Pathological Changes of Dibenzo[A,L]Pyrene-Induced Peripheral T-Cell Non-Hodgkin’s Lymphoma in Balb/C Mice

Nathera M. Al-Nuaimee1,a, Hazilawati H.1,b, Jasni S.1,c, Noordin M.M.1,d and Rosly S.M2,e

1 Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia
2 Strategic Livestock Research Centre, Malaysian Agricultural Research and Development Institute, Malaysia

a nathera16@yahoo.com, b hazila@vet.upm.edu.my, c Jasni@vet.upm.edu.my, d noordin@vet.upm.edu.my, e rosly@mardi.gov.my

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ABSTRACT

Dibenzo[a,l]pyrene (DB[a,l]P) is one of the polycyclic aromatic hydrocarbons (PAHs) family which are present in the atmosphere from combustion sources such as diesel exhaust, residential heating processes, cigarette smoke and industrial coke production. The carcinogenic effects of Dibenzo[a,l]-pyrene (DBP) in inducing lymphoma in BALB/c mice is not studied yet. The gross pathology, histopathology and immunohistochemical alterations induced by DBP in BALB/c mice were determined. Forty adult female BALB/c mice were divided equally into two groups, the first one gavaged orally using DB[a,l]P (SIGMA-ALDRICHV) dissolved in corn oil (SIGMA-ALDRICHV) at 15mg/kg of body weight while the second group was gavaged by corn oil only as a control group. Animals sacrificed after six months using ketamin: xylazin anesthesia. All animals’ organs were examined grossly for any changes in color, size, weight and the presence of any abnormal lesions. Statically significant difference appeared in lymph nodes, spleens, livers and kidneys weight compared to the control group due to enlargement of these organs in lymphoma group. Histopathological examinations showed that lymphoma induced in almost all mice organs including lymph nodes, thymus, spleen, liver, kidneys, heart, stomach, skin, small and large intestine and the brain. Immunohistochemical examinations showed that lymphoma induced by DBP in all mice organs is of T type non-Hodgkin’s lymphoma.

Keywords: Dibenzo[a,l]pyrene, Histopathological, Immunohistochemical, lymphoma

1. Introduction

Dibenzo[a,l]Pyrene is one of the air pollutant which has been detected in several environmental soil and sediment samples, in cigarette smoke condensate and in particulate matter formed during combustion of smoky coal (Mumford et al., 1987). The extreme toxicity and carcinogenicity of DB[a,l]P accompanied by its prevalence in the environment (Cavalieri et
Dibenzo[a,l]pyrene and Polycyclic aromatic hydrocarbons are reasonably anticipated to be human carcinogens based on sufficient evidences of carcinogenicity in experimental animals and in vitro studies (Binkova, et al., 2000). The term polycyclic aromatic hydrocarbons (PAHs) and Dibenzo[a,l]pyrene (DBP) is one of this family refers to a ubiquitous group of several hundred chemically-related, environmentally persistent organic compounds of various structures and varied toxicity, with two or more fused aromatic rings. (Mahadevan et al., 2005). Nakatsuru et al. (2004) reported that DBP is the most powerful carcinogenic polycyclic aromatic hydrocarbon ever tested and it is a potential toxicological hazardous chemical.

The carcinogenic effect of dibenzo[a,l]pyrene (DBP) has been well characterized in numerous animal models, a single dose of 15 mg/kg DBP to pregnant transgenic mice (B6129SF1/J (AHRb-1/d, responsive) were crossed with strain 129S1/SvIm (AHRd/d, nonresponsive) late in gestation day 17 produces an aggressive T-cell lymphoma as well as lung and liver adenoma in offspring (Castro et al., 2009). Tumor formation in DBP and carcinogenic polycyclic aromatic hydrocarbons (PAHs) is by their abilities in covalently binding to DNA in vivo with the formation of characteristic profiles of DNA adducts in the presence of cytochrome P450 to generate ultimate forms of dihydrodiols epoxides (diol-epoxides), which can covalently bind to DNA to induce PAH-DNA (induce DNA adduction). According to the structural difference in the area of epoxide formation, carcinogenic PAHs can be classified into bay and fjord region containing compounds. DBP is a PAH containing both binding sides a bay and a fjord regions And when these adducts are not repaired, or if repair introduces errors into the DNA sequence, gene mutations can be introduced upon replication of the damaged DNA which play a critical role in the carcinogenic process (Yu et al., 2006). Another findings suggest that there exists a relationship between the specific DNA adducts formed, induction of Ki-ras mutations, and the tumorigenic potential of DB[a,l]P in the lungs of strain A/J mice. Dibenzo[a,l]pyrene has been also demonstrated to be highly mutagenic in the metabolically competent MCL-5 cell line derived from human B-lymphoblastoid cells (Prahalaad et al., 1997).

