Influence of ethanol concentration in the phagocytic function of neutrophils against Klebsiella pneumoniae isolates in an experimental model

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Abstract Background/Purpose: Although the prevalence of pneumonia or other extrapulmonary infections is higher in people with alcoholism or acute alcohol intoxication, the possible relationship of acute alcohol intoxication to phagocytic function has not been investigated. Our aim was to determine whether acute alcohol intoxication suppresses phagocytic function in human neutrophils.

Methods: Twenty healthy individuals were enrolled for isolating neutrophils to evaluate the neutrophil phagocytic function at different alcohol concentrations. Klebsiella pneumoniae was isolated from clinical specimens of liver abscesses. The rate of K. pneumoniae phagocytosis (K2 and non-K1/K2 isolates) by neutrophils was determined using flow cytometry and compared among the nine groups with different alcohol concentrations.

Results: The rate of phagocytic uptake decreased significantly with increasing alcohol concentration in both the K2 and non-K1/K2 K. pneumoniae groups (r = −0.866, p = 0.03 vs. r = −0.975, p < 0.001). Moreover, the percentage of K. pneumoniae ingested by neutrophils decreased with age.
Conclusion: The ability of neutrophils to phagocytose virulent K2 K. pneumoniae was suppressed by ethanol at high concentrations. This finding may account for the higher prevalence of pneumonia or other extrapulmonary infection in people with acute alcohol intoxication. 

Methods

Participants

Neutrophils were isolated from normal, healthy individuals who were not alcoholics and had no underlying diseases, such as malignancy, diabetes mellitus, cirrhosis of the liver, renal insufficiency, or autoimmune disease. Individuals (n = 20) enrolled in the study also had reported no infections within 4 weeks. The experimental procedures were reviewed and approved by the Ethics Committee of Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan and informed written consent was obtained from each participant.

Isolation of human neutrophils

Neutrophils were separated as follows. Heparinized blood (10–60 mL) was collected and mixed with an equal volume of dextran/saline solution and allowed to sediment at room temperature for 40 minutes. The leukocyte-rich supernatant was layered over a density gradient of Ficoll-Paque (Pharmacia, Taipei, Taiwan). The samples were centrifuged at 400 g for 40 minutes at 20°C, and the pellet was collected, erythrocytes removed by hypotonic lysis, and isotonicity was restored using hypertonic saline. Each collected pellet was resuspended in ice-cold phosphate-buffered saline (PBS), and the cell concentration was adjusted to 1 × 10^7 cells/mL. We verified that cell viability was > 95% trypan blue exclusion.

Preparation of pooled serum

Pooled serum was prepared from another 10 healthy volunteers after informed consent was obtained from each participant. Heparin-free blood drawn from the volunteers was clotted at room temperature and centrifuged (1000g for 40 minutes at 20°C). The serum was removed, pooled, aliquoted, and stored at −70°C.
Fluorescence labeling of *K. pneumoniae*

The capsular serotype K2 strain isolated from a patient with a liver abscess was used in this experiment. *K. pneumoniae* strain ATCC700603 with non-K1/K2 capsular serotype was used as the control. The strains were incubated separately overnight at 37°C, and cell concentration was adjusted spectrophotometrically (Olympus, Center Valley, PA, USA) and confirmed by quantitative colony counts. Bacteria were killed by heating for 60 minutes in a 70°C water bath. The bacteria were washed with PBS and labeled with fluorescein isothiocyanate (FITC; 0.1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) in 0.10M NaHCO3 (pH 9.0) for 60 minutes at 25°C. FITC-labeled bacteria were resuspended to 2 × 10^8 cells/mL with PBS, aliquoted, and stored at −70°C. Aliquots were thawed prior to use.

Phagocytosis reactions at different alcohol concentration

Phagocytosis was measured using a standard assay. We used nine different ethanol concentration groups, with each solution created by adding alcohol to reach ethanol concentrations of 0mM, 20mM, 40mM, 60mM, 80mM, 100mM, 200mM, 400mM, and 800mM. The total volume of the final mixture was 1 mL.

Pure ethanol was introduced to a mixture pre-warmed at 37°C of 100 μL of neutrophil suspension (i.e., 1 × 10^6 cells), 100 μL of freshly thawed, pooled normal human serum (10% v/v; used for opsonization), and 600 μL PBS in 10 mm × 75 mm-polystyrene tubes (BD, Franklin Lakes, NJ, USA). After incubation at 37°C for 30 minutes, we added FITC-labeled bacteria [200 μL; 4 × 10^7 colony-forming units/mL] for a total volume of 1 mL, and the tube was agitated for 10 minutes.

