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BISPHENOL A ENHANCES GROWTH OF Hep-G2 CANCER CELLS BY UPREGULATING EXPRESSION OF PRO - INFLAMMATORY AND PRO - ANGIOGENESIS PROTEINS

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ABSTRACT: Bisphenol A (BPA) is primarily used in the manufacture of plastics and resins. There are many scientific reports that point to the adverse effects of this chemical to different animal models. There has been an increase in use of plastic during the last couple of decades and this has increased human exposure to plasticizers like Bisphenol A. But since little is known on how these endocrine disruptors especially BPA promote cancer progression, the study investigated the effect of BPA on hepatocellular carcinoma cell line Hep-G2 by evaluating the expression of molecules involved in the processes of inflammation, cancer and angiogenesis. Cell viability studies using MTT assay, RT-PCR studies and Western blotting studies were performed to check effect of Bisphenol A on liver cancer cells. Cell viability studies, RT-PCR studies and Western blotting studies showed a dose dependent increase in the expression of factors involved in cancer progression which decreased when the cells were treated with Meloxicam, a COX-2 inhibitor. The results indicate that BPA up-regulates the expression of factors involved in the inflammatory pathway thus helping in the progression of cancer.

INTRODUCTION: The increase in use of plastic during the last couple of decades have increased human exposure to plasticizers like Bisphenol A (BPA) which is a recognized endocrine disruptor. BPA is found in polycarbonate bottles, metal can coatings and other packaging materials. These plasticizers tend to mix with the food material when they come in direct contact especially when the ambient temperatures are high. BPA is reported to interact with thyroid causing its dysfunction, PPAR-γ receptor and is found to regulate neural circuits that control feeding behaviour.

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BPA works principally by mimicking the hormone oestrogen and inhibits the binding of oestrogen to its receptor. BPA is reported to bind with thyroid hormone receptor thereby interfering with the activity of the thyroid gland [1]. Bisphenol A is reported to induce liver damage by interacting with mitochondria [2]. Zhu *et al*., reported that BPA promotes growth and metastatic potential in a neuroblastoma cell line SK-N-SH [3]. But very little is known on how these endocrine disruptors especially BPA promotes cancer progression especially in case of liver cancer. Most conversions of chemicals especially xenobiotics occur in the liver and so liver is the organ which faces the first toxic effect of the xenobiotic compounds.

Our *in-vitro* study investigates the effect of BPA on hepatocellular carcinoma cell line Hep-G2 by evaluating the expression of molecules involved in

the processes of inflammation and angiogenesis. The influence of specific NSAID (Meloxicam), a COX-2 inhibitor, was also studied for its activity on BPA treated cells. The expression of Cyclo-oxygenase-2 and Akt were studied using RT-PCR and the protein expression pattern of HIF-1α and VEGF were studied using western blotting.

MATERIALS AND METHODS:

Cell Culture: The Human liver cancer (Hep G2) cells were procured from NCCS, Pune. The cells were allowed to grow in DMEM supplemented with 10% FBS, 75mg/l streptomycin and 100µ/l penicillin. Cells were grown at 37 ºC in 5% CO_2 and 95% air. All reagents were purchased from Hi-media (Mumbai, India).

Cell Viability Assay: The Hep-G2 cancer cells were seeded in 96 well microplate with 5000 cells per each well. After 24 h incubation at 37 °C, the growth medium was changed with fresh medium containing 1nm/ml, 10nm/ml and 100nm/ml of BPA and the cells were then incubated for additional 24 h. Another set of cells were also treated with selective COX-2 inhibitor, Meloxicam (40µm) along with 100nm/ml BPA for 24 h. Untreated Hep-G2 cells were taken as control. Cell viability was measured using MTT assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). The final measurement of absorbance was at 570nm [4].

After evaluating the absorbance, the % growth inhibition was determined using the formula:

% Cell Viability = (Abs (sample) / Abs (control) X 100)

Reverse Transcriptase - PCR: The expression levels mRNA of Protein kinase B (Akt) and Cycloxygenase 2 (COX-2) were studied using reverse-transcriptase PCR [5, 6]. Culture plates of 60 mm were used to grow the Hep-G2 cells. Untreated Hep-G2 cells were kept as control. A selective COX-2 inhibitor, Meloxicam (40µm) was treated along with 100nm/ml BPA. After treatment with different concentrations of BPA (1nm/ml to 100nm/ml) and Meloxicam for 24 h, the total RNA was isolated from the treated and untreated cells using manufacturer's protocol (Chromous Biotech Ltd, Bangalore, India).

