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EVALUATION OF MICROBIAL FLORA CHANGES IN BENZO (A) PYRENE INDUCED PULMONARY CARCINOMA IN *Mus musculus*

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ABSTRACT

This study mainly focused on to screen normal microbial flora changes in benzo (a) pyrene induced pulmonary carcinoma in *Mus musculus*. Benzo (a) pyrene is an important environmental carcinogenic chemical which cause number of health problems including lung cancer in human. In this present investigation there are numerous changes have been observed in normal microbial flora and newly invaded microbe in the animals subjected to lung cancer research. From our examination twenty three different bacterial species, three fungal species and four yeast species have been recorded. There was no normal flora changes have acquired in control and the animal treated with an extract of *Acanthus ilicifolius* L. which is an important mangrove associate. And slight changes with minimum microbial load only acquired in the root and root callus extract treated animals pretreated with benzo (a) pyrene, may be due to apoptosis. Extreme changes and maximum microbial load was observed from benzo (a) pyrene-treated animals. Hence the study concluded that correlation between the microbial load and environmental carcinogens induced cell proliferation, microbial load was gradually decreased along with the animal recovery treated with *Acanthus ilicifolius* L extract.

Key words: *Acanthus ilicifolius* L., benzo (a) pyrene, microbial flora, apoptosis and pulmonary carcinoma.

[I] INTRODUCTION

Tobacco causes human deaths, more than by all deaths from human immunodeficiency virus (HIV), illegal drug use, alcohol use, and motor vehicle injuries, suicides, and murders combined [1]. Smokers die 14 years earlier than non-smokers [2]. Tobacco smoking is a leading cause of human cancer and deaths which are higher in developing countries than in the developed countries. Lung cancer is an aggressive and heterogeneous disease and most common malignant tumours worldwide [3]. These organs are affected directly or indirectly by cigarette smoking and tobacco use.

Like all cancers, lung cancer cells have the ability to invade neighboring tissues and spread to distant parts of the body. Which accounts for more than 80% of lung cancers and it is the most common cause of cancer deaths worldwide [4].

Benzo(a)pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that is a byproduct of incomplete combustion or burning of organic (carbon-containing) items, e.g., cigarettes, gasoline, and wood. Pure forms of these BaP crystals are pale yellow and needle-like with faint odor [5]. BaP is commonly found with other PAHs

in cigarette smoke, in grilled and broiled foods, and as a by-product of many industrial processes [5]. It is also found in outdoor, indoor air, and in some water sources naturally [5]. BaP is metabolized (chemically modified in the body) in humans and animals to form a number of metabolites that may elicit toxicity [5]. BaP and BaP metabolites can bind to DNA forming a structure called BaP-DNA adducts. The formation of BaP-DNA adducts can interfere with or alter DNA replication (formation of DNA copies during cell division), and may be associated with an increased risk of several forms of cancer [5]. Also it is estimated that over 15% of malignancies worldwide can be attributed to infections or about 1.2 million cases per year [6]. Infections involving viruses, bacteria and schistosomes have been linked to higher risks of malignancy. Important mechanisms by which bacterial agents may induce carcinogenesis include chronic infection, immune evasion and immune suppression [7]. Tumorigenesis is initiated when cells are freed from growth restraints, later promotion results when the immune system is evaded favoring further mutations and increased loss of cell control. So the loss of immune system may be due to the microbial accumulation. Hence the present investigation aimed to identify the microbial population in benzo (a) pyrene-induced pulmonary carcinoma in *Mus musculus.*

[II] MATERIALS AND METHOD

2.1. Chemicals Used for the Study

Carcinogen Benzo (a) pyrene (B[a]P) was purchased from Sigma, USA and used for this study. All other reagents used were of analytical grade.

2.2. Experimental animal model used

The male albino mice were used as experimental animals with an age of 8-10 weeks weighing 90-110 g obtained from the central animal facility house, Annamalai University, India. The animals were housed at six per polypropylene cage and provided with standard pellet diet (Amrut rat/mice feed; M/s. Hindustan Unilever Ltd, Mumbai, India) and water *ad libitum*. The animals were maintained at a temperature of 28±2ºC with an alternating 12-h light/12-h dark cycle. The animals were maintained as per the norms provided by the Bioethic Committee of Annamalai University (**Ethical number: 160/1999/CPCSEA**).

