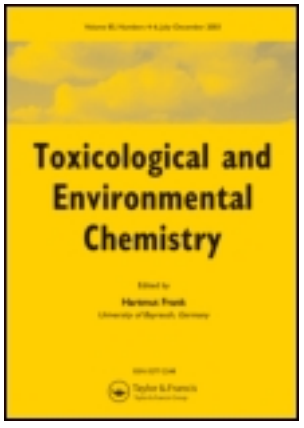

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Effects of benzo(a)pyrene on blood components, tumor markers, and oxidative status in mice

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This study was carried out to investigate the effect of long-term exposure to benzo(a)pyrene (B(a)P) in mice. Hemogram, tumor markers, oxidative status, and B(a)P residues in liver tissue were evaluated. Sixty albino Swiss mice were randomly distributed equally into three groups; the control was given 0.1 mL corn oil once a week for 8 weeks. The other two groups were given 20 and 40 mg B(a)P per kg body weight once a week orally for the same period. B(a)P-treated mice suffered from depression and ascites, and macrocytic normochromic anemia was recorded at the 16th and 30th week. There was marked leukocytosis with lymphocytosis at the early stage of the experiment, followed by leukopenia, lymphopenia, and neutropenia at the end of the experiment. Monocytes and arginase activity were elevated throughout the experiment. Alpha feto-protein was detected only in the experimental groups in the 30th week of the experiment. A marked increase in lipid peroxides associated with a decrease in reduced glutathione and glutathione-S-transferase (GST) activity was observed in liver homogenate of the B(a)P-exposed animals. Residues of B(a)P were detected in liver tissue with a concentration parallel to the B(a)P dose level. In conclusion, B(a)P caused abnormal changes in the hemogram, evidence of tumor formation through B(a)P-induced oxidative stress, and it was accumulated in the liver tissue of mice.

Keywords: benzo(a)byrene; mice; hemogram; oxidative stress; tumor markers; residues

Introduction

Benzo(a)pyrene [B(a)P] is a polycyclic aromatic hydrocarbon formed when gasoline, garbage, or any animal or plant material is burned incompletely. It was first isolated from coal tar and it exists as an environmental contaminant which can access the body through inhalation, ingestion, and skin contact (Baird, Hooen, and Mahadevan 2005; Shimada et al. 2007; Smith et al. 2007). Exposure to high concentrations of B(a)P may cause red blood cell damage, anemia, and suppression of the immune system leading to increased susceptibility to disease (ATSDR 1995).

B(a)P is metabolized in the human and animal body to form a number of metabolites that may bind to DNA forming adducts which can interfere with or alter DNA replication

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and may be associated with an increased risk of several forms of cancer (ATSDR 1995). Suppression of the body antioxidant defense by B(a)P through aryl hydrocarbon receptors leads to the generation of reactive oxygen species (Briedé et al. 2004). Metabolism or biotransformation through phase I (cytochrome *P*-450 monooxygenase enzymes) and phase II (conjugating enzymes) pathway is a requisite for detoxification and excretion of lipophilic chemicals (Goksøyr and Förlin 1992). The objective of this study was to investigate the toxic effects of B(a)P through studying hemograms, serum tumor biomarkers, oxidative status, and B(a)P residue in liver tissue of mice.

Materials and methods

This study was carried out according to guidelines for animal experimentation and approved by the Institutional Animal Care and Use Committee, National Research Centre Animal Care Unit, Dokki, Giza, Egypt.

Chemicals

B(a)P was obtained from Sigma Aldrich Sigma-Aldrich Canada Ltd. (2149 Winston Park Drive Oakville, Ontario, Canada). Glutathione (GSH) and 1-chloro-2,4-dinitrobenzene were obtained from Fluka Chemikalien, (Buchs, Switzerland). All chemicals were of analytical grade.