Total RNA (1µg) was converted to cDNA with M-MLV reverse transcriptase (Promega, Madison, WI) at 37 °C for 30 min and denatured at 90 °C for 15 minutes. The PCR was performed using GeneAmp RNA PCR kit (Perkin-Elmer Corp., Branchburg, NJ). The forward and reverse primers used for Akt, COX-2 and beta-actin amplification are specified in **Table 1**. The PCR products were separated on 1.5 % agarose gels and analyzed using a ChemiDoc XRS (Bio-Rad Laboratories Inc., Hercules, CA).

TABLE 1: FORWARD AND REVERSE PRIMERS FOR THE GENES

Genes	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)
Akt	TCTATGGCGCTGAGATTGTG	CTTAATGTGCCCGTCCTTGT
COX-2	TTCAAATGAGATTGTGGGAAAATTGCT	AGATCATCTCTGCCTGAGTATCTT
β-actin	GTTTGAGACCTTCAACACCCC	GTGGCCATCTCCTGCTCGAAGTC

Western Blot Analysis: The expression levels of Hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) proteins were studied using western blotting. Culture plates of 60 mm were used to grow the Hep-G2 cells. Cells were treated with different concentrations of BPA 1nm/ml to 100nm/ml for 24 h. Another set of cells were also treated with selective COX-2 inhibitor, Meloxicam (40µm) along with 100nm/ml BPA for 24 h. Untreated Hep-G2 cells were taken as control. The protein was separated by SDS-PAGE on a 10% separating gel and transferred to nitrocellulose membrane [7-9]. Primary mouse monoclonal anti-HIF-1α and rabbit polyclonal anti-VEGF antibodies were added to the membrane after transfer of protein from gel. The nitrocellulose membrane was blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS (pH 7.4) at 4 °C overnight to avoid non-specific binding. The dilutions used for monoclonal antibodies were 1:500 (in 3% BSA), 1:2000 (in 3% BSA) and 1:2000 (in 3% BSA) for HIF-1α, VEGF and β-actin respectively and was incubated at 4 °C overnight with gentle shaking.

The membrane was then treated with goat anti-mouse monoclonal antibody (1:3,000) for HIF-1α and goat anti-rabbit HRP tagged antibody (1:10,000) for VEGF (in 5 % non-fat milk solution)

(Santa Cruz Biotechnology, Inc., CA) for 1 hour at room temperature. The membrane was then stripped with stripping buffer (at 70 °C for 1 hour) and reproved with monoclonal antibody against β-actin to confirm the amount of protein in each well by following the manufacturer's instruction (Santa Cruz Biotechnology, Inc., CA). ECL prime western blotting detection kit (Amersham Biosciences, Piscataway, NJ) was used for detection of protein expression and was visualized using a ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA).

Statistical Analysis: Data from cell viability assay, RT-PCR and immunoblotting band intensities were analysed using Student's *t* test and the data refers to mean ± standard deviation and are average of three values per assay of three independent experiments. Significance values are represented as $p < 0.005$ (*) which is denoted in the figures.

RESULTS AND DISCUSSION:

Influence of BPA on Cell Viability: Initially, the effect of Bisphenol A on Hep-G2 cells was evaluated. The cells were treated with 1nm/ml, 10 nm/ml and 100nm/ml concentrations of BPA and checked for cell viability using MTT assay. The cells were found to be unresponsive to the concentrations 1nm/ml and 10nm/ml of BPA. But at 100nm/ml concentration, we found significant increase in cell viability in the cancer cells. Further, treatment with 40µm of Meloxicam along with 100 nm/ml of BPA reduced the viability of the cells which could be due to the selective inhibition of Cyclooxygenase-2 by Meloxicam (**Fig. 1**). Bisphenol A (BPA, 4, 4'-(propane-2,2-diyl) diphenol) is a chemical used mostly in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics.

Ambiguity still exists in understanding the consequences of BPA on cell / tissue specific actions and effects and also in understanding the receptor systems and signalling cascades in which BPA interacts [10]. BPA functions mainly by mimicking the activity of oestrogen hormone. Correlations between oestrogen and tumors have been well established and research suggests that oestrogen promotes angiogenesis as well [11]. BPA in liver gets metabolized and enters the blood circulation and finally gets excreted with urine. The highest concentration of BPA can thus be found in the liver and kidneys. And as the metabolism of BPA occurs in the liver, the effect of BPA tends to affect liver cells more.

