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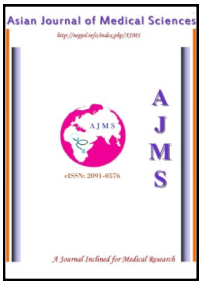

Blood levels of Polycyclic Aromatic Hydrocarbons in Women with Benign and Malignant Breast Lesions: A case-control study

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Abstract

Objective: As part of our program to investigate the possible role of environmental pollutants in the incidence of breast cancer in India, we conducted for the first time a hospital based case-control study where blood polycyclic aromatic hydrocarbons (PAHs) levels were determined in women suffering from benign and malignant breast lesions, and compared with those of disease free controls drawn from similar socioeconomic environment residing in and around New Delhi, India.

Material & Methods: Anthracene, phenanthrene, fluoranthene, naphthalene, pyrene, benzo (a) pyrene, benzo (k) fluoranthene and dibenzo (a,h) anthracene were determined by HPLC-FD.

Results: Level of total PAHs in control, benign and malignant groups (30 numbers in each) were 142.05 ± 50.84, 185.99 ± 61.97 and 200.74 ± 55.05 µg / L respectively. Mean levels of naphthalene, phenanthrene, pyrene and benzo (k) fluoranthene were higher in both malignant and benign groups than in control but the difference was not statistically significant. Of the total PAHs, 3-ringed compounds were found much higher (89%) in controls than in benign (52%) and malignant groups (54%). However, the percentage sum of 2, 4 and 5-ringed PAHs were much higher in malignant (46%) and benign (48%) groups when compared with those of controls (11%).

Conclusion: Results of the present study indicate that higher levels of PAHs (especially non-carcinogenic), though statistically non-significant, were present in cases with benign and malignant breast lesions than in those of controls.

Key Words: Polycyclic Aromatic Hydrocarbons; Breast cancer; Benign lesions; HPLC - FD

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), some of which closely resemble steroid hormones, are ubiquitous environmental contaminants produced from combustion products of fossil fuels, cigarette smoking and in grilled and smoked foods.[1] In India, PAHs were determined in air[2], water, river sediments[3], and also in some food items.[4] These compounds are lipophilic and therefore, stored in human milk, blood, placenta[5] and other fat tissues including breast fat and attain high local concentrations even at the low levels of exposure.[6] They are well established mammary carcinogens in rodents[7] Although some PAHs have been categorized by the Environmental protection agency, USA as probable or possible human carcinogens[8], their carcinogenic effects on the breast in women have not been clearly demonstrated.

On the other hand cancer of the breast is the third most common cancer in the world resulting into death of 376, 000 women annually.[9] Incidence of breast cancer is still higher in developed countries while burden of the disease in India is alarming,[10] accounting for 23% of all female cancers in metropolitan cities such as Mumbai, Calcutta and Bangalore.[11] Approximately 50% women who developed breast cancer have no identifiable risk factors beyond increasing age and gender.[12] Several reports identify certain PAHs which clearly resemble steroid hormones as example of environmental estrogens[13-15] and human exposure to non-steroidal environmental estrogens is thought to be a risk factor for endocrine disruption and development of cancer of the breast.[16] An increase in breast cancer rate is reported among communities exposed to creosote, which includes multiple compounds

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including PAH, through contamination of the water supply.[17] Several studies documented a potential role for PAH in breast carcinogenesis in women, measuring PAH-DNA adducts by 32P postlabelling method.[18-22] Our earlier studies have also shown that certain persistent environmental chemicals in blood have the potential to play a role in the development of hormone dependent non-malignant conditions such as benign breast lesions and benign prostate hyperplasia.[23] Although being endocrine disruptors, we were unable to find study in literature which reports blood PAHs levels in breast cancer patients, as well as in general population. It was therefore thought reasonable to determine blood PAHs in women identified to be suffering from benign or malignant breast lesions and to compare them with those from disease free controls.

