

Pulmonary Lesions Associated with Intratracheal Benzo(a)pyrene Instillation in Sprague Dawley Rats

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ABSTRACT

This study was conducted to assess acute exposure to very low dose of intratracheally instilled Benzo(a)pyrene (BaP) on the lungs of rats. A total of 30 rats were utilized in this study and they were randomly divided into 6 groups. The control group (G1) did not receive any treatment, whereas the rats in the remaining 5 groups were administered with 13.8 ng of BaP, which were then sacrificed at 1 hour (G2), 8 hours (G3), 16 hours (G4), 32 hours (G5), and 72 hours (G6) of post-instillation (p.i.). Morphological appearances of all the lungs of all the treated rats consisted of various degrees of congestion, mostly evident in G3 and early development of emphysema, as seen in G4. These worsened as time progressed as observed in G6. On the other hand, the histological findings of the lungs of the treated rats revealed that the lungs had underwent some changes that were characterized by progressive alveolar congestion, epithelialisation with emphysema and accompanied by infiltration of inflammatory cells predominantly with alveolar macrophages and some neutrophils. However, even with such lesions seen, there was no apparent manifestation of impairment of the pulmonary system.

Keywords: BaP, histological, intratracheally, lung, morphological

INTRODUCTION

Air pollution has been incriminated countless times as the cause of the adverse health effects reported in man (Martin *et al.*, 1997; Chauhan & Johnston, 2003). There are evidences of increased in respiratory related illnesses which have been linked to the occurrences of air pollution (Kelly, 2003; Risom *et al.*, 2005). Malaysians have suffered too many episodes of haze in the last few decades, with one of the worst in 1997, where Benzo(a)pyrene (BaP), i.e. a form of polycyclic aromatic hydrocarbon (PAH) and categorized as a particulate matter (PM), was found to be the most predominant pollutant (Zakaria *et al.*, 1998). This BaP,

which is notoriously known to possess both carcinogenic and mutagenic properties, is usually found as a product of incomplete combustion (ATSDR, 1995; Faust, 1994) ubiquitously in the environment and has extensively been used as an indicator of carcinogenic hazard in air pollution studies (Khesina, 1994). Even though many studies have been conducted using animal models and epidemiological data exist linking the presence of the hazardous BaP to cases of pulmonary insufficiency (Brauer *et al.*, 2001), the exact mechanism behind the cause and effect is still poorly understood (Lin *et al.*, 2009). There is also limited information on exposure effects to the pollutant via inhalation at such a very low dose at certain duration (ATSDR,

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1995), such as the one that was experienced by the public during the severe haze episode in 1997 in Malaysia.

MATERIALS AND METHODS

Thirty, 8-9 weeks old, male, Sprague Dawley rats weighing between 150-200g, were used as models in this study. Rats were housed in separate cages, whereby 3 rats were placed per cage. All the rats had complete access to standard rat chowder and water *ad libitum*. All the rats were allowed a week of acclimatization prior to the initiation of the treatment. As a precaution against any bacterial infections, a single long acting oxytetracycline injection (10 mg/kg) was intramuscularly administered to all rats.

The rats were equally and randomly assigned to 6 different treatment groups, encompassing the control (G1), 1 hour p.i. (G2), 8 hours p.i. (G3), 16 hours p.i. (G4), 32 hours p.i. (G5), and 72 hours p.i. (G6), whereby the rats were humanely sacrificed at the respective intervals. The rats in G1 were only sacrificed at 72 hours.

A total of 7 µl of BaP in tricapylin mixture, which is equivalent to 13.8 ng of BaP, was intratracheally instilled once, as described by Oka *et al.* (2006) under general anaesthesia to each rat in the treatment group, while the control rats did not receive any instillation. The dose was calculated based on the concentration of BaP during the 1997 haze in Malaysia which lasted for 3 months (Zakaria *et al.*, 1998).

