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Tumor Lung Cancer Model for Assessing Anti-neoplastic Effect of PMF in Rodents: Histopathological Study

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ABSTRACT

This study aimed to elaborate an experimental model of pulmonary carcinogenesis in healthy mice and to ascertain the preventive efficacy of PMF when administered against experimental lung carcinogenesis. Male Swiss Albino mice lineage was carried through an intra-peritoneal injection of the Benzo[a] pyrene diluted in corn oil, a polycyclic aromatic hydrocarbon is widely known by its power of tumoral lung induction. Four experimental groups had been used with 20 animals in each: The first is control group (without infection or treatment); the second is carcinogenic group without treatment, the third is treated carcinogenic group, the fourth is positive control group received only treatment, submitted to euthanasia 08, 16, 24 weeks after the experimental procedure. After 08 weeks, the presence of diffuse inflammatory alterations was observed in carcinogenic- non treated group with thickness of the alveolar wall after the inflammation, however, at analysis of the pulmonary tissue of the treated carcinogenic group it had been observed hyperplasic alterations (BALT hyperplasia) but in positive control group thickness of the alveolar wall was noticed. With more time, after 16, 24 weeks administration of PMF histopathological changes became lesser in the treated carcinogenic group as compared to animals treated with the B[a]P only. In conclusion, the main secondary alterations in the intra-pulmonary instillation of B[a]P of mice were: cellular proliferation, inflammatory alterations of several degrees. PMF treatment has a slightly protective effect to lung tissue along short time but with more time it improved the structure of the lung in carcinogenic treated group.

Key words: Cancer treatment, Benzo[a] pyrene, A549 cell line, BALT hyperplasia

INTRODUCTION

Annually, more than 5 million people are diagnosed with cancer and more than 3.5 million people die from cancer worldwide (Ferlay *et al*., 2000). The management of malignancies in humans still constitutes a major challenge for contemporary medicine (Coufal *et al*., 2007; Widodo *et al*., 2007). Despite improvement in therapy, the cure rate for lung cancer remains low. Chemoprevention offers an important approach to decreasing the incidence of lung cancers. Chemopreventive agents with strong efficacy against lung cancer often cause systemic toxicities and adverse effects by standard delivery modalities. Toxicities can often prevent the clinical use of these agents (Verschraegen *et al*., 2004).

According to Cragg and Newman (2000), over 50% of the drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them. In the last decades a considerable growth in scientific and medical interest for the use of herbal and traditional medicines has been observed (Gupta *et al*., 2001; Liao *et al*., 2004). Natural products play an important role in our healthcare system (Lee, 1999). They offer a valuable source of potent compounds with wide variety of biological activities and novel structures and provide important leads for the development of novel drugs (Vuorela *et al*., 2004).

Animal studies have shown that green tea, as another natural product, is a potent inhibitor of lung tumor development (Zhang *et al*., 2000). The study of the effects of green tea infusion on the spontaneous formation of lung tumors and rhabdomyosarcomas in A/J mice demonstrated that the mice given 1% green tea exhibited a significantly lower lung tumor multiplicity from 0.72/mouse to 0.41/mouse (Landau *et al*., 1998). PM 701 has been discovered to inhibit the growth of lung cancer and leukemic cells *in vitro* (Khorsid and Mushref, 2006) and to increase life span of mice bearing leukemia cells by at least 3 folds which means that it has a favorable antimitotic effect (Moshref *et al*., 2006).

Previous investigations of PM 701 (Ahmed *et al*., 2009) proved its cytotoxic activity against cancer cells, it caused selective programmed cell death of cancer cells (apoptosis) of cultured human lung cancer cells while it had reparative effect on normal human cell (foreskin) (Khorshid *et al*., 2005). It was effective in limiting of metastasic spreading of leukemia cells in animal models (Moshref *et al*., 2006). Light and electron microscopic histopathological study was carried on animal model which proved the reparative effect of this agent (Moshref, 2007).

This study aimed to confirm the previous results of present work by created lung cancer animal model using B[a]P then this animal treated by PMF and the results were compared with the results of non treated group.

MATERIALS AND METHODS

The whole project was conducted between 2007-2011 but this specific experiment on animal models was carried on in 2010.

In Tissue culture unit King Fahd Medical Research Center (KFMRC) in King Abdul Aziz University (KAU), Jeddah in Saudi Arabia.

Reagents: PM 701 is a yellowish powdered form, pH 8.3 that has sharp (offensive) odor and does not soluble in water but has good suspension with Tween 80 which was stable for at least one month. PM 701 was categorized as practically non toxic (Khorshid, 2008). PMF is the effective fraction of PM 701 as proved by El-Shahawy *et al*. (2010).

