Histopathological and Stereological Studies for Evaluation of Aeromonas Hydrophila-induced Pulmonary Structural Changes with Emphasis on the Possible Protective Effect of Inositol Hexaphosphate

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Abstract: The present study was carried out to investigate the effect of Aeromonas Hydrophila endotoxins on the lung structure and the possible protective effect of inositol hexaphosphate (phytic acid) as a safe natural product using stereological analysis as well as histopathological and ultrastructural studies. 24 albino rats were used. The rats were divided into 3 groups: first control group injected intraperitoneally with normal saline at doses and intervals parallel to the treated groups. The second group was injected intraperitoneally with the lipopolysaccharide (LPS) of A. hydrophila once a week for four weeks at dose 20mg/kg b.w. The third group was injected with LPS suspension as second group followed by synergistically oral intubation with IP6 three times/week for four successive weeks at a total dose of 40 mg/kg rat. At the end of experimental period (4 weeks), the rats were sacrificed and the lungs were prepared for stereological analysis using special stereological procedures in fixation, sampling and tissue processing. Also, some lung specimens were prepared for usual light microscopy and transmission electron microscopy. The results revealed that A. hydrophila caused diffuse alveolar damage characterized by interstitial and intra-alveolar oedema and severe damage of the air-blood barrier. Also, the results revealed the ameliorative effect of Inositol hexaphosphate (phytic acid) against the damaging effect of Aeromonas endotoxin. It was concluded that A. hydrophila represents an important risk factor for the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) and It is recommended to use of phytic acid in immunocompromised persons as a safe and effective natural product. Also, lung researchers are advised to apply stereology for better understanding lung structure and function.

Key words: Aeromonas Hydrophila • Inositol Hexaphosphate • Stereology • Ultrastructure • Endotoxemia • Rat Lung

INTRODUCTION

Aeromonas hydrophila has been described as a causal agent of respiratory tract diseases, usually when there has been aspiration or signs of bilateral diffuse disease, leading to adult respiratory distress syndrome and high mortality (40%) [1]. Adult respiratory distress syndrome (ARDS) is still a major problem in critical care medicine [2]. Among a variety of initial events that may lead to ARDS, sepsis is one of the most common causes with the poorest prognosis [3].

A. hydrophila is a species of anaerobic Gram-negative bacteria. It can be found in food as well as in aquatic environments, worldwide. It is a member of the family Aeromonadaceae and is only one of six species, Aeromonas species, that are known to be pathogenic in humans. It can cause both intestinal and nonintestinal infections in humans and can often be fatal [4, 5]. Some of the diseases that A. hydrophila can cause include: septicemia, meningitis, pneumonia and gastroenteritis [6 - 10]. It is resistant to many common antibiotics such as penicillin and ampicillin [11, 12]. However, A. hydrophila is a very prevalent species and is capable of affecting immunocompetent as well as immunocompromised individuals. Aeromonas spp. produce an array of virulence factors that include endotoxins:
lipopolysaccharides (LPS) [13]. In rodents and humans, acute inhalation of LPS induces a severe lung inflammatory response characterized by activation of alveolar macrophages, recruitment of polymorphonuclear leukocytes (PMNs) into the airway and release of proinflammatory cytokines, chemokines (e.g., macrophage inflammatory protein [MIP] ), reactive oxygen species, cytolytic proteases and lysozomal enzymes [14 - 16]. The inflammation is accompanied by increased vascular permeability, bronchoconstriction and bronchial hyper-responsiveness [14, 15, 17].

Inositol hexaphosphate (IP6), also known as phytic acid, is a naturally occurring polyphosphorylated carbohydrate that is present in substantial amounts in almost all plant and mammalian cells first identified in 1855. Vucenik and Shamsuddin [18] and Vucenik et al. [19] demonstrated that phytic acid has multiple biological functions: reducing cell proliferation and increasing differentiation of malignant cells and reversion to normal phenotype. IP6 also appears to be a natural antioxidant that can reverse the effects of damaging free radicals, fight tumor formation and enhance the body’s natural disease resistance [20, 21]. Proposed mechanisms of its action include gene alteration, enhanced immunity and antioxidant properties [22]. The anticancer action of phytic acid could be explained by its mineral chelating potential. Some studies suggest that phytic acid acts as an anti-cancer agent by reversing the proliferative effects of carcinogens [18]. The antioxidant action of phytic acid is explained by inhibiting the activity of xanthine oxidase and superoxide formation [23].

