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ORIGINAL RESEARCH

Genotoxicity of indium tin oxide by Allium and Comet tests

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Abstract Genotoxic effects of indium tin oxide (ITO) were investigated on root cells of *Allium cepa* by employing both Allium and Comet assays. *A. cepa* roots were treated with the aqueous dispersions of ITO at 5 different concentrations (12.5, 25, 50, 75, and 100 ppm) for 4 h. Exposure of ITO significantly increased mitotic index, and total chromosomal aberrations by the Allium test. While chromosome laggards, stickiness, disturbed anaphase–telophase and anaphase bridges were observed in anaphase–telophase cells, c-metaphase and binuclear cells were observed in other cells. A significant increase in DNA damage was also observed at all concentrations of ITO by the Comet assay. These results indicate that ITO exhibits genotoxic activity in *A. cepa* root meristematic cells.

Keywords Allium test · Chromosome aberration · Comet assay · DNA damage

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Introduction

ITO, composed of Indium (III) oxide (In_2O_3, 90 %) and tin (IV) oxide (SnO_2, 10 %), is used for liquid crystal display (LCDs), electrochromic displays, flat panel displays, field emission displays, touch or laptop computer screens, cell phones, energy conserving architectural windows, defogging aircraft and automobile windows, heat-reflecting coatings to increase light bulb efficiency, gas sensors, antistatic window coatings, wear resistant layers on glass, nanowires and nanorods because of its unique properties of high electrical conductivity, transparency and mechanical resistance (Copra and Das 1983; Lee et al. 1997; Alam and Cameron 2002; Patel et al. 2003; Homma et al. 2003; Jang et al. 2005; Elangovan and Ramamurthi 2005; Li et al. 2005; Lison et al. 2009). Nakajima et al. (2008) showed that indium caused tail malformations in rat fetuses by caudal hypoplasia probably due to excessive cell loss by increased apoptosis in the tailbud at the early postimplantation stage. ITO also caused chronic pulmonary toxicity when repeated intratracheal instillations were given to hamsters (Tanaka et al. 2010). Incidences of bronchiolo-alveolar adenomas and carcinomas, bronchiolo-alveolar hyperplasia, alveolar proteinosis and infiltrations of alveolar macrophages and inflammatory cells were significantly increased after inhalation exposure of rats to ITO aerosol (Nagano et al. 2011). Lison et al. (2009) found that ITO particles represent a new toxicological entity which has the potential to generate

high amounts of ROS and induce lung toxicity in experimental animals, including inflammatory and genotoxic effects. Studies of workers exposure showed that ITO induced occupational lung disease and the risk of interstitial lung damage (Homma et al. 2003, 2005; Chonan et al. 2007; Hamaguchi et al. 2008). Surfactant protein A, and surfactant protein D levels, sensitive markers of interstitial lung disease, were elevated significantly in the workers with moderately high indium exposure (Liu et al. 2012).

Higher plants (*Vicia faba*, *Tradescantia paludosa*, *Pisum sativum*, *Hordeum vulgare*, *Crepis capillaries*, *Nicotiana tabacum* and *Allium cepa* etc.) are highly reliable bioassays with a high sensitivity in general toxicity studies. Among them, *A. cepa* anaphase–telophase test is used for studying the effects of different compounds on chromosome and cell division because of its properties such as simplicity, speed, economics, large size and small number of chromosomes, good correlation with other test systems etc. (Fiskesjö 1985, 1988; Grant 1999; Rank 2003; Saxena et al. 2005; Konuk et al. 2007; Liman et al. 2010, 2012).

Comet assay is routinely used for detecting strand breaks in the DNA of single cells due to its high sensitivity, simplicity, speed, low cost, requirement of relatively small number of cells and versatility (Singh et al. 1998; Tice et al. 2000; Duez et al. 2003; Collins 2004; Olive and Banath 2006; Gichner et al. 2009). Apart from Allium test, root meristem cells of *A. cepa* are also used in different laboratories for detecting DNA damage by Comet assay which is faster, simpler and independent of mitosis (Seth et al. 2008; Chakraborty et al. 2009; Gichner et al. 2009; Ghosh et al. 2010; Liman et al. 2011; Türkoğlu 2012).

