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An In Vitro Study on the Effects of Aromatic Hydrocarbons 1- Naphthol and Dibenz[a,h]anthracene in HepG2 Cells

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Abstract: **Liver cells (HepG2) were treated with well known toxic aromatic hydrocarbons 1-Naphthol and Dibenz[a,h]anthracene. Naphthol at 20-40µg/ml affected significant increase in the levels of reactive oxygen species in the cells.. Reactive nitrogen intermediates increased to an extent of 2.4 to 3.8 fold in cells treated with naphthol over a range of 20-80 µg/ml. Expression of antioxidant enzyme Glutathione peroxidase was found to increase two fold, while catalase expression was found to decrease by 21% on exposure to 1-Naphthol. Dibenz[a,h]anthracene did not affect discernible changes in the expression of these enzymes** Induction **of** CYP1A1 mRNA **by Naphthol at 60µg/ml was around 45 fold higher than untreated cells. Dibenz anthracene at a dose of 5µg/ml was found to be a potent inducer of CYP1A1 as it elevated expression of CYP1A1 mRNA to an extent of 350 fold. . Naphthol exhibited lipogenic potential in HepG2 cells. Concommitant increase in Sterol regulatory element binding protein 1c was observed.**

Index Terms: **CYP1A1, Dibenz[a,h]anthracene, Lipogenesis, 1-Naphthol, Oxidative stress**

INTRODUCTION

Industrialization and unregulated use of chemicals has created an environment where in the living world is constantly assaulted by toxins. Aromatic hydrocarbons such as quinolines, acridines phenanthrene, naphthalenes and hydroxylated aryl hydrocarbons such as phenols, naphthols etc. are often the toxins found in water, soil and air contaminated by industrial wastes, emissions and anthropological processes (Mojiri *et al*., 2019; Cao *et al*., 2020). Exposure to these toxins is hazardous to humans and environment. Upsurge in modern age diseases such as obesity, allergies, metabolic syndrome etc. have also been partly attributed to the constant exposure to these toxins present in the environment (Poursafa *et al*., 2018; Patel *et al*., 2020; Li *et al*., 2021)

Changes in the basal functions of the cells leading to oxidative stress and induction of the intracellular proteins such as cytochrome P450 enzymes have been used as indicators/end-points to measure harmful effects of toxic compounds (Reichard *et a., 2005;* Bae *et al*, 2010; Ke *et al*., 2018; Cao *et al*, 2020)

The parameters affected by toxicity of 1-Naphthol and Dibenz[a,h] anthracene (DA) were evaluated *in-vitro*. As liver is involved in detoxification processes, liver derived HepG2 cell line was used for the study. Effect of the hydrocarbon on oxidative stress markers and CYP1A1 expression was investigated.

MATERIALS AND METHODS

Materials:

HepG2 cell line procured from NCCS, Pune, India, was maintained in MEM and RPMI medium respectively. The media contained gentamycin (40µg/ml) and penicillin-streptomycin mixture.

All the reagents used were of analytical grade. Dibenz[a,h] anthracene, Sodium palmitate, Sulforhodamine B were procured from Sigma-Aldrich. Thermofischer Nunclon flat black and Quartz 96 well plates were procured for fluorimetric and UV absorbance studies. Trizol, High Capacity RNA-to-cDNA Kit,

Taqman gene expression GAPDH and CYP1A1 assay probes and Fast advanced master mix were from Thermofischer Scientific. ELISA kits were procured from Life Technologies, Delhi, India. Absorbance was read using Tecan Infinite M microplate reader.

Effect of aryl hydrocarbon on cell viability:

HepG2 cells seeded into 96 well plates were incubated in CO_2 incubator at 37°C, the aryl hydrocarbons α-naphthol (1-naphthol) / dibenz[a,h]anthracene solubilized in DMSO were added over a range of concentration to the culture medium. Appopriate DMSO controls were maintained. After 42h, cell layer was washed with PBS and viability was studied using Sulforhodamine B (Vichai & Kirtikara, 2006). The absorbance was read at 490nm.