The rats were closely monitored for any development of clinical signs pertaining to the respiratory system dysfunction. After each interval had been reached, the rats were put under general anaesthesia using a combination of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) that were intramuscularly given and later humanely euthanized. The lungs were immediately procured and visual appraisal of the changes was noted before the lungs were fixed in 10% neutral buffered formalin for histopathological evaluations using Haematoxylin and Eosin (H&E) staining.

RESULTS AND DISCUSSION

No obvious clinical sign was observed in any of the rats. This could have been either due to the very low dose used or because the lung has a very large reserve whereby only when more than 75% of the reserve is exhausted, manifestations of the clinical sign can be noted. The expected clinical signs would generally include depression, inappetance and inactiveness, whilst signs of respiratory impairment comprised of tachypnoea, hyperventilation, dyspnoea, and in worst cases, cyanosis. However, it is believed that common air pollutants which often cause injuries to Type I alveolar cells can lead to mild to moderate permanent lung lesions. Even though large accumulations of inflammatory cells were seen in the lung which had previously been exposed to pollutants, it was subsequently insufficient to produce apparent lung lesions (Witschi, 1990) and thus, could probably explain the absence of any obvious clinical signs.

Collectively, there were a few clear gross pathological changes seen in the lungs of all the rats that were treated with BaP. The lesions include varying degrees of congestion, which were mostly distinguished in G3 rats (4/5), with emphysema observed earliest in G4 rats (5/5). However, these lesions worsened and became most apparent in G6 rats (5/5) (*Fig. 1*). On the other hand, this pulmonary congestion was not observed in any of the control rats.

Based on the findings of this study, it is clear that acute exposure to even a minute dose of BaP induced lung injuries, as can be evident from the presence of inflammatory cells, particularly the alveolar macrophage and neutrophil infiltrations in the alveolar spaces, accompanied by alveolar congestion, alveolar epithelisation, metaplasia, and dysplasia (*Fig. 2*), and followed by the early development of emphysema, as characterised by the 'thinning' appearance of the alveolar septa due to the outstretching with subsequently rupture of the alveolar wall (*Fig. 3*).

In a study done by Emre *et al.* (2007) in rats given 200 mg/kg of BaP intraperitoneally and sacrificed 24 hours later, the histological findings in the lungs showed marked thickness of alveolar septae, inflammatory cells infiltration, and

cellular debris in the lumen of the bronchioles. There was also alveolar space enlargement, which was accompanied by thinning as well as destruction of the septal wall. Similar changes in this experiment were particularly observed at 32 hr p.i with BaP (G5) (4/5).

Venugopal *et al.* (2007) reported that mice sacrificed 18 weeks after an oral treatment with BaP (50 mg/kg) were found to have developed severe alveolar changes, such as increased number of hyperchromatic, and irregular nuclei in the cells of the alveolar walls. Meanwhile, hamsters that were given BaP developed pleomorphic nucleoli and squamous metaplasia in their lungs (Smith *et al.*, 1975). All the reported observations are generally similar to the findings in this study, as shown in Fig. 2. Das *et al.* (2007) also observed that dysplasia was very prominent in the chronically BaP (0.2 mg/mouse) exposed mice 8 weeks post-exposure and onwards. Cellular dysplasia strongly indicates an early sign of cancer and is typified by the 4 major pathologic microscopic changes, namely anisocytosis (cells of unequal size), poikilocytosis (cells with abnormal shape), hyperchromatism and the presence of mitotic figure, as seen in Fig. 2.

Emphysema, as observed in this study, is also reported by others as a *sequelae* of smoking (e.g. Hautamaki *et al.*, 1997; Li *et al.*, 2003).



Fig. 1: Photograph of a rat's lung at 72 hours p.i. with BaP (G6) showing mild to moderately congested lung with very prominent emphysema at the lung lobes

Cigarette smoking also exposes an individual to the harmful effects of BaP. The findings of this study are almost similar to one by Li *et al.* (2003) who exposed rats to cigarette smoke. Rats developed inflammation in the lungs, as characterized by alveolar septal thickening, presence of RBCs and chronic inflammatory cells, mainly macrophages which were scattered within the alveolar spaces accompanied by diffused areas of emphysema involving the whole lung besides the finding of cellular debris in the bronchioles and the thickening of the blood vessel walls, and with a reduction in the size of the lumen. In this study, all the lung lobes, particularly the peripheral lung regions, were severely emphysematous and the inflammatory cells were found throughout the lungs.