Benzo[a]pyrene (B[a]P) (99% pure) (a polycyclic aromatic hydrocarbon widely known by its power of tumoral induction) was purchased from Sigma Chemical Co. (St. Louis, MO). B[a]P was prepared immediately before use in animal bioassays by dissolving in corn-oil.

Animal studies: Eighty male Swiss Albino mice lineage (5-6) weeks were obtained from animal house at KFMRC maintained in polypropylene cages of 50×33×20 cm at a constant temperature (21±1°C) and 62% relative humidity. The use of animals was according to the ethical requirements that approved by the Animals Research Ethic Committee of KAU.

Mice lung cancer models in this experiment were carried through an intra-peritoneal injection of B[a]P diluted in corn oil. Four experimental groups had been formed with 20 animals each: First

control group (no treatment to account for stress factors during mouse handling procedures affecting tumorigenesis); Second carcinogenic non treated group (50 mg B[a]P/kg/one time), third treated group (50 mg B[a]P/kg/one time)+(120 mg PMF/kg/day), fourth positive control group received the treatment only (120 mg PMF/kg/day), all animals were submitted to euthanasia 2,4,6 months after the experimental procedure.

Lung tissues were extracted and histological preparation was carried according to El-Banhawy and El-Gansory (1989). Specimens were fixed in 10% neutral formalin and the standard procedures of dehydration, clearing and embedding in wax were followed. The tissue were sectioned at 3-5 μm and processed for light microscopic investigations adopting the Hematoxylin and Eosin (H and E) staining procedure (Drury and Wallington, 1980) and submitted to the morphometrical analysis to describe the tissue alterations.

RESULTS

In this study, mice cancer models were carried through an intra-peritoneal injection of B[a]P diluted in corn oil, then a detailed histopathological examination was conducted to determine the degree of lung tumor progression related to the effect of PMF on tumor development. The results of the light microscopic investigations of the carcinogenic lungs after two months, in the group received only B[a]P suggested that B[a]P is highly toxic; all lung nodules were diagnosed as lung adenomas (Fig. 1a, b). Where after two months in the B[a]P+PMF-treated group, the mice lung tissues showed a decrease in tumor load compared to the B[a]P- group (Fig. 2a, b). After four and six months, all mice showed great tolerance to treatment with PMF and no clinical evidence of toxicity was observed as PMF was found to exhibit significant efficacy against B[a]P-induced mouse

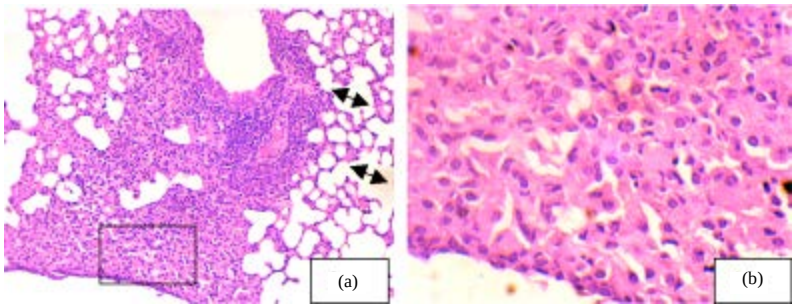

Fig. 1(a-b): Light photomicrographs representative adenomas from the B [a] P-group two mouths after the intraperitoneal injection at (a) x100 and (b) x1000 magnifications

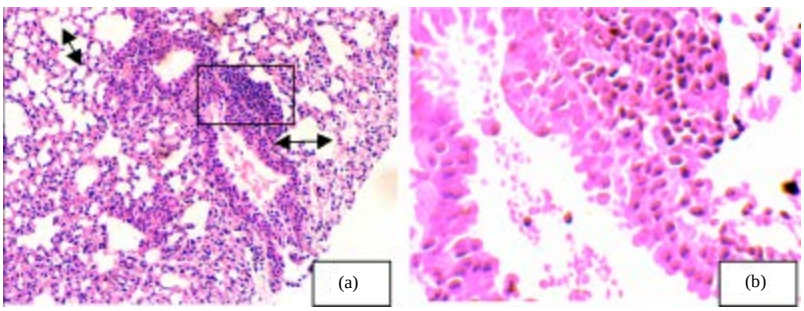

Fig. 2(a-b): Light photomicrographs representative efficacy of PMF against B[a]P-induced mouse lung tumorgensis after two months at (a) x100 and (b) x1000 magnifications

