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RESEARCH ARTICLE

STUDY ON MECHANISM OF BRONCHIAL EPITHELIAL CELL APOPTOSIS IN ACUTE LUNG INJURY INDUCED BY SMOKE INHALATION OF MOUSE MODEL BY EPITHELIAL CELL APOPTOSIS MARKERS DETECTION

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ABSTRACT

Background: The modern medical studies clarify that, it is apoptosis, not the necrosis which is actively involved in Acute Lung Injury (ALI) caused by smoke inhalation. A variety of lung apoptosis namely vascular endothelial cells, pulmonary inflammatory cells and alveolar type II cells transpire in such state. The apoptic cell population among them certainly jacked within few hours then sustained a high level.in addition, apoptic genes namely Bcl-2 and Bax was also changed in mRNA and protein expression level were materially increased. The aspiration of our dissertation is to investigate the mechanism of apoptosis induced by smoke inhalation in bronchial epithelium. **Objective:** To find out the Mechanism Of Bronchial Epithelial Cell Apoptosis In Acute Lung Injury Induced By Smoke Inhalation Of Mouse Model By Epithelial Cell Apoptosis Markers Detection. **Methods:** Twenty-two Wild-type C57BL/6 mice were subjected to smoke inhalation to formulate model after random classification into 4 groups: Control group (not exposed, n =4), 2m (exposed to smoke for 2minutes, n=6), 3m (exposed to smoke for 3minutes, n=6), 4m (exposed to smoke for 4minutes, n=6). 24hrs after the smoke exposure 10% chloral hydrate solution 350mg/kg according to body weight were administrated by intra-peritoneal injection. All the mice were sacrificed step by step at a 24hrs, 48hrs and 72hrs time interval. After anaesthesia, 1ml fresh blood was collected from the left ventricle of the mice, henceforth opening the thoracic cavity. The left lung was obtained to execute the histo-pathological investigation and grading. To reveal apoptosis and its mechanism, in situ western bloat and TUNEL were accomplished after getting the sample of bronchial epithelial cell collected from the different lobes of right lung. **Results:** Contrast with control group, haemorrhage, wall thickening, inflammation and oedema were clearly evident in lung tissue histo-pathological examination of 2m group, 3m group and 4m group. Moreover, positive expression of NF-kB, CASPASE- 3 and PARP were perceived. **Conclusions:** Bronchial epithelial cell apoptosis has a significant role in acute lung injury due to fume inhalation was evident. Positive expression of NF-kB, CASPASE- 3 and PARP indicated their involvements in acute lung injury.

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INTRODUCTION

Smoke inhalation injury is common in burn victims and significantly contributes to the morbidity and mortality of burn injuries (Lange, 2011; Toon, 2010). About 22% of burns combine with smoke inhalation injury, which increases burn-related mortality by 20% (Fraser, 2005). Flame burn victim can have inhalation injuries and the rate can be higher as 70% or even more. The basic pathological variations that occur in inhaled lung include pulmonary oedema, alveolar atrophy, and atelectasis and so on. Airway injury is caused by ventilation function obstruction and extensive pulmonary atrophy, emerging in marred ventilation and ventilatory function. Airway injury is assumed to be the leading to acute lung injury and mucosal epithelial cell injury is the chief airway injury. A numerous revisions up to now innovated that; smoke inhalation can cause a range of lung apoptosis namely vascular endothelial cells, pulmonary inflammatory cells and alveolar type II cells. Similarly, increased apoptosis of circulating lymphocytes has been reported after burn injury [4]. Nevertheless, it's undoubtedly evident that apoptosis plays an imperative role in acute lung injury, however still it is not determined whether smoke inhalation grounds endogenous or exogenous pathways of bronchial epithelial cells death, which we'll explore by passing multiple points the expression NF-kappa B, Caspase-3, and PARP, to determine the specific pathways of apoptotic bronchial epithelial cell bereavement and its mechanism of apoptosis overhaul.

METHODS AND MATERIALS

Animals: Twenty-two male Wild-type C57BL/6 mice weighing 70-85g were endowed by Laboratory Animal Centre of Henan Province [animal certification: SCXK (Yu) 2015-0004], and left to familiarize for 1 week in a temperature and light controlled (20-25C; 12-h light/dark cycle) environment, with standard food and water, afore the experimentations. This study was endorsed by the Animal Care and Use Committee of the Zhengzhou University (Henan, China), and carried out in accordance with The National Natural Science Foundation guide for the care and use of Laboratory animals.