Measurement of ROS:

Cells seeded into 24 well plates were treated with naphthol/DA for 4h in 400 microlitres of medium. After washing with PBS, the wells were loaded with 5 µM 2', 7'-dichlorofluorescein diacetate diluted in phosphate buffered saline (PBS), and incubated for 20 min at 37 °C in dark. Cells were washed twice with 500µl of PBS. The cells were trypsinized in 200µl of PBS. The suspension from each well was transferred to black 96 well plates and fluorescence was subjected to excitation wavelength of 495nm and read at emission at 520nm.

Enzymatic activities:

HepG2 cells were treated with 80µg/ml and 5µg/ml of 1-Naphthol and DA respectively. After 20h of incubation, the cells were washed, plates were kept at -80ºC for 2h, lysed using chilled 0.5% triton containing 1mM PMSF. The lysate was centrifuged and supernatant was immediately stored at -80ºC until assay. .

Catalase assay was done by incubating the lysate in 60mM of hydrogen peroxide in PBS at 27±0.5ºC. Change in absorbance at 240nm was monitored over a period of 15min. The reduction in relation to protein content was calculated.

Relative quantitation of glutathione peroxidase was measured by incubating the lysate in a reaction mixture containing 0.8mM glutathione (GSH), 0.1mM EDTA, 1.3mM Sodium azide, and 0.25mM Hydrogen peroxide in 0.08M phosphate buffer pH7.4. Care was taken to minimize oxidation of GSH. Reaction mixture was immediately incubated at 37ºC. Reaction was terminated after 5 min by addition of 1/3rd volume of 10% TCA. Time of incubation was noted. The reaction mixture was centrifuged at 12000rpm for 5min and to 20µl of the supernatant, 160 µl of 0.3M disodium hydrogen phosphate, pH10 was added followed by 20 µl DTNB [4mg of 5,5-dithio-bis-(2-nitrobenzoic acid) in 10ml 1% Tri sodium Citrate]. Absorbance was read at 412nm within 10min. Appropriate controls were maintained to account for oxidation of GSH. Consumption of GSH was calculated in relation to protein content as per Lowry's readings.

Lowry's Method:

Alkaline carbonate reagent mix was prepared by mixing 0.5 ml of 1% Potassium sodium tartrate and 0.5ml of 0.5% $CuSO_4.5H_2O$ followed by addition of 24 ml of alkaline carbonate (2% sodium carbonate in 0.1M NaOH). The lysate 30µl was added to the 250ul of alkaline carbonate reagent mix and incubated at ambient temperature (30±1ºC) for 15min. The F-C reagent diluted with equal volume of D/W, 25 µl was added, centrifuged at 13000 rpm for 5min. The supernatant was transferred to microwells and absorbance was read at 660nm.

Quantification of nitric oxide:

Cells seeded into 96 well plates at a rate of 20000/well, were treated with 80µg/ml naphthol in freshly prepared RPMI medium devoid of pH indicator dye. After 5h of incubation, the culture medium was centrifuged at 1000rpm for 5min. Concentrated Griess reagent (2x; 0.4% naphthylethylenediamine dihycrochloride and 4% Sulfanilamide in 10% phosphoric acid) was employed to accommodate higher volume of the sample. Griess reagent was added to supernatant at a ratio of 1:3. The absorbance was read at 540nm within 10min. The method was calibrated with sodium nitrite diluted in the medium. Reactive nitrogen intermediates released / Viability was noted.

Lipogenesis in HepG2 cells:

Sodium palmitate (2.8 mM stock) was prepared by mixing 2mg of the fatty acid (in 180µl 70% ethanol) and 7mg of fatty acid stripped BSA in the culture medium. The mixture was sonicated for 30 seconds and the conjugate was used for the study. HepG2 cells were treated with palmitate and naphthol in the medium containing 0.5% FBS. The wells were replaced with fresh medium after 24h. On day 3, the cell layer was subjected to SRB assay, Oil red O and SREBP1c assay.

