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RESEARCH ARTICLE

Protective potency of ascorbic acid supplementation against cytotoxicity and DNA fragmentation induced by triphenyltin on human liver carcinoma cells

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Abstract

Agrochemicals are one the most significant sources of environmental pollution. Cytotoxicity and genotoxicity are the serious side effects of fungicide. In the current study, I have evaluated acute cytotoxicity and genotoxicity of triphenyltin (TPT) on human hepatic carcinoma (HepG2) cells and the ameliorating effect of ascorbic acid for 24 h. In this experiment, I have exposed HepG2 cells to ascorbic acids (50, 100, and 200 μM) simultaneously and 24 h prior triphenyltin (TPT, 400 ng/ml) exposure for 24 h to determine the protective effect of ascorbic acid by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and NRU (neutral red uptake) assays. Oxidative stress, such as intracellular reactive oxygen species and glutathione levels, was measured in HepG2 cells. The intracellular reactive oxygen species was evaluated using fluorescent probe DCFDA (6-carboxy-2′,7′ dichloro-dihydrofluorescein diacetate). Apoptosis and genotoxicity effects of TPT in HepG2 cells were determined using flow cytometry and comet assay. The result of these experiments showed that the TPT compound (400 ng/ml) induced cytotoxicity, oxidative stress and apoptosis, and DNA damage in HepG2 cells.Ascorbic acid reduced cytotoxicity, oxidative stress, apoptosis, and genotoxicity induced by TPT. Thus, ascorbic acid is a potent antioxidant, and it showed a significant protective effect against toxicity induced by TPT in HepG2 cells.

Keywords Triphenyltin · Genotoxicity · Comet assay · Ascorbic acid · Oxidative stress

Introduction

Extensive application of fungicide and pesticide is increasing in agriculture in the world. In the current scenario, a small amount of fungicides or pesticides is consumed in daily life from vegetables, crops, grains, etc. The growing application of pesticide and fungicide to indent makes it imperative to find out the hazardous effect of these chemical compounds. Organotin have been sold since the 1930s and was applied as stabilizers for polyvinyl chloride (Antizar 2008; Carraher Jr and Roner 2009). However, the application of tributyltin and triphenyltin (TPT) as biocides in paints that entered into their large-scale introduction into the environment (Sousa et al. 2013). Some researchers reported that TPT and tributyltin are highly toxic to fish and other types of organisms (Goldberg et al. 2004; Antes et al. 2013; Sousa et al. 2013). So, the application of tributyltin was stopped in antifouling paints in 2003, and also, its application in consumer products was banned in July 2010 (Sousa et al. 2005). Despite being banned, the widespread contaminations of marine and freshwater ecosystems, and its ubiquitous occurrence in consumer products, lead to the TPT and tributyltin as a constant presence in our everyday life. Animals and human beings are exposed to tributyltin and TPT through the ingestion of contaminated food items and also through the ingestion of house dust, in which levels up to 300 ng/ml of tributyltin have already been reported (Kannan and Tanabe 2008). As a consequence, tributyltin was recently detected in human blood and serum samples (Kannan et al. 1999). Ali et al. (2008) reported chlorpyrifos-induced genotoxicity in fish. One of the best methods to screen out the cytotoxicity and genotoxicity of fungicides in the animal is in vitro study by using cell lines. Blokhina et al. (2003) and Moustafa et al. (2012) have reported that vitamin C (ascorbic acid) acts as an antioxidant and

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minimizes the generation of reactive oxygen species (ROS). Ascorbic acid and ceftriaxone showed protective potency against deltamethrin-induced nephrotoxicity in rats (Abdel-Daim and El-Ghoneimy 2015). Fungicide has toxic materials that indirectly or directly affect the living cells via the production of ROS. Some researchers have suggested that surplus intracellular ROS produced in cells after treatment of fungicides as a consequence cell toxicity occurred (Markovic and Trajkovic 2008). Most fungicides are known as a carcinogen, and mutagen is accompanied with a high risk of cancer. Excess generation of ROS in cells provoked fragmentation of nuclear material (DNA, RNA, and protein molecules) and apoptosis. The underlying mechanism of the protective effect of ascorbic acid against the genotoxic potential of TPT fungicide remains unclear. So the objective of this study was to evaluate the protective effect of ascorbic acid against TPT-induced cytotoxicity and genotoxicity in human hepatic carcinoma (HepG2) cells.

Materials and methods

Chemical and reagents

Triphenyltin (TPT) (Fig. 1e) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, MTT (1-(4, 5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, thiazolyl blue formazan), neutral red dye, DTNB, 2H2-DCFH-DA, DMSO, Annexin V FITC, and propidium iodide (PI) were obtained from Sigma-Aldrich.

Cell line and experimental design

Human hepatic carcinoma (HepG2) cells were procured from National Centre for Cell Science, Pune, India. HepG2 cells were cultured in DMEM with FBS (10%) and 100 U/ml antibiotics at CO_2 (5%) incubator at 37 °C. At about 75% confluence, cells were subcultured into 96-well plates, 6-well plates, and 25-cm^2 flasks according to design. After overnight growth, the cells were treated to ascorbic acids at the following concentration: 50, 100, and 200 Mm simultaneously and 24 h before TPT (400 ng/ml) treatment. Untreated cells have been chosen as a negative control group.