Hepatocellular carcinoma (HCC) is reported to be the second most frequent cause of cancer death in men worldwide and is extremely resistant to the chemotherapeutic drugs [12]. Initially we tried to determine the effect of BPA on the viability of liver cancer cells Hep-G2 using MTT assay. Although at 1 and 10nm/ml concentrations of BPA no marked difference in viability was observed, we found that at 100nm/ml concentration, BPA increases the viability of the cells significantly compared to the untreated control. As BPA is found to mimic oestrogen and also to induce inflammation, we hypothesied that BPA might be regulating the factors linking inflammation and cancer progression.

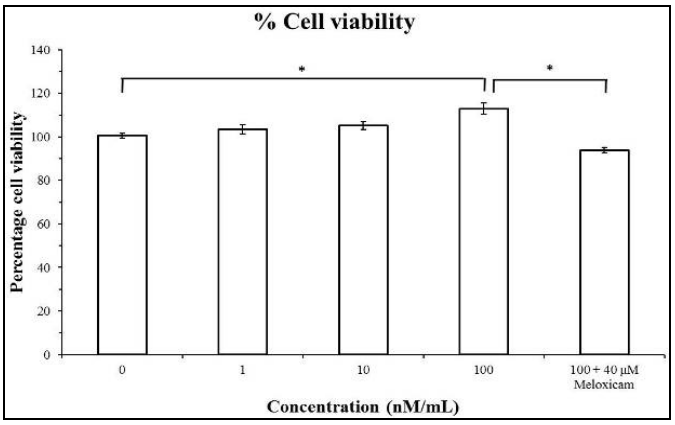

FIG. 1: DOSE DEPENDENT RESPONSE OF BPA ALONE AND WITH MELOXICAM ON THE CELL VIABILITY OF Hep-G2 CELLS

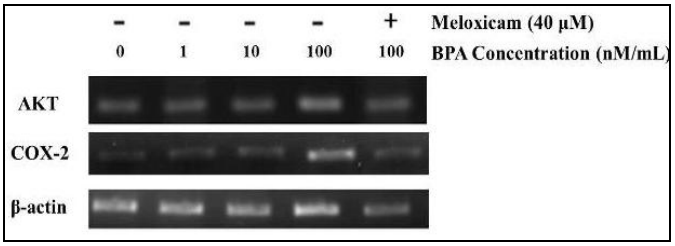

FIG. 2: RT-PCR GEL SHOWING mRNA EXPRESSION PATTERN OF Akt AND COX-2 AFTER TREATMENT WITH DIFFERENT CONCENTRATIONS OF BPA ALONE AND WITH MELOXICAM

Effect of BPA on Pro-inflammatory Genes: The transcriptional level expressions of Akt and COX-2 were found to increase in the BPA treated Hep-G2 cells. There was significant increase in the 100 nm/ml BPA treated cells compared to the untreated control cells. But when the cells were treated with

40µm Meloxicam along with 100nm/ml BPA, the expressions of both Akt and COX-2 was found to decrease significantly (**Fig. 2** and **Fig. 3**). This shows that BPA induces upregulation of pro-inflammatory molecules which could be reversed by using a selective COX-2 inhibitor.