For oil red O assay, cells were washed with PBS and fixed with 4% Formaldehyde in cold PBS for 30 min followed by staining with 0.3% Oil red in 60% isopropanol for 15min. After 5 washes with D/W, the stained oil droplets were dissolved with isopropanol and absorbance was read at 490nm.

For measurement of SREBP 1c, the cells subjected to freeze thaw were lysed using cold 0.5% triton containing 1mM PMSF. The lysate was collected, pooled and centrifuged at 13000rpm for 5min at 4ºC. The supernatant 20 µl was analysed for SREBP 1c by kit method (Life Technologies, Delhi, India) as per manufacturer's instructions. Protein content was determined by Lowry's method.

Quantification of CYP1A1 m-RNA expression by QPCR:

Cells exposed to naphthol/DA at 60µg/ml and 5µg/ml respectively for 24h was subjected to RNA isolation by Trizol

method. Isolated RNA was quantified using NanoQuant system (Tecan Infinite M microplate reader. The cDNA preparation followed by QPCR for GAPDH and CYP1A1 gene expression was carried out as per the manufacturer's instructions. Ratio of cycle threshold (Ct) values of CYP1A1/GAPDH was noted. Expression level was quantified by Delta-Delta Ct method.

Statistical Analysis:

Results are expressed as mean ± S.D. (n≥3). The data analyzed using paired t test was used with one tailed distribution to compute the value of significance. The p≤ 0.05 was considered significant. The p value for effect of test compound (naphthol/DA) was calculated in relation to untreated cells

RESULTS AND DISCUSSION

Aromatic hydrocarbon pollutants added to the environment by industrial activities and anthropogenic processes, have potentially deleterious effects on organisms. Researchers have attributed oxidative stress and/ AhR mediated ligand activation pathways as possible mechanisms for toxic effects of these compounds (Cao *et al*, 2020; Reichard *et al., 2005*; Bae *et al*, 2010; Yazdani, 2020). Deregulation of cell proliferation by Benzo (α) pyrene has been reported by Pliskova et al, 2005 in MCF 7 cells. Thus, polycyclic aromatic hydrocarbons are reported to be highly mutagenic, carcinogenic and immunotoxicogenic (Patel et al., 2020, Reichard et al., 2005).

Dibenz[a,h]anthracene is a polycyclic aromatic hydrocarbon often released into the environment through incomplete combustion of gasoline, cigarettes, and coal tar (Kim and Kim, 2016). DA dissolved in DMSO (1mg/ml) was found to be insoluble when added to culture medium at concentrations of 30µg/ml and higher. It was therefore used at lower concentrations. It was found that at concentrations of 3 t0 6 µg/ml, the loss of viability in HepG2 cells occurred to an extent of 43- 50%.

The metabolite of carbaryl insecticides and naphthalenes, 1-Naphthol is an extensively used chemical in chemical industries (Croera *et al*., 2008; Wilson *et al*., 1996; Tilak *et al*., 1981). Being a hydroxy aromatic hydrocarbon 1- Naphthol was relatively more soluble and exhibited cytotoxicity resulting in loss of viability to an extent of 30 to 60% in 42h in the range of 31 to 62µg/ml, (Fig. 1A).

Toxicity of chemicals results in oxidative stress affecting increased production of reactive oxygen species (ROS) and alteration in expression of antioxidant enzymes. The levels of ROS induced by the test compounds over a range of their respective cytotoxic concentrations were measured using DCFDA