Morphological analysis of cells

HepG2 cells (1×10^4) were treated to different concentrations of ascorbic acids (50, 100, and 200 μM) simultaneously and 24 h prior to TPT treatment (400 ng/ml) for 24 h. After treatment, the morphology of HepG2 cells was observed by using a phase-contrast microscope (Nikon Eclipse Ti-S Japan).

MTT assay

The toxicity of TPT was measured by using the MTT assay as described by the manufacture's protocol. In short, 1×10^4 cells/well were seeded in culture plate (96-well) and exposed to ascorbic acids (50, 100, and 200 μM) simultaneously and 24 h before TPT treatment (400 ng/ml) for 24 h. After exposure, the culture media were removed from plates and loaded 100 μl MTT solution (5 mg/ml) per well and incubated at

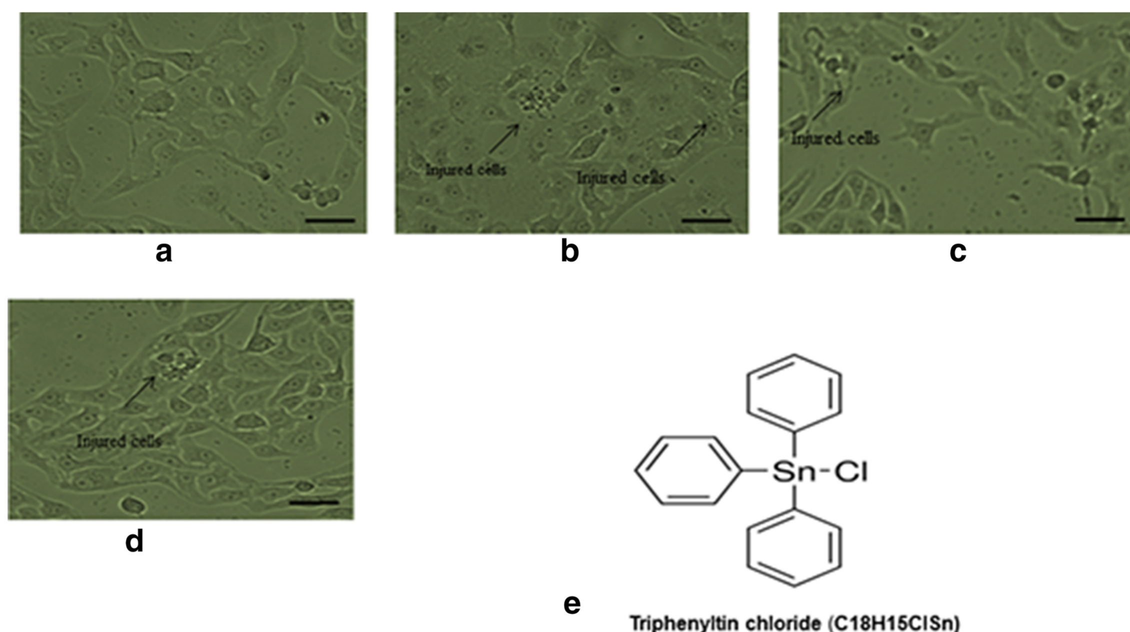

Fig. 1 Morphological changes in HepG2 cells after treatment of Triphenyltin (TPT) without/with different dose of ascorbic acid (AA) for 24 h (A). Control cells (B) Treated cell (Triphenyltin (TPT) (C). Pre exposed cell (Triphenyltin (TPT) + 200μM Ascorbic acid) (D). Co-exposed cell (Triphenyltin (TPT) +200μM Ascorbic acid). (E). Chemical structure of Triphenyltin compound. Scale bar is 200 μm

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37 °C for a minimum of 3 h and 30 min. After incubation, the MTT solution was discarded, and culture plates were washed with cold PBS, and formazan crystal was dissolved in DMSO, and the optical density was observed at 570 nm using a microplate reader (Synergy-HT; BioTek, Winooski, VT, USA).

Neutral red uptake (NRU) test

The toxicity of TPT with/without ascorbic acid on HepG2 cells for 24 h was evaluated by neutral red uptake test according to method (Borenfreund and Puerner 1985). After exposures to ascorbic acids (50, 100, and 200 μM) simultaneously and 24 h before TPT treatment (400 ng/ml) for 24 h, the cells were washed with chilled saline buffer and added neutral red dye (100 μl) with DMEM for 4 h and after incubation, and the cells were washed with fixative and simultaneously dye extractor solution. The optical density was measured at 540 nm.

Reactive oxygen species (ROS)

The production of intracellular ROS in HepG2 cells after co-/pre-exposure of ascorbic acids (50, 100, and 200 μM) and TPT (TPT) treatment (400 ng/ml) for 24 h was determined by Domico et al. (2007). In brief, I have grown the cells (1×10^4 cells/well) in 96-well black plates and then co-/pre-exposed of ascorbic acids (50, 100, and 200 Mm) and TPT treatment (400 ng/ml) for 24 h. After exposure, cells were incubated with H2DCF-DA for 30 min. After incubation, the plate was read at 480 nm (excitation wavelength) and 530 nm (emission wavelength) by using a fluorescence microplate reader (Spectra MAX Gemini EM, Molecular Devices).