As shown from the previous studies that Dibenzo[a,l]pyrene is highly carcinogenic in lab animals and to the knowledge its carcinogenic effect in BALB/c mice is not studied yet. Thus, the study was carried out to evaluate its carcinogenicity in BALB/c mice which is cheap and available as a lab model other than the transgenic strains which are highly expensive. In addition the immunohistochemical study was also conducted to determine the lymphoma type induced in all mice organs.

2. Methodology

2.1 Animals and Management

All animal experiments and animal care were preformed according to the Guidelines for the Care and Use of Laboratory Animals (National Academy Press, Washington DC, 1996). The protocol was reviewed and approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (Ref: UPM/FPV/PS/3.2.1.551/AUP-R28 and AUP No 38). Forty female BALB/c mice 6-8 weeks old, of 30-35 gm bw, were divided into two groups of twenty animals each, and housed
randomly composing of five mice per cage, in separated polypropylene plastic cages with wire grid lids to accommodate placement of water bottles and food. The mice were allowed to acclimatize to the housing conditions and lighting (12 hours light/dark per day) during the 24 weeks of experimental period. Mice were fed with 15g/mouse of grinded commercial chow daily and drinking water was made available ad libitum for two weeks prior to study treatments.

2.2 Chemical Preparation, Solvent Used, Dosage and Administration

Dibenzo[a,l]pyrene CAS-number is 000191-30-0, of molecular weight 302.38 and purity of 99% was available in vial of 100 mg and the solvent used which was corn oil were both of the highest purity (SIGMA-ALDRICH). Dibenzo[a,l]pyrene solution was freshly prepared immediately by dissolving 15 mg/kg bw of DBP in corn oil (5 ml/kg bw) (SIGMA-ALDRICH) before administered to the mice. Dibenzo[a,l]pyrene solution was administered by oral gavages using oral feeding stainless steel needle (22 gm x 1 inch) to the experiment animals. Corn oil alone was also gavaged to the control group at a dose of 5 mL/kg bw.

2.3 Treatment

Dibenzo[a,l]pyrene dose was 15 mg/kg bw once orally dissolved in corn oil according to the previous studies of (Yu et al., 2006), the dose which induced leukemia and lymphoma in transgenic mice offspring.

2.4 Experimental Design

At the beginning of the experiment, forty adult female BALB/c mice were divided equally into two groups (n=20); group A (control group) and group B (DBP treated group). The mice in group A were administered once orally with corn oil only at a dose of 5ml/kg bw at the beginning of the study (Yu et al., 2006). The mice of groups B received freshly prepared DBP once orally at a dose of 15mg/kg bw at the beginning of the study (Yu et al., 2006).

2.5 Pathology

2.5.1 Animals Euthanasia

All mice were euthanized for necropsy by the end of the experimental period (24 weeks) post-administration of DB[a,l]P using ketamine:xylazine mixture of 0.03 ml.

2.5.2 Gross Pathology

Postmortem was conducted on the euthanized mice and all mice organs were examined carefully for any changes to evaluate the gross abnormalities post treatment. These changes include the presence of any tumor masses, size and color changes of organs which include: the lymph nodes, spleen, liver, kidneys, small and large intestine, brain, skin, muscles and heart. The lymph nodes, spleen, liver and kidneys were then blotted dry and weighed.
2.5.3 Histopathological Examination

For histopathological examination, portions of auxiliary, mesenteric and submandibular lymph nodes, spleen, liver, lung, kidneys, thymus, brain, heart, stomach, small and large intestines sample (~ 0.5 cm3) were removed and fixed in 10% neutral buffered formalin for 24 to 48 hours. Routine histological processes were conducted according to standard procedures (Luna, 1968). The severity of lesions was determined with an image analyzer microscope (Olympus BX 51).