Stereology is a method with which to obtain quantitative structural data of irregular three-dimensional objects on the basis of measurements made on two-dimensional sections. It depends, mainly, on special rules of sampling and tissue processing and specifically designed computer programs. Volumes estimated by computer-assisted stereology can be determined with high precision and accuracy [24]. Stereology is the method of choice for microscopy-based lung morphometry [25]. It is an essential tool for making statistically valid comparisons in experimental studies of the ultrastructural alterations that are seen in acute lung injury [26].

The present study was designed to investigate the effect of Aeromonas Hydrophila endotoxins on the lung structure and the possible protective effect of inositol hexaphosphate (phytic acid) as a safe natural product using stereological analysis as well as histopathological and ultrastructural studies.

**MATERIALS AND METHODS**

**Animals:** Twenty-four male Wistar albino rats of 375±30g were used. The animals were housed in stainless-steel cages under strict hygienic conditions and maintained at standard laboratory conditions of temperature, relative humidity and light/dark cycle and diet and water were provided ad libitum.

**Preparation of Aeromonas Hydrophila Endotoxin (LPS):** The strain of bacteria used in the present study is a subculture slant identified as A. hydrophila (A-47) from that purchased from American Type Cell Culture, a Global Biosource Center, USA (ATCC; Cat. # 7966). The bacterial suspension of A. hydrophila was prepared according to Schill et al. [27] and modified by Austin & Austin [28]. Extraction of LPS was performed using the phenol-water method according to Westphal and Jann [29]. The LPS was purified from nucleic acids by ultracentrifugation.

**Preparation of Inositol Hexaphosphate (IP6):** The pure inositol hexaphosphate (IP6, 98%) dodecasodium salt (C6H6O6P6Na12), produced by Sigma Co. USA (Cat # P-8810) was dissolved in sterile saline to the desired total dose of 40 mg/kg according to Vucenik et al. [30, 31].

**Experimental Design:** The animals were divided into three groups, each group contained 8 animals; first group remained as a control group that injected intraperitoneally (i.p.) with phosphate-buffered saline (PBS) (pH 7.4) 0.2 ml/rat at intervals parallel to the treated groups. The second group was injected i.p. with LPS at a dose of 20 mg/kg in 0.2 ml PBS once a week for four weeks as described by Parsey et al. [32]. The third group was injected with LPS suspension as second group followed by synergistically oral intubation with IP6 three times/week for four successive weeks at a total dose of 40 mg/kg/rat [30, 31]. At the end of experimental period (4 weeks), the animals were anaesthetized and the lungs were removed for further fixation.

**Stereology:** Lung preparation protocols, from fixation over dehydration and embedding to sectioning, were performed very carefully to avoid alterations of the tissue dimensions [26]. Emphasis was taken for the sample design in order to be accurate and efficient with taken in consideration bias related to the orientation of the structures in the lung [25, 33].
Fixation: Primary chemical fixation by controlled vascular perfusion with a glutaraldehyde-containing fixative followed by a phospholipid-stabilising protocol was used as it was reported that it is the method of choice when subsequent stereological analysis of edema and surfactant has to be performed [34, 35]. Fixation of the lungs was performed by vascular perfusion as described by Ochs et al. [36].

Sampling: Stereology is basically a sampling theory and, thus, the steps to obtain the tissue samples are an integral part of a stereological study. The most efficient way to achieve this is known as systematic uniform random sampling [26]. Systematic uniform random sampling performed at each sampling step yields samples that are representative of the whole organ [37]. Samples were collected according to standard methods as described by Fehrenbach and Ochs, [35]. 5–11 blocks were obtained from each single lung.

Tissue Processing: Processing of tissue blocks was performed as described by Fehrenbach and Ochs, [35]. For histological analysis, semithin sections (0.5 µm thick) were stained with methylene blue and examined by light microscopy. For ultrastructural study, ultrathin sections were cut and stained with lead citrate, using an Ultrostainer.

Stereological Analysis: Semithin and ultrathin sections were analyzed by established stereological methods [37]. At the light microscopic as well as at the electron microscopic level a systematic quadrats subsampling scheme [38], was applied to generate test fields over the whole section, distributed in a systematic random fashion. All parameters were determined on-line by means of point and intersection counting. For light microscopy an eyepiece containing an integration plate (100/25 points) was used at a magnifications of X100, X200 and X1,000. For transmission electron microscopy a coherent multipurpose test system with 192 test points and 96 test lines was applied at a magnification of X6,500.