The generation of ROS due to co-/pre-exposure of ascorbic acids (50, 100, and 200 μM) and TPT (400 ng/ml) in HepG2 cells was determined by fluorescence image analysis by using a fluorescent microscope (Olympus CKX41; Olympus: Center Valley, Pennsylvania, USA), with images taken at ×10 magnification.

Cell lysate

After co-exposure and pre-exposure of ascorbic acids (50, 100, and 200 μM) and TPT (TPT, 400 ng/ml) in HepG2 cells were washed with cold phosphate buffer saline and collected in an Eppendorf tube through scraping. The cells were scraped in N-morpholino ethanesulfonic (MES) buffer and sonicated for 15 min and centrifuged at 13000 rpm for 10 min at 4 °C, and the supernatant (cell lysate) was collected in a new tube and stored at − 80 °C for reduced glutathione (GSH) and lipid peroxide (LPO) test.

The amount of protein in cell lysate was determined by the Bradford method (Bradford 1976) using bovine serum albumin as the standard.

Glutathione (GSH) test

The GSH level was determined according to the instructions of colorimetric GSH kit (Cayman Chemical kit, Item no. 703002, Ann Arbor MI USA). The quantity of glutathione was represented as nM GSH/mg protein.

Lipid peroxide test

Lipid peroxide (LPO) was determined according to the instructions of colorimetric LPO kit (Cayman Chemical kit, Item no. 705002, Ann Arbor MI USA). The quantity of LPO was represented as nM MDA (malondialdehyde)/mg protein.

Assessment of chromosome condensation and caspase-3 activity

The effect of co-exposure and pre-exposure of TPT with different concentrations of ascorbic acid on chromatin condensation in HepG2 cells was evaluated by using Hoechst 33342 staining. I have observed fragmented chromatin in TPT-treated cells as described by Gao et al. (2016).

Caspase-3 activity in HepG2 cells after exposure to TPT was measured by using Cayman Chemical colorimetric assay kits.

Annexin V-FITC/PI staining

To find out the early apoptotic and late apoptotic cells, co-exposure and pre-exposure of TPT with ascorbic acid (100 μM) in HepG2 cells were evaluated by using Annexin V-FITC/PI staining method by flow cytometer (FACS Canto TM II, BD BioSciences, CA, USA). Briefly, all treated and untreated cells collected by trypsinization and washed with cold PBS and re-suspended in binding buffer (500 μl). PI (10 μl) and Annexin V-FITC (5 μl) were added in cell suspension (500 μl) and incubated for 30 min in the dark at room temperature. After incubation, the stained cells were examined by FACS and detected as green (Annexin V-bound FITC) event and red (DNA-bound propidium iodide). Data was presented by FACS Diva 6.1.2 software.

Alkaline single cell gel electrophoresis

DNA damage in HepG2 cells was observed by comet assay according to Singh et al. (1988). The cells (50,000 cells per well) in the 6-well plate were cultured and exposed to co-exposure and pre-exposure of TPT with different concentration of ascorbic acid for 24 h.

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Statistical analysis

The significant value of the present data was analyzed by using a one-way analysis of variance (ANOVA, SPSS software), and the significant value was fixed at $^{a}p < 0.05$ control and $^{*}p < 0.05$, $^{**}p < 0.01$ vs. TPT 400 ng/ml exposure. Data were expressed as the average value of the triplicate-independent experiment.

Results

Alteration in the morphology of HepG2 cells

HepG2 cells were exposed to TPT (400 ng/ml, Fig. 1e) as consequence of the morphology of cells was changed in spherical, and cells were detached from culture disc (Fig. 1b). On another hand, there is a little change in morphology of cells in co- and pre-exposure of ascorbic acid (Fig. 1c, d) in comparison to control cells (Fig. 1a).

Cytotoxicity

The cell toxicity of TPT (400 ng/ml) and ameliorating effects of ascorbic acid for 24 h were done by using MTT and NRU methods, respectively. The cell viability was decreased 33.06% and 42.28% in pre-exposure and co-exposure of TPT, and the viability of cells was increased 87.76% and 73.5% with ascorbic acid (200 μM) pre-exposure and co-exposure conditions (Fig. 2a). Data of NRU assay was accorded with the MTT assay (Fig. 2b). The correlation between MTT and NRU results during pre-exposure and co-exposure were presented in Fig. 2c, d), respectively. Thus, it was confirmed by this correlation that the cytotoxicity of TPT was inhibited due to application of ascorbic acid on concentration-dependent manner (Fig. 2c, d).

Oxidative stress

The intracellular-free radicals' formation was determined by using the DCFDA method, and the radicals are produced significantly after exposure to TPT (400 ng/ml) for 24 h and reduced the generation in presence of ascorbic acid (Fig. 3a–e).