2.5.4 Immunohistochemistry

The immunohistochemistry analysis was performed using a Dako Envision®+Dual Link System-HRP (DAB+) kit (Dako K4965, USA) and as per manufacturer’s instruction. The kit contained a blocking agent, secondary antibody labeled to horseradish peroxidase and diaminobenzidine (DAB). The primary antibodies used were rabbit polyclonal to CD3 primary antibody (ABcam ab5690, UK) and rabbit polyclonal antibody to CD79α primary antibody (ABcam ab5691, UK) which were directed against CD3 (T-lymphocyte marker) and CD79 α (B-lymphocytes marker) to identify the type of lymphoma. Paraffin sections of tissue samples histopathologically (3.2.5) identified as lymphoma were deparaffinized in two changes (10 minutes each) of xylene and rehydrated in three changes of 3 minutes each of 100%, 90% and 70% ethanol respectively. The tissue sections were rinsed in distilled water and immersed in Tris-buffered saline (pH 9) (TBS) (Dako S3001, USA) bath for 5 minutes.

The tissue sections were placed in a glass jar filled with target retrieval solution (Dako S3001, USA) pre-heated at 60oC and incubated in a microwave oven at 97oC for 25 minutes. Then the tissue sections were left in a glass jar to cool to room temperature for 20 minutes, before the section were rinsed in distilled water and later immersed in TBS buffer bath for 5 minutes. The excessive buffer on the tissue section was tapped off and wiped gently with a tissue paper around the sections. The tissue sections on the glass slides were surrounded by a circle of wax using a special pen (Dako S2002, USA) to confine the reagent only on the section on the slide. The tissue sections were flooded with dual endogenous enzyme (Dako K4065, USA) as blocking reagent to cover the sections and further incubated in a humidity chamber for 8 minutes, before the sections were rinsed in distilled water and immersed in TBS buffer (pH 9) bath for 5 minutes.

The excess buffer on the tissue sections was removed by tapping the slides and wiped gently with a tissue paper around the sections. The sections were applied with adequate (0.2-0.5 ml) CD3 primary antibody (ABcam ab5690, UK) or CD79α primary antibody (ABcam ab5691, UK) reagents and incubated in a humidity chamber at room temperature for 30 minutes, before rinsing in distilled water and immersed in TBS buffer (pH 9) bath for 5 minutes. Following that, the excessive buffer on the tissue sections was tapped off and wiped gently with a tissue paper around the sections. The tissue sections were applied with (0.2-0.5 ml) labeled polymer-HRP reagent (Dako K4065, USA) and incubated in a humidity chamber at room temperature for 45 minutes, then rinsed in distilled water before being immersed in TBS buffer (pH 9) bath for 5 minutes.

After that, the excessive buffer on the sections were removed by tapping and wiped gently with a tissue paper around the sections. The tissue sections were applied with DAB+
substrate-chromogen solution (Dako K4065, USA) and incubated in a humidity chamber for 5 minutes. Then, the tissue sections were rinsed in distilled water before being immersed in TBS buffer bath for 5 minutes.

The tissue sections were then immersed in Myer’s haematoxylin dye (counter stain) for 3 minutes, before they were rinsed in tap water. The tissue sections were dehydrated in three changes of 3 minutes each of 70%, 90% and 100% ethanol, respectively. Then the tissue sections were immersed in two changes (10 minutes each) of xylene and mounted with cover slip using DPX. The tissue sections were examined using an image analyzer microscope (Olympus BX 51).

3. Results

3.1 Pathological results:

Sacrificed mice were examined for gross and microscopic lesions.

3.1.1 Gross Pathology:

Gross findings include, enlargement of the lymph nodes, splenomegaly and hepatomegaly, the kidneys in group B mice compared to the sizes of organs in group A mice appeared within normal size (Fig.1). The enlarged liver in group B mice developed tense capsule, rounded borders and accentuated lobular pattern.