Experimental animals

Sixty female Swiss albino mice weighing 23–27 g and 3–4 weeks of age were obtained from the Animal House, National Research Center, Giza, Egypt. The animals were housed in a well-ventilated animal room under standardized conditions of $20 \pm 3^{\circ}C$, relative humidity $50 \pm 5\%$, and 12 h light/dark cycle. Feed and water were supplied *ad libitum* to meet the requirements of the NRC (1995). Mice were acclimatized for 15 days before the start of the experiment.

General layout of the experiment

Mice were randomly allocated into three equal groups. The first group was kept as control and received 0.1 mL kg^{-1} body weight (b.wt.) corn oil once a week for 8 weeks. The second and third groups were orally given B(a)P 20 and 40 mg kg^{-1}.b.wt. once a week for 8 weeks, respectively. Clinical signs and body weight were recorded for all groups during the period of the experiment. Blood was collected by retro orbital venous plexus by puncturing seven mice of each group on the 8th, 16th, and 30th week of the experiment and divided into two portions. The first portion was anticoagulated with EDTA and used for hematological evaluation. The other portion was used for serum separation and determination of tumor biomarkers. *Post-mortem* examination was performed at the end of the experiment. Liver specimens were collected from seven mice in each group at the end of the experiment for evaluation of the oxidative parameters and determination of B(a)P residue. The relative organ weights (organ to body weight ratio) were calculated for liver, lung, spleen, and stomach according to the method of Sellers et al. (2007) using the following equation:

$$\text{Relative organ weight} = \text{organ weight(g)}/\text{body weight(g)} \times 100.$$

Hemogram

Erythrogram and leukogram counts were performed by standard manual methods, according to Feldman, Zinkl, and Jain (2000).

Tumor markers

Serum alpha feto-protein was determined quantitatively with an enzyme immunoassay, according to Chan and Miao (1986), using commercial kits (BioCheck, Inc. Foster City, CA 94404 U.S.A.) and a microplate reader (EL_X 800 Universal Microplate ELISA Reader, Bio-TEK, Instruments INC, Vermont, USA). Determination of serum arginase activity was performed according to the method of Marsch, Fingerhut, and Miller (1965) using commercial kits (Bio-diagnostic Co., Dkki, Giza, Egypt).

Detection of liver oxidative status

Preparation of liver homogenate

One gram of liver tissue was collected from each mouse of the different groups at the end of the experiment. The liver tissue was washed in ice-cold 0.9% NaCl and homogenized in ice-cold 1.15% solution of potassium chloride in 50 mmol L^{-1} potassium phosphate buffer solution (pH 7.4) to yield a liver homogenate of 10% (W/V; Weight of liver tissue, g per Volume of buffer, mL). Homogenization was performed using a sonicator, (4710 Ultrasonics Homogenizer, Cole-Parmer Instrument Co., USA). The homogenate was centrifuged at $4000 \times g$ for 5 min at 4°C. The supernatant was collected and used for determination of the concentrations of reduced GSH, the activities of glutathione-S-transferase (GST), and lipid peroxidation by-products.

Determination of reduced GSH

GSH content in liver homogenate was determined according to Ellman (1959). The reduced chromogen was directly proportional to the GSH concentration and its absorbance was measured at 405 nm using a spectrophotometer (Model, JASCO 7800, UV/VIS, Japan). The concentrations of GSH were expressed as μmol g^{-1} tissue.

Determination of GST activity

The activity of GST in liver homogenate was determined according to Habig, Pabst, and Jakoby (1974). The enzyme activity was determined by monitoring changes in the absorbance at 340 nm using a spectrophotometer (Model, JASCO 7800, UV/VIS, Japan) and expressed as nmol min^{-1} mg^{-1} protein. Determination of total proteins in liver homogenate was performed according to Tietz (1995).

Determination of lipid peroxidation by-products

Lipid peroxidation by-products in liver tissue homogenate were performed according to Ohkawa, Ohishi, and Yagi (1979), based on the reaction of thiobarbituric acid with malondialdehyde in acidic media at 95°C for 45 min to form thiobarbituric acid-reactive substances (TBARS). The resulting pink-colored reaction product was extracted with *n*-butanol and the absorbance was determined at 535 nm using a spectrophotometer

(Model, JASCO 7800, UV/VIS, Japan). Level of lipid peroxides was expressed as nmol g^{-1} wet liver tissue.