2.3. Callus Induction

Plant tissue culture technique is not only used to protect the plant species also it is used to enhance the production of plant metabolites. In this study root and root callus of *Acanthus ilicifolius* L. have been used as drug to cure the benzo (a) pyrene-induced pulmonary carcinoma. For callus induction the young root explants aseptically inoculated after surface sterilization on MS medium supplemented with different growth regulators like NAA, IAA, IBA and 2,4-D form the ratio of 0.1 to 3.0 mg/L individually and in combination. Then the developed root callus and root extracts with 8:2 ratio of methanol:chloroform were subjected to pulmonary cancer treatment.

2.4. Design of the experiment

The animals were randomized into experimental, control groups, and divided into six groups of six animals each. Animals in group I was treated as untreated control (olive oil). The animals in group II were administered with benzo (a) pyrene (B[a]P) (50mg/kg body weight dissolved in olive oil) using a No. 0.6 mm feeding tube twice a week for 4 weeks. Group III animals were given with benzo (a) pyrene as in group II, in addition to that they were administered with root extracts of *Acanthus ilicifolius* L. at a dose of 10 mg/kg body weight daily for 16 weeks. Group IV animals were given with benzo (a) pyrene as in group II; in addition, the animals were administered with root-callus extracts of *Acanthus ilicifolius* L. at a dose of 10 mg/kg body weight daily for 16 weeks. Group V animals received only the root extracts of *Acanthus ilicifolius* L. at a dose of 10 mg/kg body weight daily for 16 weeks. Group VI animals were

given with root-callus extracts of *Acanthus ilicifolius* L. at a dose of 10 mg/kg body weight daily for 16 weeks. The experiment was terminated at the end of 16th week and the samples were analyzed.

2.4.1. Animal weight:

Animal weight was recorded in every week.

Animal weight = initial weight of the animal – final weight of the animal

Tumor volume: Tumor volume was calculated by the water displacement method.

Tumor burden:

Tumor burden = number of tumor × volume of tumor.

2.5. Sampling, microbial isolation and identification

Qualitative screening of aerobic and facultative anaerobic organisms from animals induced with pulmonary carcinoma has been done. Saliva, mucosa-associated biofilm samples from upper respiratory tracts and cancerous cell lesions were collected and microorganisms were detected by cultural-morphological characteristics, biochemical analyses and polymerase chain reaction (PCR).

2.5.1. Plating: Animals were sacrificed under sterile condition and samples were taken aseptically from upper respiratory tract and lower respiratory tract (cancerous lung tissues) under laminar flow hood and transferred to a solution consisting of 500 µL of PBS (phosphate buffered solution) and 500µL of ultra pure water. The samples were processed within 2 hours. Homogenized samples were inoculated in peptone water, potato dextrose broth and Sabouraud dextrose broth with 100 µg/ml of chloramphenicol (HIMEDIA, Mumbai, India) and incubated for 3-7 days at room temperature. Subsequently, from microbial growth observed in peptone water, serially diluted and aliquots of 0.1 ml were transferred to Mannitol salt agar, Brain Heart Infusion agar supplemented with defibrinated sheep blood, Cetrimide agar, Eosin Methylene Blue agar, Deoxycholate Citrate agar, Loeffler serum medium, MacConkey agar, Xylose Lysine Deoxycholate agar and Bile Esculin agar and incubated in aerobiosis for 48 h at 37°C. Aliquots of 0.1 ml from pre-inoculated broth were also transferred to Lowenstein Jensen medium and incubated for 1-2 weeks at 42°C. Aliquots of 0.1 ml from pre-inoculated potato dextrose broth were transferred to potato dextrose agar and incubated at room temperature, for 3-7 days. Samples were also inoculated onto Sabouraud dextrose agar with 100 µg/ml of chloramphenicol and incubated at room temperature, for 3-7 days, for yeasts isolation.