The possible pathomechanism behind the occurrence of emphysema was believed to have been due to the alveolar macrophages releasing many proteolytic enzymes where the macrophages were accounted for more than 90% of the inflammatory cells in the smoker's

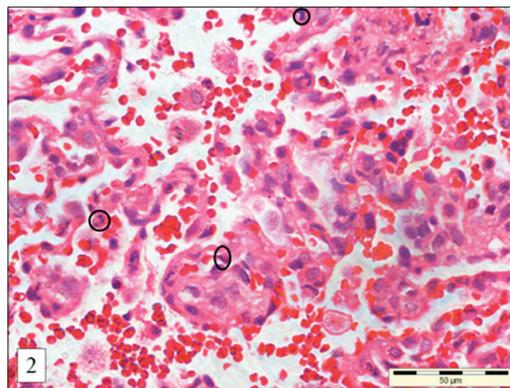


Fig. 2: A photomicrograph of the histological section of the lung of a BaP treated rat at 8 hours p.i. (G3) depicting a lesser dense area of the lung with severe inflammatory reactions predominantly by alveolar macrophages and mild neutrophils infiltration, and accompanied by severe haemorrhages in alveolar spaces and thickened alveoli. Some of the cells were also hyperchromatic and had irregular nuclei. There is also a presence of degenerated and necrotic cells. Mitotic figure was also visibly seen (indicated by black circles) [H&E; x400]

lungs, as evident from this study. This is on the contrary to the belief that only neutrophils are responsible in secreting elastase which results in the breakdown of elastic fibres. Macrophages can release a specific enzyme, known as macrophage elastase, i.e. a metalloproteinase that can solubilise various extracellular matrix proteins and elastin. This enzyme is also believed to be responsible in generating monocytes chemotactic activity which causes recruitment of more monocytes in the lungs. In addition, this enzyme can also make α_1 -antitrypsin inactive, which will indirectly enhance the elastase activity of neutrophils (Hautamaki *et al.*, 1997; Lucattelli *et al.*, 2003).

The elastase released by leukocytes encompasses both the alveolar macrophages and neutrophils which can be cytotoxic to the endothelial cells and may subsequently lead to increase vascular permeability or vasculitis (Lee & Downey, 2001). In view of the fact that blood vessel wall is made of elastic and collagen fibres; this possibly makes it vulnerable to be digested by elastase. Thus, there could very well be change in the permeability or necrosis leading to the escape of erythrocytes

into the alveolar spaces, as illustrated in *Fig. 3*. Prolonged secretion of elastase and other neutral proteinases can lead to breakdown of elastic tissues and damage to blood vessels (Werb & Gordon, 1975).

Recently, there has been much interest in the theory of oxidative stress caused by air pollutants, resulting in the degeneration and necrosis of cells. This is due to the disruption in the integrity of a cell from the attack of free radicals on the very vulnerable phospholipid layers (Kelly, 2003; Kooter, 2004). Recent evidences suggest that BaP may be able to induce oxidative stress conditions in living systems and therefore causes oxidative damage to macromolecules such as protein, lipid, and DNA (Garcon *et al.*, 2001).

