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Modulatory Effects of Fresh Garlic Extract on Chrysotile Asbestos Induced Genotoxicity: An *In Vitro* Study

S. Yadav,[1] Q. Rahman,[1] S. Geh,[2] A. B. Pant[1]

[1] Industrial Toxicology Research Centre, Post Box 80, M. G. Marg, Lucknow, India
[2] Institute for Hygiene and Occupational Health, University of Essen, Germany

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Asbestos is an important environmental carcinogen and remains the primary occupational concern in many developing countries. Studies conducted in exposed population displayed the genetic damage caused by asbestos (Dusinska *et al.*, 2004). The mechanisms of fiber-induced genotoxicity are not yet clear, but indirect mechanism via production of reactive oxygen species (ROS) has been proposed (Xu *et al.*, 2002). Two pathways of ROS generation by asbestos are well cited firstly, via the involvement of redox active iron (Fe^{++}, Fe^{+++}) present as an impurity on the surface of asbestos fiber, catalyzes the formation of OH^{*} radicals through the Haber- Weiss reaction (Macord and Wong, 1979). Secondly, through phagocytosis of fibers by inflammatory cells such a pulmonary macrophages, neutrophils *etc.* (Kamp and Weitzman 1999).

Glutathione (GSH) and the glutathione redox system are important defense systems against oxidative damage in mammalian cells (Afaq *et al.*, 2000). Earlier studies have revealed a marked depletion in the level of GSH in the experimental systems after exposure to asbestos fibers (Abidi *et al.*, 1999). Garlic (Allium sativum L., *Liliaceae*) contains 33 sulfur compounds, several enzymes, 17 amino acids and several minerals. Both oil- and water-soluble allyl sulfur compounds of garlic possess anticancer, antitumorgenic and antimicrobial properties (Druesne *et al.*, 2004; Canizares *et al.*, 2004). Diallyl sulfide (DAS) and Diallyl-disulfide (DADS), are sulphur rich constituents of garlic and known to induce activities of Phase II enzymes *e.g.* glutathione *S*-transferase and quinone reductase, which in turn reduce the genotoxicity of several carcinogens (Guyonnet *et al.*, 2002).

The present investigation was carried out to examine the modulatory effect of garlic extract (*Allium Sativum*) supplementation on chrysotile asbestos induced genotoxicity in human peripheral blood lymphocytes, using cytokinesis block micronucleus assay, under *in-vitro* conditions. Further, electron spin resonance (ESR) assay was performed to assess the generation of hydroxyl radical.

MATERIALS AND METHODS

For human peripheral blood lymphocytes culture, intravenous blood samples from healthy human volunteers were collected and cultured following the protocol of

Correspondence to: A. B. Pant

Moorehead *et al.* (1960). In Brief, 0.5 ml of blood was mixed with 5.0 ml of RPMI 1640 medium supplemented with 20% fetal calf serum, 0.2% sodium bicarbonate, 0.1% antibiotic-antimycotic solution. Then 2% phytohemagglutinin–M was added to the culture to induce the lymphocyte specific mitogenecity. Cultures were treated with various concentrations of chrysotile fibers *i.e.* 0.5µg/ml, 1µg/ml, 5µg/ml, 10µg/ml (UICC standard). Fresh garlic extract was prepared by the method described by Ameen *et al.* (2003). Freshly prepared garlic homogenate (5.0 %) was applied in the culture (2µl/ml and 5µl/ml).

For the treatment, the average length and diameter of fibers 10µm and 2.24-µm were determined respectively. Lymphocyte cultures were exposed to various concentrations chrysotile *i.e.* 0.5µg/ml, 1µg/ml, 5µg/ml, 10µg/ml. Untreated and negative controls (Gypsum 1.0µg/ml) also run parallel under identical conditions. Fresh garlic extract was prepared by the method described by Ameen *et al.* (2003). Freshly prepared garlic homogenate (5.0 %) was applied in the culture (2µl/ml and 5µl/ml).

For micronucleus assay, human peripheral blood lymphocytes cultures were incubated at 37^0C for 72 h. After the incubation of 44h cultures were supplemented with cytochalasin B to block cytokinesis (Fenech, 2005). The cells were harvested by hypotonic 0.075 M KCl for 5 min, at 37^0C temperature, and fixed in Carnoy's fixative (methanol/acetic acid, 3:1). Finally, Cells were dropped onto the slides and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 5 min. About 1000 binucleated cells from each case were examined for micronucleus assay.