2.5.2. Identification:

Bacterial species were identified using the following methods: colonial characteristics, Gram-staining, acid fast staining, growth at 10% sodium chloride, production of gas from glucose, and biochemical tests using the HiIMVIC™ commercial kit (HIMEDIA, Mumbai, India) and were identified by following the keys of Bergy's manual of determinative bacteriology [8, 9]. The yeast identification was performed by carbon and nitrogen assimilation tests, fermentation of carbohydrates, germ tube formation (at 37°C and at 39°C), colonial morphology on Candida agar (HIMEDIA, Mumbai, India), and growth at 37°C and 42°C. The isolates with inconclusive identification to species level were performed by DNA extraction and amplification following the methodology previously described, using PCR with universal bacterial primers [10].

2.5.3. PCR assay

1.5 ml of overnight grown bacterial culture was taken and DNA was extracted according to the method of Sambrook and Russell [11]. The genomic DNA extracted from the bacterial strain was PCR amplified for 16S rRNA gene using the universal bacterial primers: 27F (5'-AGAGTT TGA TCM TGG CTC AG- 3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3').

Concentrations of bacterial DNA were determined with a spectrophotometer (Beckman Instruments, Richmond, Washington, USA) at A_{260} nm. Amplification reaction was carried out in a 0.2 ml optical-grade PCR tube (Tarsons, India). 50 nanogram of DNA was added to a final volume of 50 µl of PCR reaction mixture containing 1.5mM $MgCl_2$, 1X Reaction buffer (without $MgCl_2$) (GeNei), 200µM of each dNTPs (GeNei), 100 pM of each primer and 1.5U Taq DNA polymerase (GeNei). PCR was performed in a thermalcycler (Tech Gene) with an initial denaturation at 95°C for 5 min. followed by 30 cycles of 95°C for 30sec (denaturation), 52°C for 45sec (annealing), 72°C for 90 sec (extension) and 72°C for 10min (final extension). Amplification product was run on 1.5% agarose in 1X TAE buffer [40mM Tris, 20mM Acetic acid, 1mM EDTA (pH8.0)] stained with ethidium bromide (0.5 mg/mL). The PCR product was purified using the QIAGEN PCR purification kit and DNA sequencing was carried out by using 3730 Genetic analyser, Applied biosystems, USA (Ramachandra Innovis, Chennai, India).

[III] RESULTS

This is the study mainly focused on to screen the microbial population present in the benzo (a) pyrene-induced lung cancer of *Mus musculus.* In which root and root derived callus extract were used as drug to cure the benzo (a) pyrene-induced pulmonary carcinoma. The calli were developed from the MS medium fortified with 2.5 mg/l 2,4-D and 0.5 mg/L NAA.

In this examination number of microbial flora like bacteria, fungi and yeasts have been identified and confirmed using cultural-morphology, biochemical assays and polymerase chain reaction (PCR), the results were presented in the table 1. The normal bacterial flora like *Streptococcus pyogenes, Staphylococcus aureus, Micrococcus* sp., *Bacillus cereus, Escherichia coli, Enterobacter aerogenes, Pseudomonas aeruginosa, Salmonella* sp., *Actinomyces* sp. and *Corynebacterium* sp. were higher in benzo (a) pyrene-induced lung cancer animals group 2 and some of these organisms were higher in group 5 in which the animals were treated with root extract of *Acanthus ilicifolius* L. These bacterial species were significantly higher than in the olive oil control group 1. Similarly higher load of fungi like *Trichoderma* sp. and *Aspergillus fumigatus* were found in the group 2 and five as like bacterial examinations. The yeasts like *Candida* sp. and *Saccharomyces boulardii* were significantly higher in group 2.

Apart from the normal micro flora studied in the experimental animals, some of the new species also were identified in the animals subjected to this present investigation especially from group 2 to 4, which included *Vibrio vulnificus, Citrobacter rodentium, Proteus mirabilis, Mycobacterium* sp., *Haemophilus* sp., *Aeromonas hydrophila, Chlamydia pneumoniae, Helicobacter* sp. Except *Proteus mirabilis, Haemophilus* sp. and *Chlamydia pneumoniae* these all were present in very minimum number. Fungal species like *Aspergillus flavus* and yeasts species *Saccharomyces boulardii* and *Cryptococcus* sp. have been identified in the group 2 to 4. These new microbial flora were not found in the control group 1, root and root-callus extracts treated group 5 and 6. Among the total bacterial species *Chlamydia pneumoniae* has been found to be the predominant.