Lobo et al. (2010) suggested that antioxidants, free radicals, or reactive oxygen species (ROS) can cause direct impairment to carbohydrates, proteins, and lipids. The ratio of MDA, which is a final product of LPO, was significantly increased after exposure to TPT (400 ng/ml) for 24 h, but it was decreased with ascorbic acid exposure (Fig. 4a). On the other hand, GSH level was reduced after exposure to TPT (400 ng/ml) for 24 h, but it was increased with ascorbic acid exposure (Fig. 4b).

Apoptosis

In the current study, I have evaluated the apoptotic effect of TPT and ameliorating the apoptotic effect of ascorbic acid on HepG2 cells by using three biomarkers, e.g., chromosome condensation, caspase-3 activities, and apoptotic and necrotic cell sorting by flow cytometer. Control cells showed an intact nucleus with blue fluorescence, but apoptotic cells have fragmented chromatin with high blue intensity (Fig. 5a–d).

The activity of caspase-3 (trademark of apoptosis) is increased in TPT treated cells as compared to TPT with ascorbic acid exposed cells (Fig. 5e).

I have used Annexin V-FITC and PI staining to examine apoptotic and necrotic cells after exposure to TPT alone and with ascorbic acid. As a result, TPT (400 ng/ml)-treated cells produced more apoptotic and necrotic cells as compared to the negative control (Fig. 6a) and TPT (400 ng/ml) with ascorbic acid exposure (Fig. 6b–f).

DNA damage

DNA damage after exposure of TPT alone and with ascorbic acid was measured by using single-cell gel electrophoresis method and as a result of TPT alone increased DNA damage more in comparison to negative control (Fig. 7a–c) and with ascorbic acid exposure (Fig. 7b, c).

In TPT exposed cells, the % tail DNA was found (26.78%) (Fig. 7a, b) in comparison to the negative control (4.35%) (Fig. 7a–c) and with pre-exposure of ascorbic id acid (100 mM) 12.5% (Fig. 7a). Also, the same pattern of DNA damage was observed in the co-exposure condition of TPT and ascorbic acid (Fig. 7a–f).

Discussion

In this study, human hepatic carcinoma (HepG2) cells were applied as an in vitro model to determine the toxic potential of TPT and the ameliorating effect of ascorbic acid. I have observed that ascorbic acid significantly decreased the toxicity of TPT on HepG2 cells. The intracellular-free radicals are produced and affected on cell organelles and cellular substances such as carbohydrate, fats, proteins, and DNA molecules (Gaetke and Chow 2003).

I have determined lipid peroxide level with and without ascorbic acid; our results indicated that TPT could induce HepG2 cells impairment, which led to the increase of MDA and reduction of GSH activity. Oxidative stress induced by intracellular-free radicals (ROS) is in control for different types of cells damage and is the most important mechanism of cell injury. Due to oxidative stress, the surplus generation of ROS and successively the reduction of antioxidants affected the total breakdown of the endogenous antioxidant defense

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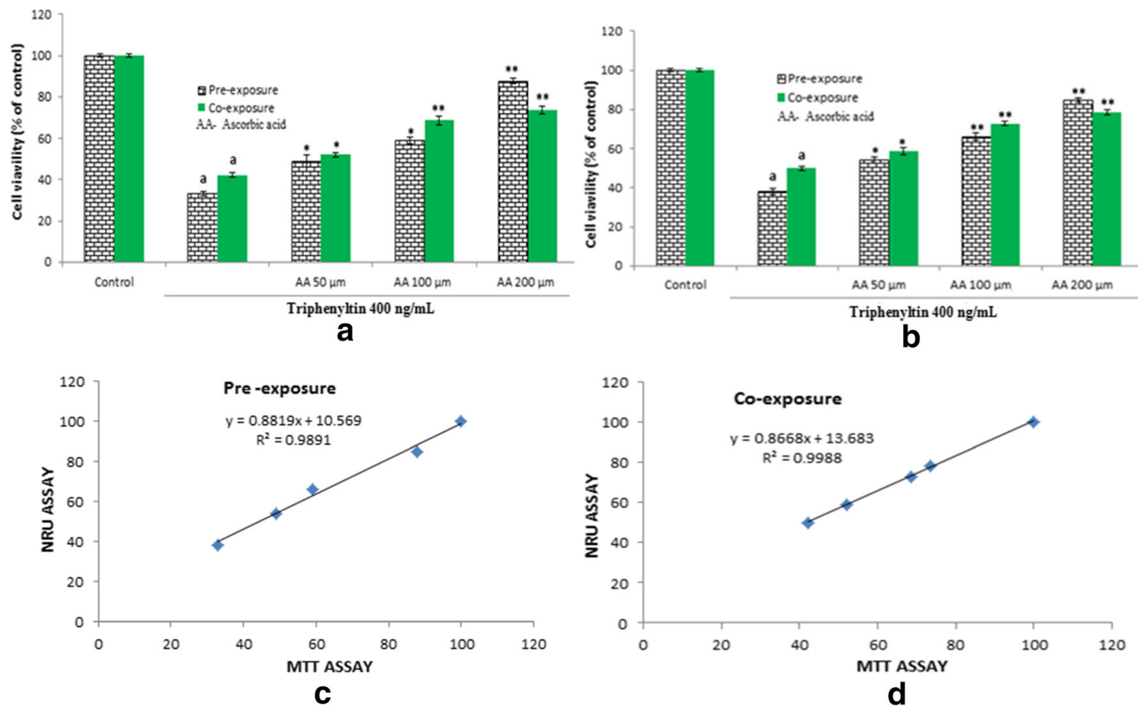

