In vivo efficacy of sitafloxacin in a new murine model of nontypable *Haemophilus influenzae* pneumonia by sterile intratracheal tube

Shigeki Nakamura[1,*], Katsunori Yanagihara[1,2], Nobuko Araki[2], Yoshitomo Morinaga[1,2], Koichi Izumikawa[1], Masafumi Seki[1], Hiroshi Kakeya[1], Yoshihiro Yamamoto[1], Shimeru Kamihira[2], and Shigeru Kohno[1,3]

[1]Second Department of Internal Medicine, [2]Department of Laboratory Medicine, and [3]Division of Molecular & Clinical Microbiology, Department of Molecular Microbiology & Immunology, Nagasaki University Graduate School of Medical Science, Nagasaki, Japan

Address correspondence to:

Katsunori Yanagihara

Department of Laboratory Medicine, Nagasaki University School of Medicine

1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Tel: +81 – 95 – 819 – 7276; Fax: +81 – 95 – 849 – 7285

E-mail: k-yanagi@nagasaki-u.ac.jp

ABSTRACT

A novel murine model of non-typable *Haemophilus influenzae* (NTHi) pneumonia was established. A plastic tube was inserted into the trachea 7 days before bacterial inoculation. Numbers of NTHi recovered from the lungs and trachea were maintainedfor 7 days. Histologically, bronchioles and adjacent alveoli in the intubation group were filled with numerous inflammatory cells. Additionally, we evaluated the efficacy of sitafloxacin compared to ciprofloxacin using our new murine pneumonia model. Our data suggest that sitafloxacin displays equivalent efficacy to ciprofloxacin against *H. influenzae* pneumonia. Our new murine NTHi pneumonia model appears useful not only for *in vivo* evaluation of antibiotics, but also for analysis of the pathogenesis of *H. influenzae* pneumonia.

Key words: *Haemophilus influenzae*, nontypable, sterile intratracheal tube, sitafloxacin

INTRODUCTION

Haemophilus influenzae is recognized as a frequent cause of a variety of infections, including acute otitis media, sinusitis, acute purulent exacerbation of chronic bronchitis and pneumonia. With the recent overuse of antibiotics such as cephalosporin, the incidence of drug-resistant *H. influenzae* in Japan has been increasing steadily, particularly for β-lactamase-negative, ampicillin-resistant *H. influenzae* (BLNAR). Clinically, we have encountered *H. influenzae* pneumonia in patients with chronic respiratory diseases such as bronchiectasis, diffuse panbronchiolitis and cystic fibrosis. Murine models of *H. influenzae* pneumonia have been reported using agar beads and cell-bound organisms[1-5]. We describe herein a novel murine model of NTHi pneumonia that uses an indwelling intratracheal tube to escape from the normal host defense mechanisms and cause pulmonary infection. We also evaluated the *in vivo* efficacies of sitafloxacin, a new fluoroquinolone, using this new murine model.

MATERIALS AND METHODS

Bacteria

NTHi strain ATCC51907, which had been stored in tripticase soy broth with 10% glycerol stocks maintained at -80 °C, was spread on chocolate α agar plates (Nissui Pharmaceutical, Tokyo, Japan) and incubated overnight (18-24 h) at 37 °C in 5% CO_2.

Laboratory animals

Six-week-old male ddY-specific pathogen-free mice (body weight, 16-20 g) were purchased from SLC Japan (Tokyo, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University (Nagasaki, Japan). Experimental protocols were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University.