The following stereological parameters were estimated:

(1) At the first level (LM 100X):
- Total parenchyma volume.
- Total nonparenchyma volume.

(2) At the second level (LM 200X):
- Volumes of nonparenchyma components:
  - Air ways (wall & lumen).
  - Vessels (wall & lumen).
  - Peribronchovascular space.

(3) At the third level (LM 1000X):
- Volumes of parenchyma components:
  - Alveolar space (air & edema fluid).
  - Alveolar septum (capillaries & tissue).
- Volume fraction of alveolar epithelial surface.

(4) At the fourth level (TEM 6,500X):
- Volumes of alveolar septum tissues:
  - Endothelium.
  - Interstitium.
  - Epithelium.
- Volume fraction of type I pneumocytes:
  - Normal.
  - Swollen.
  - Fragmented.

Histopathological Investigation: For histopathological analysis, semithin sections (0.5 µm thick) from three randomly chosen samples per lung were stained with hematoxyline and eosin and examined by light microscopy.

Ultrastructural Investigation: From the previously prepared blocks, ultrathin sections were counterstained with uranyl acetate and lead citrate using a standard procedure and examined by transmission electron microscopy.

Statistical Analysis: Data referring to individual lungs are given as discrete values obtained according to standard stereological formulas. Mean values are given±SD. Differences between control and experimental animals were tested for significance using analysis of variance (ANOVA). All statistical analyses were performed using the PC-STAT analysis of variance program [39] followed by LSD analysis. P<0.05 was considered to be statistically significant.

RESULTS

Histopathological Investigation: The lungs of rats in the control group showed normal alveolar architecture, normal open intra-alveolar spaces with normal terminal bronchioles. There was no evident lesion or inflammatory cells (Fig. 1A).
Fig. 1: A: a photomicrograph of section of control lung showing normal alveolar architecture and normal open intra-alveolar spaces. B & C: sections of bacteria infected lung showing diffuse alveolar damage, disruption and loss of both epithelial and endothelial cells. There were perivascular/peribronchial acute inflammation. There were hemorrhage and exudation of protein-rich edema. D: a section of bacteria infected lung showing emphysematous alterations; there were bursting interalveolar septa and emphysematous blebs (arrows). E: a section of phytic acid treated lung showing normal lung architecture. There were mild inflammation of peribronchial and perialveolar tissues with moderate edema of interalveolar spaces and minimal fibrotic changes. (H & E 400X).

Examination of the second group (bacteria infected group), exhibited marked lung histopathologic abnormalities, characterized by diffuse alveolar damage, disruption and loss of both epithelial and endothelial cells. There were perivascular edema and perivascular/peribronchial acute inflammation. There were hemorrhage and exudation of protein-rich edema into the alveolar spaces. The interalveolar septum showed focal thickness and fibrosis. The bronchioles showed local detachment of epithelium (Figs. 1B&C). Microscopic images also, revealed focal emphysematous alterations in a form of emphysematous blebs. The blebs showed thinned septa, atrophy of blood vessels and projecting spines, being a sign of bursting interalveolar septa (Fig. 1D).

Microscopic images of the third group (phytic acid treated group) showed great improvement in the histopathological picture, which nearly showed normal lung architecture, but there were mild inflammation of peribronchial and perialveolar tissues with moderate edema of interalveolar spaces and minimal fibrotic changes (Fig. 1E).

Ultrastructural Investigation: At the ultrastructural level, images of the control group showed regular morphology and normal size of air-blood barrier (Fig. 2A).

Images of the second group (bacteria infected group), showed swelling and focal fragmentation of the capillary endothelial cells and type I alveolar epithelial cells, leading to a denudation of the basal lamina (Fig. 2B&C). There was organization with proliferation of type II alveolar epithelial cells (cuboidal metaplasia). Alveolar septal thickening and finally fibrosis were present (Fig. 2B&C).

In the phytic acid treated group, the ultrastructural picture showed nearly normal size of the air-blood septum with minimal fibrotic changes. There was focal endothelial and epithelial swelling.

Stereological Analysis: The stereological data of parenchymal and nonparenchymal components are shown in the Table (1).

Fluid accumulation resulted in a significantly higher (P<0.05) total volume (mm³) of the parenchyma and nonparenchyma lung in the bacteria infected group (1.981 ± 498 and 339 ± 177, respectively) as compared with the control group (1.496±212 and 130±68, respectively).