Smoke Exposure by self-made smoke machine: Four mice were arbitrarily selected as the control group; the other eighteen were bare to smoke inhalation. 150g dry pine wood shavings and 30ml kerosene were mixed meticulously in a specially made metal container with two channels for air entry and smoke exhaustion, besides with a heater underneath. After turning on the heating device 5mins before the experiment, the smoke was fanned from the container to a 45cm×30cm×40cm glass container using an air blower. After the apparatus was loaded with smoke, six mice were organised in a 35×25×20cm wire cage and settled into the container. The air was blown into the container last for 10 seconds, with an interval of 15 seconds, this cycle was repeated six times. After a minute of inhalation of smoke, eventually the mice were taken out of the glass box, and keep them 5 minutes in standard atmosphere, replication the above procedure for 2 times, for the 2m group, 3times for 3m group and 4 times for the 4m group. During the entire technique, the temperature inside the container was steadily below 40 ℃. Four mice died during this progression, the mortality rate was 18.2%.

After finishing smoke inhalation procedure,24 hours after the injury, and 10% chloral hydrate solution at a dose of 350mg / kg were administrated through intra-peritoneal injection just before the sample collection. Blood samples were collected through a puncture in the left ventricle, keep for 60 min to clot and centrifuged at 5,000r/min for 10 minutes, and the left lung was acquired to perform the histopathological examination and grading. Simultaneously, the expression of NF-kB, CASPASE-3 and PARP was detected by Western blot and ELISA, in the right upper lobe. And right middle lobe was collected to assess the DNA degradation of apoptic cells by using TUNEL assay.

Histopathological Examination and grading: After the left lung tissue fixing with 10% formalin for 24 h, lung tissues were dehydrated in ascending series of ethanol solutions (70%, 80%, 95% and 100% alcohol), embedded in paraffin and sectioned with 5mm thickness, stained by hematoxylin-eosin (HE). Two experienced pathologists in a blinded manner appraised the tissue features by a microscopic inspection. The severity of lung injury was graded by the criteria of haemorrhage, neutrophil infiltration, oedema, bronchiole epithelial desquamation, and hyaline membrane formation. The degrees of lung injury were ranged from 0 to 4, briefly, with 0 indicating absent, 1 mild, 2 moderate, 3 severe, and 4 the most severe. The results were added to the lung histopathological score.

Western Bloat examination to detect the expression of NF-kB, CASPASE-3 and PARP: The protein expression of NF-kB, CASPASE-3 and PARP in lung tissue was detected by Western Blot. Right upper lobe lung tissue protein was extracted by using RIPA lysis buffer and phenylmethylsulfonyl fluoride (PMSF), and the supernatant was collected. Protein concentration was measured by BCA kit (Biotechwell Company Shanghai China). Protein of 30μg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Membranes were washed with TBS (140 mmol/L NaCl, 50 mmol/L Tris-HCl; pH 7.2) containing 0.1% Tween 20 (TBST) and 5% skimmed milk to block nonspecific protein binding. Membranes were incubated with rabbit anti- NF-kBpolyclonalantibody(5μg/ml Abcam USA), rabbit anti-Caspase-3 polyclonal antibody (1:100Abcam USA) rabbit anti-βactin polyclonal antibody(1:500 Abcam USA) in TBST for overnight at 4℃, washed five times with TBST, and then incubated with goat anti-rabbit IgG horseradish peroxidase–conjugated secondary antibody (1:2000Zhongshan Biotech, Beijing,China) for 1.5 h at room temperature. The detection of the band was performed using the enhanced chemiluminescence (ECL) detection system. The Versadoc Imaging System and Quantity One Software (Bio-Rad Laboratories, CA) were used to develop and analyse the membranes. With β-actin as a reference protein, the grey value ratio reflects the expression of the target protein.

