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In-vitro Analysis of Benzene Cytotoxicity and Effect of p53 a Tumor Suppressor Protein on LL-24 Normal Lung Epithelium Cell, A-549 Lung Cancer Cell Line and MCF-7 Cell Line

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Abstract The current research explains the *in-vitro* cytotoxicity study of the benzene exposure and expression of p53 in normal lung epithelium, A-549 lung cancer cells and MCF-7 cell lines. Worldwide the benzene exposure causes toxicity and lethal effects on human beings. The main objective of this study is to analyze the benzene exposure level and induction of various proteins, which are present on nasal passage of human cells. The benzene was prepared in different concentrations and exposed to normal lung (LL24), a lung carcinoma (A-549) and MCF-7 cell lines. At first low dose of benzene was exposed 0.2 ppm and then followed by low dose, they were exposed up to high dose. To find out the effects of benzene, a cytotoxicity assay and RT-PCR was carried out. Results from the cytotoxicity clearly stated that, A-549, MCF-7 were more tolerate and resisted to the benzene exposure at 0.6, 0.8 ppm concentrations, than the LL-24 cells does. More over the LL -24 was affected from 0.6 to at high dosage 1.0 ppm benzene exposure, while there was no effect was reported in the A-549 lung carcinoma up to 0.8 ppm. Compared with LL-24 and A-549, the breast cancer cells MCF-7 was seen as more tolerate throughout the concentration 0.2 to 1.0 ppm. Although the results from RT-PCR clearly stated that, an increased expression of p53 and caspase 3 due to with increased concentration of benzene on LL-24. But p53 negative regulator mdm-2 was not expressed. Hence the Mdm-2 frequently reported on A-549 and MCF-7 than the LL-24. So finally we came to concluded that, different concentrations of benzene induced p53 and caspase 3 levels on LL-24 and does affect mdm-2 expression in cancer cells. So finally the study exhibited the effect of carcinogens, Chemical tolerance of cancer cell proliferation through mdm-2 expression, and the preventive activity of p53 on normal cell line rather than cancer is confirmed.

Keywords *Benzene, Toxicity, p53, mRNA, cDNA, Cytotoxicity, Reverse transcriptase PCR.*

1. INTRODUCTION

Exposure of the volatile chemicals is a chief occupational and ecological health distress. Volatile solvents are widely used as degreasing agents, industrial solvents, and fuel constituents etc. They existing an increasing risk of occupational exposure through nasal and dermal absorption, which are able to leads to toxicological effects on the human (Rogers and Denison, 2000).Safety evaluation of chemicals is performed *in-vivo* on laboratory animal models (Huff, 2002). The carcinogen related assays are the landmarks for protection and prevention of occupationally association (Bakand et *al*., 2006). In this study we have selected industrial volatile compound benzene and It is an aromatic hydrocarbon type and an intermediate chemical in the production of wide range of economical important products such as commercial drugs, rubber industries, tannery industries, degreasing industries, enamel, paint industries and petroleum refineries etc .But while handling of the volatile solvents, they

will easily get exposed to the current environment and makes some considerable changes on the ecology and living things. The effects may be a lethal and mutational depending on the exposing site accordingly. From the report of EPA (Environmental protection agency. 2000) the exposure of benzene through the oral, dermal and nasal route are an important health concern.

A short time exposure may causes some immediate changes in the nasal cells and longtime exposure will definitely cause chronic effects, which leads to the permanent damage on it. Because the benzene is classified as Groug1A carcinogen by IARC (International agency in research on cancer) and EPA have reported that the exposure to benzene definitely will cause nasal cell damage and hematological abnormality. Normally the benzene is metabolized in the liver by the enzyme cytochrome 2E1 (Cyp2E1) (Ramaldi et *al*., 1998). So when a person gets exposed to benzene, there will be a more expression levels on Cyp2E1 and converted into the catechol. But some defense mechanisms protect the cell from the chemical stress and oxidative damage through detoxifying enzymes for example SOD (Super oxide dismutase). Although instead of those enzymes, a few oncogenic proteins preventing the nucleic acid damage from the chemical stress namely an important guardian of the cell p53 .The p53maintains stability and apoptosis through activation (Maximov and Maximov, 2008). More over importantly the function starts chemical stress signal from the ATM kinase enzymes and the N-terminal TAD of p53 get phosphorylated, which activates enzymes cascades (Maximov and Maximov, 2008). This p53 is essential to maintain the cell cycle and to preventing the cell from mutation and tumorogenesis by chemical stress (Mandriani, B. *et al*. 2016). In the LL-24 cells the tumor suppressor levels are being normal compared with activated cells. Because there is no stress signal was arose for activation in the normal cell cycle. But in the lung cancer cells (A-549) p53 is idol, because a cancer already has evolved and adapted to inhibit the expression of p53 to prevent the caspase mediated apoptosis (Shi D and Gu W .,2012) . These inhibition and over proliferation can be done through the activation of main down regulator Mdm-2 protein. Perhaps if mdm-2 gets deactivated, a possible way is there to get p53 activation or otherwise none of them can activate p53 in the lung cancer cells. Also compared with lung cancer cells, a normal lung cells (LL-24) maintaining the balance of p53 neither activation during stress nor degradation by Mdm-dependent ubiquitination. This balance makes the differentiation between normal and cancer cells to respond their chemical environment. Here this research has evaluated the differentiation behavior of above mentioned cell, while treating with different concentration of benzene through cytotoxicity assay and also studies the expression levels of p53 in stress activated cells through reverse transcriptase PCR.

