver nitrate solution after appropriate fixing. The whole procedure was carried out in dim light to minimize artifactual. DNA damage analysis was performed at a magnification of × 100 using a light microscope after coding the slides. A total of 100 cells were screened per slide.

Statistics

The statistical analysis of experimental values in the SCE, PI and comet tests was performed by one-way analysis of variance (ANOVA) and Duncan's test using the S.P.S.S. 13.0 software. The statistical analysis of MN frequencies was performed by use of the χ^2 test. And the level of 0.05 was regarded as indicative of statistical significance for all tests.

Results

The results of the present study showed that AA at tested concentrations did not induce significant number of SCEs. Moreover, the presence of 4.87 and 9.73 μM AA during the treatment of the cells with TiO_2 reduced the number of SCEs significantly ($P < 0.05$) (Fig. 1).

Proliferation index is used to verify study results and provide additional information on cell viability and toxicity. Regarding PI values, our results showed that treatments with AA (at both doses) and TiO_2 (3 and 5 μM) alone did not alter PI values compared to the control (Fig. 2). Whereas, a statistically important decrease in the rate of PI was observed after treatment with 10 μM TiO_2. However, the concomitant treatments with AA and TiO_2 (10 μM) increased the rate of PI as compared to the group treated with titanium alone.

The ability of TiO_2 to induce MN in cytokinesis blocked cells, as well as a decrease in the MN frequency in cultures treated with AA is reflected in Fig. 3. A dose dependence in the elevation of MN was observed after exposure to TiO_2 for 72 h, and it became statistically significant at concentrations of 5 and 10 μM. The positive effect of AA in decreasing the incidence of MN in comparison with an unprotected level was attained when cultures were treated simultaneously with TiO_2 and AA ($P < 0.05$).

The effect of AA on DNA damage in human lymphocytes induced by TiO_2 was determined by SCGE assay. As shown from the results presented in Fig. 4, the comet tail length increased from 0.196 to 0.605 μm with the increase in the concentration of titanium from 0 to 10 μM and AA had an inhibitory effect on DNA damage in human lymphocytes induced by TiO_2. At two (4.87 and 9.73 μM) concentrations of AA, there were significant ($P < 0.05$) inhibitory effects on DNA damage. This result shows that the DNA damage in human lymphocytes induced by TiO_2 was reduced by the AA application.