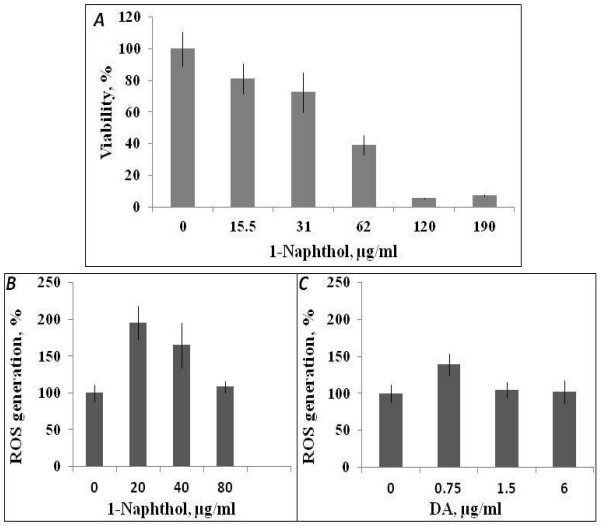

Fig 1 Effect of aromatic hydrocarbons on HepG2 cells; *NL-Naphthol; A- Effect of 1-Naphthol on viability of HepG2 cells; B & C- Effect of Naphthol and DA on generation of ROS*

Naphthol affected a significant increase in ROS to an extent of 1.9 and 1.6 fold relative to its basal level at 20 and 40µg/ml respectively. Interestingly, both the compounds exhibited dose dependent decrease in ROS levels (Fig. 1B & 1C). Libalova *et al* (2018) have reported similar response for benzo[a]pyrene, and 3-nitrobenzanthrone in human lung cell lines and have attributed this response to possible exhaustion of total antioxidant capacity of the cells and activation of antioxidant mechanisms. Glutathione peroxidase (GPx) and Catalases are the key enzymes involved in the first line of defense in the anti oxidant mechanisms of the cells (Ighodaro & Akinloye, 2018). The oxidized and reduced forms of Glutathione act as redox buffers in the cells and this buffer system is mainly involved in regulation of hydrogen peroxide. Catalase is known to catalyse the breakdown of hydrogen peroxide. The lysates obtained from HepG2 cells treated with naphthol at 80µg/ml showed 2 fold increase in the expression of GPx, while Catalase expression/activity was found to reduce by around 20.5% (Fig. 2A). Increased expression of GPx and reduction in catalase expression under oxidative stress is reported by investigators. Balance between GPx and catalase reportedly plays an important role in amelioration and regulation of oxidative assaults of reactive oxygen radicals. (Baud *et al*., 2004; Simic *et al*., 2006).

DA did not influence the expression of these enzymes to a discernible extent. The results indicate that GPx plays an important role in regulation of naphthol mediated oxidative stress.

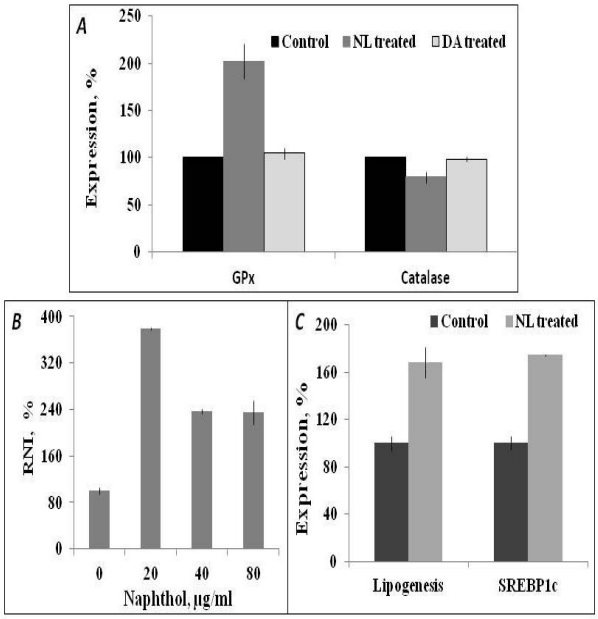

Fig 2 Influence on various cytotoxic parameters in Hepg2 cells; *NL-Naphthol. A- effect on expression of antioxidant enzymes GPx & Catalase; B- Effect on generation of RNI; C-Effect of naphthol on lipid accumulation*

Generation of reactive nitrogen intermediates (RNI) is induced as a consequence of oxidative stress on exposure to aromatic hydrocarbons (Myhre & Fonnum, 2001). Nitric oxide is a highly reactive and diffusible molecule which can damage the surrounding cells. As naphthol was found to affect significant alterations in ROS level and expression GPx, its effect on formation reactive nitrogen intermediates (RNI; nitric oxide derivatives) was studied. As shown in Fig. 2B, 2.4 to 3.8 fold increase in RNI content was observed. RNI level decreased with increasing concentration of naphthol and the results are in concordance with the trend observed in ROS level.