Hydroxyl radical formation by chrysotile was measured idiotically by electron spin resonance (ESR). 100 µl of the chrysotile suspension (80 mg/ml) was mixed with 200 µl of the spin trap 5,5 dimethyl-1-pyroline N oxide (DMPO, 0.05 M in double distilled water) and 100 µl H_2O_2 (0.5 M in PBS). To measure without H_2O_2, 125 µl of the chrysotile suspension (200 mg/ml) was mixed with 200µl DMPO 0.2 M in double distilled water. As values were taken 100 µl, 125 µl double distilled and AAS tested water instead of the suspension. The suspension was incubated for 15 mins. (5 mins. without H_2O_2) at 37^0 C in a water bath and filtered through a 0.2 µm filter (acrodisc 25 mm syringe filter pall gelman, laboratory, Ann Arbor USA). The filtrate was immediately transferred to a capillary and measured with a microscope ESR spectrometer (Magnettech), the ESR spectra was recorded at room temperature using the following instrumental conditions: magnetic field 3360 G, sweep with 100 G, scan time 3, modulation amplitude 1.975 G, receiver gain 1000. Quantification was done by accumulation of two different spectra each are ranging two different scans. All four peaks were the amplitude. Outcomes are expressed as the total amplitude in arbitrary units (AU) (Shi *et al.*, 2003).

STATISTICAL ANALYSIS

Data were evaluated for statistical significance using two-way analysis of variance to compare mean percent change in the rate of inducing of MN in different treatment groups from control taking duration for chemical exposure and treatment of garlic extract as independent factors. Prior to analysis, the homogeneity of variance between the treatment groups was ascertained.

RESULTS AND DISCUSSION

In the present investigations, freshly prepared aqueous extracts of garlic were used to see its protective effect against chrysotile asbestos induced MN formation in human peripheral blood lymphocytes. Data of MN assay and ESR are summarized in Fig. 1,2 and 3 respectively. Chrysotile asbestos (0.5μg/ml, 1μg/ml, 5μg/ml) was found to induce statistically significant ($p<0.05$) increase in the micronuclei count in cultured human peripheral blood lymphocytes against the untreated controls, a concentration dependent increase in the MN count was observed 21.5±1.41 and 23.0±2.82 MN/1000 cells at 0.5μg/ml and 1.0 μg/ml of chrysotile asbestos respectively.

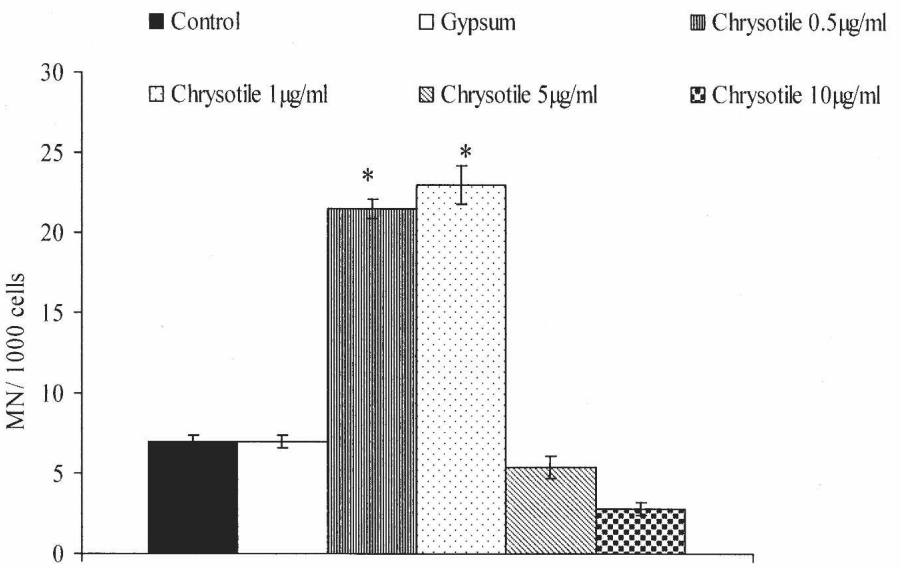

Figure 1. MN formation in human peripheral blood lymphocytes after exposure to various concentrations of chrysotile asbestos (0.5μg/ml, 1μg/ml, 5μg/ml, 10μg/ml). * Significance level $p<0.05$.

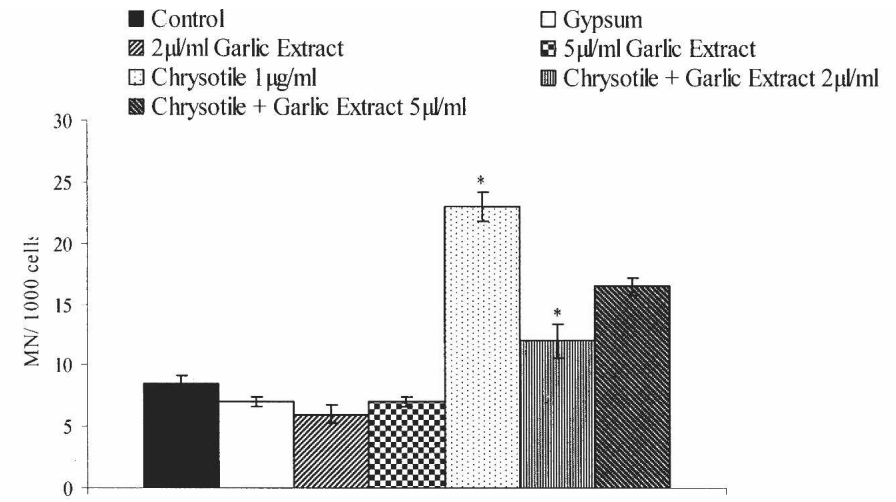

Figure 2. MN formation in human peripheral blood lymphocytes after exposure to chrysotile asbestos (1µg/ml) and treatment with fresh garlic extract (5µl/ml and 10 µl/ml). * Significance level $p < 0.05$.