The objective of this study was to investigate the effects of ITO on MI (mitotic index), mitotic phases, CAs (chromosome abberations) by Allium test and DNA damage by Comet assay in the root meristem cells of *A. cepa*.

Materials and methods

Organism

Allium cepa (2n = 16) onion bulbs, 25–30 mm diameter, without any treatment, were purchased from a local supermarket.

Chemicals

Indium tin oxide (CAS No. 50926-11-9, nano powder, <50 nm particle size, In_2O_3, 90 %; SnO_2, 10 %), methyl methanesulfonate (MMS, CAS No. 67-27-3), normal melting point agarose (NMPA), low melting point agarose (LMPA), di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), Trizma base, Tris HCl, Triton X-100 and SDS were purchased from Sigma Aldrich (Munich, Germany).

Allium cepa anaphase–telophase test

ITO was suspended directly in distilled water and dispersed by ultrasonic vibration (130 W, 20 kHz) for 30 min to prepare five required dispersions (12.5, 25, 50, 75, and 100 ppm). All concentrations were selected arbitrarily. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. Six healthy onion bulbs were grown in the dark at room temperature (~21 °C ± 4 °C) for 24 h. After this time, they were treated with different concentrations of ITO for 4 h. The root tips (4–5 mm) were collected and immediately fixed in aceto:alcohol (1:3) for 24 h. They were then transferred to 70 % ethyl alcohol and stored at 4 °C until use. Fixation and staining of the root tip cells were carried out as reported earlier (Liman 2013).

Slides were randomly coded and scored blindly. The MI and the frequencies of CAs were carried out according to Saxena et al. (2005). In order to obtain MI, 5,000–6,000 cells (1,000 cells in each of the five slides) were observed for each sample. MI is calculated as: $MI = total\ number\ of\ dividing\ cells/total\ cell\ number \times 100$. In chromosome aberration test, 100 cells in anaphase or telophase were examined for CAs per slide if possible. Types of aberrations scored included disturbed anaphase–telophase, chromosome laggards, stickiness and anaphase bridges.

Application of the Comet assay (single cell gel electrophoresis)

Root meristem cells of *A. cepa* were exposed to similar concentrations of ITO as used for cytogenetic analysis. 20–30 seedlings were placed in a petri dish kept on ice and spread with 500 µL of ice-cold Tris-$MgCl_2$ buffer (0.2 M Tris, pH 7.5; 4 mM $MgCl_2$-$6H_2O$; 0.5 % w/v

Triton X-100). The roots were immediately chopped with a fresh razor blade and isolated root nuclei were collected in the buffer. Each microscope slide was pre-coated with a layer of 1 % NMPA and thoroughly dried at room temperature. Next, 100 µL of 0.8 % LMPA at 37 °C was mixed with 20 µL of the nuclear suspension and dropped on top of the first layer. The slides were allowed to solidify for 5 min on an ice-cooled tray and were then immersed in ice-cold lysing solution (1 M NaCl; 30 mM NaOH, 0.5 % w/v SDS, pH 12.3) for 1 h. Subsequent to lysing, the slides were placed in a horizontal gel electrophoresis chamber and the DNA was allowed to unwind for 1 h in the electrophoretic buffer, containing 30 mM NaOH and 1.5 mM EDTA at pH > 12.3. Electrophoresis was then conducted for 20 min at 25 V (1 V cm^{-1}) in the chamber cooled on ice. Following electrophoresis, the slides were rinsed three times with cold distilled water for neutralization and stained with 60 µL EtBr (20 µg ml^{-1}) and covered with a cover slip (Rucińska et al. 2004). Fifty comets (50 comets/slide) were scored visually as belonging to one of five classes (0—undamaged, 1—mild damage, 2—moderate damage, 3—severe damage, 4—complete damage) using a fluorescence microscope (Collins 2004). Thus, the total score for 50 comets could range from 0 (all undamaged) to 200 (all damaged). The percentage of damaged cells was calculated and statistically analyzed. Arbitrary Unit used to express the extent of DNA damage was calculated as follows Eq. 1):

$$\text{Arbitrary unit} = \sum_{i=0}^{4} Ni \times i \quad (1)$$

where Ni = Number of cells in i degree; i = degree of damage (0, 1, 2, 3, 4).

