Genotoxicity of chrysotile asbestos on *Allium cepa* L. meristematic root tip cells

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Genotoxic and mutagenic potential of chrysotile asbestos on animals and humans has been extensively documented, but limited information is available regarding its effects on plants. In the present study, *Allium cepa* L. bulbs were exposed to four concentrations (0.5, 1.0, 2.0 and 5.0 µg/ml) of chrysotile asbestos under laboratory conditions and alterations in certain cytogenetic parameters were studied at 24 h intervals till 96 h.

In control plants a time and concentration-dependent decrease in the number of cells in prophase was found, but in exposed plants progression of prophase stage was found to be slow. In control plants, the number of cells in metaphase was found to decrease with time. On the contrary, increase was recorded in exposed plants. In control plants, increase in the number of cells in anaphase and telophase stage was found with time, but in exposed plants a gradual decrease was recorded. A time and concentration-dependent decrease in mitotic index was found in exposed plants compared to respective controls. An increase in the number of interphase nuclei, spindle disturbances, chromosome stickiness and micronuclei formation was also recorded in exposed plants. The present findings report genotoxicity of chrysotile asbestos in plants.

Keywords: Chrysotile asbestos, mitotic index, micronuclei genotoxicity.

ASBESTOS is present in serpentine areas in five states of India, viz. Andhra Pradesh, Jharkhand, Karnataka, Rajasthan and Uttarakhand[1]. On the basis of their crystal structure, asbestos fibres have been grouped into chrysotile and amphibole[2]. Chrysotile fibre exposure causes various deleterious and carcinogenic health effects in man and laboratory mammals[3]. However, potential ecological impact of this material has been largely ignored[4]. Schreir and Timmenga[5] have shown that in acidic soil environments magnesium and trace metals present in fibre composition are released and their concentration locally increases. In aquatic ecosystems, it adversely affects growth and physiology[6] and also causes damage to the antioxidative system of aquatic plants[7] due to reactive oxygen species (ROS)-mediated toxicity. Ripple effects throughout the food chain are unclear as are long-term impacts on entire ecosystems. Efforts to develop an integrated approach to ecological and human health risk assessment are desirable for efficacy, cost-effectiveness and to garner public support[8,9]. Moreover, the ultimate goal of ecotoxicology is to serve in predicting or assessing the risk of ecological effects occurring due to environmental pollutants[10].

Allium cepa test is a quick and inexpensive biological test for ecological and genetic risk assessment[11]. The test has been used since late 1930s and has been standardized[12]. It is a sensitive and reliable plant assay in environmental monitoring. The test is based on the assessment of the toxic and genotoxic potential of chemicals in species of genus *Allium* by measuring the mean root growth, recording mitotic activity (mitotic index) and mitotic abnormalities in meristematic root tip cells[13]. There are several studies using *Allium* in genotoxicity evaluation. Among them, the original form[12] involves germination of onion bulbs in tap water of known quality to a length of 1–2 cm and thereafter the specific treatment. A modification of the test involves root growth in a test liquid without pre-growth period in tap water[12]. Nowadays, thousands of chemicals are released and find their way into the environment, air, land, groundwater and surface water by industrial activity, agricultural practices, domestic activity, etc. Most of these compounds eventually descend to the ground and therefore the surface soil may be contaminated with these potentially genotoxic compounds[14]. For maintaining biodiversity and sustainable agriculture their ecological impact should be assessed properly.

The physical and chemical agents present in soil can cause major alterations to the genetic material of eukaryotic cells[15]. Evidence suggests that chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level – for example, chromosome breaks may result from unrepaired double-strand breaks in DNA and chromosome rearrangements may result from misrepair of strand breaks in DNA[16]. In the classical cytogenetic techniques, chromosomes are studied directly by observing and counting aberrations in metaphase[17]. This is a complex, time-consuming and laborious process. Heddle[18] and Schmid[19] proposed independently an alternative and simpler approach to assess chromosome damage

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in vivo, i.e. to measure micronuclei (MN; also known as Howell–Jolly bodies to haematologists) in dividing cell populations. Micronuclei are expressed in dividing cells that either contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term 'micronucleus'. Micronuclei provide a convenient and reliable index of both chromosome breakage and chromosome loss. Micronuclei are expressed in cells that have completed nuclear division, they are ideally scored in binucleate stage of the cell cycle[20,21]. In the present study genotoxicity of chrysotile asbestos was evaluated in a plant model by both the tests, i.e. chromosome aberration assay and micronucleus assay along with studies on different phases of mitosis, mitotic index (MI) and spindle disturbances. Any effect of asbestos contamination on crops may adversely affect agricultural production as well as plant genetic resources by causing genome instability. Therefore, it is important to study the genotoxic effects of chrysotile asbestos contamination on plant model.