Murine model of *H. influenzae* respiratory infection

We used as reference the intubation model of chronic airway infection in mice using the methods described by Yanagihara *et al*[6] (Fig. 1). Briefly, disposable, sterile, plastic cut-down intravenous catheters (3-Fr, 1.0-mm diameter; Atom, Tokyo, Japan) were

used for tracheal intubation. Tubes were 3.0 mm in length, with slits at the proximal end to prevent blockage by oral secretions. The intubation procedure was performed under pentobarbital anesthesia. The blunted end of the inner needle of an intravenous catheter (Angiocath; Becton Dickinson Vascular Access, Sandy, UT, USA) was inserted through the oral cavity with the outer sheath and attached tube at the tip. The tube was advanced through the vocal cords into the trachea. The inner needle was then removed, and the outer sheath was gently pushed to place the plastic tube into the main bronchus. The intubation model in this study is different from the reference (6) from the timing of inoculation. Unfortunately, we could not establish the pneumonia by using the bacterial precoated tube preparing just like reference (6). For the purpose of impairment the bronchial surface and the delay in bacterial clearance, organisms were instilled 7 days after intubation. *H. influenzae* were cultured on chocolate Ⅱ agar plates (Nissui Pharmaceutical) and incubated overnight (18-24 h) at 37 °C in 5% CO_2, then the organisms were suspended in normal saline. Final numbers of bacteria prepared were approximately 2×10^9 colony-forming units (cfu)/ml, as determined by the optical density method. Infection was induced by intratracheally inoculation of 0.05 ml of bacterial suspension under anesthesia with pentobarbital sodium.

Bacteriological and histopathological examination

Mice were divided into 3 groups: mice that were inoculated with *H. influenzae* after intubation; mice that were not intubated and only inoculated with *H. influenzae*; and mice that were intubated without inoculation. Non-intubated group was inoculated intratracheally at the same time when the intubated group was inoculated 7 days after intubation. Mice were sacrificed on days 1, 3, 5 and 7 after inoculation. The tube was removed and the lungs were excised under aseptic conditions when mice were sacrificed. Lungs were homogenized in 1.0 ml of phosphate-buffered saline and cultured quantitatively by serial dilution on chocolate α agar plates (Nissui Pharmaceutical), then incubated overnight (18-24 h) at 37 °C in 5% CO_2. For histopathological examinations, lung specimens were fixed in 10% formalin-buffered solution.

Bronchoalveolar lavage and cytokine enzyme-linked immunosorbent assay (ELISA)

Bronchoalveolar lavage (BAL) was performed as described previously[7]. Briefly, mice (n=10, each groups) were sacrificed on day 1, 3, 5 or 7 after inoculation. The chest was opened to expose the lungs and a disposable sterile plastic cutdown intravenous catheter

was inserted into the trachea. The intratracheal tube was not removed for BAL procedure. BAL was performed 3 times sequentially using 1.0 ml of saline each time. Recovered fluid fractions were pooled for each animal. Total cell counts were performed by Turk staining. For differential cell counts, cells were centrifuged at 850 rpm for 2 min onto slides, which were then stained with Diff-Quick stain. Differential cell counts were performed by counting 100 cells. Concentrations of macrophage inflammatory protein (MIP)-2 and interleukin (IL)-1β in BAL fluid (BALF) were assayed using mouse cytokine ELISA test kits (R&D Systems, Minneapolis, MN, USA).

Antimicrobial agents

Sitafloxacin (STFX) was kindly provided by Daiichi-Sankyo (Tokyo, Japan), and ciprofloxacin (CPFX) was kindly provided by Bayer Corporation (Tokyo, Japan). These agents were prepared according to the instructions of the manufacturer and frozen at -80 °C until use.

Antibiotic susceptibility testing

Susceptibilities of *H. influenzae* to sitafloxacin and ciprofloxacin were tested in duplicate for each isolates at inocula of 10^5 cfu/ml and determined using a broth dilution method with *Haemophilus* Test Medium according to Clinical and Laboratory Standards Institute (CLSI) recommendations[8].

Antibiotics examinations

From 24 h after infection, antibiotics were administered orally twice a day to the STFX treatment group and CPFX treatment group for 3 days. Each single dose was 10 mg/kg[9]. Each group was killed by cervical dislocation 12 h after the last drug administration. Bacteriological examinations and BALF analysis were performed using the methods described above.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Differences between numbers of viable bacteria in lungs were evaluated by analysis of variance. Values of $P<0.05$ were considered statistically significant.