The findings of the cellular debris in the lumen of the bronchioles (see *Fig. 4*) can be explained as the result of degeneration as well as necrosis of the epithelial cells lining the bronchioles leading to the sloughing of dead cells into the lumen. Since BaP was introduced intratracheally, the bronchioles have direct contact with the pollutant and in view of the fact that the cell membrane (which consists

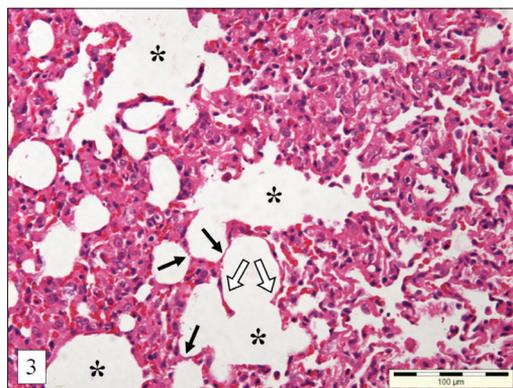


Fig. 3: A photomicrograph of the histological section of the lung of a rat treated with BaP at 32 hours p.i. (G5) at a less dense area near the lung periphery, exhibiting a moderate alveolar congestion, clusters of alveolar macrophages, stretching of alveolar wall (arrow) and rupture of alveoli (arrowheads) with emphysema (asterisks). [H&E; x200]

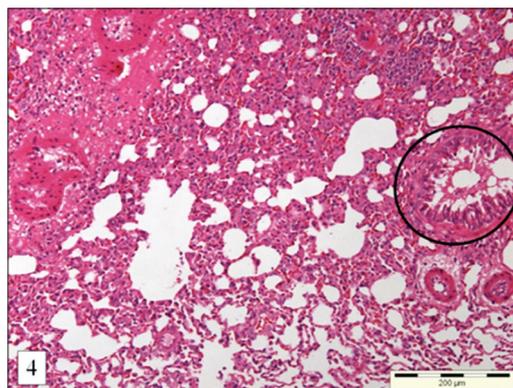


Fig. 4: A photomicrograph of the histological section of the lung of a BaP treated rat at 72 hours p.i. (G6), exhibiting alveolar hyperplasia at the top, whereas emphysema was seen to have distributed all over the lung mainly at the bottom area. Notice the cellular debris in the bronchiole (marked by black circles) [H&E; x100]

of phospholipid layers) is very susceptible to the attack of free radicals generated by BaP, it could have resulted in bronchiolitis. Werb & Gordon (1975) commonly observed recurrent bronchiolitis as a primary response to dusts and air pollutants.

The results of this study also demonstrated that BaP might induce an intense influx of immune cells in the lungs at around 8 to 16 hours p.i with BaP. Emre *et al.* (2007), Kooter (2004) and Kelly (2003) stated that immune cells influx is a prominent feature in response to exposure to PM. The blood-air barrier migration behaviour by the inflammatory cells might have triggered a proliferative response in the alveolar epithelium (Witschi, 1990).

The most vulnerable cell to toxic agents, particularly from air pollutants in the lung, is Type I alveolar cell (Kooter, 2004; Kelly, 2003; Rahman & MacNee, 2000). Owing to its odd shape and localization in the alveolar epithelium which precludes it from undergoing cellular division to replace damaged cells. Thus, the damaged cells are slowly being replaced by Type II alveolar cells that have taken up the shape and functional role after undergoing the division process.

It is believed that common air pollutants often cause injuries to Type I alveolar cells and will usually leave only mild to moderate permanent lung lesions (Witschi, 1990). This could have resulted in the architectural changes and cell proliferation, as well as cellular changes seen from the histopathological findings of lungs of rats which had received the BaP treatment in this study. The transition of Type I cells to form Type II alveolar cells is a strong indication that the lung is responsive and adapting to the assaults by BaP.

CONCLUSION

In conclusion, it has been shown that acute exposures of the lung to BaP can illicit damaging pulmonary changes, even at very minute dose. This is apparent from the morphological changes which include congestion of the lungs with a

subsequent development of emphysema, whereas histological alterations were characterized by the progressive alveolar congestion, epithelialisation with emphysema, and accompanied by the influx of inflammatory cells, mainly by alveolar macrophages and some neutrophils. However, these lesions occurred without the manifestation of clinical signs pertaining to pulmonary system dysfunction. Nevertheless, more studies need to be done to establish the possibility of the involvement of oxidative stress caused by BaP in disrupting cellular viability which could have led to the occurrence of degeneration and necrosis of exposed cells.

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