Materials and methods

Test plant

Approximately equal sized and untreated bulbs (40–60 g) of *A. cepa* L. with chromosome complement of $2n = 2x = 16$ were used. After harvest the bulbs were stored for a few months in darkness under dry conditions at 6–10°C (winter rest) till the experiments were started.

Test chemical

Chrysotile asbestos fibres of size < 30 μm were used for the study. Four suspensions of chrysotile asbestos containing 0.5, 1.0, 2.0 and 5.0 μg/ml of this mineral were prepared in double-distilled water with constant stirring. Control plants were grown in the double-distilled water without chrysotile fibre.

Toxicity test procedure

Chromosomal aberration assays were carried out as described by Fiskesjö[12,22,23]. The basal plates of the bulbs were immersed in tap water and allowed to produce roots until they reached a length of 1.5–2.0 cm. The bulbs were then transferred to tubes containing different concentrations of asbestos suspension and exposed for 24, 48, 72 and 96 h, as *A. cepa* cell cycle is of 24 h (ref. 24). Next microscopic slides were prepared. Five onion bulbs were prepared for each concentration of the test suspension. Tap water which had been left overnight to allow evaporation chlorine was used as a negative control and for diluting the test suspensions. Fresh test suspensions and control water were used every day. Experiments were performed at relatively constant temperature (20°C), controlled humidity (60%) and protected from direct sunlight.

Preparation of microscope slides

Roots were cut-off after exposure and fixed in ethanol : acetic acid (3 : 1) for 24 h. Next, the roots were hydrolysed in 1 M hydrochloric acid at 60°C for 10 min and stained with Schiff's reagent for 2 h. Root tip segments (1–1.5 mm starting from the root tip after removing the root cap) were cut-off and squashed in a drop of 45% acetic acid. For each concentration tested, five root tips were squashed for five slides. Permanent slides were prepared using liquid carbon dioxide[25] air-dried overnight and mounted in DePex mounting medium. Permanent slides were studied under the light microscope with an oil-immersion objective and a 1000 × magnification.

For each treatment and negative control 5000 cells were scored. For cytotoxicity assessment the frequencies of mitotic phases, MI, chromatin abnormalities (stickiness), spindle disturbances and micronuclei assay were used as end-points. MI was calculated as the ratio between dividing cells and total number of cells scored. The frequency of each mitotic phase, mitotic abnormalities and stickiness were expressed as the percentage with respect to the total number of cells in mitosis.

Examination of slides and assessment of micronuclei frequency

Slides were examined at 1000 × magnification using a fluorescence microscope. To achieve this magnification, 10 × eye piece and 100 × objective were used (Leica DM2500B Model Microscope). Slides were coded before analysis so that the scorer was not aware of the identity of the slide. A score was obtained for slides from each treatment. The number of micronuclei in 1000 binucleate (BN) cells was scored and the frequency of micronuclei per 1000 BN cells was calculated.

Experiments were conducted in five replications. A series of five samples were prepared for each concentration of test suspension. Data presented are mean of three independent experiments. Data for each parameter were evaluated for statistical significance using two-way analysis of variance (ANOVA) to compare the means considering the duration of exposure and concentration as independent variables. The individual treatment between the two groups was assessed by computation of least

significant difference taking t values for error d.f. at the 1% and 5% level of significance.

Results and discussion

High concentration of any chemical may affect (inhibit or stimulate) the cell cycle[26]. If exposure affects different stages of the cell cycle, it may be deleterious for the crop and may cause mutations. In the present study a decrease in the number of cells in prophase was found with time in control plants and in chrysotile asbestos-exposed plants the progression of prophase was slow compared to control (Figure 1). This indicates that asbestos exposure delays progression of cell cycle and treated cells remain in prophase for a longer time. The reason may be impaired development of prophase bands due to inhibition of RNA synthesis in root meristem cells because microtubular orientation in the meristematic cells depends on long-lived RNA species[27]. Contrary to control plants where a time-dependent decrease in metaphase cells was found, in treated plants a time- and concentration-dependent increase was recorded which indicates metaphase arrest of the cells (Figure 2). Physiological studies indicate that calcium is involved in the metaphase/anaphase transition[28]. Deficit in intracellular calcium retards the onset of anaphase[29]. But the role of calcium in asbestos-mediated toxicity in *A. cepa* is yet to be properly explored. In control plants, with increase in time accumulation of cells in anaphase was found, but a time- and concentration-dependent decrease was recorded in exposed plants (Figure 3). Similarly, in control plants a significant increase in the number of cells in telophase stage was found with time, while a time- and concentration-dependent significant decrease was recorded in exposed plants (Figure 4). Chrysotile-meditated toxicity on cell division might be either due to its surface charge or ROS formation. Chrysotile fibres carry a positive surface charge at pH < 11.8 (ref. 30). These charged fibres presumably would be attracted to negatively charged protein groups in cell membranes. The chrysotile fibre would then be surrounded by proteins and submerge into the cell. This series of events could be a mechanism by which fibres could gain entry into the cell[31]. Moreover, a common effect of many toxicants/pollutants is to generate free radicals and reduced forms of oxygen that may damage cellular components such as lipids, proteins or DNA[32]. The importance of ROS in contributing asbestos-associated cytotoxicity has been reported[33]. It has been shown that fibre-cell interaction is not necessary for production of ROS. For example, chrysotile and crocidolite asbestos in cell-free solutions