[IV] DISCUSSION

In general cancer is defined as the uncontrolled proliferation and accumulation of abnormal cells that have enough DNA damage to be freed from the normal restraints of the cell cycle [12]. Several pathogenic bacteria, particularly those that can establish a persistent, infection, can promote or initiate abnormal cell growth by evading the immune system [13]. Bacterial species or their toxins can alter host cell cycles or stimulate the production of inflammatory substances linked to DNA damage [14]. Also several micro-organisms having capable of converting alcohol, cigarette

smoke and some other organic non-toxic chemicals into carcinogen [15]. Benzo (a) pyrene is a second most important carcinogenic chemical abundantly present in the cigarette smoke.

Though the pulmonary carcinoma is induced by the environmental carcinogen benzo (a) pyrene, the present study revealed that the microbial interactions also, which may play a role in the severe cell proliferation, suppressing the apoptosis and further infections Fig. 1, as supporting evidence of these results also reported in *Helicobacter pylori* induced gastric epithelial cell apoptosis [16]. In addition to this evidence a substantial number of bacterial pathogens have been putatively linked to cancer. As early as 1772, *Mycobacterium* species was thought to cause malignancy (17). It was observed from our experiment that the *Mycobacterium* species have been obtained in group 2, benzo (a) pyrene treated animals. The bacterial species associated with cancer etiology are diverse; however, the infections they cause share common characteristics [7]. Similar to other cancers the pulmonary carcinoma also showed almost same characteristics like microbial association, when the cancer burden decreased microbial load also gradually decreased especially the cancer risks were significantly higher microbial association in benzo (a) pyrene treated animals compared to control and animals treated with root and root callus alone (group 1, 5 and 6). This association gradually decreased in the group 3 and 4 pretreated with benzo (a) pyrene. In contrast the species *Chlamydia pneumoniae* has found to be the most predominant among the total microbes were detected from this present study. Similarly the association of *Chlamydia pneumoniae* with pulmonary carcinoma has been found in earlier research reports [18-20]. In addition to the normal flora so many new microbial populations were detected, it may also competently involved in the DNA adduct formation and cell proliferations.