RESULTS

In vitro susceptibility

For *H. influenzae* ATCC51907, minimum inhibitory concentrations (MICs) of STFX, and CPFX were 0.0006 mg/L and 0.003 mg/L, respectively. Both antimicrobial agents showed good potency against *H. influenzae* according to CLSI recommendations.

Changes in viable bacterial number over time (Fig. 2)

Respiratory infection occurred in all intubated mice with inoculation. The number of viable bacteria increased to 7.1±1.0 ($\log_{10}$ cfu/ml) 3 days after inoculation. Conversely, the number of viable bacterial decreased immediately and no colonies were isolated by 5 days after inoculation in non-intubated mice. The contaminated samples were excluded.

BALF analysis

Total cell counts (Table 1A) and neutrophil counts (Table 1B) were significantly higher in intubated mice than in non-intubated mice after inoculation. Peak total cell counts and neutrophil counts were seen on day 1 after inoculation and gradually decreased in intubated group. To estimate further differences, inflammatory cytokine levels in BALF were analyzed. MIP-2 (Table 2A), IL-1β (Table 2B) were significantly higher in intubated mice than in non-intubated mice after inoculation. These data imply that the acute inflammation induced by *H. influenzae* was strongest in the early days of infection and can persist for 7 days in intubated mice. Total cell counts, neutrophil counts and inflammatory cytokines increased slightly with intubation alone (data not shown).

Histopathological examination

In mice infected with intratracheal tube, bronchioles and adjacent alveoli were filled with numerous neutrophils and epithelial cells, and invasion of inflammatory cells occurred on day 1 and persisted to day 7 after infection (Fig. 3A-D). The inflammatory changes were shown in both lungs. Conversely, in non-intubated infected mice, mild inflammatory changes were evident on day 1 after infection, but inflammation was greatly improved by day 3 (Fig. 3E-H). Intubation alone did not produce inflammatory

changes in the mouse lung.

In *vivo* efficacy of STFX against *H. influenzae*

We divided into 3 groups to estimate the in vivo efficacy of STFX using this new murine *H. influenzae* pneumonia model; mice that were untreated; mice were treated by STFX; mice that were treated by CPFX; and all mice were intubated and inoculated. In the bacteriological study, mean viable bacterial counts in the lungs of STFX- and CPFX-treated mice and untreated control mice were 1.8±0.4 $\log_{10}$ cfu/ml, 1.9±0.3 $\log_{10}$ cfu/ml and 7.3 ±0.4 $\log_{10}$ cfu/ml at 3 days after administration of antimicrobial agents, respectively. STFX and CPFX each completely eradicated viable bacteria from all mouse lungs (Fig. 4A). Histopathological studies of lung tissue from control mice showed the presence of progressive pneumonia (Fig. 5A). In mice with antimicrobial therapy, total cell counts and neutrophil counts in BALF decreased (Figs. 4B, 4C). In addition, only a small number of neutrophils were seen in alveolar walls, and inflammatory changes were apparently improved (Figs. 5B, 5C). These data confirmed that STFX displays good potency that is not inferior to CPFX against *H. influenzae* pneumonia.

DISCUSSION

H. influenzae is a frequent pulmonary pathogen in humans, causing community-acquired infections such as chronic bronchitis, pneumonia, meningitis and sinusitis[1,10-12]. Despite the high case-fatality rates reported for acute and chronic *H. influenzae* infection, little information on pathogenesis and therapy has been gained from experimental models. Although murine models of *H. influenzae* infection have been created using agar beads and cell-bound organisms, few reports have described experimental models[1-5]. These methods were established to prevent rapid removal by mucociliary clearance and examined the reasons for the characteristics of *H. influenzae* pneumonia induced by microaspiration of the intracellular bacteria. Clinically, *H. influenzae* pneumonia is well known to arise in patients who have chronic pulmonary diseases such as chronic obstructive pulmonary disease or bronchiectasis[15,16]. The present study established a new murine model of *H. influenzae* pneumonia using a sterile intratracheal indwelling catheter. Intratracheal tube was removed when mice were sacrificed under aseptic conditions, and *H. influenzae* was isolated from the removed tube (3.2 ± 1.0 $\log_{10}$ cfu/ml). Since this model contributed to semipermanent escape from the normal host defense mechanisms for the pathogens, and caused lower respiratory tract infection. In this model, an intratracheal tube is essential for induction