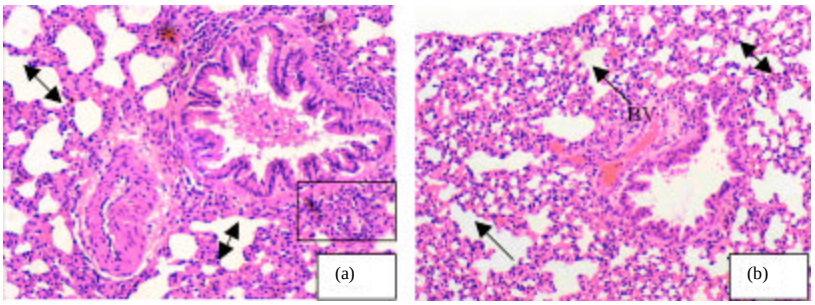

Fig. 3(a-b): (a) Light photomicrographs representative efficacy of PMF against B[a]P-induced mouse lung tumorigenesis after four months at x400 magnification and (b) Light photomicrographs representative mice were subjected to PMF treatment for 2 months, note alveolar widened (ulceration) and thick alveolar septa (arrow). Congested blood vessel (BV) at ×100 magnifications

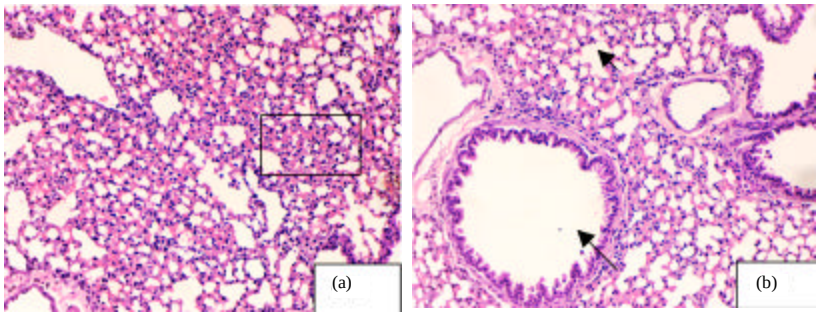

Fig. 4(a-b): (a) Light photomicrographs representative mice were subjected to PMF treatment for 4 months, note metaplasia in the epithelium of the lung at ×100 magnifications and (b) Light photomicrographs of mice were subjected to PMF treatment for 6 months note normal cytoarchitecture with well preserved alveoli (arrows) and alveolar septa (arrow-head) at x400 magnification

lung tumorigenesis with still manage to reveal the type II pneumocytes that had elongated nucleus with prominent nucleolus and scanty cytoplasmic contents (Fig. 3a). The PMF inhibited tumor load which is commonly interpreted as tumor growth during tumor progression compared with the group received only B[a]P.

As shown by this experiment the cytotoxicity of PMF well recorded in the group treated only with PMF, where lung congestion and widened alveolar septum, obviously, the first two months are more serious (Fig. 3b), after four months of the treatment the histopathological observation showed an alteration in the cytoarchitecture of the epithelium of the lung of the PMF group, these alterations were characterized by ulceration of the alveoli and desquamation (Fig. 4a). But after six months might be adaptation occur as, the lung tissue cells missing the morphological changes in the structure and the type II pneumocytes keep its normal shape with elongated basophilic nucleus with less prominent nucleolus and scanty acidophilic cytoplasm (Fig. 4b). Whereas, the histopathological observations of carcinogenic animals showed a great deal of alteration in the

cytoarchitecture of the epithelium of the lung, these alterations were characterized by tumorgenesis in addition to ulceration in the alveoli and metaplasis which may affect the functional capacity of the studied tissue.

DISCUSSION

The results of the light microscopic investigations of the carcinogenic lungs after two months, in the group received only B[a]P suggested that B[a]P is carsinogenetic, all lung nodules were diagnosed as lung adenomas with agree with Castro *et al.* (2008) who mentioned that [B[a]P]-induced mice lung carcinogenesis without significant systemic toxicity. The histopathological observations of carcinogenic animals showed a great deal of alteration in the cytoarchitecture of the epithelium of the lung, these alterations were characterized by tumorgenesis in addition to ulceration in the alveoli and metaplasis which may affect the functional capacity of the studied tissue. The mechanism by which B[a]P brought the ulceration of alveoli was by excavation of the surface epithelium and supporting tissues of the alveolar wall. The destruction of epithelial cells will diminish the secretion of surfactant which reduces surface tension within the alveoli preventing alveolar collapse during respiration. This will in turn reduce the easy flow of air from the terminal bronchioles in to the alveoli thus, reducing the alveoli ventilation, as described by Nioya *et al.* (2009).