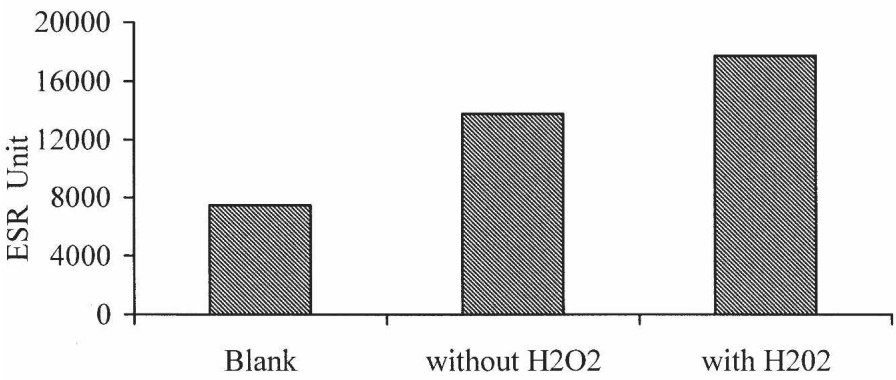

Figure 3. Quantification of the 5,5 dimethyl-1-pyroline N oxide (DMPO-OH) signal induced by chrysotile asbestos in the absence and presence of hydrogen peroxide measured by Electron Spin Resonance (ESR).

The exposure concentrations (5.0 µg/ml and 10.0 µg/ml) of asbestos used in the study have shown statistically significant decrease in cell viability due to cytotoxicity (data not shown). Eventually, a reduction in MN induction was observed i.e., 4.8±0.75 MN/1000 cells and 3±0.45 MN/1000 cells on the exposure

of these concentrations i.e., 5.0 µg/ml and 10.0 µg/ml respectively. Thus, the cytotoxic responses of asbestos at higher concentrations are suggestive the necrotic cell death in cultured lymphocytes at large and few cells were remained in the culture for MN analysis. However, the cells left were found comparatively healthier than the cells exposed to lower concentrations, which might be the region to get lower number of MN induction. (Fig.-1). Our this finding is well in accordance to the study of Dopp and Schiffmann (1998), where they have reported the similar cytotoxic responses of asbestos at higher concentrations. Further, it has also been reported that ROS generation also play a key role in asbestos fiber induced genetic damages (Dusinska *et al.*, 2004). Freshly prepared garlic extract (5%) has shown reduction in MN count in chrysotile asbestos (1µg/ml) exposed cultured human peripheral blood lymphocytes against the asbestos exposed only (1µg/ml). Interestingly, the higher concentration of garlic (5µl/ml) was found to be comparatively lesser effective. This might be attributed through the increasing concentration of diallyl sulfide (DAS), a primary constituent of garlic and well known to have cytotoxic and mild genotoxic potential at higher concentrations (Musk *et al.*, 1997). So, it can be well speculated that at 5µl/ml concentration of garlic extract, the concentration of DAS must reached to the level, where it shows some genotoxic response and masking of the protective effects could be seen. As such, both the concentrations of garlic used in the study were found to be inert when supplemented alone (Fig.-2). A statistically significant ($p<0.05$) protective effect was observed with 2µl/ml concentration of freshly prepared 5% aqueous solution of garlic. Bhattacharya *et al.* (2004), also shown the antigenotoxic effect of garlic extract. Evidently proofs are available in the literature demonstrating the involvement of oxidative stress as one of the important key mechanisms in the asbestos mediated genetic damages (Dopp *et al.*, 2005).

In the present study, attempts were made to further strengthen the hypothesis for the oxidative damage by chrysotile and protective effect of garlic extracts by antioxidant mechanism. Electron Spin Resonance (ESR), an analytical technique was used, which directly measures free radicals by electron spin resonance spectrometry. The spin trapping is involves in the ESR by accumulation of a compound known as a spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) that reacts rapidly with free radicals to form radical-adducts that are much more stable and longer lived than the original species. Chrysotile asbestos was found to induce almost 2 folds ESR signals abiotically under experimental conditions when compared with the control set run parallel under identical conditions except the presence of H_2O_2 (Fig.-3). These findings of ESR are suggestive that chrysotile causes the damage through generation of free radicals. Thus, the protective effect of garlic extract observed in the present study can be attributed to antoxidative mechanism. Since, the antioxidant properties of garlic and its ingredients are well known in the literature.

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