of pneumonia. We inoculated bacteria 7 days after intubation, because we expected the impairment of the bronchial surface and the delay in bacterial clearance. In addition, although histopathological findings in the lungs of animals intubated with sterile tubes showed localized inflammation close to the site of tube attachment (data not shown), we expected mucociliary clearance in the airways would be impaired by the plastic tube. Moreover, this method implied that bacteria could persist for a long period in the airways because of the protective environment of the intratracheal tube. In our study, many viable bacteria could be recovered from the respiratory tract for 7 days. These data indicate that pretreatment with an intratracheal tube plays an important role in allowing *H. influenzae* to persist in the lower respiratory tract for a long period. Histopathologically, in non-intubated mice, mild inflammatory changes were seen on day 1, but improved immediately by day 3. In addition, in intubated mice, the high amounts of cytokine (MIP-2 and IL-1β) were released into the BALF although we didn't analyze the protease-level. This improvement is presumably facilitated by the rapid clearance of bacteria from the airways in the absence of intubation.

The current study also illustrated the utility of this new experimental model in evaluating therapeutic efficacy. In comparison with controls, antibiotic-treated mice showed significantly improved bacterial clearance from the lungs and trachea (Fig. 4).

Several experimental studies have suggested that CPFX and other quinolones are therapeutically active in the treatment of respiratory infection[17]. Sitafloxacin is a new fluoroquinolone offering a broader spectrum and more potent activity against gram-positive, gram-negative and atypical pathogens than other quinolones such as ofloxacin (OFLX), ciprofloxacin (CPFX) and sparafloxacin (SPFX)[18,19]. The in *vivo* activity of STFX against *H. influenzae* was compared to that of CPFX, through the evaluation of BALF analysis and bacteriological and histopathological changes (Figs. 4 and 5). In STFX-treated mice, *H. influenzae* was decreased by 3 days after starting oral administration of STFX (Fig. 4A). In addition, total cell counts and neutrophil counts in BALF were considerably decreased, and histopathologically, inflammatory changes were greatly improved with STFX treatment (Figs. 4 and 5). These results resembled those for CPFX. There are some considerable reasons. First, in present study, we used the immunocompetent, healty mice, and the fluoroquinolone well-susceptible strain. We consider that if the mice had the destroyed lung or the bacteria was drug resistant strains, STFX may be able to show the effectiveness better than CPFX because it was reported that STFX could show the higher tissue concentration than CPFX[9]. Second, it is also considered that the drug levels of STFX and CPFX, respectively, in lung-tissue of mice may be different from the human origin so that the differences could not be shown in

this study.

In conclusion, we demonstrated that STFX offers a high degree of efficacy against *H. influenzae* using our new murine model of *H. influenzae* pneumonia. We expect that our newly established model will have wide potential use as an animal model of *H. influenzae* to investigate not only the efficacy of antibiotics, but also the pathogenesis of *H. influenzae*.

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The first two authors (S.N and K.Y) contributed equally to this work.

Transparency declarations

None to declare

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FIGURE LEGENDS

Figure 1: Schematic of the intubation tube and intubation procedure in the mouse bronchus.

Figure 2: Number of viable organisms in lower respiratory organs. Mice were inoculated with 1×10^8 cfu/ml of *H. influenzae* ATCC51907. On days 1, 3, 5 and 7 after infection, numbers of viable bacteria were isolated, but in the non-intubated group, complete eradication was achieved within 5 days. $^*P<0.001$ compared with the non-intubated group. ●, Intubated group; ■, Non-intubated group.