Statistical analysis

The MI, mitotic phases and CAs (expressed as percents) and comet scores were presented as mean ± standard deviation. The levels of significance in different treatment groups were analyzed using the Duncan multiple range tests by using SPSS 18.0 version for Windows software. $p < 0.05$ was set as statistical significance.

Results and discussion

The Allium test was carried out for detecting genotoxic effects and DNA damage of Indium tin oxide. The effect of ITO on MI and mitotic phases in the root meristematic cells of *A. cepa* treated for 4 h is summarized in Table 1. At all concentrations used in the incubations of root increased MI was observed compared to negative control. The highest values were obtained with 100 ppm of ITO (25.1 ± 0.75), and the lowest one with 10 ppm of MMS (15.83 ± 1.48). The increase of MI showed statistically significant results ($p < 0.05$) in all concentrations. MI increased in a dose dependent manner ($r = 0.599$, $p < 0.01$). The cytotoxicity levels of an agent can be determined by the increase or decrease in the MI (Fernandes et al. 2007). MIs lower than the negative control may indicate that the growth and development of exposed organisms have been affected by the test compounds. On the other hand, MIs above those of the negative control may be result of the induction of increased cell division, which may characterize an event detrimental to cells, leading to uncontrolled proliferation and even tumor formation

Table 1 The effects of ITO on mitotic index and mitotic phase in *A. cepa* root meristem cells

Concentration (ppm)		CCN	Mitotic Index ± SD	Mitotic phases (%) ± Standard deviation (SD)*			
				Prophase	Metaphase	Anaphase	Telophase
Control	–	5,181	18.96 ± 1.06a	78.78 ± 2.46a	9.15 ± 1.91ad	4.98 ± 1.92a	7.09 ± 1.94a
MMS	10	5,231	15.83 ± 1.48b	77.91 ± 0.87ab	19.81 ± 0.56b	0.96 ± 0.29b	1.32 ± 0.16b
Indium tin oxide	12.5	5,263	23.32 ± 1.15c	77.39 ± 1.12ab	7.09 ± 1.19c	4.74 ± 1.67a	10.78 ± 1.43c
	25	5,295	23.63 ± 0.7cd	76.69 ± 0.8b	8.25 ± 1.36ac	8.26 ± 0.95cd	6.8 ± 0.94a
	50	5,248	23.72 ± 0.3cd	78.08 ± 1.26ab	7.42 ± 1.13c	6.64 ± 0.9c	7.86 ± 0.55ad
	75	5,201	24.69 ± 1.43cd	73.41 ± 1.23c	10.62 ± 1.14de	8.76 ± 1.32d	7.21 ± 1.59a
	100	5,182	25.1 ± 0.75d	68.65 ± 0.54d	11.82 ± 0.87e	10.39 ± 0.84e	9.14 ± 0.69d

CCN counting cell number

* Means with the same letter do not differ statistically at the level of 0.05

(Hoshina 2002) or could be a consequence of delayed mitosis (Tkalec et al. 2009) The increased cell proliferation activity can be the consequence of a reduction of the time necessary for DNA repair (Evseeva et al. 2005). The characteristic effect caused by the tested preparations of ITO was a decrease of prophase index (especially at 100 ppm) and metaphase index (except at 75 and 100 ppm), simultaneous increase of anaphase (except at 12.5 ppm) and telophase index (except at 25 ppm) when compared to control. Some of the increased and decreased phase indices showed statistically significant results ($p < 0.05$). This may indicate an alteration of the cell cycle with more cells entering and exiting mitosis than in the controls. According to Hasegawa et al. (2012), ITO may affect cytosolic signal transduction elements to interfere with the homeostatic regulatory system, causing abnormal cell proliferation.