TUNEL: The lung tissues were fixed in 4% buffered formalin and embedded in paraffin. Five-micrometerthick sections were attached to slides, deparaffinized, and stained with haematoxylin and eosin to evaluate morphologic changes. *In situ* terminal deoxynucleotidyl transferased UTP nick-end labelling (TUNEL) assay was executed using an *in situ* apoptosis detection kit (Apotag, Oncor Corp., Gaithersburg, MD).

All stages were completed conferring to the supplier's instructions. Briefly, paraffin-embedded sections were de-waxed and rehydrated and then incubated with proteinase K (20 mg/ml in 100 mMTris and 50 mMethylenediaminetetraacetic acid) for 15 minutes at 25°C. After the slides were washed four times with distilled water, the sections were incubated in equilibration buffer for 5 minutes. The sections were then incubated with the labelling solution containing terminal deoxynucleotidyltransferase (TdT) in a humidified chamber for 1 hour at 37°C. The reactions were terminated by rinsing the sections in a wash buffer. The units were incubated with antidigoxigenin fluorescein for 30 minutes at room temperature and then rinsed three times in phosphate-buffered saline. The fluorescent TUNEL-labelled slides were photographed using a fluorescence microscope.

Statistical analysis: All values are stated as means ±SD, data collected were imperilled to statistical analysis using IBM SPSS Statistics version 22 software. The homogeneity of variance was first checked with Levene test. Analysis of variance (ANOVA) was used for an overall comparison between the groups followed by LSD t-test (when the homogeneity of variance assumption was met) or by Games Howelltest (when the homogeneity of variance assumption was not met), A value of $P<0.05$ was regarded as statistically significant.

RESULTS

Histo-pathological Examination and grading: Light microscopic examination shows, the normal lung tissue alveolar structure is complete, uniform alveolar septums, alveolar cavity has neither exudates, nor leukocyte infiltration (Fig-01). Lung tissue exposed to the smoke including the 2m group, 3m group and 4m group showed haemorrhage, alveolar septal edema, and extensive neutrophil infiltration in different degrees. Alveolar cavity is observable in a large number of inflammatory cells and erythrocyte exudation (Fig-02, 03 and 04.). Compared with control group, the pathological score of 2m group, 3m group and 4m group were decreased (P <0.05).

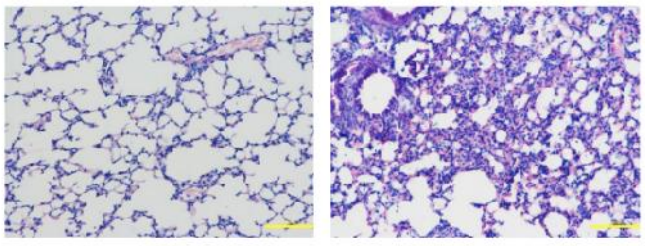


Fig-01 Fig-02

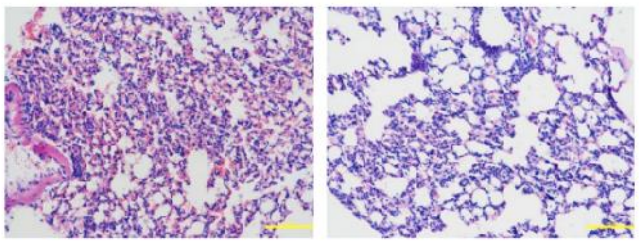


Fig- 03 Fig- 04

Western bloat examination: Compared with the control group, the expression of Caspase3(Fig.1 & Fig.2) and NF-kB (Fig.3 & Fig.4) in the 2m group, 3m group and 4m group increased(P<0.05). Compared with 24hours result, it increased in 48 and 72 hours in lung tissue.