2. MATERIAL AND METHODS

2.1 Culturing of cell line (Carlisle *et al*., 2000, Lieber *et al*., 1976)**:** The Normal lung epithelial cell line LL-24, lung carcinoma A-549 and MCF-7 was purchased from the NCCS pune, Mumbai. Then cell culture was maintained as adherent type with modifier Ham's F-12K (Kaighn's modification) (Hi-media labs, India) medium containing 10% fetal bovine serum (Gibco, Milan, Italy) supplemented with standard anti-biotic such as 100 IU/ml penicillin, 100µg/ml streptomycin and anti-fungal 100 IU/ml amphotericin-B was added to maintain the sterility of the medium, while growing the culture at 37°C, 5% $CO_2$and 95% air. The culture was subsequently checked for confluence and after the monolayer formation of cells within 2-3 days, sub-culture into new 25 ml T-flask with suitable medium. Prior to treatment the benzene was prepared at the concentration from 0.2 ppm to 1.0 ppm respectively. Then for the treatment of the benzene the cells were trypsinized and transferred into a new well plate for 90% cell formation. After formation cells treated with benzene in dose dependent manner and observed 12, 18 and 24 hrs. Then the medium was discarded and the cells were harvested by trypsin digestion and finally pelleted by centrifuge.

2.2 Cytotoxicity Assay (Mossmann et *al*., 1983): *In-vitro* cytotoxicity assay was performed for the measurement of cell proliferation and toxicity of benzene on normal and lung cancer cells, which leads apoptosis, necrosis, and a reduction in cell

viability. Cells were cultured in flat-bottomed; 96-well tissue culture plates for 24hrs and a suspension of two set of different concentration of benzene were treated. In the first set benzene was treated as (0.1, to 0.5) ppm respectively and followed by first, a second dose was treated (0.5 to 1.0ppm). The cells were incubated for 24 hrs 37° C, 5% CO_2 and 95% air. Finally the tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazol-ium bromide) was added to the wells and the cells are incubated 3hrs. Here MTT is reduced by metabolically active cells to insoluble purple Formazan dye crystals. Then the crystals solubilized and the absorbance was read using a micro plate reader (Bio-Rad, United).

2.3 Isolation of mRNA and cDNA synthesis (Chomczynski, P., 1993)**:** The mRNA was isolated by TRIZol (Invitrogen) protocol from monolayer of the normal and lung cancer cells. The protocol was proficient and often used in isolation of total mRNA from tissue, monolayer, cell suspension and frozen tissue. All the mRNA isolation procedures were carried out according to the instruction of the manufacturer, from which the cDNA (New England labs) was synthesized according the manufacturer instruction for the downstream processes. DNA contamination was removed by the DNase (Thermo Fisher Scientific).

2.4 RT-PCR amplification (Petra Baresova *et al.*, 2014): The RT-PCR amplification was performed to cDNA for p53, caspase and Mdm-2 gene expression using specific primers and all the primers are employed in this study corresponding to the appropriate gene.

3. RESULTS AND DISCUSSION

From the results obtained in cytotoxicity assay the cell proliferation was denoted as absorbance intensity of the MTT dye, which reduced into the purple Formazan crystals. At first the MTT was performed and % of cell viability was calculated by plotting concentration versus % Abs (absorbance intensity O.D) values (Fig: 1). According to the LL24, there was no toxicity was recorded in the concentration at 0.2 and 0.4 ppm of benzene. But when the LL-24 exposed to the remaining concentrations 0.6, 0.8 and 1.0 ppm, toxicity and stress stimulus was seen especially at 0.6 ppm (Fig:1A, Fig:2 A2) concentration and considered as starting point of the stress stimulus, and then it was lead apoptosis proved through p53 and caspase expression in RT-PCR.