Accumulation of edema fluid was observed in different compartments of the lung: in the peribronchovascular space, in the alveolar space and in the interstitium of the alveolar septum. The edema fluid in
Fig. 2: A: a representative electron micrograph of lung of control animals showing normal air-blood barrier with electron-dense alveolar epithelium (Ep) and capillary endothelium (En) and a very small interstitial space between epithelium and endothelium. Note the appearance of intra-alveolar surfactant (arrow). Scale bar 2 µm. B: an electron micrograph of lung of bacteria infected animals revealed the presence of edematous fluid (arrow) in the alveoli (Alv). The capillary lumen (Cap) contains an erythrocyte (Er). Scale bar 2 µm. C: an electron micrograph of air-blood barrier showing swollen and fragmented epithelium (Ep) and endothelium (En) Scale bar: 500 nm. D: a light micrograph of methylene blue stained section from lung of control group demonstrate almost normal lung architecture. Scale bar: 200 µm. E: a light micrograph of methylene blue stained section from lung of infected group demonstrate intra-alveolar fluid (arrows). Scale bar: 100 µm. Ep: epithelium. En: endothelium. Alv: alveolus. Cap: blood capillary. Er: erythrocyte.

Table 1: Stereological data of parenchymal and nonparenchymal components

<table>
<thead>
<tr>
<th>Stereological parameter</th>
<th>CONTROL</th>
<th>BACTERIA</th>
<th>BACTERIA+PHYTIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mm³)</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Total Nonparenchyma</td>
<td>130±68</td>
<td>339±177**</td>
<td>137±62</td>
</tr>
<tr>
<td>Air way</td>
<td>45±33</td>
<td>68±59</td>
<td>47±29</td>
</tr>
<tr>
<td>Wall</td>
<td>14±6</td>
<td>23±17</td>
<td>15±8</td>
</tr>
<tr>
<td>Lumen</td>
<td>31±22</td>
<td>45±42</td>
<td>33±21</td>
</tr>
<tr>
<td>Vessels</td>
<td>57±32</td>
<td>94±32</td>
<td>60±35</td>
</tr>
<tr>
<td>Wall</td>
<td>12±5</td>
<td>27±11</td>
<td>13±5</td>
</tr>
<tr>
<td>Lumen</td>
<td>44±27</td>
<td>68±33</td>
<td>47±32</td>
</tr>
<tr>
<td>Peribronchovascular sp.</td>
<td>28±12</td>
<td>177±99**</td>
<td>30±10</td>
</tr>
<tr>
<td>Total parenchyma volume</td>
<td>1.496±212</td>
<td>1.981±498**</td>
<td>1.502±234</td>
</tr>
<tr>
<td>Alveolar space</td>
<td>1.188±178</td>
<td>1.503±412</td>
<td>1.198±188</td>
</tr>
<tr>
<td>Air</td>
<td>1.167±191</td>
<td>1.049±299</td>
<td>1.173±183</td>
</tr>
<tr>
<td>Edema fluid</td>
<td>21±19</td>
<td>453±275**</td>
<td>25±18</td>
</tr>
<tr>
<td>Alveolar septum</td>
<td>308±65</td>
<td>479±108**</td>
<td>304±81</td>
</tr>
<tr>
<td>Capillaries</td>
<td>131±32</td>
<td>190±52</td>
<td>130±33</td>
</tr>
<tr>
<td>Tissue</td>
<td>177±43</td>
<td>288±69**</td>
<td>174±39</td>
</tr>
<tr>
<td>Epithelium</td>
<td>27±6</td>
<td>50±18**</td>
<td>25±3</td>
</tr>
<tr>
<td>Interstitium</td>
<td>103±22</td>
<td>176±49**</td>
<td>102±30</td>
</tr>
<tr>
<td>Endothelium</td>
<td>47±17</td>
<td>62±13</td>
<td>47±20</td>
</tr>
<tr>
<td>Fraction of alveolar epithelium</td>
<td>5.0±5.20</td>
<td>55.0±14.8**</td>
<td>7.4±6</td>
</tr>
<tr>
<td>Fraction of type I pneumocytes</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>normal</td>
<td>82.2±11.2</td>
<td>49.5±17.8**</td>
<td>79±17</td>
</tr>
<tr>
<td>swollen</td>
<td>13.3±12.0</td>
<td>19.2±9.2</td>
<td>12.8±10.8</td>
</tr>
<tr>
<td>fragmented</td>
<td>3.3±3.6</td>
<td>25.6±18.2**</td>
<td>7.2±3.5</td>
</tr>
</tbody>
</table>

Values were expressed as mean±SD. Differences between control and experimental animals were tested for significance using (ANOVA). All statistical analyses were performed using the PC-STAT analysis of variance program, followed by LSD analysis. (n = eight animals per group). ** Statistically significant at P<0.05.