2. Material and Methods

2.1. Sampling

This investigation consisted of three subject groups, one designated as study group (n=30) with a final diagnosis of malignant breast disease; the other with a final diagnosis of benign breast lumps (n=30) and the third group consisting of disease free controls (n=30) drawn from the similar socioeconomic status, in order to obtain the preliminary data on the blood PAHs levels in these groups of women, as a pilot study. The females who presented to the General Surgery and Surgical Oncology OPD at Sir Ganga Ram Hospital, New Delhi (Jan 2002 - Dec 2003) with a palpable lump in the breast were included in the study. The inclusion criteria for the study group were females with palpable lump in the breast indicating the excision of lump, consent for surgery and histopathology to confirm benign or malignant lesion. The control group included those subjects who were not suffering from any chronic disease (s) like cardiovascular, kidney, diabetes and hypertension etc. Since breast cancer is generally a disease of the elderly, being rare below the age of 35 yrs, we included only patients above 30 yrs in the control group to allow a proper age matching of the study and the control groups. There was however, no upper age limit for either group. These women were from the population residing in and around New Delhi and were representative of the population.

2.2. Examination of subjects

Personal details of the subjects were recorded including the factors that influence the risk of breast cancer like age at menarche and menopause, total duration of breast feeding, history of any hyper-estrogenic states like endometrial carcinoma, ovarian carcinoma or endometriosis by medical collaborator of the study from Sir Ganga Ram Hospital, New Delhi. Family history if any, of breast carcinoma was noted together with addiction of tobacco and alcohol. A detailed clinical examination was performed by qualified medical persons. The lump, the skin, the nipple areola complex, chest wall, axilla for lymph node, abdomen and any sites of bone pain were thoroughly examined. A clinical diagnosis of a benign breast disease or breast carcinoma and the stage were formulated. A fine needle aspiration cytology (FNAC) and mammography was performed to confirm the nature of the lesion. Hypotheses and other details of the study were described to participants before sample collection and their consent was obtained. Additionally Institutional ethics committee clearance was also obtained for collecting the human blood samples.

2.3. Sample collection

Approximately 2 ml of blood was withdrawn from all subjects and stored in pre-heparinized glass vials. All the samples were coded and transported under ice-cold condition to the Analytical Toxicology Lab, ITRC, Lucknow for PAH analysis. The samples were stored at -20 ºC until analysis. The blood sample was thawed in water bath just prior to the analysis. The analytical toxicologist was totally blind to the medical history and final diagnosis of the subjects.

2.4 Chemicals and reagents

All the chemicals, solvents and water used were of analytical or HPLC grade. Hexane, acetonitrile and SPE cartridges LiChrosep RP 18 (500 mg) were procured from Merck, Darmstadt, Germany. Individual standards of all 16 polycyclic aromatic hydrocarbons (EPA Priority list) were purchased from Supelco, Bellefonte, (PA, USA). Purity of PAH standards were in the range of 93.4 - 99.7%. All standards were dissolved separately in acetonitrile to make stock solution; working standard solution was made by mixing stock solution of each compound at different concentration in amber colored volumetric flask (to prevent light exposure) and stored at 4 ºC in refrigerator till analysis.

2.5. Analyses of PAHs

Extraction of polycyclic aromatic hydrocarbons from blood was carried out according to the method reported by Van Schooten et al.[24] Liquid-liquid extraction was performed by n-hexane and before the HPLC analysis, n-hexane phase was changed to acetonitrile. SPE cartridges, conditioned with acetonitrile were used for clean up

process. The aliquot of final sample was analyzed on HPLC -FD at A RP (Reverse Phase) C-18 ODS analytical column (75 mm x 4.6 mm i.d., 3.5 mm particle size), with a pre-column of the same phase from Waters (Water Milford, MA, USA) for different PAHs other than acenaphthylene (due to no response by FD). The elution conditions and detection wavelength program was same as reported by Barranco et al.[25] during polycyclic aromatic hydrocarbons analysis in edible oils. Solvents that constituted the mobile phase were acetonitrile (A) and water (B). The elution conditions were: 0–10 min, 50% A isocratic; 10–24 min, linear gradient 50% A–100% A; 24–35 min, 100% A isocratic; and finally, back to the initial conditions and recondition the column. An aliquot from same sample was reanalyzed in isocratic mode using UV detector (254 nm) only for acenaphthylene. The flow rate was maintained at 1.0 ml / min and the injection volume was 20 µl. Recoveries, which were calculated by using observed and spiked concentrations for PAHs ranged from 78 - 94% for all PAH compounds.