PMF is a fraction of a natural product, readily available, cheap, sterile and non-toxic according to chemical and microbiological testing and proved effectiveness of this agent is reproducible on both *in vitro* and *in vivo* models as previously shown by Khorshid (2008 and 2009) and Khorshid *et al.* (2011). In addition to the previous characters for PMF, we found that after two months of treatment with PMF the lung tissues showed a decrease in tumor load compared to non treated group. After four and six months, all mice showed great tolerance to treatment with PMF and no clinical evidence of toxicity was observed but PMF was found to exhibit significant efficacy against B[a]P-induced mouse lung tumorigenesis with still manage to reveal the type II pneumocytes that had elongated nucleus with prominent nucleolus and scanty cytoplasmic contents. The PMF inhibited tumor load which is commonly interpreted as tumor growth during tumor progression compared with the group received only B[a]P.

As shown by this experiment the PMF made few alterations at the first two months in the group treated only with PMF like lung congestion and widened alveolar septum, after four months of the treatment the histopathological observation showed an alteration in the cytoarchitecture of the epithelium of the lung of the PMF group like ulceration of the alveoli and desquamation. But after six months might adaptation occur as, the lung tissue cells missing the morphological changes in the structure and the type II pneumocytes keep its normal shape with elongated basophilic nucleus, less prominent nucleolus and scanty acidophilic cytoplasm.

Previous results of cells count experiments showed severe drop of human lung cancer (A549) cells number when incubated in PMF compared with the number of control cells (cancer non-treated) that incubated in MEM media. The activity of PMF appeared here is due to the antiproliferative effect and apoptotic effect of this substance on different cancer cells as shown by Khorshid *et al.* (2009). It is likely that some degree of apoptosis and inhibition of cell proliferation might contribute to decreases in tumor load as occurred in present experiment where PMF might contribute to decreases in tumor load along six months, some of these results were described by Mahboub and Khorshid (2010) where they investigated the role of green tea extract on the proliferation of human ovarian cancer cells.

PMF as extracted from PM 701 contains cupper and Zn as elemental analysis and some amino acids as theronine, cysteine, tyrosine and methionine, also contains S-Methylglutathione as mentioned by El-Shahawy *et al.* (2010). Many previous studies may contributed to present findings here by explaining the role of Zn as an essential trace mineral that plays a key role in many important body processes such as binding DNA and RNA producing energy, regulating the immune system and cell metabolism. Zn as antioxidant that blocks the action of activated oxygen atoms which are known as free radicals and can damage cells (Galan *et al.*, 2005). Other studies indicated that Zn affects various enzymes and transcription factors which are important for normal cell proliferation and differentiation; it modulates DNA replication, protein synthesis and cellular signaling pathways which described by Khorshid (2004) and Wong and Abu Baker (2008).

Also Cup and Zn elements are essential for several biological functions throughout life such as repairing cells and protecting them from damage as mentioned by Sandstead and William (2007). Copper is a trace mineral that is needed for many important body processes. Animal studies have shown that copper is useful in maintaining antioxidant defenses that block the action of activated oxygen atoms which are known as free radical and can damage cells (Araya *et al.*, 2005). Also it protects rat liver from cancer damage and the intake increase of copper has been found to reduce the occurrence of cancer in the test of animals (Coates *et al.*, 1989). All these studies explained the reparative effects of PMF in treated mice, where PMF contains Zn and Cu.

In addition the PMF contains some amino acids as theronine, cysteine, tyrosine and methionine which are very important for damage the proliferated cancer cells as stated by Vuorela *et al.* (2004) suggested the presence of both peptide and receptor has been found to bind OGFr (opiod growth factor) and hence a reduction in OGFr-OGFr interactions that would repress cell replication. S-Methylglutathione in PMF extracted content acts as an important defense mechanism against certain toxic compounds such as drugs and carcinogens, these results were consistent with Shimizu *et al.* (2005).

CONCLUSION

In the present study, PMF was the target for its anticancer effectiveness as it was capable of killing cancer cells. Animal model (mice) for cancer was induced chemically using Benzo pyerine given to animal via IP. The success in achieving metastasing lung cancer was evident. Also, the safety of PMF was proved here, where control group was also included for finding any side effects. The results were encouraging, animal survival was more in treated group. Histopathological examination of the affected tissues showed marked deceased in tumor size in lung as inducing apoptosis was proved to be the main effect of the treatment.

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