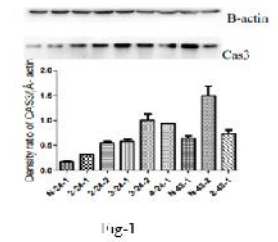


Fig-1

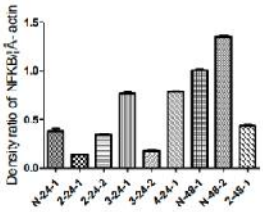


Fig-3

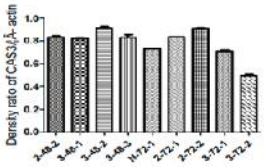


Fig-2

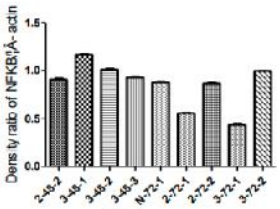
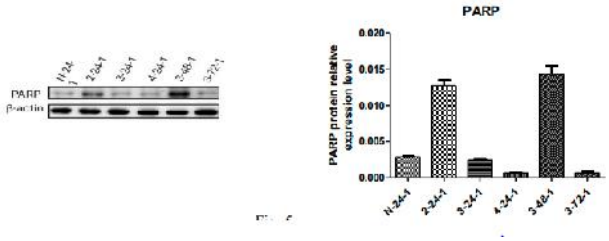


Fig 4

Paralleled with the control group, the expression of PARP (Fig.5) in the 2m group in 24hrs and 3m group in 48hrs increased (P<0.05). Other results are not changed ominously.



TUNEL assessment: Compared with control group (Fig-1), DNA fragments prominently increased in 2m, 3m and 4m groups (Fig-2,3a,3b and 4)which indicate a vibrant association with apoptosis.

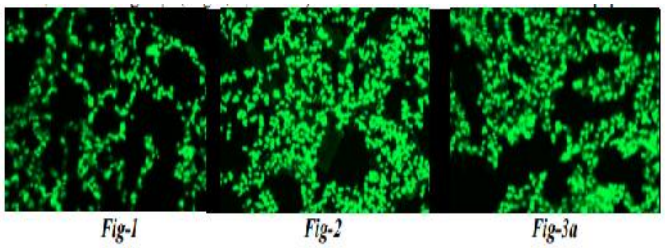


Fig-1 Fig-2 Fig-3a

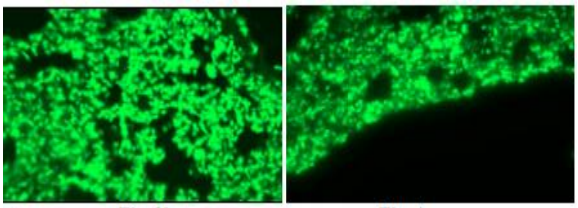


Fig-3b Fig-4

DISCUSSION

According to the WHO, more than 1 billion people develop airway and pulmonary inflammation as a result of inhaled smoke from indoor cooking fires, forest fires and burning of crops (Schwela, 1999; Schwela, 1997).

In addition, smoke toxicity is increasing because industrial products have shifted from woods and natural materials towards lighter construction materials, synthetics and petrochemicals, which ignite and burn two- to three-times hotter and faster. Thus, the probability that fire victims will breath in smoke and toxic gases is increased, because they have less time to escape (Sebastian Rehberg, 2009). Cell death occurs by two general mechanisms, necrosis and apoptosis, and we have an incomplete understanding about the mechanisms that determine which of these two processes occurs (Thomas, 2005). Apoptosis is a form of regulated cell death in which activation of specific intracellular serine rich proteases (caspases) leads to DNA cleavage and cell death. Apoptosis is an essential feature of development, and provides a mechanism for tissue remodelling in specific regions such as the interdigital spaces of fingers and toes. In general, apoptosis occurs without the release of intracellular products, whereas necrosis is associated with cellular swelling, membrane rupture, and the escape of intracellular products into the local environment. Apoptosis occurs in response to activation of specific cell membrane receptors, termed "death receptors," as well as in response to the release of mitochondrial products such as cytochrome C (Thome, 2001) (Figure 1).