Detection of B(a)P residue in liver tissue

The test was performed utilizing a gas chromatograph (Hewlett-Packard HP 5890 series II Plus) equipped with a flame ionization detector and HP-1 analytical capillary column (30 m × 0.53 mm), film thickness (0.88 um), cross-linked methyl silicone gum for the separation of B(a)P. The operational conditions of gas chromatography were detector temperature (300°C), injection port temperature (250°C), column initial temperature (220°C), initial time (2 min), rate of temperature increase (5°C min^{-1}), upper temperature (280°C), and upper time (2 min). The flow rate of the carrier gas nitrogen was 4 mL min^{-1} and the injection volume was 3 μL throughout.

Statistical analysis

The data were presented as means ± standard deviations. Differences between the control and treated groups were tested for significance using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant at $p < 0.05$ level (Snedecor and Cochran, 1989) using SPSS version 10 computer program downloaded from the website http://www.spss.com.

Results

No clinical signs were observed in the control group throughout the experimental period. The mice of groups treated with low and high doses of B(a)P suffered from depression and ascites. At the end of the experiment, *post-mortem* examination revealed hepato-splenomegaly in both the treated groups, especially those treated with the high dose.

The weight of the relative organs of mice in low- and high-dose B(a)P-treated groups showed significant ($p < 0.01$) increase compared to the control group. The relative stomach weight was within the normal range in the mice which had received B(a)P at the low dose (Table 1).

The results of the hemogram during the experimental period are shown in Tables 2 and 3. The erythrocyte count was significantly ($p < 0.05$) decreased in mice that received 20 or 40 mg B(a)P kg^{-1} b.wt. at the 30th week compared to the control group. The values of the mean corpuscular volume (MCV) were increased after 16 weeks of administration of either dose of B(a)P. No statistical differences were recorded in hemoglobin (Hb) concentration, packed cell volume (PCV) %, and mean corpuscular hemoglobin concentration (MCHC) in all the treated mice (Table 2). Total leukocytic and lymphocytic counts were significantly ($p < 0.05$) increased at the 8th and 16th week of the experiment in both the B(a)P-treated groups compared to the control group. At the 30th week of the experiment, a significant ($p < 0.05$) decrease in total leukocytic and lymphocytic counts was recorded in both the B(a)P-treated groups in comparison with the control group. Monocytosis was noticed in both the B(a)P-treated groups. The neutrophil counts showed a significant ($p < 0.05$) decrease starting after 16 weeks of B(a)P administration, compared to the control group (Table 3).

Table 1. Effect of B(a)P on organs relative weight (%) of mice after 30 weeks of the experiment (mean ± SD, $N = 7$).

Groups organs	Control	B(a)P at dose	
		20 mg kg^{-1}	40 mg kg^{-1}
Liver	4.3 ± 0.3^{c}	6.9 ± 0.1^{b}	8.9 ± 0.4^{a}
Spleen	0.5 ± 0.1^{c}	1.4 ± 0.3^{b}	2.1 ± 0.3^{a}
Lung	0.8 ± 0.1^{b}	1.0 ± 0.1^{a}	1.0 ± 0.1^{a}
Stomach	0.9 ± 0.0^{b}	0.8 ± 0.0^{b}	1.8 ± 0.1^{a}

Note: Means with different letters at the same row are significantly different at $p<0.05$.

Table 2. Effect of B(a)P on the erythrogram of mice (mean ± SD, $N = 7$).

