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ORIGINAL ARTICLE

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. pneumonia* 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. pneumonia* 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.

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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.

Introduction

Alcoholism is a leading preventable cause of death in the United States[1] and has substantial public health impact on American Indian and Alaska Native populations.[2] Alcoholism is a major public health problem worldwide, constitutes the leading risk factor in developing countries with low mortality rates, and ranks third in developed countries according to the World Health Report 2002.[3]

Excessive alcohol consumption leads to increasing risk of infectious diseases and certain cancers. Acute and chronic alcoholics are more susceptible to infection, especially pulmonary infections, specifically pneumonia and tuberculosis, and treatment is often less effective in alcoholic patients.[4–6] The incidence and mortality of pneumonia and tuberculosis is approximately two-fold that of nondrinkers or light drinkers.[5–7] Two large epidemiological studies involving 600 critically ill patients showed that a patient with a history of alcohol abuse was more susceptible to development of acute respiratory distress syndrome.[7–9] The most common etiologic agents of pneumonia among alcoholics are *Pneumococcus*, *Klebsiella pneumoniae*, or *Haemophilus*, of which *K. pneumoniae* pneumonia has been most extensively evaluated and studied.[10–16] The incidence of extrapulmonary infections, such as spontaneous bacteremia and bacterial peritonitis, is also higher in alcoholic patients and is particularly prevalent in patients with alcoholic cirrhosis.[17–19] Regarding the etiology of spontaneous bacterial peritonitis, *Escherichia coli* and *K. pneumoniae* are the most frequently isolated microorganisms.[20] Lifestyle and high rates of exposure to infectious organisms may contribute to the high incidence of infections in the alcoholic population.[21–23] However, numerous studies now support the contention that alcohol itself can modulate the immune system at various levels.[24]

Previous studies showed that ethanol suppressed several leukocyte functions, including adhesion, chemotaxis, and oxygen metabolism.[25,26] However, the precise effect of acute ethanol intoxication on phagocytosis remains unknown. According to Zhang et al,[27] acute ethanol intoxication suppresses circulating polymorphonuclear leukocyte (PMN) phagocytosis. However, the animal study conducted by Sabino et al[28] revealed no phagocytic function change in mice with acute ethanol intoxication. To determine whether acute alcohol intoxication impairs the phagocytic function of neutrophils, this study compared the rate of human neutrophil phagocytosis of *K. pneumonia*, one of the most common pathogens found in both pneumonia and extrapulmonary infections in alcoholic patients, under different alcohol concentrations in an experimental model.

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 (1000*g* 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 $NaHCO_3$ (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.[29] 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-polypropylene 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.[30] 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 K2 *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%. Thus, 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%. Thus, 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

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

Alcohol concentration (mM)	Phagocytosis (%)	
	K2	Non-K1/K2
0	56.15 ± 3.52	77.80 ± 4.91
20	55.59 ± 2.96	74.01 ± 3.57
40	54.19 ± 3.55	71.42 ± 3.74
60	50.76 ± 4.51	73.55 ± 4.38
80	55.58 ± 2.34	70.25 ± 3.77
100	57.30 ± 2.50	75.28 ± 4.20
200	53.54 ± 3.21	68.25 ± 4.43
400	54.88 ± 3.75	62.77 ± 4.23
800	30.00 ± 2.60	49.15 ± 4.50

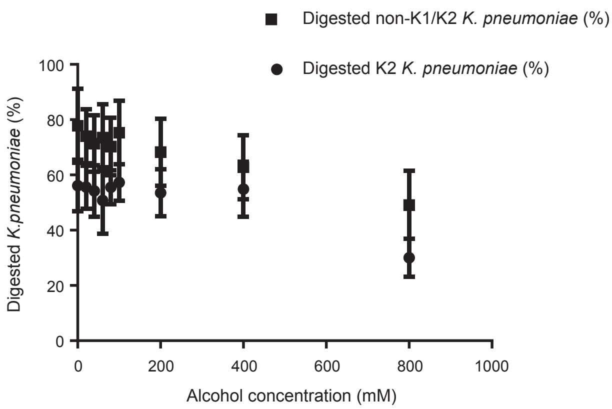

Figure 1. The percentage of human neutrophil phagocytosis K2 *Klebsiella pneumoniae* and non-K1/K2 *K. pneumoniae* under different alcohol concentrations. The neutrophil phagocytosis of *K. pneumoniae* decreased as alcohol concentration increased in both the K2 and non-K1/K2 *K. pneumoniae* groups.