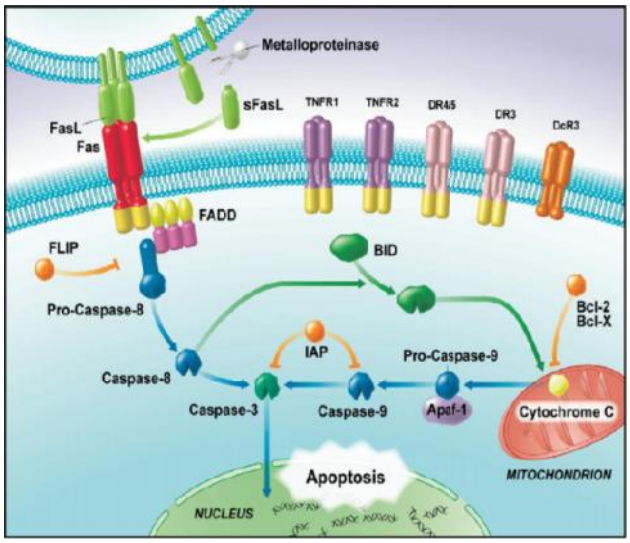


Figure 1. Cellular pathways that mediate apoptosis. A family of death receptors can initiate apoptosis, and their relative importance depends on the cell type. Two major pathways are shown here: the Fas receptor pathway and the receptor-independent mitochondrial pathway. The various membrane receptors share some components of the signalling pathways shown. DcR3 is unique, however, as it is a truncated receptor that does not signal. The soluble form of DcR3 functions as a decoy receptor that blocks receptor/ligand-mediated apoptosis.

Apoptosis is frequently increased during inflammation and can be induced in both lymphoid and parenchymal tissues by cytokines such as TNF-a or Fas ligand (FasL) (Nagata, 1995) Increased apoptosis in solid organs has also been reported in sepsis (Wang, 1994; Norimatsu, 1995). TNF-a signalling by means of its type I receptor and FasLsignalling by CD95/Fas/Apo1 induce concatermization of signal transduction proteins with death domains, and activation of a novel family of cysteineaspartate proteases (caspases) (Alnemri, 1996). Caspase-3 is probably the best understood of the mammalian Caspases in terms of its specificity and roles in apoptosis. Recent progress has generally confirmed the notion of multiple, complex death pathways (some of which require caspase-3 in specific cell types) that converge on common events including cell shrinkage, blabbing, chromatin condensation and DNA fragmentation (Alan, 1999). Several studies has revealed that caspase-3 is important for cell death in a remarkable tissue-, cell type- or death stimulus-specific manner, and is essential for some of the characteristic changes in cell morphology and certain biochemical events associated with the execution and completion of apoptosis. According to a group of scientists, increased apoptosis was observed primarily in lymphoid organs, such as spleen and thymus, but was not seen in the liver or lungs (Kunitaro Fukuzuka). Moreover, Caspase-3, a member of the caspase family of 13 aspartate specific cysteine proteases that play a central role in the execution of the apoptotic program (Alnemri, 1996; Cohen, 1997), is primarily responsible for the cleavage of PARP during cell death (Cryns, 1998; Nicholson, 1995; Tewari, 1995). It has been proposed that RNS (reactive nitrogen species)- mediated injury is related to DNA damage and the consequential activation of the nuclear enzyme PARP (Zhang, 1994). PARP is a chromatin-bound enzyme constitutively expressed in most cell types [31] and is involved in DNA repair (Szabo, 1996). PARP is cleaved by caspase-3 early during apoptosis in many different cell lines. The cleavage of PARP between Asp214 and Gly215 results in the separation of the two zinc-finger DNA-binding motifs in the NH2-terminal region of the enzyme from the auto-modification and catalytic domains, thus preventing the recruitment of the catalytic domain to sites of DNA damage. This cleavage of PARP has been suggested to occur in order to prevent depletion of energy (NAD and ATP) that is thought to be required for later stages of apoptosis. It is also thought that PARP cleavage serves to prevent futile repair of DNA strand breaks during the apoptotic program (Hamid Boulares, 1999). It has been shown that NF-κB in muscle is activated by disuse (Hunter, 2002), and sepsis (Penner, 2001) and in various tissues including the liver and heart, after burn injury (Nishiura, 2000; Maass, 2002). The cellular host response to burn injury plays a pivotal role in determining the intensity and duration of the inflammatory response. NF-κB, a ubiquitous nuclear transcription factor, plays a key role in regulating inflammation as well as other immune responses (Single Hind Limb Burn Injury to Mice Alters NF Kappa, 2014). In this present study, our goal was to find out the appropriate mechanism of bronchial epithelial cell apoptosis induced by smoke inhalation and its role in acute lung injury by detecting several proteins which are involved different pathways of cellular damage.

Conclusion

This scientific study reveals that, epithelial cell apoptosis has an obvious connection in ALI due to inhalation injury. Positive expression of NF-kB, CASPASE- 3 and PARP indicated their involvements in acute lung injury.

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