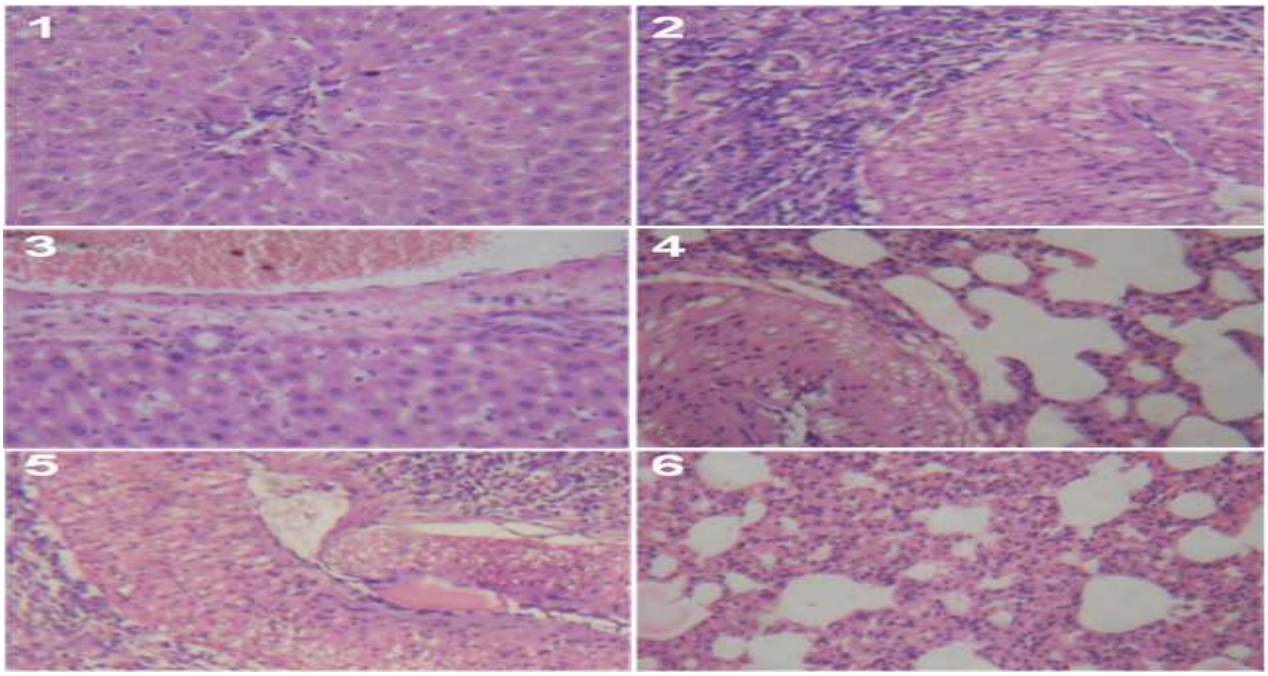

Fig.1. Histological changes in the lung tissue of Mus musculus. (Group 1) Control, (Group 2) treated with benzo (a) pyrene, (Group 3) treated with benzo (a) pyrene + root extract of *A. ilicifolius*, (Group 4) treated with benzo (a) pyrene + root-callus extract of *A. ilicifolius*, (Group 5) treated with root extract of *A. ilicifolius* alone, (Group 6) treated with root-callus extract of *A. ilicifolius* alone (magnification 40 x).

ANIMAL MODEL	1	2	3	4	5	6
Treatment	**Control**	**Benzo (a) pyrene**	**Root extract + B(a)P**	**Root Callus extract + B(a)P**	**Root extract**	**Root Callus extract**
Animal body weight (gm)	**110**	**78**	**92**	**98**	**105**	**104**
Tumour Volume (ml)	**0.00**	**3.85**	**2.15**	**1.15**	**0.00**	**0.00**
Streptococcus pyogenes	+	+++	++	-	++	+
Streptococcus salivarius	-	+	+	-	+	-
Staphylococcus aureus	+	+++	+	+	+	+
Staphylococcus saprophyticus	-	+	-	-	+	-
Micrococcus sp.,	++	+++	++	++	+++	+
Bacillus cereus	+	++	+	+	++	+
Bacillus subtilis	+	+	-	-	++	+
Escherichis coli	+	++	-	-	+	-
Enterobacter aerogenes	+	++	+	+	+	-
Klebsiella oxytoca	-	++	-	-	+	-
Pseudomonas aeruginosa	++	+++	++	++	+	+
Pseudomonas fluorescens	+	+	-	-	-	+
Vibrio vulnificus	-	+	-	-	+	-
Citrobacter rodentium	-	+	+	-	+	+
Salmonella sp.,	+	++	+	-	++	-
Proteus mirabilis	-	+++	-	-	+++	+
Mycobacterium sp.,	-	+	+	-	+	+
Haemophilus sp.,	-	+++	-	-	-	-
Actinomyces sp.,	+	++	+	+	+++	++
Aeromonas hydrophila	-	++	+	+	+	-
Chlamydia pneumoniae	-	+++	+++	+	+	-
Helicobacter sp.,	+	++	+	+	-	-
Corynebacterium sp.,	+	+++	++	+	+	-
Aspergillus fumigatus	+	+	-	-	++	+
Aspergillus flavus	-	++	-	-	++	-
Trichoderma sp.,	+	+++	+	+	+++	+
Candida sp.,	+	+++	+	-	+	-
Saccharomyces albicans	+	++	++	++	+	-
Saccharomyces boulardii	-	+++	+	+	-	+
Cryptococcus sp.,	-	+++	-	-	-	-

Table 1. Investigation of microbial flora changes in benzo [a] pyrene-induced pulmonary carcinoma