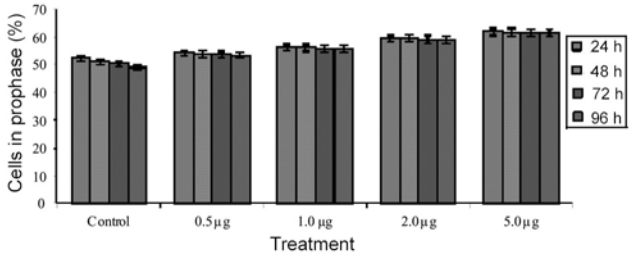

Figure 1. Effect of chrysotile asbestos exposure on prophase stage in *Allium cepa* L. root meristems. Values presented are mean ± SE. Control values were 52.28, 51.02, 50.34 and 48.96 at 24, 48, 72 and 96 h respectively.

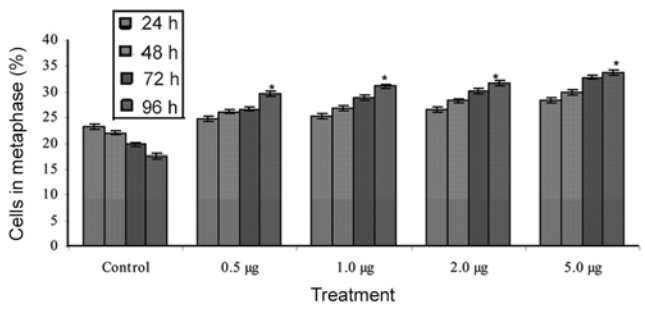

Figure 2. Effect of chrysotile asbestos exposure on metaphase stage in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 23.24, 21.98, 19.83 and 17.56 at 24, 48, 72 and 96 h respectively. *$P \leq 0.05$.

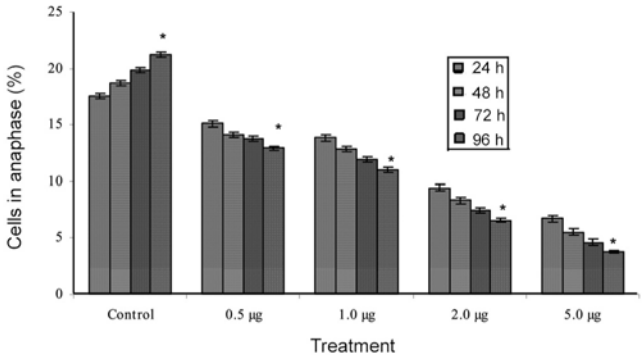

Figure 3. Effect of chrysotile asbestos exposure on anaphase stage in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 17.58, 18.64, 19.84 and 21.16 at 24, 48, 72 and 96 h respectively. *$P \leq 0.05$.

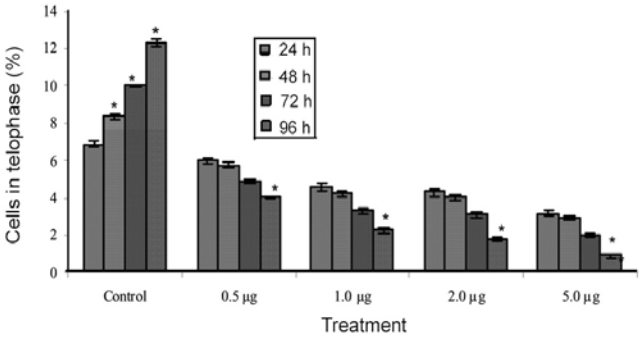

Figure 4. Effect of chrysotile asbestos exposure on telophase stage in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 6.9, 8.36, 9.99 and 12.32 at 24, 48, 72 and 96 h respectively. *$P \leq 0.05$.

of water or physiological saline spontaneously generate superoxide ($O_3^{\bullet}$) or hydroxyl radical ($OH^{\bullet}$)[34]. In animal model also, i.e. rat pleural mesothelial cells *in vitro* oxygen derivatives are involved in the cytotoxicity and DNA damage produced by asbestos[35].