Groups parameters	Weeks	Control	B(a)P at dose	
			20 mg kg^{-1}	40 mg kg^{-1}
RBCs count ($\times 10^{12}$ L^{-1})	8	10.6 ± 0.2^{a}	10.3 ± 0.1^{a}	10.5 ± 0.9^{a}
	16	9.2 ± 0.1^{a}	9.0 ± 0.1^{a}	8.9 ± 0.1^{a}
	30	9.1 ± 0.2^{a}	8.9 ± 0.1^{b}	8.9 ± 0.0^{b}
Hb concentration (g L^{-1})	8	126.6 ± 0.6^{a}	129.4 ± 3.3^{a}	128.2 ± 1.9^{a}
	16	128.0 ± 1.3^{a}	132.0 ± 0.3^{a}	134.0 ± 1.6^{a}
	30	133.0 ± 1.3^{a}	130.9 ± 0.2^{a}	131.1 ± 1.4^{a}
PCV (%)	8	44.0 ± 0.8^{a}	44.3 ± 0.6^{a}	45.0 ± 0.4^{a}
	16	44.1 ± 0.9^{a}	44.5 ± 0.5^{a}	44.0 ± 0.8^{a}
	30	44.4 ± 0.6^{a}	43.7 ± 0.3^{a}	42.9 ± 0.4^{a}
MCV (fL)	8	41.5 ± 0.2^{a}	43.0 ± 0.1^{a}	42.7 ± 0.0^{a}
	16	48.2 ± 0.4^{b}	49.4 ± 0.1^{a}	49.2 ± 0.4^{a}
	30	49.1 ± 0.4^{b}	51.8 ± 0.9^{a}	50.3 ± 0.7^{a}
MCHC (g L^{-1})	8	288.0 ± 3.8^{a}	291.8 ± 4.2^{a}	279.5 ± 3.3^{a}
	16	290.5 ± 2.8^{a}	296.4 ± 3.9^{a}	304.8 ± 2.3^{a}
	30	299.3 ± 1.4^{a}	293.5 ± 2.3^{a}	305.3 ± 0.4^{a}

Notes: Means with different letters at the same row are significantly different at $p<0.05$. RBCs = Red blood cells. Hb = Hemoglobin. PCV = Packed cell volume. MCV = Mean corpuscular volume. MCHC = Mean corpuscular hemoglobin concentration.

α-Feto-protein was detected only in the 30th week of the experiment in mice that received high dose of B(a)P. The activity of arginase was markedly ($p < 0.001$) increased in the B(a)P-treated groups compared to the control group (Table 4).

The levels of reduced GSH and the activities of GST activity were significantly ($p < 0.01$) decreased in liver homogenates of both the B(a)P-treated mice, compared to the control group. Lipid peroxides in liver homogenates from the B(a)P-treated mice were significantly ($p < 0.01$) increased as compared to the control group. The B(a)P residues in liver tissue of the treated mice was 4.1 ± 0.5 and 11.5 ± 0.1 µg g^{-1} liver tissue in mice treated with B(a)P at doses 20 and 40 mg kg^{-1}.b.wt., respectively (Table 5).

Table 3. Effect of B(a)P on the leukogram of mice (mean ± SD, $N = 7$).

Groups parameters	Weeks	Control	B(a)P at dose	
			20 mg kg^{-1}	40 mg kg^{-1}
Total leukocytic count ($\times 10^9$ L^{-1})	8	12.3 ± 0.2^b	13.2 ± 0.4^a	13.4 ± 0.4^a
	16	11.9 ± 0.3^b	14.2 ± 0.3^a	14.2 ± 0.4^a
	30	10.6 ± 0.1^a	8.7 ± 0.2^b	8.7 ± 0.1^b
Lymphocytes ($\times 10^9$ L^{-1})	8	9.6 ± 0.5^b	10.7 ± 0.6^a	10.9 ± 0.6^a
	16	9.6 ± 0.5^b	12.5 ± 0.6^a	12.7 ± 0.7^a
	30	8.4 ± 0.4^a	5.8 ± 0.3^b	4.9 ± 0.6^c
Monocytes ($\times 10^9$ L^{-1})	8	0.1 ± 0.0^c	0.2 ± 0.0^b	0.3 ± 0.1^a
	16	0.2 ± 0.1^b	0.3 ± 0.2^b	0.7 ± 0.1^a
	30	0.2 ± 0.1^c	2.2 ± 0.3^b	2.9 ± 0.2^a
Neutrophils ($\times 10^9$ L^{-1})	8	2.6 ± 0.3^a	2.3 ± 0.3^a	2.2 ± 0.2^a
	16	2.1 ± 0.3^a	1.4 ± 0.3^b	0.8 ± 0.2^c
	30	2.0 ± 0.2^a	0.7 ± 0.2^b	0.8 ± 0.1^b