Further confirmation was done by Gas chromatography – mass spectrometer using model auto system XL (Perkin-Elmer, USA) coupled with a Turbo Mass detector. GC conditions and temperature programming was same as described by Poon et al.[26] The mass detector operated in electron impact at 70 eV in full scan. The target compounds were quantified in the selected ion monitoring (SIM) mode, using the molecular ion and one qualifier ions for each compound as described by king et al.[27]

2.6. Statistical analysis

The variation in PAHs values in the three groups was found to be heterogeneous. So in order to make the data follow normal distribution, the different PAH values were transferred to, Y (transformed variable) = Log10 (x+10) {where x is the original value}

One-way analysis of variance (ANOVA) was used to test the statistically significant differences of continuous co-variants among control, benign and malignant groups presented in Table-1. For discrete variables Chi Square test was applied to see the statistical significance.

3. Results

Table-1 demonstrates the characteristics of the subjects. Demographic characteristics heights, weight, BMI (body mass index) were not different in the three groups.

Reproductive features like age at menarche, age at menopause, breast-feeding months were also not significantly different. However, age of the subjects was found to be significantly different among three groups ($p<0.05$).

Table-1: Characteristics of the subjects with benign & malignant breast lesions and disease free control

Variables		Control (n = 30) Mean ± SD	Benign (n = 30) Mean ± SD	Malignant (n = 30) Mean ± SD
Age (years)		43.55 ± 12.32	44.2 ± 9.36	51.36 ± 11.32*
Height (cm)		148.38 ± 9.77	155.68 ± 6.18	156.18 ± 14.48
Weight (Kg)		56.26 ± 9.39	57.2 ± 9.96	61.8 ± 10.6
BMI (Kg/m2)		25.52 ± 5.79	23.41 ± 3.41	26.13 ± 3.88
Age at menarche (years)		14.21 ± 1.39	13.93 ± 1.33	13.89 ± 1.37
Age at meno-pause (years)		46.8 ± 4.68	43.7 ± 6.40	45.43 ± 3.64
Breast Feeding (Months)		33.56 ± 23.08	33.44 ± 26.32	36.04 ± 35.48
Family History		0	0	0
Lump size (cm)		-	3.33 ± 1.71	4.91 ± 2.54*
Lump Side	Left	-	18 (60%)	17 (57%)
	Right	-	12 (40%)	13 (43%)
Addiction (Tobacco/ alcohol)		0	0	0
Area of living	Rural	7 (23%)	0 (0%)	2 (7%)
	Urban	23 (77%)	30 (100%)*	28 (93%)

Values represent mean ± SD or No. of subjects (%).One-way ANOVA was applied for determining statistical significance of mean values among the subjects with benign and malignant breast lesions and disease free controls. Student t-test was applied to compare the size of lump between benign and malignant. Chi-square was used to test the significance of area of living among the groups.*$p<0.05$

Table-2: Frequency of blood PAHs in the subjects with benign and malignant breast lesions and disease free control

Frequency of Blood PAHs	Control Number (%)	Benign Number (%)	Malignant Number (%)
Anthracene	(12) 39%	(16) 52 %	(4) 13%
Phenanthrene	(8) 26%	(9) 29%	(7) 23%
Fluoranthene	(7) 23%	(2) 6%	(7) 23%
Naphthalene	(7) 23%	(8) 26%	(8) 26%
Acenapthylene	(5) 16%	(3) 9%	(8) 26%
Pyrene	(2) 6%	(3) 9%	(4) 13%
Benzo (a) pyrene	(1) 3%	(2) 6%	ND
Benzo (k) fluoranthene	(1) 3%	(2) 6%	(1) 3%
Dibenz (a, h) anthra-cene	ND	ND	(2)6%

No subject from any group was found addicted to alcohol / tobacco and no one had the family history of any breast disease.

Size of the lump was found higher in malignant than benign group (4.91 vs 3.33 cm, $p<0.05$). 60% benign and

57% malignant cases had the lump left side, while 40% in benign and 43% in malignant group had it right side. All the subjects in benign group were urban- being significantly different from those of controls ($p<0.05$) having 77% urban.

Table-2 shows the distribution of different PAHs detected in the blood of three groups of women. Non-carcinogenic PAHs like anthracene, phenanthrene, fluoranthene, acenaphthylene, and pyrene were found in all three groups. Among carcinogenic PAHs naphthalene and benzo (k) fluoranthene was found in all three groups, however dibenzo (a, h) anthracene was found only in two cases (6%) of malignant group.

The frequency of non-carcinogenic PAHs, anthracene and phenanthrene were highest (52% and 29% respectively) in benign group.