Figure 3: High-power magnification of the lung (×200, hematoxylin and eosin). A-D) Intubated group; E-H) Non-intubated group. A, E) Day 1; B, F) Day 3; C, G) Day 5; D, H) Day 7. In intubated mice that were infected, bronchioles and alveoli were filled with neutrophils from day 1 to day 7 after infection.

Figure 4: Viable bacteria (A) were completely eradicated by 3 days after starting oral STFX or CPFX administration. Total cell counts (B) and neutrophil counts (C) in BALF were markedly decreased by 3 days after beginning oral STFX or CPFX administration. $^*P<0.05$ compared with control group. STFX, oral administration, 10 mg/kg, twice/day;

CPFX, oral administration, 10 mg/kg, twice/day.

Figure 5: High-power magnification of the lung after 3 days treatment (×200, hematoxylin and eosin). (A) Control, untreated; (B) STFX group, oral administration, 10 mg/kg, twice/day; (C) CPFX group, oral administration, 10 mg/kg, twice/day. Inflammatory changes improved immediately after starting treatment in STFX and CPFX treatment groups.

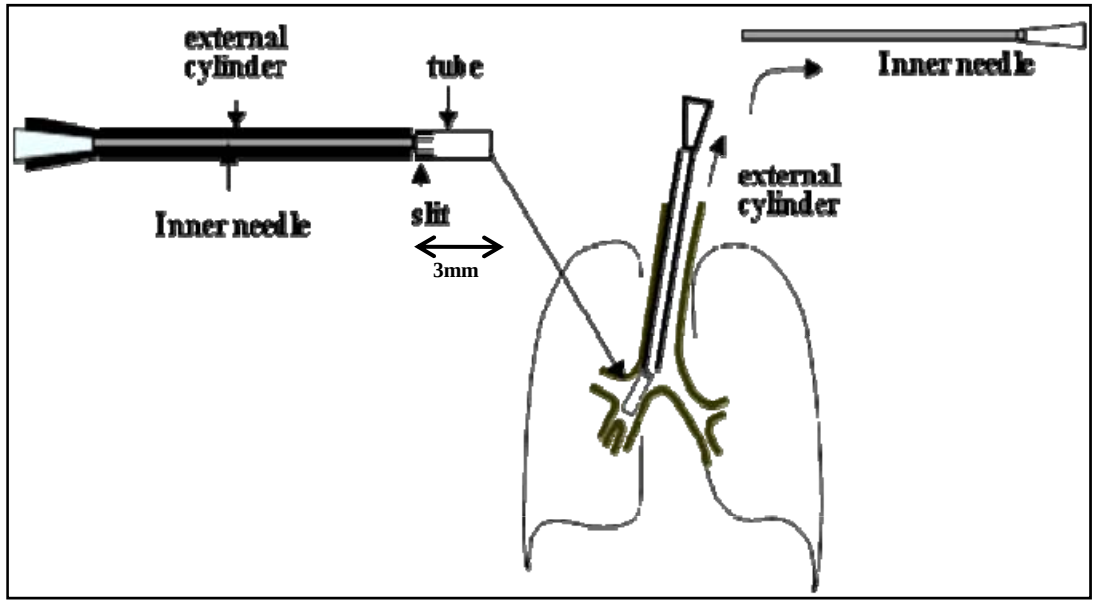
external
cylinder
tube
Inner needle
Inner needle
slit
3mm
external
cylinder

