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Cytotoxicity, oxidative stress and genotoxicity induced by glass fibers on human alveolar epithelial cell line A549

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ABSTRACT

Man-made vitreous fibers have been widely used as insulation material as asbestos substitutes; however their morphology and composition raises concerns. In 1988 the International Agency for Research on Cancer classified fiberglass, rock wool, slag wool, and ceramic fibers as Group 2B, i.e. possibly carcinogenic to humans. In 2002 it reassigned fiberglass, rock and slag wool, and continuous glass filaments to Group 3, not classifiable as carcinogenic to humans. The aim of this study was to verify the cytotoxic and genotoxic effects and oxidative stress production induced by *in vitro* exposure of human alveolar epithelial cells A549 to glass fibers with a predominant diameter <3 μm (97%) and length >5 μm (93%). A549 cells were incubated with 5, 50, or 100 μg/ml (2.1, 21, and 42 μg/cm^2, respectively) of glass fibers for 72 h. Cytotoxicity and DNA damage were tested by the MTT and the Comet assay, respectively. Oxidative stress was determined by measuring inducible nitric oxide synthase (iNOS) expression by Western blotting, production of nitric oxide (NO) with Griess reagent, and concentration of reactive oxygen species by fluorescent quantitative analysis with 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). The results showed that glass fiber exposure significantly reduced cell viability and increased DNA damage and oxidative stress production in a concentration-dependent manner, demonstrating that glass fibers exert cytotoxic and genotoxic effects related to increased oxidative stress on the human alveolar cell line A549.

1. Introduction

Man-made vitreous fibers (MMVFs) are inorganic materials with an amorphous molecular structure obtained from several types of minerals. MMVFs, including glass fiber, glass filament, rock/slag wool, and less common materials like refractory ceramic fibers, are widely used as thermal and acoustic insulation materials in building and industrial applications (Lunn et al., 2009; Marsh et al., 2011). MMVFs are non-crystalline, fibrous inorganic substances that like asbestos are silicate fibers.

It has been estimated that more than 9 million ton MMVFs are manufactured in the world each year. MMVF products can release airborne respirable fibers during manufacturing, use and removal (Baan and Grosse, 2004). In a recent study of an office building in Helsinki (Finland), MMVFs were found in the dust collected from surfaces in over 60% of the offices tested (Salonen et al., 2009).

In 1988, the International Agency for Research on Cancer (IARC) classified glass fiber, rock and slag wool, and ceramic fibers as Group 2B, possibly carcinogenic to humans (IARC, 2002). It subsequently reassigned glass fiber, rock and slag wool, and continuous glass filament to Group 3, not classifiable as to carcinogenic to humans, and confirmed refractory ceramic fibers as Group 2B (IARC, 1988).

Abbreviations: ANOVA, variance analysis; DMEM, Dulbecco's modified Eagle's medium; HA, head area; HI, head intensity; HL, head length; IARC, International Agency for Research on Cancer; iNOS, inducible nitric oxide synthase; LMA, low melting point agarose; MMVFs, man-made vitreous fibers; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMA, standard melting point agarose; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; SCGE, single-cell gel electrophoresis; TA, tail area; TD, the product of tail/head distance; TDNA, percentage of fragmented DNA; TI, tail intensity; TL, tail length; TMOM, tail moment.

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A number of epidemiological studies have found an increased risk of respiratory system cancer in cohorts of MMVFs-exposed workers (Lee et al., 1995; Boffetta et al., 1999; Marsh et al., 2001; LeMasters et al., 2003; Stone et al., 2004; Baccarelli et al., 2006; Shannon et al., 2005). In addition, several studies showed that MMVFs induce cancer in various experimental animals (IARC, 1988; Hesterberg and Hart, 2001; Kamstrup et al., 1998). Some *in vivo* (Hesterberg and Hart, 2001; Bunn et al., 1993; Kamstrup et al., 2002) and *in vitro* (Hart et al., 1994; Okayasu et al., 1999; Wang et al., 1999; Dörger et al., 2000; Kim et al., 2001; Elias et al., 2002) toxicity studies found that MMVFs exert cytotoxic and carcinogenic effects, whereas other investigations showed oxidative effects induced especially by rock wool and refractory ceramic fibers (Cavallo et al., 2004; Lutz and Krajewska, 1995; Gilmour et al., 1997; Murata-Kamiya et al., 1997; Brown et al., 1998; Ruotsalainen et al., 1999), and genotoxic effects exerted by glass fibers (Zhong et al., 1997). Cytotoxic effects at the level of the cell membrane were demonstrated in alveolar macrophages of rats exposed to respirable-sized MMVFs (Luoto et al., 1994) and in human mesothelial cells exposed to rock wool (Cavallo et al., 2004). In addition, it has been reported that fibers may induce carcinogenic effects by production of oxygen reactive species (ROS), resulting in induction of oxidative DNA damage and cell transformation (Brown et al., 1998; Fubini, 1996). Fiber size, shape, chemical composition and biopersistence have been found to be important factors related to cytotoxic and carcinogenic actions of fibrous materials (Lippmann, 1990; Lippmann, 1993; Hill et al., 1995; Fubini, 1996). Findings from *in vitro* and inhalation studies suggest that longer and thinner fibers induce greater toxicity (Everitt, 1994).