Table-3: Comparison of PAHs levels detected in the subjects suffering from benign and malignant breast disease with controls

PAHs (In ppb)	Control Mean ± SE (n = 30)	Benign Mean ± SE (n = 30)	Malignant Mean ± SE (n = 30)	P value
Naphthalene	6.07±3.34	60.99±29.43	49.65±24.27	0.306
Acenaphthylene	109.42±50.09	70.75±42.08	91.28±46.18	0.659
Phenanthrene	9.91±5.38	16.79±7.19	15.22±6.94	0.756
Anthracene	6.48±3.17	9.35±3.73	3.52±8.46	0.329
Fluoranthene	5.84±4.11	3.44±2.49	17.22±8.07	0.255
Pyrene	1.15±0.88	23.34±15.86	10.48±6.61	0.442
Benzo (k) fluoranthene	0.22±0.22	1.04±0.74	0.45±0.45	0.595
Benzo(a) pyrene	2.95±2.95	0.27±0.20	ND	0.359
Dibenzo(a, h) anthracene	ND	ND	12.89±12.27	0.159
Total PAHs	142.05±50.84	185.99±61.97	200.74±55.05	0.699
Non-carcinogenic PAHs	138.87±51.05	184.67±62.07	187.40±50.94	0.903
Carcinogenic PAHs	3.17±2.95	1.31±0.91	13.34±12.29	0.374

Table-3 represents the mean (SE) values of 9 detected PAHs in the blood of all three groups. Mean level of acenaphthylene (109.42 ± 50.09 ppb) in control group was highest, while benzo (a) pyrene (0.27 ± 0.20 ppb) in benign group was the lowest among all detected PAHs. Levels of naphthalene, phenanthrene, anthracene, pyrene, and benzo (k) fluoranthene were higher in benign group than in controls; however acenaphthylene and fluoranthene were higher in malignant group in comparison to benign group. Di benzo (a, h) anthracene was not detected in control and benign groups.

Levels of naphthalene, pyrene and phenanthrene, the most abundant PAHs in air, were found higher in both benign and malignant groups compared to control but it did not reach the nominal significant level. Mean levels of non-carcinogenic PAHs were found higher than the carcinogenic PAHs in all groups. Total PAHs were higher in both malignant and benign groups when compared with those of control group, but differences were not statistically significant probably due to very large heterogeneous distribution.

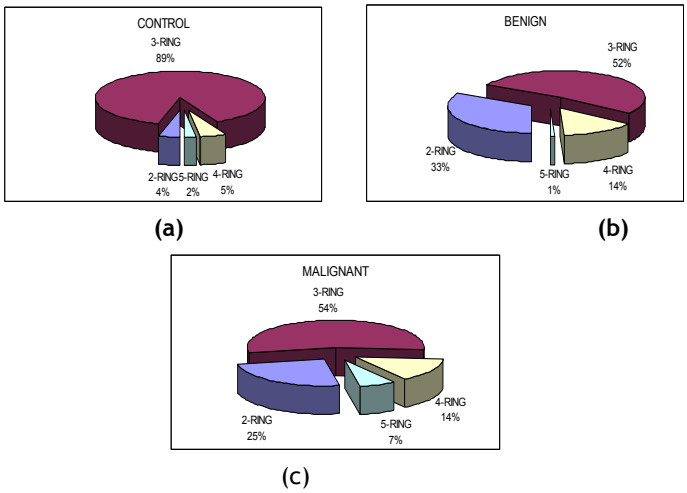

Figure -1: Contribution of the different groups of PAHs to the total PAHs burden in (a) Control (b) Benign and (c) Malignant groups

Interestingly, distribution of PAHs according to the number of rings (Fig-1) in their structure indicated that 3-ringed PAH was 89% in controls as compared to only 52% in benign and 54% in malignant group. However, contribution of 2-ringed PAHs was lowest in the control group i.e. 4% as against 33% and 25% among benign and malignant groups respectively. Similarly, there were five percent 4-ringed PAHs in the control group as compared to 14% in both benign and malignant groups. Distribution of 5-ringed PAHs was highest (7%) in the malignant group followed by 2% in controls and benign group respectively.