In the A-549, the cell damage and DNA break was reported at 1.0 ppm of benzene, but prior to cell damage, a stress stimulus was seen at same ppm (Fig:1B,Fig:2 B3). But in the MCF-7 there was no changes were made like apoptosis, necrosis etc., throughout the concentration up to 1.0 ppm (Fig 1C, Fig: 2 C3). Compared with MCF-7, at 1.0 ppm concentration, the cell proliferation was disrupted and continued to the apoptosis through stress stimuli on both A-549 and LL-24. But A-549 does not expressed p53, but they expressed caspase 3 as seen in LL-24 at 0.8 and 1.0 ppm concentrations (Fig:2 A2,A3 and B2,B3).

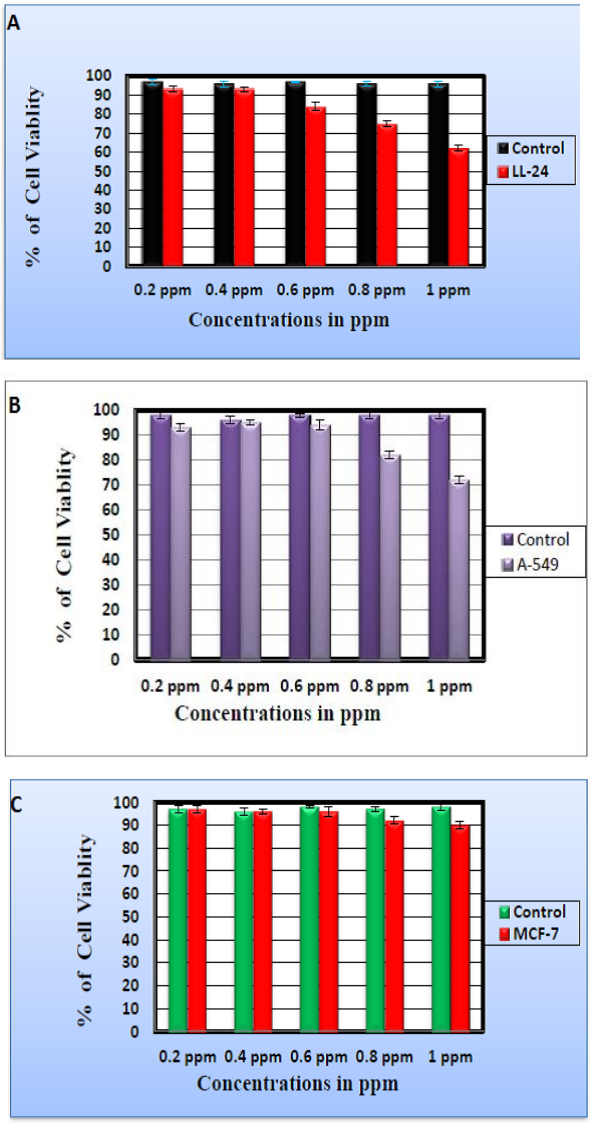

Fig: 1 Cytotoxicity assay A. LL-24, B. A-549, C. MCF-7

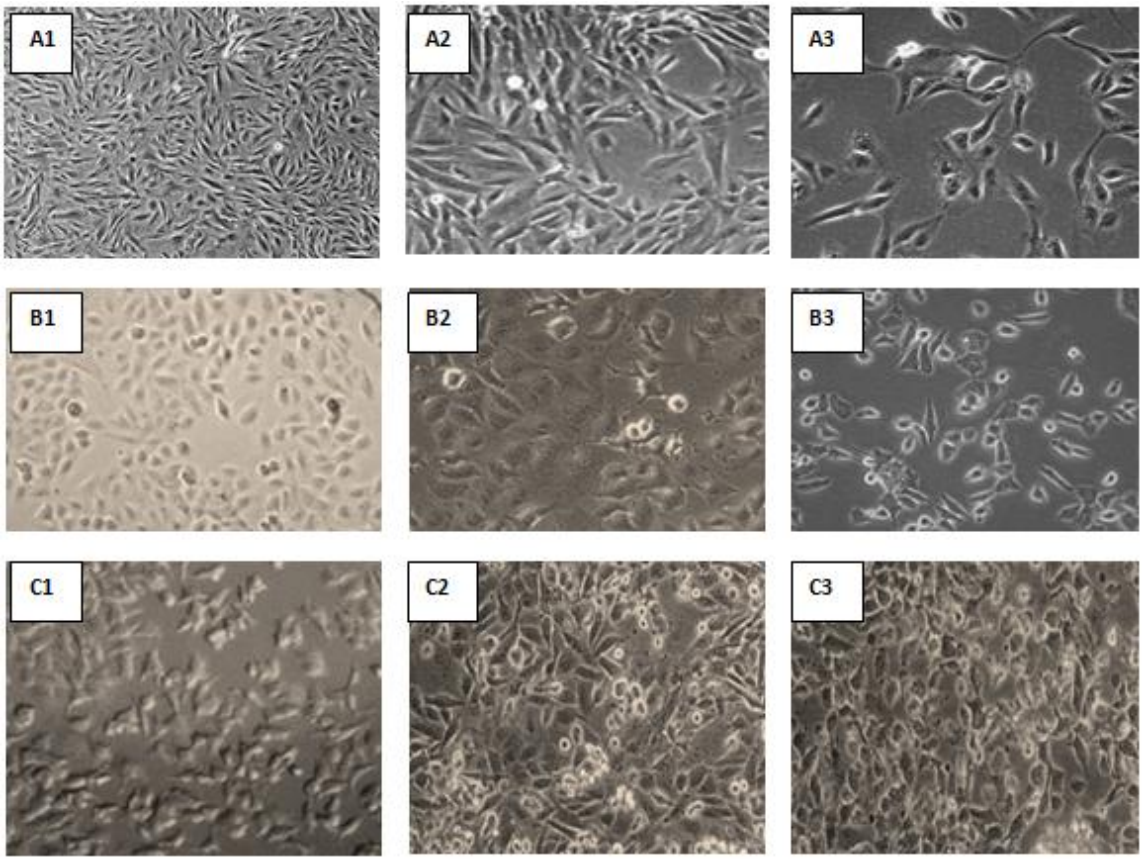

Fig: 2 Inverted phase contrast images of Cytotoxicity assay (LL-24 of A1, A2, A3 (Concentration Control, 0.8 and 1.0 ppm respectively), A-549 of Image B1, B2, B3 (Concentration Control, 0.8 and 1.0 ppm respectively), MCF-7 Image of C1, C2, C3 (Concentration Control, 0.8 and 1.0 ppm respectively).

According to the results from RT-PCR, a p53 expression was seen as low at 0.2 ppm and it was increased with increasing the concentration of benzene from 0.6 to 1.0 ppm on LL-24 cells. Especially at concentrations 0.8 and 1.0 ppm LL-24 had an expression of p53 and caspase 3 (Fig: 3 A1, A2). In the A-549 had a high expression of Mdm-2 throughout the concentration up to 0.8 ppm and hereafter caspase 3 was expressed at 1.0 ppm, but it does not expressed p53 at any ppm (Fig:3 B1,B2). Compared with LL-24 and A-549, in