In the *A. cepa* anaphase–telophase chromosome aberration test to investigate the genotoxic potential of the ITO conducted with root meristematic cells of *A. cepa* is shown in Table 2. Four types of aberrations (especially chromosome laggards, stickiness, disturbed anaphase–telophase and anaphase bridges) were observed in anaphase–telophase cells. The effect of ITO concentrations on CA was significantly different ($p < 0.05$) compared to the negative control and higher than MMS at 75 and 100 ppm of ITO. Total aberrations for ITO was found in a dose dependent manner ($r = 0.852$, $p < 0.01$). Among above aberrations, disturbed anaphase–telophase and chromosome laggards (especially at 75 and 100 ppm) could occur by the effect of ITO on microtubule formations (Amer and Ali 1986; Kumari et al. 2009). Such spindle malfunctioning may arise due to inhibition of tubulin polymerization (Kuriyama and Sakai 1974) or could be changes in the cytoskeleton proteins after incubation with ITO (Kwee et al. 2001; Tkalec et al. 2009). Chromosome laggards at anaphase may due to the failure of the chromosomes or acentric chromosome fragments to move to either of the pole and may cause delayed prophase and/or metaphase and lead to an increased mitotic index (Evseeva et al. 2005). Stickiness (especially at 25 ppm), a chromatid type of aberration, indicates highly irreversible type of toxic effect of ITO, and its occurrences during the study could be due to sub-chromatid linkage between chromosomes and probably leads to cell death (Chauhan et al. 1986; Ajay and Sarbhoy 1988; Kovalchuk et al. 1998; Barbério et al. 2011). Anaphase bridges indicating structural chromosomal mutations could happen during the translocation of unequal chromatid exchange or can be due to dicentric chromosome presence due to the breakage and fusion of chromosomes and chromatids, or due to less active replication enzymes (Badr et al. 1992; El-Ghamery et al. 2000; Luo et al. 2004). In addition to these anomalies, other anomalies (especially c-metaphase and binuclear cell) were also observed. While the lowest anomalies were observed with 0.08 ± 0.03 % at the 100 ppm for ITO, the highest one was observed with 2.93 ± 0.41 % at the 10 ppm of MMS. Statistically significant ($p < 0.05$) frequencies of other anomalies were recorded for 25 and 50 ppm for ITO. C-metaphase, a

Table 2 Percentage of chromosome aberrations of ITO at different times and concentrations obtained for the *A. cepa* anaphase telophase test

Concentration (ppm)		Anaphase–telophase anomalies %						Other anomalies %				
		CCN	DAT	CL	S	AB	TA ± SD*	CCN	CM	BNC	*p*	TA ± SD*
Control	–	500	6.2	6.8	9.6	1.2	**23.8 ± 3.03a**	5,181	0.06	0.06	–	**0.12 ± 0.05a**
MMS	10	139	3.8	39.67	0.63	–	**44.1 ± 2.62bd**	5,202	2.93	–	–	**2.93 ± 0.41b**
Indium tin oxide	12.5	500	3.8	18.8	7.4	1.8	**31.8 ± 4.32c**	5,263	0.23	0.02	–	**0.25 ± 0.08ac**
	25	500	6.2	17.4	11.2	1	**35.8 ± 2.58c**	5,295	0.4	0.02	0.02	**0.44 ± 0.1c**
	50	500	8.2	24	3.4	4.6	**40.2 ± 3.11d**	5,248	0.34	0.08	–	**0.42 ± 0.13c**
	75	500	6	27.8	8.4	2.2	**44.4 ± 3.57bd**	5,201	0.27	0.06	–	**0.33 ± 0.19ac**
	100	500	4.4	28.6	8	4.2	**45.2 ± 2.28b**	5,182	0.08	–	–	**0.08 ± 0.03a**