Although there is a positive correlation between amount of DNA per nucleus and longer minimum time required for the mitotic cycle[36], in the present study mitotic cycle of the same plant, i.e. *A. cepa* meristematic root tip cells having the same DNA content in each nucleus was delayed in exposed plants; this may be due to genotoxicity of chrysotile asbestos. Furthermore, gradual decrease in MI was also found in exposed plants compared to respective control (Figure 5). This clearly indicates that the treatment has a mito-depressive effect and results in the inhibition of access of cells to mitosis[37]. A disturbance of the normal cell cycle process by prevention of biosynthesis of DNA and/or microtubule formation[38] is known as antimitotic effect[39,40]. Furthermore, increase in the spindle disturbances (Figure 6) and chromosome stickiness (Figure 7) is an explicit indication of the mutagenic effects of asbestos exposure on plants. Earlier, similar effects were found following exposure of plants with pesticides and other chemicals[41]. Chromosome stickiness may result from changes in specific non-histone proteins (topoisomerase II and the peripheral proteins) that are integral components of the chromosome and whose function is necessary for separation and segregation of chromatids, the changes being caused either by mutation in structural genes for the proteins (heritable stickiness) or by direct action of mutagens on the proteins (induced stickiness). It may occur in various degrees (slight, moderate, severe, extreme) that are determined by the number of target protein molecules affected. A certain number (threshold) of affected molecules at a given site on a chromosome is required to resist the forces of anaphase movement in order to produce microscopically detectable stickiness. Chromosome stickiness may also result from molecular events that can occur at several phases of the cell cycle (including interphase), but can only be recognized at prometaphase, metaphase and anaphase. Moreover, chromosome stickiness may cause chromosome aberrations by the physical stretching and breaking of chromatids at the sticky sites; hence the breakage resulting from stickiness is a secondary effect that requires anaphase movement, in contrast to breakage resulting from direct action of mutagens on DNA[42].

Mutagenic nature of asbestos exposure on *A. cepa* was unambiguously confirmed by induction of micronuclei formation in exposed plants (Figure 8). Micronuclei are

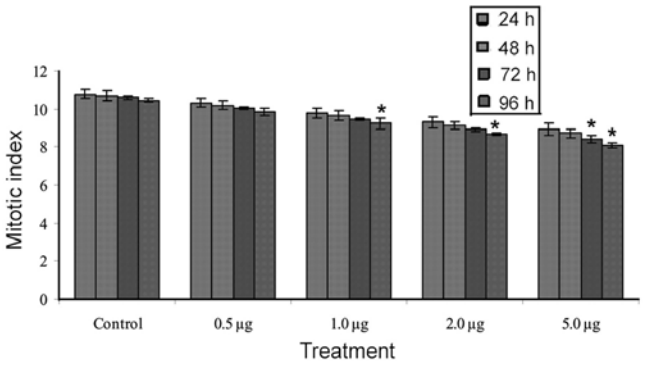

Figure 5. Effect of chrysotile asbestos exposure on mitotic index in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 10.78, 10.7, 10.6 and 10.45 at 24, 48, 72 and 96 h respectively. *$P \leq 0.05$.

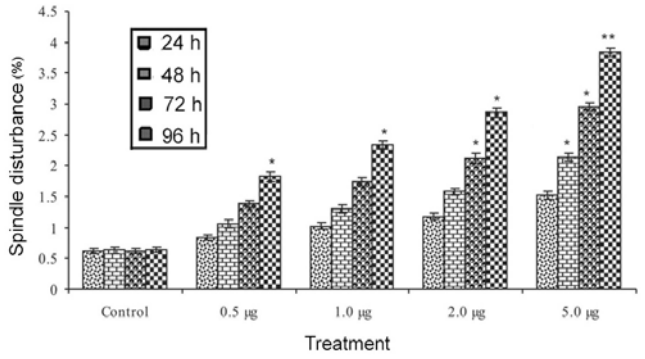

Figure 6. Effect of chrysotile asbestos exposure on spindle disturbance in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 0.62, 0.63, 0.62 and 0.64 at 24, 48, 72 and 96 h respectively. *$P \leq 0.05$, **$P \leq 0.01$.