[V] CONCLUSION

The present study clearly indicates the contribution of microbial population in pulmonary carcinoma incidence along with benzo (a) pyrene. And increase and decrease of microbial load and invasion of new microbial flora is a literal evidence of pulmonary carcinogenesis and its severity. The decrease of microbial load may be due to apoptosis by our natural drugs like root and root-callus of *Acanthus ilicifolius* L.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

[1] McGinnis, J, Foege, W.H, (1993), Actual causes of death in the United States, J Am Med Assoc. 270, 2207-2212

[2] Center for Disease Control and Prevention (CDCP), (2002), Annual smoking attributable mortality, years of potential life lost, and economic costs - United States, 1995-1999. Morbid Mortal Weekly Report 51, 300-303

[3] Dickson, S.A, (1954), Panacea or precious bane. Tobacco in 16th century literature. New York, New York Public Library.

[4] Feinhandler, S.J, Fleming, H.C, Monahan, J.M, (1979), Pre-Columbian tobaccos in the Pacific, Economic Botany. 33, 213-226

[5] U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR), (1995), "Toxicological Profile for Polyaromatic Hydrocarbons (PAHs)." http://www.atsdr.cdc.gov/toxprofiles/tp69.html

[6] Pisani, P, Parkin, D.M, Munoz, N, Ferlay, J, (1997), Cancer and infection: estimates of the attributable fraction in 1990, Cancer Epidemiol Biomarkers Prev. 6, 387-400

[7] Kuper, H, Adami, H.O, Trichopoulos, D, (2000), Infections as a major preventable cause of human cancer, J Intern Med. 248, 171-83

[8] Holt, J.G, Krieg, R.N, Sneath, P.H.A, Staley, J.T, Williams, S.T, (1983), Bergey's Manual System, Bacteriology. 4, 440

[9] Oliver, J.D, (1982), Instruments and methods: taxonomic scheme for the identification of marine bacteria, Deep Sea Res. 29, 795-798

[10] Weisburg, W.G, Barns, S.M, Pelletier, D.A, Lane, D.J, (1991), 16S Ribosomal DNA Amplification for Phylogenetic Study, J Bacteriol. 173(2), 697-703

[11] Sambrook, J, Russel, D.W, (2001), *Molecular Cloning*. 3rd edition. 3 vol. Cold Spring Harbor Laboratory Press, New York.

[12] Mager, D.L, (2006), Bacteria and cancer: cause, coincidence or cure? A review, Journal of Translational Medicine. 4(14), 1-18

[13] Tsujii, M, DuBois, R.N, (1995), Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2, Cell. 83, 493-501

[14] Parsonnet, J, (1995), The incidence of *Helicobacter pylori* infection, Aliment Pharmacol Ther. 9(2), 45-51

[15] Meurman, J.H, (2010), Oral microbiota and cancer, J Oral Microbiol. 2, 5195-5204.

[16] Cover, T.L, Krishna, U.S, Israel, D.A, Peek, R.M, Jr, (2003), Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin, Cancer Res. 63, 951-7

[17] Onuigbo, W.I, (1975), Some nineteenth century ideas on links between tuberculous and cancerous diseases of the lung, Br J Dis Chest. 69, 207-210

[18] Littman, A.J, White, E, Jackson, L.A, Thornquist, M.D, Gaydos, C.A, Goodman, G.E, Vaughan, T.L, (2004), Chlamydia pneumoniae infection and risk of lung cancer, Cancer Epidemiol Biomarkers Prev. 13, 1624-1630

[19] Koyi, H, Branden, E, Gnarpe, J, Gnarpe, H, Steen, B, (2001), An association between chronic infection with Chlamydia pneumoniae and lung cancer. A prospective 2-year study, APMIS. 109, 572-580

[20] Anttila, T, Koskela, P, Leinonen, M, Laukkanen, P, Hakulinen, T, Lehtinen, M, Pukkala, E, Paavonen, J, Saikku, P, (2003), Chlamydia pneumoniae infection and the risk of female early-onset lung cancer, Int J Cancer. 107, 681-682.