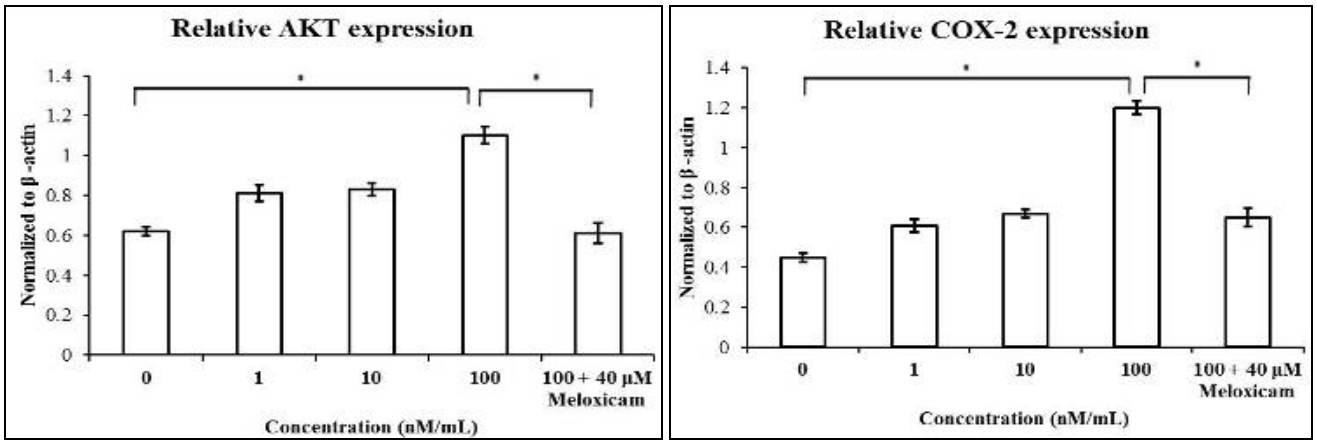

FIG. 3: RELATIVE PROTEIN EXPRESSION OF Akt AND COX-2 COMPARED TO β-ACTIN

A major component linking inflammation and cancer is Akt or Protein kinase B and COX-2 which are both pro-inflammatory in nature generally. Over-expression of both Akt and COX-2 is frequently observed in many human cancers especially liver cancers. Akt is reported to regulate the processes of tumorigenesis and cancer progression by triggering cell proliferation and inhibiting apoptosis. Activation of Akt is found to be triggered by the over-expression of COX-2 [13]. The first evidence of a potential relationship between COX-2 and human cancers was reported in 1994 by Eberhart *et al*., [14]. COX-2 has been shown to be over-expressed in various carcinomas and to have central role in tumorigenesis. The expression pattern of COX-2 protein has been well correlated with the differentiation grade in hepatocellular carcinoma, indicating that abnormal COX-2 expression plays an important role in hepato-carcinogenesis. Both tumor and stromally derived COX-2 could influence tumor angiogenesis and/or immune function [15].

In our study, we found that BPA treatment induced over-expression of both Akt and COX-2 in a dose dependent manner which would help in cancer progression. Meloxicam, used in our study, is found to target the COX-2 / MMP-2 / E-cadherin, Akt, apoptotic and autophagic pathways in COX-2-dependent and independent pathways and cause anti-tumor activity. Meloxicam also inhibits cell autophagy which helps in overcoming the resistance to meloxicam-induced apoptosis in HCC. Meloxicam (40µM) was used to inhibit COX-2 expression since at this concentration it was found to induce 25% growth inhibition in the Hep-G2 cells [16]. We used Meloxicam to selectively inhibit COX-2 expression to check the effect it had on the viability of the cells. It was observed that Meloxicam treatment significantly reduced the cell viability which was found to be increased with BPA treatment. This showed that BPA induced the COX-2 / Akt pathway to enhance cell viability and can be inhibited by using selective COX-2 inhibitor. Targeting COX-2 appears to be a promising strategy in prevention and treatment of cancer [17].

Effect of BPA on Pro-angiogenesis Proteins: The expression studies of proteins HIF-1α and VEGF showed that cells treated with increasing concentrations of BPA (1 to 100nm/ml) induced higher expression of these proteins. Further, treatment of cells with both 100nm/ml and 40µm Meloxicam showed a significant decrease in the expression of both proteins (**Fig. 4** and **Fig. 5**).

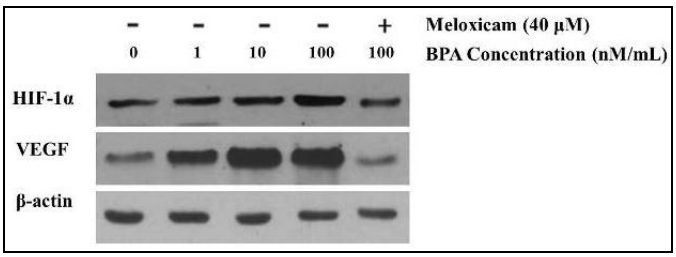

FIG. 4: WESTERN BLOT OF HIF-1α AND VEGF AFTER TREATMENT WITH DIFFERENT CONCENTRATIONS OF BPA ALONE AND WITH MELOXICAM

This result indicates that upstream inhibition of COX-2 using a selective COX-2 inhibitor, Meloxicam in the current study, down regulates the expression of pro-angiogenesis proteins.