Aromatic hydrocarbons are known to influence lipid metabolism (Kassotis *et al.*, 2021). Hydroxylated aromatic hydrocarbon Tetra bromobisphenol A is reported to induce accumalation of oil droplets in human mesenchymal stem cells (Kakutani *et al*., 2018). The highly non polar Polycyclic aromatic hydrocarbon tetrachlorodibenzo-p-dioxin (TCDD) however, downregulated lipogenesis. 1-Naphthol being an aromatic hydrocarbon, its lipogenic potential was studied in HepG2 cells. Palmitate mediated fat accumulation is reported in cells of hepatic origin (Bucher *et al*., 2018, Zhao *et al*., 2021). Thus effect of naphthol on palmitate induced lipogenesis was studied. Fig.3 presents microscopic observation of lipid accumulation in HepG2 cells treated with 200µM palmitate and 20µg/ml naphthol. Palmitate treated cells were stained slight pink indicating presence of lipid droplets. Under influence of naphthol, the conversion of palmitate to triglycerides appears to have enhanced as Fig.3C shows distinct pink colored cells. Palmitic acid is known to affect viability of cells and hence, it was observed that palmitate treated cells tend to shrink and get easily detached from the surface.

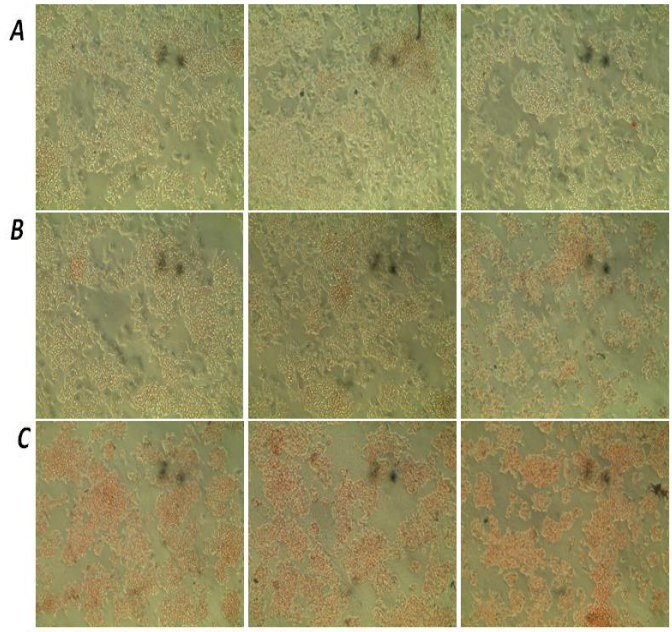

Fig 3 Effect of 1-Naphthol on Palmitate mediated lipid accumulation in HepG2; *A-Untreated control cells; B- Palmitate treated control cells; C- Palmitate treated cells in presence of naphthol*

Lipogenesis mediated by naphthol in presence of 100µM of the fatty acid was assessed. Involvement of sterol regulatory element binding proteins (SREBP-1) in promoting the expression of Fatty Acid Synthase in HepG2 cells is reported (Kim and Chung, 2018; Bai *et al*., 2019). Quantification of accumulated triglycerides and SREBP-c protein was carried out and the result is presented in Fig. 2C. Naphthol was found to enhance lipogenesis to a significant extent. Good correlation was found between lipid accumulation and expression of SREBP 1c, as both increased 1.67-1.75 fold in presence of naphthol.