Note; Means with different letters at the same row are significantly different at $p<0.05$.

Table 4. Effect of B(a)P on tumor markers in serum of mice (Mean ± SD, $N = 7$).

Groups parameters	Weeks	Control	B(a)P at dose	
			20 mg kg^{-1}	40 mg kg^{-1}
Alpha feto-protein (μg L^{-1})	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	16	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	30	0.0 ± 0.0^c	22.0 ± 0.9^b	27.6 ± 0.7^a
Arginase (IU L^{-1})	8	23.0 ± 1.9^c	38.6 ± 2.7^b	45.1 ± 2.5^a
	16	22.4 ± 1.0^c	35.0 ± 2.2^b	40.7 ± 1.0^a
	30	25.3 ± 0.9^b	43.3 ± 0.8^a	46.4 ± 1.7^a

Note: Means with different letters at the same row are significantly different at $p<0.05$.

Table 5. Effect of B(a)P on oxidant/antioxidant parameters of liver mice at the 30th week of the experiment (Mean ± SD, $N = 7$).

Groups parameters	Control	B(a)P at dose	
		20 mg kg^{-1}	40 mg kg^{-1}
GSH reduced (μmol g^{-1} liver homogenate)	0.3 ± 0.0^a	0.2 ± 0.0^b	0.2 ± 0.0^b
GST (nmol min^{-1} mg^{-1} protein)	0.4 ± 0.1^a	0.1 ± 0.0^b	0.1 ± 0.0^b
Lipid peroxides (nmol g^{-1} liver homogenate)	11.7 ± 0.5^b	21.8 ± 0.8^a	23.5 ± 1.5^a
B(a)P (μg g^{-1} liver tissue)	0.0 ± 0.0^c	4.1 ± 0.5^b	11.5 ± 0.1^a

Note: Means with different letters at the same row are significantly different at $p < 0.05$.

Discussion

The present experiment showed that ascites and depression were the common clinical signs in the B(a)P-treated mice. Ascites correlates with the reported degenerative changes in the hepatocytes. In this work, depression and ascites were the only apparent clinical signs reported in the B(a)P-treated mice. In this respect, ferrets exposed orally to B(a)P at a dose level of 240 mg kg^{-1} b.wt. thrice per week for 10 weeks showed no clinical signs (Helden et al. 2007).

Post-mortem examination revealed splenomegaly and hepatomegaly in both the B(a)P-treated groups which correlates with the reported increased relative weight of liver, spleen, lung, and stomach of the treated mice. The increase in the weight of the relative organs were accompanied with cancer formation in these organs, confirmed by histopathological examination as lymphosarcoma in liver, spleen, and lung with metastatic adenocarcinoma in lung and squamous cell carcinoma in the stomach (under publication). Increase in spleen weight may also be attributed to induction of extra-medullary hematopoiesis (Booker and White 2005).

Erythrogram of the treated groups revealed significant decrease of red blood cells at the end of the experiment, increase of MCV value at the 16th and 30th weeks of the experiment with no change in MCHC in both the B(a)P-treated groups, suggesting the presence of macrocytic normochromic anemia. It has been reported that B(a)P metabolism yields quinones which make red blood cells more susceptible to lysis leading to anemia and splenomegaly (Booker and White JR 2005; Matiasovic et al. 2008). Extra-medullary hematopoiesis has also been reported by Hakura et al. (1998) in the B(a)P-treated mice.