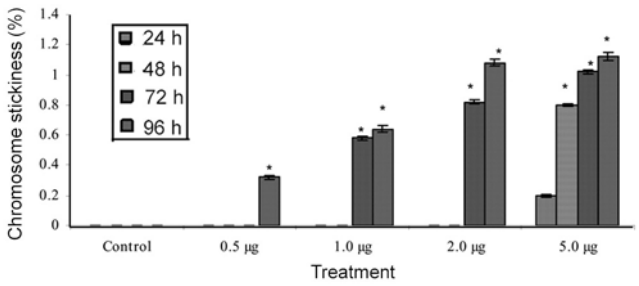

Figure 7. Effect of chrysotile asbestos exposure on chromosome stickiness in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 0 at 24, 48, 72 and 96 h. *$P \leq 0.05$.

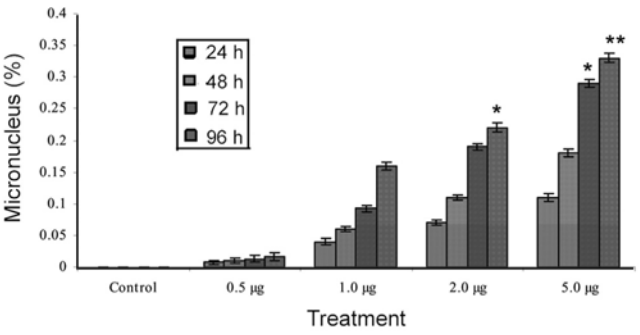

Figure 8. Effect of chrysotile asbestos exposure on micronucleus induction in *A. cepa* L. root meristems. Values presented are mean ± SE. Control values were 0 at 24, 48, 72 and 96 h. *$P \leq 0.05$.

morphologically identical to but smaller than nuclei. The diameter of micronuclei varies between 1/16 and 1/3 of the mean diameter of the main nucleus, which corresponds to 1/256 and 1/9 of the area of the main nucleus in a BN cell. Micronuclei are non-refractile and they can therefore be readily distinguished from artifacts such as staining particles. These are not linked or connected to the main nucleus. They may touch but not overlap the main nucleus and the micronuclear boundary should be distinguishable from the nuclear boundary. Usually micronuclei have the same staining intensity as the main nucleus, but occasionally may be more intense. Based on these characters of micronuclei, the micronucleus assay has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably. Micronuclei are formed as a consequence of chromosome breakage (elastogenicity) and disturbance of the spindle apparatus (aneuploidy). Scoring for micronuclei is easier than for chromosomal alterations and several comparative studies indicate that micronucleus assays is as sensitive for the detection of genotoxins as chromosome aberration experiments[43]. Therefore, in this study micronucleus assay was used to confirm the genotoxicity of chrysotile asbestos in a plant model. In lung cancer cells also chrysotile causes increase in micronucleated cells[44]. In animal cells chrysotile fibres cause carcinogenicity, catalysis of radical species (such as ROS) resulting in direct or indirect DNA mutations[45,46]. Furthermore, one of the great generalizations of cell biology is that the cells of higher organisms, whether from plants or animals, are fundamentally similar[47]. Therefore, in chrysoltile asbestos-exposed plant cells may also express similar effects.

Asbestos can be activated or inactivated by surface charge alterations[1]. Surface charges depend on the environmental conditions in which asbestos is present; therefore, the effects may vary in different environmental conditions as well as in different plant species. Total plant growth and development depends on mitotic processes in plant meristematic regions. Cell division is a multistep process that demands the precise and well-coordinated functioning of different cell organelles and products of many genes for its completion. Therefore, there are many potential targets for chemicals that can alter the efficiency of the process without disrupting it[48], as has been observed in the present study. An increase of the genotoxic load in ecosystems due to release of genotoxic chemicals will affect mutation frequencies as well as fertility of organisms. These adverse effects may lead to the formation of mutant plants, a decline in the population size of affected plant species and finally (in extreme cases) may even cause extinction of species.

The present findings show the genotoxicity of chrysotile asbestos on *A. cepa* root meristems, i.e. on plant model confirmed by chromosomal aberration assay as well as micronucleus assay. This may be the reason of neoendemism (insula) and palaeoendemism (depleted taxa) in plants growing in serpentenitic environments. Because this material is naturally present in five states of India as well as in several other countries, it may adversely affect ecology, agricultural productivity and may cause mutations in the available plant genetic resources. Therefore, in order to conserve biodiversity precautionary measures need to be adopted on priority basis in such areas.

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