Fig. 2 Cytotoxicity of triphenyltin (TPT) 400 ng/mL without/with different dose of ascorbic acid (AA), for 24 h in HepG2 cells, as evaluated by (A). MTT (B). NRU tests. (C) Correlation of MTT and NRU tests in pre exposure experiments (D). Correlation of MTT and NRU test in co-exposure experiments. Each value represents the mean ±SE of three experiments. n=3, [a]p<0.05 control and *p <0.05, **p<0.01 vs. triphenyltin (TPT) 400 ng/mL exposure

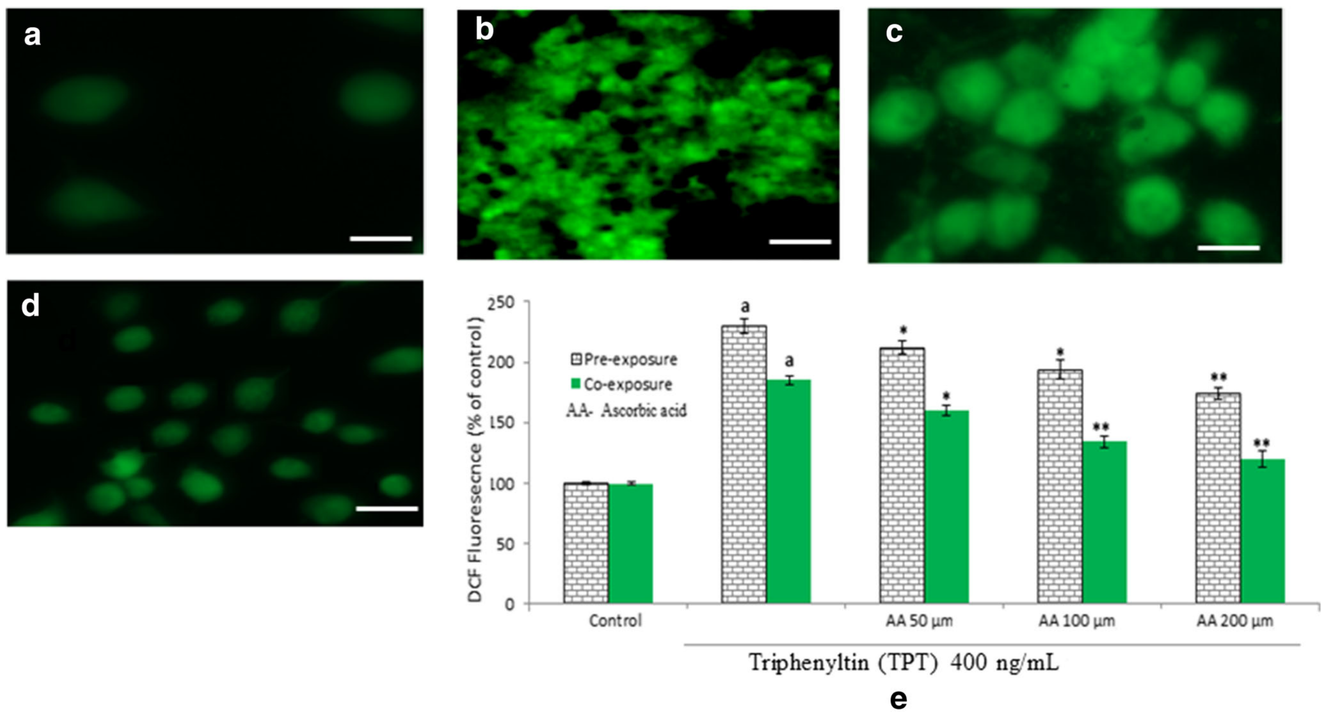

Fig. 3 Intra cellular ROS generation in HepG2 cells after treatment of TPT400 ng/mL without/with different dose of ascorbic acid (AA), for 24 h (A). Control cell (B). Treated cell TPT400 ng/mL (C). Pre exposed cell (TPT400 ng/mL+)200µM Ascorbic acid). (D). Co-exposed cell (TPT400 ng/mL+)200µM Ascorbic acid) (E) %ROS production due to after treatment of TPT400 ng/mL µg/mL without/with different dose of ascorbic acid (AA), for 24 h in HepG2 cells. Each value represents the mean ±SE of three experiments. [a]p<0.05 control and *p<0.05,0.01 vs TPT400 ng/mL exposure. Scalebar is 200 µm