Phagocytosis assay using flow cytometry

FITC-labeled neutrophils were analyzed using a FACScan with an argon-ion laser (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) as previously described. A total of 10,000 neutrophils were processed using Cellquest version 1.0 software (Becton Dickinson Immunocytometry Systems). By analyzing mixtures of labeled and unlabeled bacteria, the boundary between positive and negative fluorescence was determined. The percentage of ingested bacteria was assessed after the addition of ethidium bromide.

Statistical analysis

Between-group differences in the phagocytic function of neutrophils at different alcohol concentrations were examined via one-way analysis of variance with repeated measures. Pearson’s correlation was used to evaluate the relationship between alcohol concentration and rate of phagocytic uptake. Linear regression was used to evaluate the relationship between age groups and rate of phagocytic uptake. Differences were considered to be significant at p < 0.05, and all statistical tests were two sided. Data are presented as mean ± standard error of the mean (SEM).

Results

To determine whether acute alcohol intoxication impairs the phagocytic function of neutrophils, we compared the rate of human neutrophil phagocytosis of *K. pneumoniae* under different alcohol concentrations. *K. pneumoniae* capsular serotype K2 was chosen as the bacterial strain, and the non-K1/K2 strain was chosen as the control. Table 1 revealed the rate of human neutrophil phagocytosis of K2 and non-K1/K2 *K. pneumoniae* under different alcohol concentrations. Data are presented as mean ± SEM.

The *K. pneumoniae* strain reportedly has a high resistance to neutrophil phagocytosis. The mean percentage of neutrophil phagocytosis of K2 *K. pneumoniae* strain without alcohol was 40–50%. Data from samples exhibiting higher percentages (> 65%) of neutrophil phagocytosis of the K2 *K. pneumoniae* strain in the absence of alcohol were abandoned. The non-K1/K2 *K. pneumoniae* strain was also reported to be sensitive to neutrophil phagocytosis. The mean percentage of neutrophil phagocytosis of the non-K1/K2 *K. pneumoniae* strain in the absence of alcohol was 75–85%. Data from samples exhibiting low percentages (< 60%) of neutrophil phagocytosis of the non-K1/K2 *K. pneumoniae* strain in the absence of alcohol were abandoned.

Overall, the trend toward decreased rates of phagocytosis in the presence of increased alcohol concentrations appeared much more significant at 800mM in both the K2 and non-K1/K2 *K. pneumoniae* groups. No significant difference in the rate of phagocytosis at concentrations from 0mM to 400mM was observed between either the K2 or non-K1/K2 *K. pneumoniae* groups. Evidence of dead cells in some samples at an alcohol concentration of 800mM was observed, and these samples were abandoned. The neutrophil phagocytosis of *K. pneumoniae* was significantly lower in the 800mM group, while the rate of neutrophil *K. pneumoniae* phagocytosis was similar at all other alcohol concentrations. Pearson’s correlation analysis of the relationship between K2 *K. pneumoniae* phagocytosis and alcohol concentration revealed that the rate of phagocytic uptake decreased significantly with increasing alcohol concentration (r = −0.866, p = 0.03), while similar analysis of non-K1/K2 *K. pneumoniae* phagocytosis revealed that the rate of phagocytic uptake decreased significantly with increasing alcohol concentration (r = −0.780, p = 0.04).

Table 1 Percentage of human neutrophil phagocytosis of K2 and non-K1/K2 *Klebsiella pneumoniae* isolates under different alcohol concentration.

<table>
<thead>
<tr>
<th>Alcohol concentration (mM)</th>
<th>Phagocytosis (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>K2</td>
</tr>
<tr>
<td>0</td>
<td>56.15 ± 3.52</td>
</tr>
<tr>
<td>20</td>
<td>55.59 ± 2.96</td>
</tr>
<tr>
<td>40</td>
<td>54.19 ± 3.55</td>
</tr>
<tr>
<td>60</td>
<td>50.76 ± 4.51</td>
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<tr>
<td>80</td>
<td>55.58 ± 2.34</td>
</tr>
<tr>
<td>100</td>
<td>57.30 ± 2.50</td>
</tr>
<tr>
<td>200</td>
<td>53.54 ± 3.21</td>
</tr>
<tr>
<td>400</td>
<td>54.88 ± 3.75</td>
</tr>
<tr>
<td>800</td>
<td>30.00 ± 2.60</td>
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</table>
The percentage of human neutrophil phagocytosis in *Klebsiella pneumoniae* and non-K1/K2 *K. pneumoniae* under different alcohol concentrations. The neutrophil phagocytosis of *K. pneumoniae* decreased as alcohol concentration increased in both the K2 and non-K1/K2 *K. pneumoniae* groups.

**Table 2** Comparison of the percentage of human neutrophil phagocytosis of K2 and non-K1/K2 *Klebsiella pneumoniae* isolates by age group.