the breast cancer cell lines MCF-7 had not expressed p53 and caspase at any concentration, at the same time they have expressed mdm-2 at higher level in all concentrations (Fig:3 C1). From the results we are able to discuss that, the toxicity of the benzene was not a lethal at very low dose, but when the cell exposed to high concentration, which leads to the oxidative and chemical stress into the cell (Bates S, and Vousden KH., 1999). The Chemical stress will be handled by different enzyme systems, especially ATM kinese playing important role in chemical system (Colman MS *et al*., 2000). When the chemical stress arises, it signals p53 through the chk1 and chk2 to prevent the damage (Ljungman M., 2000). Then p53 get activated through the phosphorylation and induce p21 to binds with cyclin D to stop the cell cycle (Oren M., 1999). If DNA found irreparable this induces programmed cell (Lakin ND, Jackson SP., 1999). Here the benzene concentration has at 0.6, 0.8 and 1.0 ppm on LL-24.this concentration have positive expression of p53 to prevent the cell and DNA (Ashcroft M *et al*.,1999). But 0.8 ppm gave positive expression on p53 and caspase 3, where cell has started to struggle against stress condition (Bringold F and Serrano M., 2000). Then the cell getting ready to lyse itself at 1.0ppm thorough programmed cell death due to the benzene toxicity and gave positive expression on p53 and caspase 3 (Fig:3 A1,A2). Next the A-549 gave positive expression on mdm-2 a negative regulator of p53 from 0.2 to 0.8 ppm .but it does not expresses the p53, due