The reported leukocytosis and lymphocytosis may be attributed to the formation of B(a)P quinones during B(a)P metabolism through direct and enzymatic oxidation which affects bone marrow stromal cells and then reflects on the hematopoiesis (Galvan et al. 2006; Latif et al. 2010). The observed leukopenia, lymphopenia, and neutropenia in the 30th week of the experiment may be due to toxins secreted from the tumor cells (lymphosarcoma in liver, spleen, and lung, adenocarcinoma in lung, and squamous cell carcinoma in stomach (under publication)), leading to an increase in the rate of destruction of circulating leukocytes which affect bone marrow (Uno et al. 2004). The significant decrease in neutrophils may be attributed to lowered immunity as a result of B(a)P effects on the bone marrow (Ramesh et al. 2001).

Alpha feto-protein and the activity of arginase are two biomarkers that were detected in elevated quantities in the blood, body tissue, or urine, and could be used as indicators of tumor formation (Malleske et al. 2006; Mielczarek et al. 2006; Deignan et al. 2007). Elevation of marker enzymes could be attributed to destruction of the neoplastic tissue (Deignan et al. 2007). Alpha feto-protein was only detected in the 30th week of the present experiment, especially in the high-dose treated group. Elevation of serum alpha feto-protein could indicate the formation of malignant disease (Erisir et al. 2005). A direct relationship has been observed between the incidence of elevated alpha feto-protein levels and the stage of the disease.

Arginase is one of the essential enzymes in the terminal stages of urea cycle in the liver that participates in the elimination of ammonia from the body. Significant increase of arginase was shown in both the B(a)P-treated groups during the whole experimental period. It has been reported that serum arginase activity increased in all liver diseases. Hepatocellular carcinoma and liver cirrhosis were associated with elevated serum arginase, and it is used in the diagnosis of liver metastasis (Malleske et al. 2006; Mielczarek

et al. 2006). Arginase is more reliable than alpha feto-protein for early diagnosis of cancer (Ashamiss et al. 2004).

The present data revealed decrease in GSH-levels and GST-actvities, and an increase in MDA concentrations in the B(a)P-treated mice. The increased MDA concentration may be attributed to its excessive formation as a result of membrane lipid peroxidation by B(a)P. An increase in MDA indicates serious damage to cell membranes and the inhibition of several enzymes and cellular functions (Selvendiran, Banu, and Sakthisekaran 2005; Badary et al. 2007). Lipid peroxidation is a cellular process that commonly takes place under normal physiological conditions. Under excessive oxidative stress, the level of lipid peroxidation can become so high that it may be involved in carcinogenesis (Selvendiran, Banu, and Sakthisekaran 2004; Senthilnathan et al. 2006). Free radicals also attack proteins damaging several amino acids. The decrease of GSH and GST may be explained by excessive utilization of these antioxidants from tumor cell proliferation in different organs (Sorokina, Solyanik, and Pyatchanina 2010).

B(a)P residues were detected in the liver tissue of the treated groups. The cumulative effect of B(a)P on the liver is due to direct transfer of this compound after oral administration from the intestine via the portal vein. Lower hepatic metabolism of B(a)P with poor excretion leads to its accumulation in the hepatic tissue (Galvan et al. 2005). B(a)P and its metabolites are lipophilic compounds stored in lipid-rich tissues as a result of repeated exposure. Such compounds are biotransformed and redistributed even after the cessation of exposure (Ramesh and Knuckles 2006). Release and redistribution of B(a)P from storage sites cause an increase in non-metabolized B(a)P in the liver (Smith et al. 2007), as the liver is the principal detoxifying organ of B(a)P.

In conclusion, low- and high-dose exposure to B(a)P (20 and 40 mg kg^{-1} b.wt., respectively) caused abnormal changes in the hemogram, and is also evidence of tumor formation through B(a)P-induced oxidative stress. This is in addition to accumulation of B(a)P in the liver tissue of experimental mice.

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