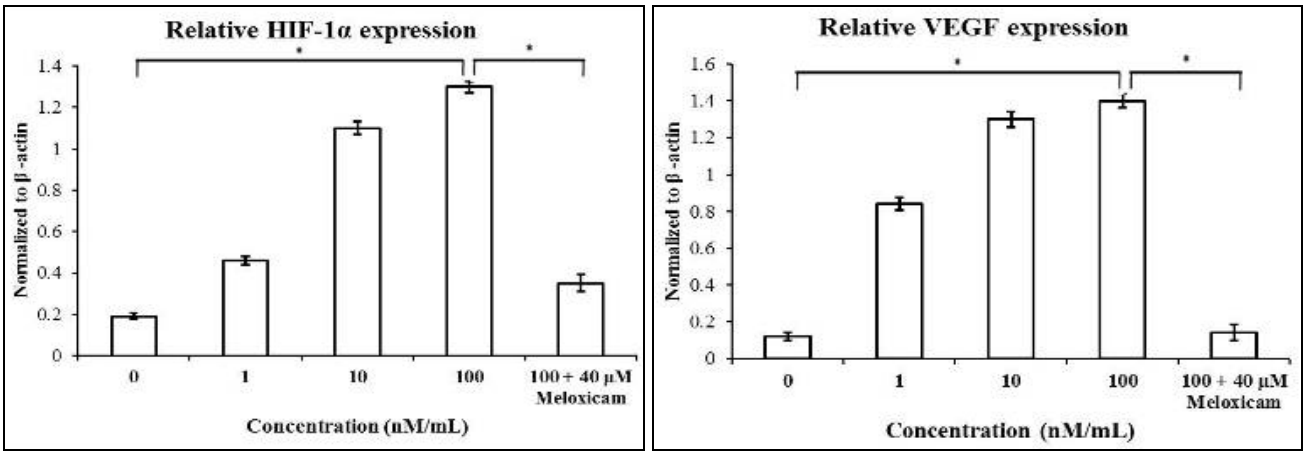

FIG. 5: RELATIVE PROTEIN EXPRESSION OF HIF-1α AND VEGF COMPARED TO β-ACTIN

HIF-1α expression in HCC is considered to be a negative prognostic factor for clinical outcome after surgery and is associated with metastatic potential [18]. A plethora of pathways that regulate cell metabolism, proliferation, angiogenesis and metastasis are found to be regulated by HIF-1α. HIF-1α is also reported to directly induce up-regulation of vascular endothelial growth factor (VEGF) which has a major part in the process of angiogenesis [19]. Vascular endothelial growth factor (VEGF) is widely reported to be an inducer of angiogenesis. It acts as a highly specific mitogen for endothelial cells. VEGF induced signal transduction proceeds by the binding of these factors to tyrosine kinase receptors resulting in endothelial cell proliferation, migration, and angiogenesis [20]. The immunoblotting result confirms the up-regulation of HIF-1α and VEGF in the BPA treated cells whereas expressions of HIF-1α and VEGF were found to be significantly inhibited in the BPA + Meloxicam treated cells.

In the present study we found that BPA increases cell viability of cancer cells at lower concentrations and induces the overexpression of Akt, COX-2, HIF-1α and VEGF, which are generally found to aid cancer progression. The treatment with Meloxicam, a COX-2 inhibitor, reduced the cell viability in HepG2 cells and treatment with Meloxicam was found to down regulate the expression of Akt, COX-2, HIF-1α and VEGF, thus, corroborating our hypothesis that BPA helps in cancer progression by up regulating the factors that link inflammation and cancer progression.

CONCLUSION: There are ambiguities still existing in understanding how Bisphenol A interacts with cells and tissues. Our study focussed on understanding the mechanism of action of BPA and how it interacts and regulates the major molecules involved in inflammation and cancer progression. The study shows that BPA at very low concentrations up regulates the expression of pro-inflammatory and pro-angiogenesis proteins namely, Akt, COX-2, HIF-1α and VEGF in the liver cancer cells. The use of a selective COX-2 inhibitor, Meloxicam, was found to decrease the expression of these proteins significantly. Taken together, these results indicate that Bisphenol A acts on targets in the inflammatory pathway and up regulates their expression thereby helping in cancer progression and poor prognosis in liver cancers.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interests.

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