Figure 1

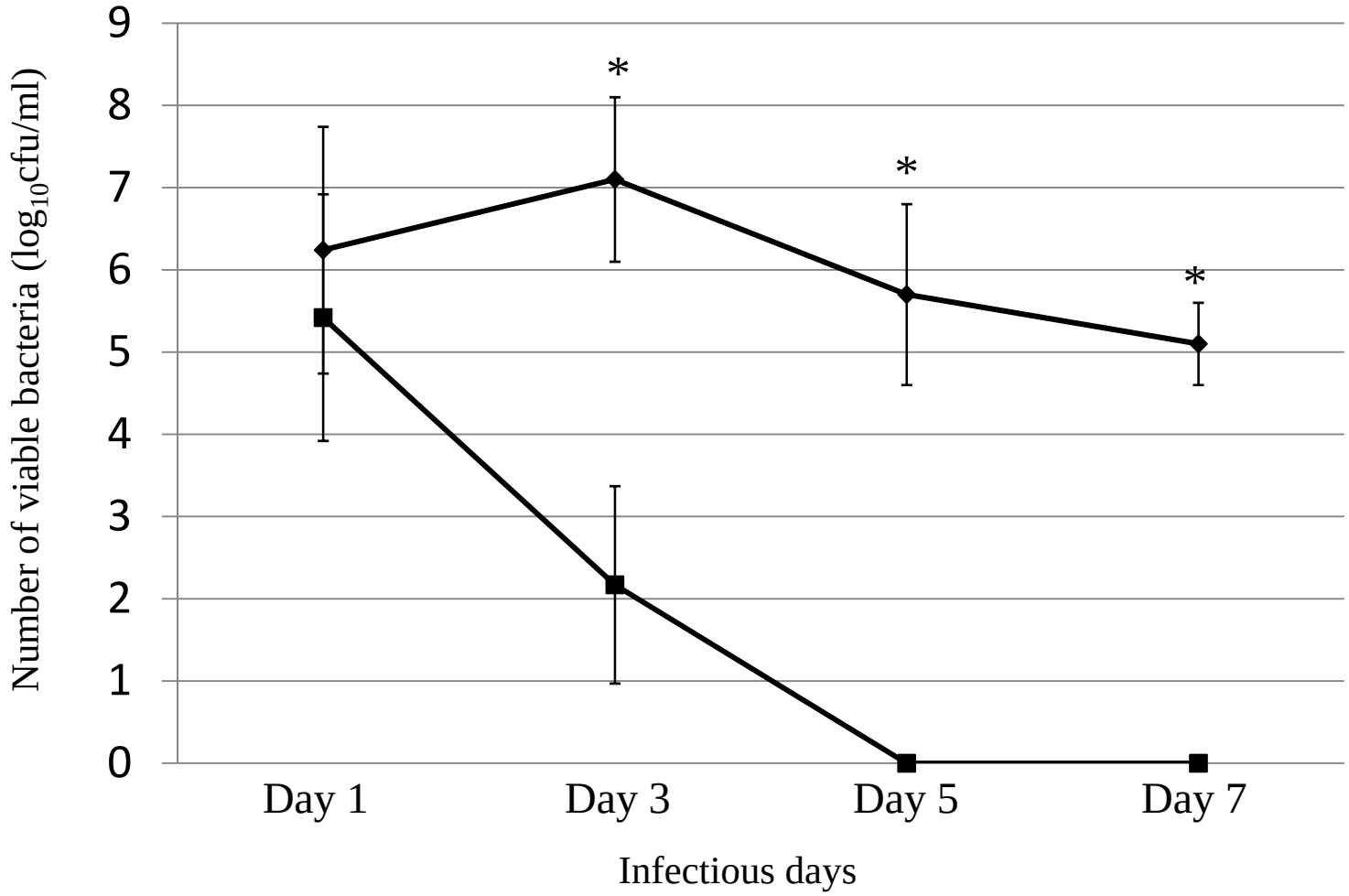
Number of viable bacteria (log_{10}cfu/ml)
9
8
7
6
5
4
3
2
1
0
*
*
*
Day 1
Day 3
Day 5
Day 7
Infectious days