to the inhibition of p53 by mdm-2 (Borresen and Dale., 2003).then cell attained toxicity at 1.0 ppm and gave a positive gene expression on caspase 3, meanwhile the A-549 was not able to expresses mdm-2 at this concentration. Because mdm-2 is also a regulatory protein like p53.The reason to expression more mdm-2 in A-549 is to block p53 activation, which leads controlled cell growth (Vassilev, 2007). Although when they reached maximum toxicity level it cannot be prolifereated.Because of this A-549 could not able to survive at 1.0 ppm of benzene and induces p53 independent cell death (Fig:3 B1, B2). Compared with LL-24 and A-549, the breast cancer cell MCF-7 has a good proliferation throughout the concentration of benzene from 0.2 to 1.0. Because benzene and its derivates are named for estrogen induction and makes way to breast cancer cell profeliration normally without any disruption (Sørlie T *et al.* 2001). Even higher concentration of benzene treatment, the MCF-7 only can survive. At last the benzene can affect the normal and cancer cells at higher concentration and induces apoptosis through p53 dependent and independent way. But if the cancer cell died after the treating of benzene, is not due to the anti-cancer (Amundson SA *et al.*, 1998). Logically we cannot come to conclude that, the benzene possess anticancer activity. The cell death was reported on A-549 cells are due to extreme toxicity of benzene.

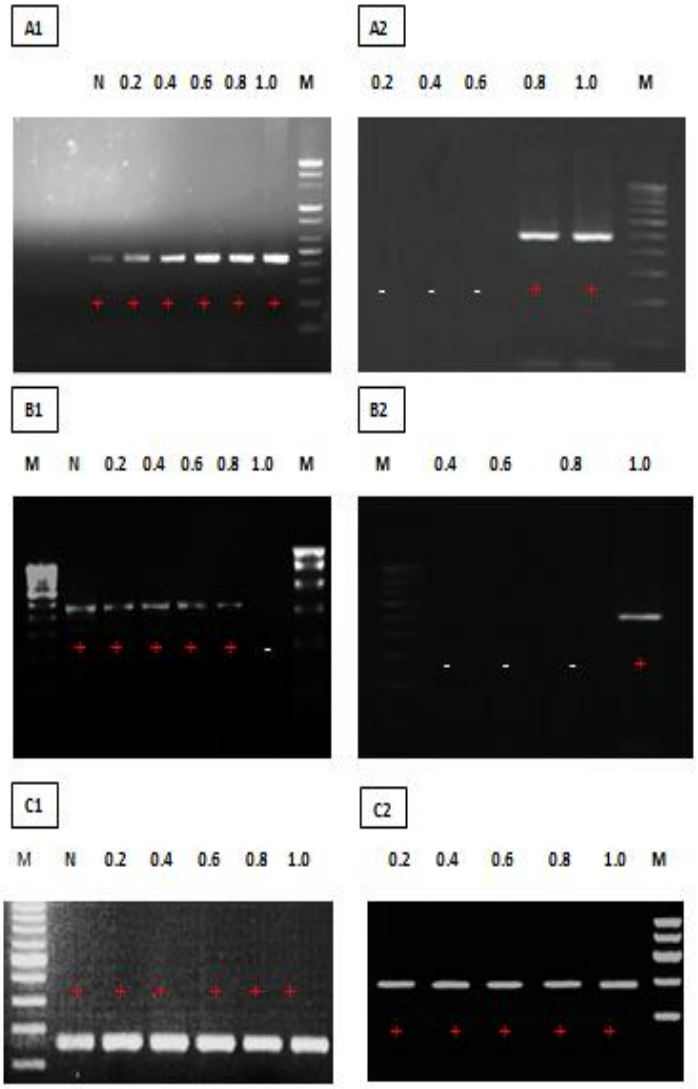

Fig: 3. RT-PCR expression of p53, caspase 3 and mdm2

p53 and caspase expression on LL-24 (A1 p53 and A2 Caspase 3). Mdm-2 and caspase 3 expressions on A-549 cells (B1- Mdm-2 and B2-Caspase 3), Mdm-2 and caspase expression on MCF-7 cells (C1-Mdm-2). beta actin expression on normal cells C2- beta actin).

4. CONCLUSION

Finally we came conclude that the benzene is severe pollutant to environment and living things. Here in the lung epithelial cell (LL-24) p53 has struggled to tackle chemical stress, while exposing to the benzene environment up to the concentrations 0.6ppm. But not able give stable control in cell proliferation. So caspase were expressed and the programmed cell death attained. One of immortalized lung cancer A-549 also affected too and not able proliferates at 0.8 ppm concentration. But the MCF-7 getting proliferated more than others. So person, who are suffered by breast cancer, they have more cancer proliferation duo to the estrogen induction by benzene. Also the people who are working in industries consuming benzene, they also have more chance to develop toxicity and it leads to organ damage. In this study, the p53 confirmed that, the toxicity of benzene at high concentrations (0.8 and 1.0 ppm) is extremely lethal to living things.

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