<table>
<thead>
<tr>
<th>Alcohol concentration (mM)</th>
<th>Phagocytosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K2</td>
</tr>
<tr>
<td></td>
<td>Young group (&lt;40 y)</td>
</tr>
<tr>
<td>0</td>
<td>58.52</td>
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<tr>
<td>20</td>
<td>59.4</td>
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<td>40</td>
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<td>80</td>
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<td>200</td>
<td>56.33</td>
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<tr>
<td>400</td>
<td>60.04</td>
</tr>
<tr>
<td>800</td>
<td>31.48</td>
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</tbody>
</table>

**Figure 1.** The percentage of human neutrophil phagocytosis of *Klebsiella pneumoniae* and non-K1/K2 *K. pneumoniae* under different alcohol concentrations. The neutrophil phagocytosis of *K. pneumoniae* decreased as alcohol concentration increased in both the K2 and non-K1/K2 *K. pneumoniae* groups.

**Discussion**

Factors that contribute to the high incidence of infection among alcoholics include dulled mental function, breakdown of local protective barriers, aspiration, exposure, and malnutrition.31 Immune abnormalities in chronic alcoholics due to malnutrition, vitamin deficiency, and advanced liver cirrhosis were also thought to affect high infection rates.24 Moreover, alcohol itself is also considered a potent modulator of the immune system. Increasing evidence from *in vivo* human and animal studies, as well as *in vitro* experiments, suggested that alcohol use can modulate the immune system at various levels.24,32–37

Pulmonary infection is the most common infection site associated with alcoholism. The integrity of the pulmonary host-defense system is mainly maintained by resident alveolar macrophages and polymorphonuclear leukocytes that are recruited into the alveoli from systemic circulation in response to an invading pathogen. Therefore, phagocytosis and bactericidal activity of alveolar macrophages and circulating neutrophils may play important roles in the susceptibility to infection in alcoholic individuals. Numerous studies reported impaired mechanisms associated with immune system functions in alcoholics, including the phagocytic and bactericidal functions of macrophages and monocytes.24–27 The animal study conducted by Aroor and Baker38 also revealed inhibition of phagocytosis and superoxide anion production by microglia in mice with ethanol intoxication.
The microbicidal activity provided by pulmonary-recruited PMNs also contributes an essential component to defense of the lower respiratory tract. Previous studies showed that acute ethanol intoxication impaired PMN migration to the lungs and suppressed pulmonary microbicidal function in animals challenged with intrapulmonary bacteria or endotoxin.39–41 Precise phagocytosis rates exhibited by circulating neutrophils remain unknown. Although the animal study conducted by Sabino et al.28 revealed phagocytic activity involving technetium-labeled colloids did not change in mice with acute ethanol intoxication, our study revealed that acute ethanol intoxication may be capable of suppressing human circulating PMN phagocytic activity against K. pneumoniae. The differences in results may be due to different neutrophil sources, different organisms used to react with PMN leukocytes, different methods for measuring neutrophil function, and the ratio of stimulating agents to cells. Our study demonstrated the phagocytic activity of human neutrophils against K. pneumoniae using a standard assay and a fixed ratio of stimulating agents to cells rather than the phagocytic activity of rat neutrophils to colloids without fixed ratios of stimulating agents to cells.29 Our method may reflect human neutrophil phagocytic activity much more accurately.

K. pneumoniae produce virulence factors, such as smooth lipopolysaccharide (LPS; with O antigen), pili for adhesion to host cells, and capsules (K antigen) that are antiphagocytic, siderophores that aid the bacterium in its competition with the host for iron uptake.42 Greater understanding of the virulent determinants associated with K. pneumoniae has focused on the capsule serotypes. Our previous study revealed that isolates with capsule serotypes K1 and K2 were more resistant to phagocytosis compared with non-K1/K2 strains and were also more virulent.43 Patients with diabetes and older age are susceptible to K. pneumoniae infection. Our previous studies also demonstrated that poor glycemic control and aging contributed to impaired neutrophil phagocytosis of K1/K2 K. pneumoniae strains, but did not significantly affect the phagocytosis of non-K1/K2 K. pneumoniae strains.44 In the current study, we chose serotype K2 K. pneumoniae, the second most prevalent serotype next to serotype K1 as a cause of pyogenic liver abscesses and involved in community-onset pneumonia in Taiwan,45 as the strain used in this study and non-K1/K2 K. pneumoniae as the control.45,46 We found that ethanol concentration influenced neutrophil phagocytosis of both K2 and non-K1/K2 strains, and that aging could impair phagocytosis of both strains under alcohol treatment.

In conclusion, our study demonstrated that the phagocytic function of circulating neutrophils, as well as other neutrophil functions, such as adhesion, chemotaxis, and oxygen metabolism, could be suppressed in human with acute ethanol intoxication.

Acknowledgments

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References