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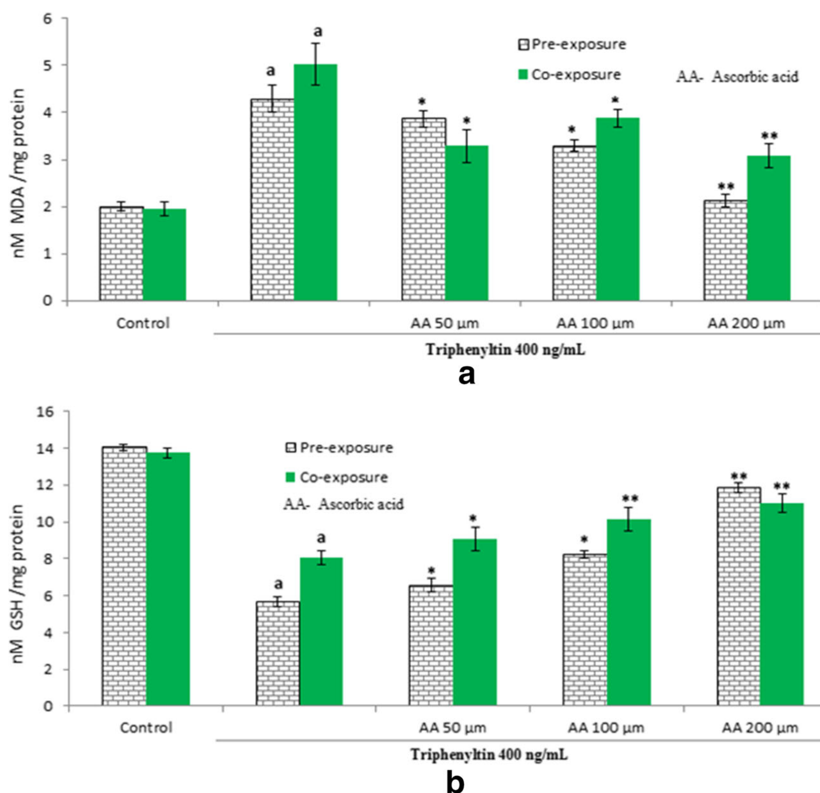
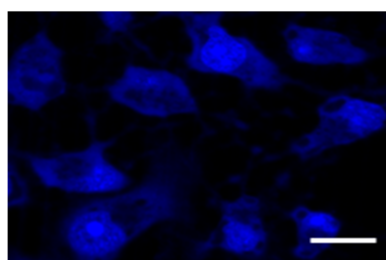


Fig. 4 After treatment of triphenyltin (TPT) 400 ng?mL without/with different dose of ascorbic acid (AA), for 24 h in HepG2 cells (A). Levels of LPO (B). GSH. Each value represents the mean ±SE of three experiments. n=3, [a]p0.05 control and *p<0.05, **<0.01 vd triphenyltin (TPT) 400 ng/mL exposure



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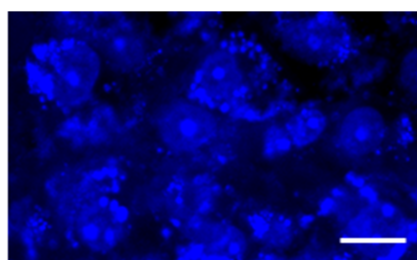

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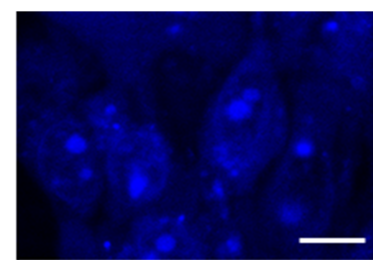

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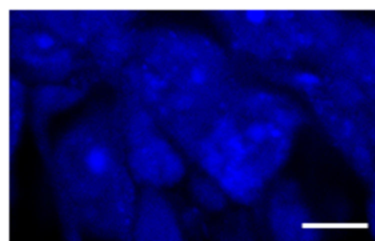

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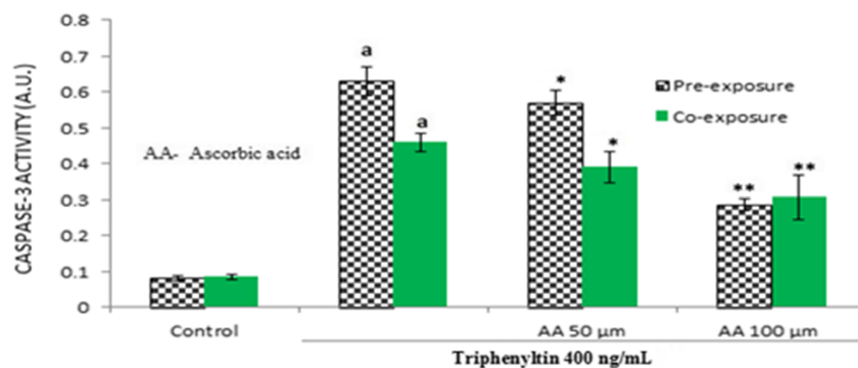

e

Fig. 5 Chromosomal condensation in HepG2 cells after treatment of Triphenyltin (TPT) 400 ng/mL without/with different dose of ascorbic acid (AA), for 24 h. Control cell (B). Treated cell (Triphenyltin (TPT) 400 ng/mL) (C). Pre exposed cell (Triphenyltin (TPT) 400 ng/mL + 100µM Ascorbic acid) (D) Co-exposed cell (Triphenyltin (TPT) 400 ng/mL + 100µM Ascorbic acid) (E) Induction of caspase-3 activity in HepG2 cells afyer treatment of Triphenyltin (TPT) 400 ng/mL without/with different dose of ascorbic acid (AA), for 24 h. Each value represents the mean ±SE of three experiments. [a]p <0.05 control and *p<0.05, **p<0.01 vs. Triphenyltin (TPT) 400 ng/mL exposure. Scale bar is 200 µm