3.1.2 Statistical Analysis:

The body and mice organs weight was analyzed statistically using SPSS version (Independent-Samples T Test) and differences at p<0.05 were considered as significant. There was a significant decreased (p>0.05) in the body weight of group B mice and the animals had rough hair coat compared with group A (control) mice, while organs weights including the spleen, lymph nodes, kidneys and liver of the group B mice showed higher significant difference (p>0.05) compared to the organs of group A (control) mice (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Spleen weight</th>
<th>Liver weight</th>
<th>Kidney weight</th>
<th>Lymph node weight</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.15±0.04</td>
<td>1.55±0.64</td>
<td>0.68±0.34</td>
<td>0.005±0.001</td>
<td>39.31±4.6</td>
</tr>
<tr>
<td>B</td>
<td>0.46±0.17*</td>
<td>2.96±1.00*</td>
<td>1.00±0.18*</td>
<td>0.041±0.008*</td>
<td>30.09±3.9*</td>
</tr>
</tbody>
</table>

*Significant difference

*n= Groups A and B animals number are twenty mouse in each group.
Fig.1. (A, B, C, D) Photograph of mice organs (group B) and control mice organs (group A). The spleen, lymph nodes, liver and kidneys of the DBP treated (group B) mouse appeared enlarged compared with the same organs of the control (group A) mice which were appeared of normal sizes.

3.2 Histopathology

The malignant lymphocytes were seen to be highly variable in morphology (pleomorphism). Cell size usually ranges from small to medium to large sized cells, or a mixture of these. A monomorphic cellular composition was also seen. The neoplastic cells also showed irregular nuclei and vary considerably in sizes and shapes, with vesicular or hyperchromatic chromatin and prominent nucleoli. The cytoplasm appeared clear, pale, eosinophilic or basophilic. The neoplastic cells showed diffused infiltration pattern and proliferation which resulted in the loss of the normal architecture of the pre-existing cellular arrangement in the lymph nodes, spleen, lungs, liver, kidneys, brain, digestive (esophagus, stomach, small and large intestines) and the skin. Degeneration and necrosis were detected in the host cells of the liver, spleen, lung and digestive system with congestion of their blood vessels. Giant tumor cells were observed in the lymph nodes, spleen, liver and skin. Mitosis was observed in all affected organs with lymphoma. Lymphoma lesion appeared vascular due to angiogenesis with admixed plasma cells and eosin-ophils. In the skin, the pleomorphic malignant lymphocytes infiltrated all layers of the skin (epidermis, dermis and hypodermis) and the skin adnexia which include the sebaceous glands. The neoplastic lymphocytes severely infiltrated the lung tissue. In the digestive system, lymphoma lesions were seen in the esophagus, stomach, small and large intestine involved all the layers in these organs in group B mice. Neoplastic lymphocytes were seen infiltrated the lamina propria, between the glands and the muscularis mucosa and in the mucosa, submucosa and the muscularis externa layers of the digestive system parts in group B mice.
In the small and large intestine, the pleomorphic neoplastic lymphocytes were seen infiltrated the mucosa at the crypts of Lieberkuhn in the lamina propria and the intestinal villi. The neoplastic lymphocytes severely infiltrated the colon mucosa and submucosa causing thickening of the colon which led to this space occupying lesion in group B mice. In the central nervous system, the pleomorphic malignant lymphocytes infiltrated the cerebellum and the cerebral cortex of the brain. The neoplastic lymphocytes infiltrated the white matter of the cerebral cortex especially around the blood vessels and caused demyelination of the myeline sheaths of the myelinated axons. The focal infiltration of the malignant lymphocytes in the cerebellum caused degenerative changes which led to loss of its normal architecture. No lesions were seen in the thymus and heart (Figs 2 & 3).

3.3 Immunohistochemistry (IHC)

Immunohistochemical studies on lymphoma affected organs showed that the cancer cells in the organs with lymphoma in groups B mice after immunephentypotyping expressed T cell marker positively (anti-CD3 primary antibody) (brown color) and showed negative expression to B cell (anti-CD79α primary antibody) (blue color) marker. Other normal lymphocytes in the involved organs expressed B cell marker positively (blue color) (Figs.4 & 5).