DISCUSSION

The first justification for lung morphometry derives from the need of structural information in setting up models that allow one to assess the importance of structural design for functional performance. Stereological
methods are very efficient and provide lung researchers with tools that are essential to obtain valid data for the quantitative assessment of the ultrastructural alterations that are seen in acute lung injury. It thus, is the method of choice for microscopy-based lung morphometry [25, 26].

*A. hydrophila* infection in mice causes septicemia, endotoxic shock and death [40]. *Corrin* [41] observed that the lungs of human bacterial-shock victims are the most frequently damaged organs. In the present study, LPS induced septicemia followed by acute lung injury (ALI) in the form of diffuse alveolar damage characterized by interstitial and intra-alveolar edema and severe damage of the air-blood barrier. These findings are corresponding to those reported by others [42 - 48]. Generally, it was believed that the histopathological features of ALI reflect a typical response pattern to lung injury irrespective of the underlying cause [49].

According to our results, there was a highly significant increase of the volume of interstitial and intra-alveolar edema in the bacteria infected group compared with that of control. *Ochs et. al.* [50], *Muhlfeld et. al.* [51] and *Fehrenbach et. al.* [52] reported that intra-alveolar edema is the functionally most significant. These studies have also shown that edema assessment by stereology better reflects the functional status of the lung than does wet/dry ratio analysis. Damage to the alveolar capillary endothelium is a well-established cause of the influx of protein-rich fluid into the alveolar compartment [53].

The present study revealed that air-blood barrier and its component parts exhibited significant increase in thickness in the bacteria infected group compared with that of control animals. According to Fick’s law of diffusion, oxygen flow across a tissue barrier is directly proportional to the cross-sectional surface area and inversely proportional to the thickness of the barrier. Intra-alveolar and interstitial edema, as well as edematous swelling of the alveolar epithelium or capillary endothelium, increase the effective barrier thickness, thereby leading to decreased oxygenation in ALI [26].

A significant increase in alveolar epithelial cell injury was seen in the bacteria infected group compared with the control group as well as significant increase in the surface fraction of swollen and fragmented type I pneumocytes while in turn the surface fraction of normal type I pneumocytes decreased. These findings are in line with other studies comparing different experimental groups [42, 44, 45]. *Kinnula et al.* [54] and *Heffner* [55], reported that oxygen reactive species (ORS) can damage epithelial and endothelial cells lining the blood-air barrier by lipid peroxidation. In addition, the permeability of the blood-air barrier is augmented in acute lung injury as a result of basal lamina disruption, most likely mediated by matrix metalloproteinases [56]. The clinical symptoms of ALI are a direct consequence of severe damage to the blood-air barrier and subsequent protein-rich (permeability) pulmonary edema [57]. Intra-alveolar edema leads to alterations in the pulmonary surfactant system, which, in turn, lead to the decreased compliance that can be observed in ALI patients [58, 59].

Oral administration of phytic acid (IP6) leads to less severe edema formation and better fine structural preservation of the blood-air barrier. IP6, in particular, attenuated the extent of edema formation in all three compartments investigated (intra-alveolar, peribronchovascular, septal). Moreover, the ultrastructural appearance and thickness of all components of the blood-air barrier (alveolar epithelium, interstitium, capillary endothelium) were nearly similar to the control group. The ameliorative effect of IP6 could be explained by the role of IP6 in regulating vital cellular functions, including cell proliferation and differentiation as well as its antioxidant and anticancer properties [30, 31, 60]. Also, the recovering features in the histopathological sections mediated by IP6 may be not only because IP6 is a natural antioxidant, but also because it is a precursor/store of intracellular inositol phosphates that are important for various cellular functions [61].

**CONCLUSION**

The present study revealed that *Aeromonas hydrophila* bacteria represent an important risk factor for the development of acute lung injury (ALI). Also, we revealed the ameliorative effects of phytic acid on the lung after intoxication with the *A. hydrophila* endotoxin and advise its use in cases of immunocompromised persons. Eventually, physician-scientists should be encouraged to apply stereology to better understand lung structure and function in health and disease.

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REFERENCES