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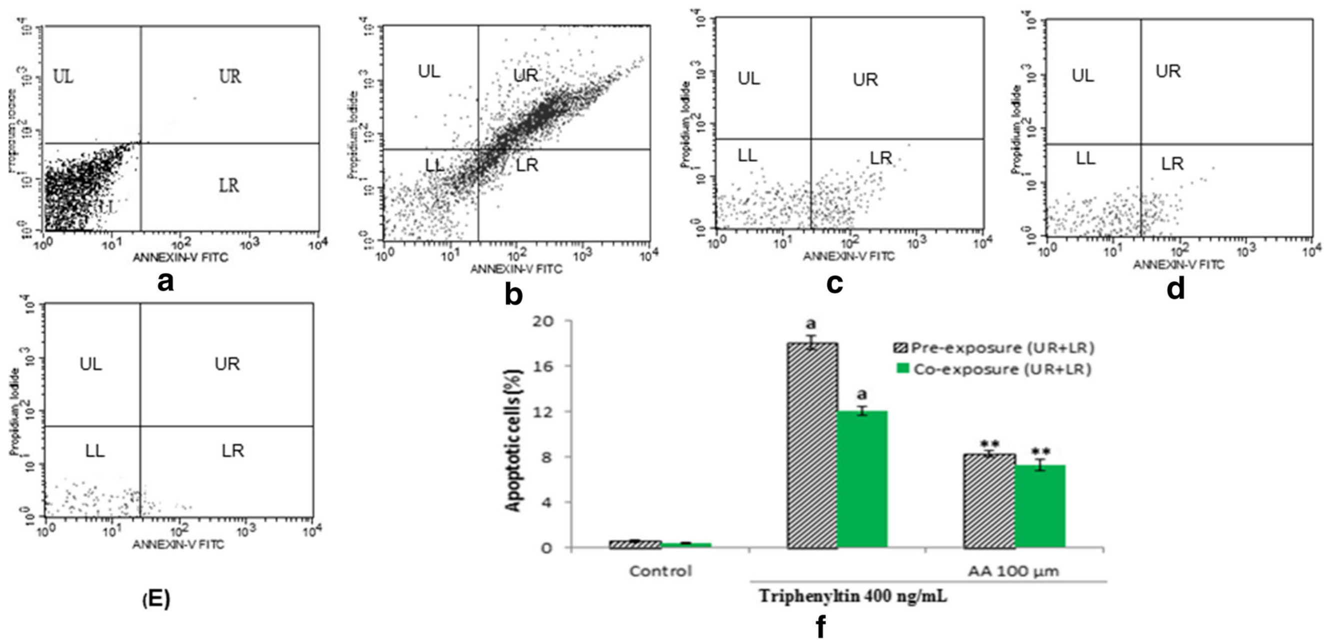
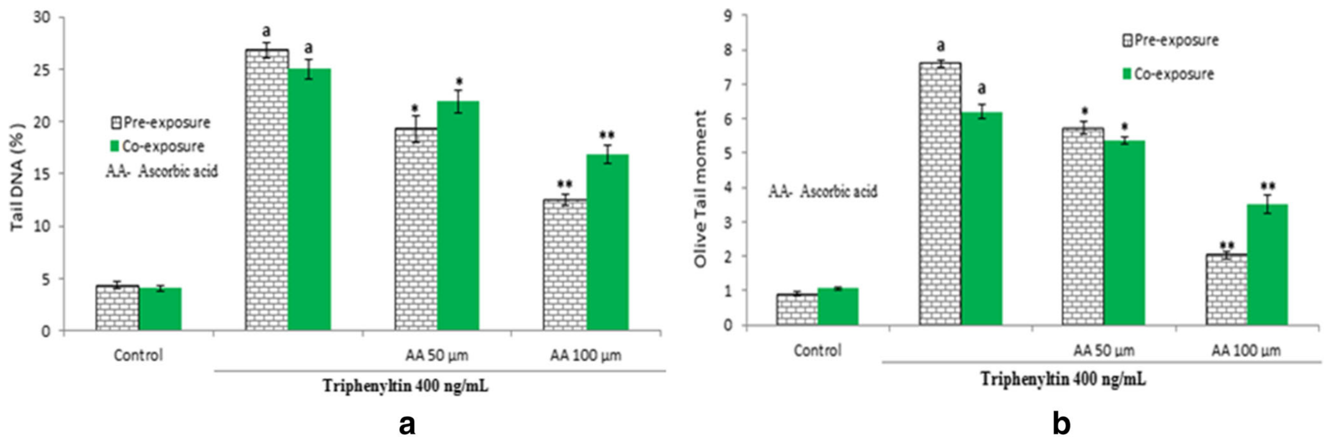