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

Alcohol concentration (mM)	Phagocytosis (%)			
	K2		non-K1/K2	
	Young group (< 40 y)	Old group (> 40 y)	Young group (< 40 y)	Old group (> 40 y)
0	58.52	52.99	80.48	73.78
20	59.4	50.48	75.09	72.38
40	59.99	46.46	71.78	70.87
60	57.15	42.24	71.28	76.96
80	59.99	49.7	69.57	71.27
100	59.71	54.08	74.48	76.49
200	56.33	49.82	69.79	65.96
400	60.04	47.99	65.68	58.4
800	31.48	28.03	54.02	41.86

also decreased significantly with increasing alcohol concentration ($r = -0.975$, $p < 0.001$; Figure 1). Both groups showed a negative correlation between phagocytosis rate and alcohol concentration. These findings may account for the higher prevalence of pneumonia or other extrapulmonary infections in patients with acute alcohol intoxication.

The rate of *K. pneumoniae* phagocytosis was compared between two age groups: < 40 years and > 40 years (Table 2). The percentage of K2 and non-K1/K2 *K. pneumoniae* ingested by neutrophils was higher in the < 40 years age group as compared with the >40 years age group ($p = 0.001$; Figure 2). The percentage of phagocytosed K2 *K. pneumoniae* at alcohol concentrations of 0mM, 20mM, 40mM, 60mM, 80mM, and 100mM in both groups (< 40 years vs. >40 years) was 58.52% versus 52.99%, 59.4% versus 50.48%, 59.99% versus 46.46%, 57.15% versus 42.24%, 59.99% versus 49.7%, 59.71% versus 54.08%, 56.33% versus 49.82%, 60.04% versus 47.99%, and 31.48% versus 28.03%, respectively. The percentage of phagocytosed non-K1/K2 *K. pneumoniae* at alcohol concentrations of 0mM, 20mM, 40mM, 60mM, 80mM, and 100mM in both groups (< 40 years vs. > 40 years) was 80.48% versus 73.78%, 75.09% versus 72.38%, 71.78% versus 70.87%, 71.28% versus 76.96%, 69.57% versus 71.27%, 74.48% versus 76.49%, 69.79% versus 65.96%, 65.68% versus 58.4%, 54.02% versus 41.86%.

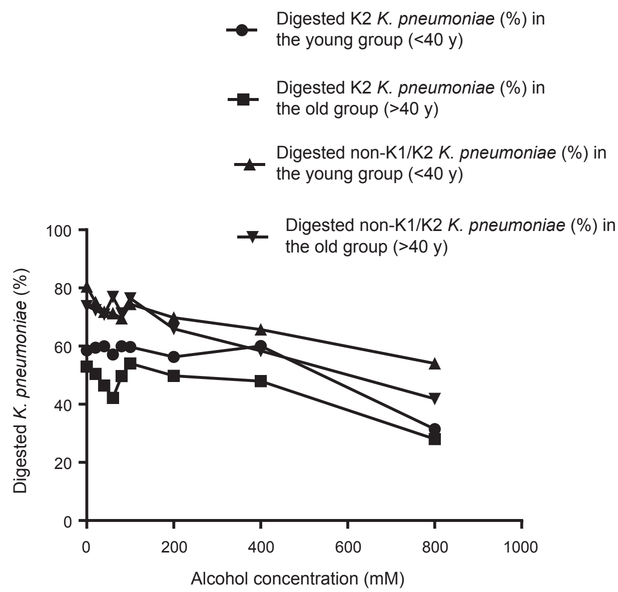

Figure 2. The percentage of *Klebsiella pneumoniae* ingested by neutrophils was higher in the < 40 years group as compared to the > 40 years group.

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]

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.[32–37] The animal study conducted by Aroor and Baker[38] 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 as 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.

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