Figure 2

Table 1A: Serial changes in total cell counts in BALF*

Day	Total cell counts in BALF ($\times 10^6$)	
	Intubation group with infection (n=5)	Non-intubation group with infection (n=5)
1	22.53±7.9	2.42±1.21**
3	20.35±3.37	2.52±0.88**
5	12.7±3.47	1.15±0.95**
7	5.2±1.3	0.95±0.74**

Table 1B: Serial changes in number of neutrophils in BALF*

Day	Neutrophils in BALF ($\times 10^6$) (n=5)	
	Intubation group with infection (n=5)	Non-intubation group with infection (n=5)
1	18.14±3.11	0.12±0.03**
3	7.24±2.02	0.08±0.01**
5	3.07±0.92	0.06±0.01**
7	0.15±0.04	0.06±0.04**

*Values are expressed as mean±SD.
** $p<0.01$ compared with non-intubation group.

Table 2A: Levels of MIP-2 in BALF*

Day	MIP-2 (pg/ml)	
	Intubation group with infection (n=5)	Non-intubation group with infection (n=5)
1	188.67±68.6	52.14±40.1**
3	97.27±20.2	18.15±1.7**
5	29.76±10.71	10.03±2.9**
7	23.04±1.4	5.23±1.1**

Table 2B: Levels of IL-1β in BALF*

Day	IL-1β (pg/ml)	
	Intubation group with infection (n=5)	Non-intubation group with infection (n=5)
1	236.59±64.9	149.48±120.27**
3	183.89±88.8	36.86±4.54**
5	144.76±134.15	40.29±8.85**
7	48.85±4.62	8.51±2.2**

*Values are expressed as mean±SD.
** $p<0.01$ compared with non-intubation group.