Polycyclic aromatic hydrocarbons are known to bind to aromatic hydrocarbon receptors (AhR) leading to ligand mediated activation of phase I cytochrome P450 enzymes. CYP1A1 is one such protein induced upon activation of AhR (Kim *et al*., 2021). CYP1A1 is responsible for the detoxification of polycyclic aromatic hydrocarbons by oxidizing them into reactive intermediates. The intermediates if not metabolized, may lead to mutagenicity. CYP1A1 is also believed to be involved in various cell signaling pathways. Higher inducibility of the enzyme is suspected to lead to carcinogenesis (Reichard *et al*., 2005; Croera *et al.*, 2008; Gastelum *et al*., 2020). Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and TCDD have been reported to upregulate CYP1A1 activity hundred to thousand fold in HepG2 cells

(Ibrahim *et al*., 2020).

QPCR was carried out to study mRNA expression in both naphthol and DA treated HepG2 cells. GAPDH was employed as the house keeping gene. Figure 4A, represents the amplification plot for mRNA expression in naphthol treated cells and the respective control. Ct value for CYP1A1 was significantly higher in comparison to GAPDH in both treated and untreated (control) cells. Ct values for CYP1A1 in naphthol treated cells and control was around 21 and 27 respectively, indicating induction of mRNA in response to naphthol.

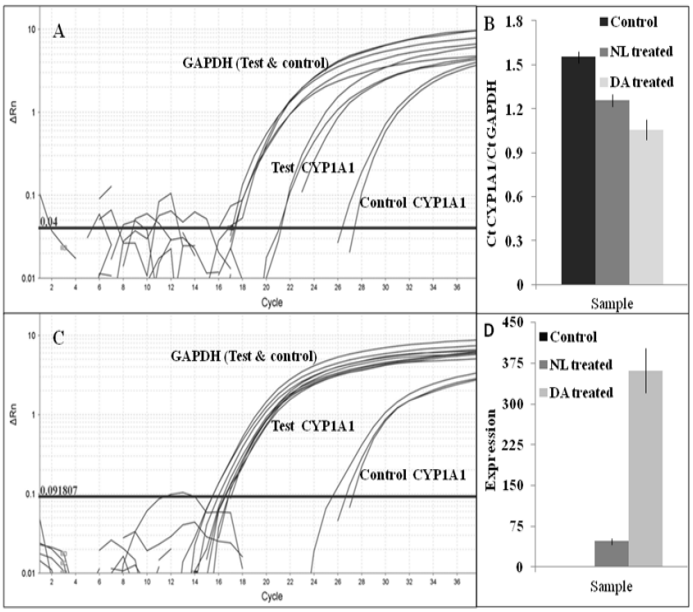

Fig 4 QPCR for expression of CYP1A1 in Naphthol / DA treated HepG2 cells; *A- Amplification plot for Naphthol treated cells (Cycle no. on X-axis and ΔRn on Y-axis, Rn represents baseline signal); B- Comparison of ratio of Ct CYP1A1/GAPDH; C- Amplification plot for DA treated cells; D- Fold change expression of CYP1A1*

The ratio of Ct values of the test gene to that of GAPDH is shown in figure 4B. The ratio indicates that DA is a strong inducer of CYP1A1 gene. Amplification plot (Fig. 4C) showed that the Ct values for CYP1A1 reduced significantly in comparison to respective controls. In the present work, exposure to naphthol affected around 45 fold induction of CYP1A1. Upregulation of CYP1A expression was found to be significantly higher (350 times) in DA treated HepG2 cells (Fig. 4D). DA being a polycyclic aromatic hydrocarbon, the results are in accordance with the functionality of CYP1A1**,** as the enzyme is involved in phase I xenobiotic and drug metabolism.

CONCLUSION

Naphthol can be used as the aryl hydrocarbon for cytotoxicity studies. HepG2 was found to be sensitive to Dibenz[a,h]anthracene mediated toxicity. Naphthol affected the factors involved in oxidative stress to a significant extent. DA appears to affect the cells via CYP1A1 mediated mechanisms.

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