SD standard deviation

* Means with the same letter do not differ statistically at the level of 0.05

CCN counting cell numbers, *DAT* disturbed anaphase–telophase, *CL* chromosome laggards, *S* stickiness, *AB* anaphase bridge, *TA* total anomalies, *CM* C-metaphase, *BNC* binuclear cell, *P* polyploidy

possibly reversible effect, might occur due to disturbed microtubules by ITO or an imbalance of the proteins responsible for the structure of nuclear chromatin and can result in multinuclear cells (Fiskesjö 1988; Shahin and El-Amoodi 1991; Odeigah et al. 1997; Kurás et al. 2006; Fernandes et al. 2007). Binuclear cells might occur as a result of inhibition of cell plate formation or even mitotic irregularities (Grant 1978). Such anomalies in interphases may lead to the induction of cell death process (Leme and Marin-Morales 2008). Unlike our results, Asakura et al. (2009) showed that indium was not genotoxic by chromosomal aberration tests in cultured mammalian cells because average particle size of indium (45 μm) was larger than cell size. It was found that ITO particles induced an increased frequency of micronuclei in type II pneumocytes in vivo but not in lung epithelial cells in vitro, suggesting the preponderance of a secondary genotoxic mechanism (Lison et al. 2009). ITO also increased mutation in rat alveolar type II cells (Driscoll et al. 1997) and carcinogenicity in male and female rats (Nagano et al. 2011). Indium chloride also induced micronuclei formation (Takagi et al. 2011; Lin et al. 2011).

Due the ability of the Comet assay to detect low levels of DNA damage in different cell types, the Comet assay represents a powerful tool with which to identify DNA damage. Results obtained from the Comet assay are summarized in Table 3. Results of the chromosomal aberrations and MI show a good correlation with that of the Comet assay. As it can be seen, exposure of ITO increased the DNA damage at all concentrations in a dose dependent manner ($r = 0.923$, $p < 0.01$). Comet assay results showed that DNA damage was significantly higher at all concentrations of ITO compared to negative control. While the highest genotoxic activity was observed in the positive control (130 ± 3.46), the lowest one was observed in the negative control (34 ± 5.29). The DNA damaging activity of ITO could be associated with the generation of free radicals. Free radicals from ITO are most probably generated at reactive sites caused by the introduction of substitutional Sn in the overall crystal structure, where electron density is high (Fan and Goodenough 1977). ITO showed the capacity to cause a rupture of C–H bonds, with the consequent release of carbon centered radicals (Lison et al. 2009). ITO showed mutagenic activities via generating DNA damage (Emerit et al. 2001), or bind to nucleoprotein, nucleotide or functional proteins owing to longer contact time (Hasegawa et al. 2012). Gottschling et al. (2001) suggested that indium phosphide-induced oxidative stress may play an important role in the pulmonary carcinogenesis of indium phosphide. Bustamente et al. (1997) found that Indium can induce apoptosis and necrosis in T lymphocytes in a dose-dependent manner.

Table 3 Detection of DNA damage in nuclei of *A. cepa* root meristem cells exposure to ITO using the Comet assay

Compounds	Concentration (ppm)	DNA Damage (Arbitrary Unit ± SD)*
Negative control	–	34 ± 5.29a
MMS	10	130 ± 3.46b
Indium tin oxide	12.5	50 ± 4c
	25	56.66 ± 2.3d
	50	95.33 ± 4.08e
	75	100.66 ± 4.16ef
	100	104 ± 2f

SD standard deviation

* Means with the same letter do not differ statistically at the level of 0.05

Conclusions

As a result, ITO has a genotoxic effect by increasing CAs and DNA damage and it also induced cell division by increasing the MI in *A. cepa* root meristematic cells. Therefore further studies should be conducted to better understand the molecular mechanisms involved in the genotoxicity of ITO. It is essential to pay much greater attention to indium compounds.

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