![Fig.2](image)

**Fig.2.** Section of an auxiliary lymph node of group A, B, & D mice. A) The cells in the lymph node of the control group (group A) appeared to have normal size, shape and distribution. B) Lymphoma lesion in an auxiliary lymph node of a mouse of group B. Notice the development of newly formed blood vessels (black arrowheads) and neoplastic lymphocytes and red blood cells are observed in the lumen of some blood vessels (red arrows). C) The proliferated neoplastic lymphocytes showed with pleomorphism. The large cells showed vesicular nuclei and prominent nucleoli (black arrowheads).Eosinophils are also seen in the lymphoma lesion (yellow arrowheads). A, B & C, H&E stained. Bar: 50μm.
Fig. 3. Sections of Lymphoma lesions of mice in group B. A) Lymphoma lesion in the spleen of a mouse, notice the proliferation of undifferentiated and pleomorphic neoplastic lymphocytes. Some of these cells showed large vesicular nuclei and prominent one or two nucleoli (arrowheads). Eosinophils (arrows) are also observed in the lymphoma lesion which appeared highly vascular. B) Lymphoma lesion in the spleen of a mouse. Notice the pleomorphism of the proliferates neoplastic lymphocytes which in the red pulp and evidence of giant tumor cells (arrowheads) (composed of 3-5 vesicular nuclei with prominent nucleoli). C) Section of a Lymphoma lesion in the skin of a mouse. Bizzare tumor cell (arrowhead) and other malignant lymphocytes can be seen in a haemorrhagic area in the hypodermis. D) Section of the colon of a mouse showed. lymphoma lesion within the mucosa and submucosa where the neoplastic lymphocytes infiltrated these parts (L). The lymphoma zone of infiltration in the colonic mucosa and submucosa is wide causing thickening of the colon wall (double head arrows) compared to the normal mucosa and submucosa above (M).

Fig. 4. Section of lymph node in group A mice. A) Positive control of lymph node in group A mouse. B) Negative control of lymph node in group A mouse. (C & D) Severe diffused proliferation of T neoplastic lymphocytes showing positive expression to CD3 (T cell marker) (brown color) of the lymph node (cortical and paracortical regions) B lymphocytes shows negative expression to CD3 (blue color) especially in the germinal center of the lymphatic nodule (arrowheads) in A, C and D. A: CD3 marker & hematoxylin-IHC stained, B: without CD3 marker & hematoxylin-IHC stained, C & D CD3 marker & hematoxylin-IHC stained, A Bar: 200μm, B Bar: 100μm, C Bar 500μm, D Bar: 50μm.
Fig.5. A) Section of the spleen of (group A) mice Negative control of the spleen of a group A. mouse. Neoplastic lymphocytes showed negative expression to CD79α marker B cell marker. B) Section of lymphoma lesion in the spleen of (group B) mice. Neoplastic lymphocytes which proliferates in the white pulp shows positive expression to CD3 (T cell marker) (dark brown color) (yellow arrowheads). Normal B lymphocytes showing negative expression to CD3 marker (blue colour) (red arrowheads). C) Neoplastic lymphocytes in a mouse liver which expressed CD3 T cell marker positively (brown color). B lymphocytes shows negative expression to CD3 (blue colour) (white arrowheads). D) Section of lymphoma lesion in the kidneys of group B mice. Neoplastic lymphocytes which expressed CD3 T cell marker positively appeared brown in color in the renal cortex near the renal artery (red arrowhead), proximal convoluted tubules, and the glomerulus (black arrowhead). A CD3 marker & hematoxylin-IHC stained, B, C & D CD79α marker & hematoxylin IHC stained. A Bar: 200μm. B & C Bar: 50μm. D Bar: 200μm.
4. Discussion

Dibenzo[a,l]pyrene is one of the most potent carcinogenic polycyclic aromatic hydrocarbons that had been proven to induce cancers in laboratory animals and in non mammalian species such as medaka fish (Cavalieri et al., 1991; Higginbotham et al., 1993; Reddy et al., 1999; Mahadevan et al., 2005; Castro et al., 2009).