However, little is known about the mechanisms involved in the toxic effects of borosilicate glass fibers on human alveolar epithelial cells, the first level of lung exposure to the fibers. These fibers are widely used in chemical laboratory equipment, cookware, lighting and windows for their superior durability, chemical and heat resistance properties.

In this study, we investigated the induction of cytotoxicity, genotoxicity and oxidative stress by glass fibers in human alveolar epithelial cells A549. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and evaluation of oxidative stress markers such as ROS, inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production. The Comet assay was applied to assess the DNA damage exerted by glass fibers.

2. Materials and methods

2.1. Characterization of glass fibers

Qualitative analysis of glass fibers was performed using a Microanalysis IXRF System (model 500, San Francisco, USA). Fibers were borosilicate glass with high content (%/weight) of SiO_2 and B_2O_3 and low content of alkaline oxide. Their oxide composition in terms of percent weight is reported in Table 1.

Table 1
Composition of the glass fibers used in the study by percent weight of component oxides.

Component	Percent weight
SiO_2	80.6
B_2O_3	13.0
Na_2O	4.0
Al_2O_3	2.3
K_2O	0.1

For experiments, about 1 g of sample was suspended in distilled water by ultrasonic agitation (frequency 40 kHz and 20 min, 4×) (Digital Ultrasonic Cleaner, Qsonica, LLC, Newtown, Connecticut, USA), and a small amount of each material was filtered on a polycarbonate membrane (Whatman® Nuclepore™ Track-Etched Membranes, size 25 mm, pore size 0.2 μm), according to Cavallo et al. (2004).

The glass fibers selected by the polycarbonate filters were characterized by measuring the diameter and length of 300 fibers using a LEO1430 scanning electron microscope (Zeiss, GöttingenGermany) at a magnification that allowed each time precise measurement of fiber size in the 300–80k× range. Glass fibers were characterized by a predominant diameter <3 μm (97%) and length >5 μm (93%) (Table 2). Distribution was lognormal as estimated by the Kolmogorov–Smirnov normality test. The values of zeta-potential at 25 °C were −50.5 ± 2.95 and −10.2 ± 0.72 in water and culture medium, respectively (Zetasizer Nano ZSP – Malvern Instruments, WR14 1XZ, UK).

2.2. Cell cultures and treatments

A549 cells – human bronchoalveolar carcinoma-derived cells with some features characteristic of alveolar epithelial type II cells, obtained from the American Type Culture Collection (Rockville, MD, USA) – were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Milan, Italy) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml fungizone, and incubated at 37 °C in humidified atmosphere containing 95% air/5% CO_2. Cells were routinely split (1:2) each week and used between the 4th and 5th passage. Cells from confluent cultures were detached using 0.25% trypsin in 1 mM EDTA solution and seeded in complete DMEM medium. For the experiments, cells were trypsinized, counted in a hemocytometer, and plated onto 96-well plates (for cytotoxicity experiments), 6-well plates (for ROS, iNOS and NO determination), or 100 mm Petri dishes (for the Comet assay) before incubation with 5, 50, or 100 μg/ml (2.1, 21, or 42 μg/cm^2, respectively) of glass fibers for 72 h.

2.3. Determination of cytotoxicity

Cytotoxicity was tested by the MTT assay as reported by Graziano et al. (2012). It is a colorimetric assay where cell viability and proliferation are measured by the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. Since the reduction requires active mitochondrial reductase enzymes, conversion can be directly related to the number of viable cells. Comparison of the amount of purple formazan produced by cells treated with an agent with the amount produced by untreated control cells allows calculation of the effectiveness of the agent in causing cell death by a dose–response curve. Briefly, cells were seeded at an initial density of 8×10^3 cells/microwell (0.32 cm^2 surface area) in 200 μl flat-bottomed microplates and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h (60–70% confluence) they were treated with 5, 50, or 100 μg/ml of glass fibers

Table 2
Comet test results: A549 cells after 72 h incubation with glass fibers.

	TDNA	TMOM
Control	10 ± 2.3	23 ± 1.5
5 μg/ml	24 ± 4.6	71 ± 6.4
50 μg/ml	65 ± 12.5	1.560 ± 75
100 μg/ml	78 ± 9.8	2.496 ± 62