Fig. 6 Triphenyltin (TPT) 400 ng/mL without/with different dose of ascorbic acid (AA), induced apoptosis in HepG2 cell for 24 h. (A-E) Annexin V-FITC and PI flourenscence were measured using flow cytometer with PI and Annexin VIFTC filters, respectively. LL, living cells (Annexin V^-/PI^-); LR, primary apoptic cells (Annexin V^+/PI^-); UR, late apoptotic and secondary apoptotic cells (Annexin V^+/PI^+); dose of ascorbic acid (AA, 100μM), induced apoptotosis in HepG2 cell for 24 h. Data represent average ±SE of triplicate experiments. [a]p <0.05 control and *p<0.05, **p<0.01 vd triphenyltin (TPT) 400ng/mL exposure



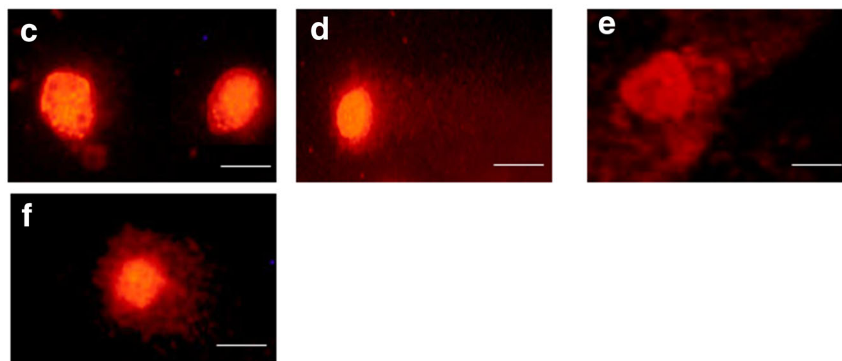


Fig. 7 DNA damage in HepG2 cells after treatment of triphenyltin (TPT) 400 ng/mL without/with different dose of ascorbic acid (AA), for 24 h (A). Tail DNA (%) (B). Olive tail moment (C) =. Control cell (D), Treated cell (triphenyltin (TPT) 400 ng/mL) (E). Pre exposed cell (triphenyltin (TPT) 400 ng/mL + 100μM Ascorbic acid) (F). Co-exposed cell (triphenyltin (TPT) 400 ng/mL +100μM Ascorbic acid). Each value represents the mean ±SE of three experiments. [a]p <0.05 control and *p <0.05, **p <0.01 vs. triphenyltin (TPT) 400 ng/mL exposure. Scale bar is 200 μm

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mechanisms, culminating in failure to protect cells from oxidative damage.

MDA and GSH are more susceptible indicators of oxidative stress (Frijhoff et al. 2015). MDA is the end product of lipid peroxidation (Ayala et al. 2014), and MDA levels reflect the extent of cell damage due to oxidative stress. TPT may induce the production of intracellular ROS at mitochondria which have been applied as a model exogenous oxidative stress–mediated experiment in hepatocellular apoptosis. Ascorbic acid pre- and co-exposure significantly protected HepG2 cells from TPT produced the damage. Ascorbic acid increases the level of GSH and decreases the MDA level and inhibited the apoptosis and DNA fragmentation induced by TPT.

Ascorbic acid is a naturally occurring antioxidant compound and present in animal and plant cells, a highly effective antioxidant, acting as a potent geneprotector and antitumor agent (Packer and Fuchs 1997). Also, this powerful molecule exhibits a key role against DNA damage induced by both oxidative mutagens and alkylating agents. It is suggested that the protective effect of ascorbic acid against DNA damage correlated with its free radical-scavenging properties.

In this study, TPT has been used to induce toxicity and improve the protective effects of ascorbic acid. Previously some researchers reported natural antioxidant improved protective effects of melatonin against DNA. TPT increased the caspase-3 activity and number of chromatin-condensed hepatic cells. Also, the quantity of fragmented DNA was increased in exposed cells as compared to control cells. To find out the probable cause of TPT-induced cell death and the protective effect of ascorbic acid, I examined the alteration of different biomarkers involved in apoptosis. Susin et al. (1998) suggested that mitochondria play an important role in apoptosis, and compromise of mitochondrial integrity may be prevented by various biomarkers of apoptosis. Oxidative stress leads the activation of caspase enzymes via the involvement of cytochrome-c in the intermembrane space into the cytoplasm (Yuan et al. 2003). The findings from the present study revealed that oxidative stress is an involved mechanism in TPT-induced genotoxicity in HepG2 cells. On the other hand, this study supports the hypothesis that ascorbic acid exposure is an effective antioxidant in the reduction of TPT-induced oxidative damage and genotoxicity as evident by (1) reduced level of intracellular ROS, (2) increased GSH level, and (3) decreased DNA damage using the comet assay. Thus, TPT induced apoptosis via mitochondrial and caspase-3 dependent in HepG2 cells. Ascorbic acid attenuation of TPT induced oxidative stress and genotoxicity in the HepG2 cell line.

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