The present study showed that oral administration of DBP induced non-Hodgkin’s lymphoma of T type in adult female BALB/c mice. The tumor response in this model is a function of the Cyp1b1 which is the most efficient cytochrome P450 enzyme in bioactivation of DBP and catalyzed the formation of fjord region (DB[a,l]PDE) which both bind extensively to deoxyadenosine residues in DNA, forming DB[a,l]-PDE-DNA adducts which is the critical step in DB[a,l]P-mediated carcinogenesis in mice. Previous studies with knockout mice showed that Cyp1b1 was the most efficient cytochrome P450 enzyme in bioactivation of DBP and other PAHs carcinogenesis (Buters et al., 1999; Heidel et al., 2000; Shimada et al., 2001; Luch et al., 2002; Buters et al., 2003; Kleiner et al., 2004).

These results are consistent with other studies using DBP orally in the same dose to pregnant transgenic mice, in their attempts to develop a pregnant mouse model in which exposure to the polycyclic aromatic hydrocarbon (PAH), DBP during late gestation produces an aggressive T-cell lymphoma in offspring between 3-6 months of age and the males offspring survivors exhibited multiple lung and liver tumors (Yu et al., 2006; Castro et al., 2008). Systemic exposure to DBP caused DNA damage in mouse lung tissue. DNA adducts formed in the lungs of C57BL/6 mice frequently was detected when these mice were gavaged orally with 20 mg DBP/kg body weight, daily for 10 days, which was contributed to (−)-anti-DBPDE formation (Mahadevan et al., 2005).

In the present study, lymphoma was observed in the lymph nodes, spleen, liver, kidneys, skin, digestive and central nervous system (CNS) of group B (DBP treated) mice which suggest the fact that malignant T-lymphocytes within the lymphatic tissue can divide rapidly and replicate themselves uncontrollably, causing lesions or growths that cause lymph nodes enlargement and destroy the normal structure of organs where these cells had metastasized. Through the lymphatic and vessels the malignant lymphocytes metastasized quickly and invaded other organs such as the spleen (splenomegaly), liver (hepatomegaly), kidneys (renomegaly), skin, digestive organs (esophagus, stomach, small and large intestine) and CNS. The rapid replication which occurred in lymphoma was due to numerous genetic abnormalities that cause malfunction of the normal control of cell replication. This control system involved two critical proteins in lymphoma development which are the bcl-2 and bcl-6 proteins. Malfunction of these proteins that control cell growth and cell death leads to permanent cell division and the insensitivity of these cells to normal signals to die (Kuppers et al., 2002; Croce, 2008; Young-Uk et al., 2009). In the present study, non-Hodgkin’s lymphoma lesion was absent in the heart and thymus. This may be related to the difference in animal model species and possibly due to organs structural difference, organs blood supply and the animal’s age with other environmental differences. Experimental T-cell lymphoma in transgenic mice offspring after 3-6 months of study also did not occur in the heart (Yu et al., 2006).
In the present study metastasis to the central nervous system (CNS) (cerebral cortex and cerebellum) was reported for the first time in laboratory mice. Metastasis occurred due to break away of the cancer cells from the tumor primary sites to the circulatory system blood stream (arteries and veins). Often, the metastatic location is noticed to be the nearest cluster of small blood vessels found by the circulating cancer cells. Neoplastic cells were seen in the lumen of small blood vessels in the cerebral cortex and the cerebellum. Other metastasis can occur through the lymph system or the spinal fluid between the layers of the meninges. Non-Hodgkin's lymphoma of the central nervous system in human which were either primary central nervous system lymphoma or metastasized from other primary sites of the body to the brain had been documented (Nelson et al., 1992; O'Neill et al., 1995).

Immunohistochemical staining in the present study showed that the cancer cells can be recognized by their aberrant expression of T cell marker positively CD3+ and expressed B cell marker negatively because they generally carries aberrant T-cell marker expression. In a previous study their results were focused on a fact that unspecific peripheral T-cell lymphoma is usually characterized by an aberrant T-cell phenotype (Cho et al., 2009) and their studies also accommodate with the results in the present study (Strickler et al., 1987; Hastrup et al., 1989; Yamazaki, 2002; Zettl et al., 2004; Ballester et al., 2006; Alizadeh and Advani, 2008).

5. Conclusion

the oral administration of dibenzo[a,l]pyrene at a dose of 15 mg/kg body weight successfully induced unspecific peripheral T cell non-Hodgkin’s lymphoma in various organs of the female BALB/c mice in DBP treated group (group B). Gross and histopathology observations depicted the tissue and cellular changes associated with this carcinogen.

References