4. Discussion

PAHs and their metabolites are distributed to different organs and tissues by transport through the blood. Surrogate markers that estimate levels in target organs, such as protein[28] and hemoglobin[29] adducts in blood might reflect target organ levels because these macromolecules are more abundant in blood than DNA. For these reasons, present study, first of its kind was designed to estimate PAHs levels (both carcinogenic as well as non-carcinogenic) in blood samples of women suffering from benign and malignant breast lesions and these results were compared with the levels present in controls. We hypothesized that, if the higher PAHs levels are present in blood then there will be higher biological effective dose available for accumulation (being PAHs lipophilic in nature), activation and pathophysiological

changes resulting in mammary tumor formation. In vitro studies also show that human breast epithelial tissue has the ability to metabolize PAHs to their ultimate mutagenic / carcinogenic moieties that can induces mutations, oxidative stress and altered transcriptional activity. That is believed to be primary requirement for tumor initiation during carcinogenesis.[30-32]

Present study shows higher levels of non-carcinogenic than the carcinogenic PAHs levels in all the three groups. One reason for this may be their higher concentration in ambient air and among different congeners of PAHs, two-ring and three-ring PAHs dominated the distribution in biological samples, such patterns are properties of PAH mixtures generated from petrogenic pollution.[33] The risk associated with human exposure to atmospheric PAHs is highest in cities, considering the density of population, increasing vehicular traffic, and scarce dispersion of the atmospheric pollutants. Air and water monitoring of some of the major cities in India showed high PAHs levels in urban environment. Ambient air PAHs in Delhi ranged between 668 ± 399 and 672 ± 388 ng m3 in the years 2002 and 2003, while in Lucknow water levels of PAHs were ranged 0.04-65.85 µg / L.[2, 3] These values are higher than the standard limits of 5 ng/m3 (CPCB, India) and 0.2 µg / L (BIS, India) respectively.[34, 35] Also higher PAH levels have been reported in several common Indian oil fried and pyrolysed food items.[4] On the other hand, rural population especially women are supposed to get exposure from burning of coal, wood and biomass fuel during their routine food preparation. These common activities have been identified as among the major contributors to the PAHs release.[36] None of the subjects in all three groups (i.e. control, benign and malignant) reported addiction to smoking (one major source of PAH exposure) and alcoholism, which is not very common in Indian women. Among non-carcinogenic PAHs pyrene, phenanthrene and fluoranthene were found higher in malignant and benign groups than the control but are non-significant. These three PAHs with anthracene constitute the major portion of the total PAHs in environment.[37] Dibenzo (a,h) anthracene, a carcinogenic PAHs was found only in the subjects of malignant group. Although, total blood PAHs level was found higher in malignant group than the other two groups i.e. benign and control, but no any individual PAH or their combination was found significantly higher in any group. Results of the present study are consistent with previous studies reported higher PAH-DNA adduct levels in blood among breast cancer cases as compared with controls.[21, 38, 39]

Due to the variable composition of PAHs mixtures from different sources, benzo(a)pyrene or other single compound may not be representative of all exposure conditions.[40] Benzo(a)pyrene is just one carcinogenic compound in a mixture of carcinogens present in the atmosphere and people are unlikely to be exposed to a single PAH compound. Thus, the benzo(a)pyrene approach which ignores the other PAHs will yield an inadequate evaluation of the risk of PAHs mixture. Interestingly, our data shows the distribution of 2, 4 and 5-ringed PAHs were found higher in benign and malignant groups in comparison to controls. This is important finding because the compounds with higher molecular weight (contain more rings structure) have more stability and resist more to degrade than the other low molecular structures and persisted for a long duration. Also, some 2-ringed PAHs like naphthalene has been considered as possible carcinogenic to humans[41] and we have found higher levels of 2-ringed PAHs compounds in study groups. Higher solubility of naphthalene than other congeners with a larger molecular weight of PAHs may be responsible for that.[33] Fang et al also indicated that the health risk of gaseous-phase PAHs (enriched in low molecular weight compounds) was higher than the particle phase.[42] We opined that, these factors may play some possible role in PAHs related health risk and should also be consider for further studies.

We did not find any significant difference in the levels of blood PAHs among all three groups of women but the results of this pilot study with small sample size and limited statistical power suggests and paves the way for study with large sample size for a statistically sound conclusion. Our results of present study, first of its kind from India show that blood PAHs levels could be suitable evidence to environmental exposure to PAHs and recommend future assessment of the health risk associated with total PAH exposure. The results will highly beneficial for the further relevant studies.

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