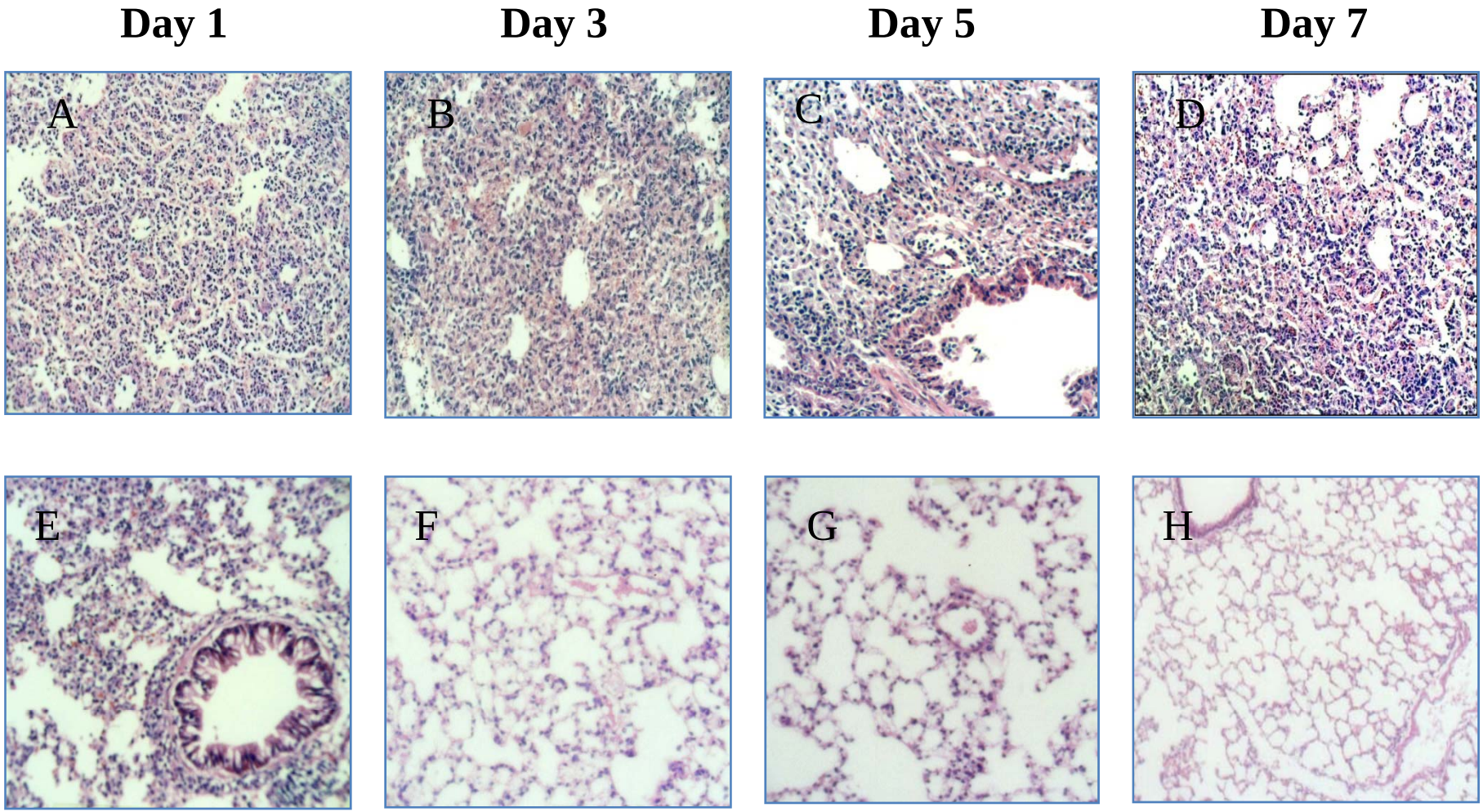

Figure 3

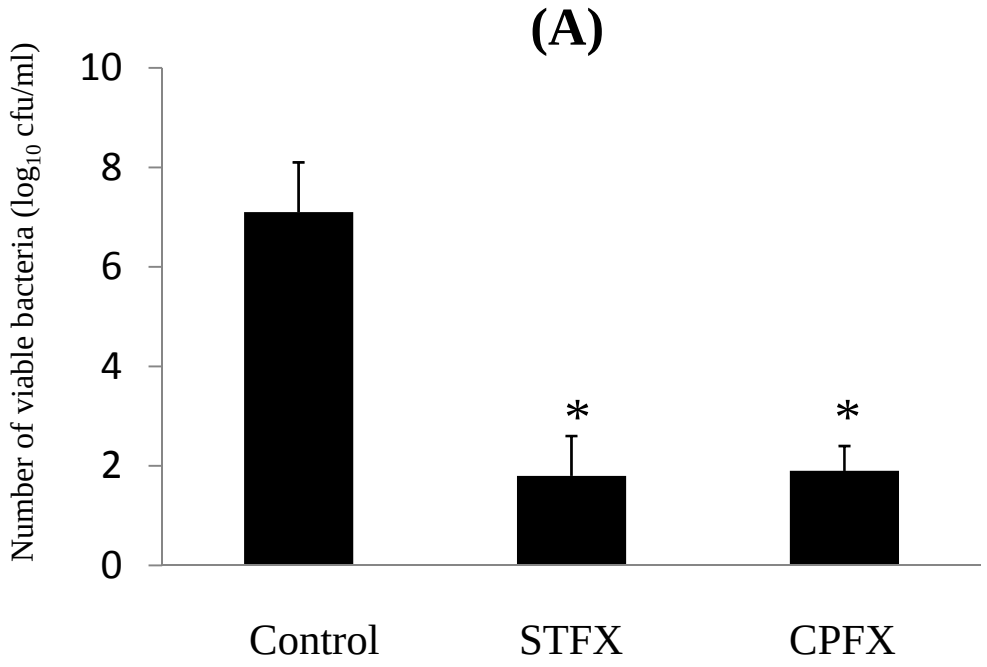
(A)
Number of viable bacteria (log_{10} cfu/ml)
10
8
6
4
2
0
*
*
Control
STFX
CPFX

Figure 4

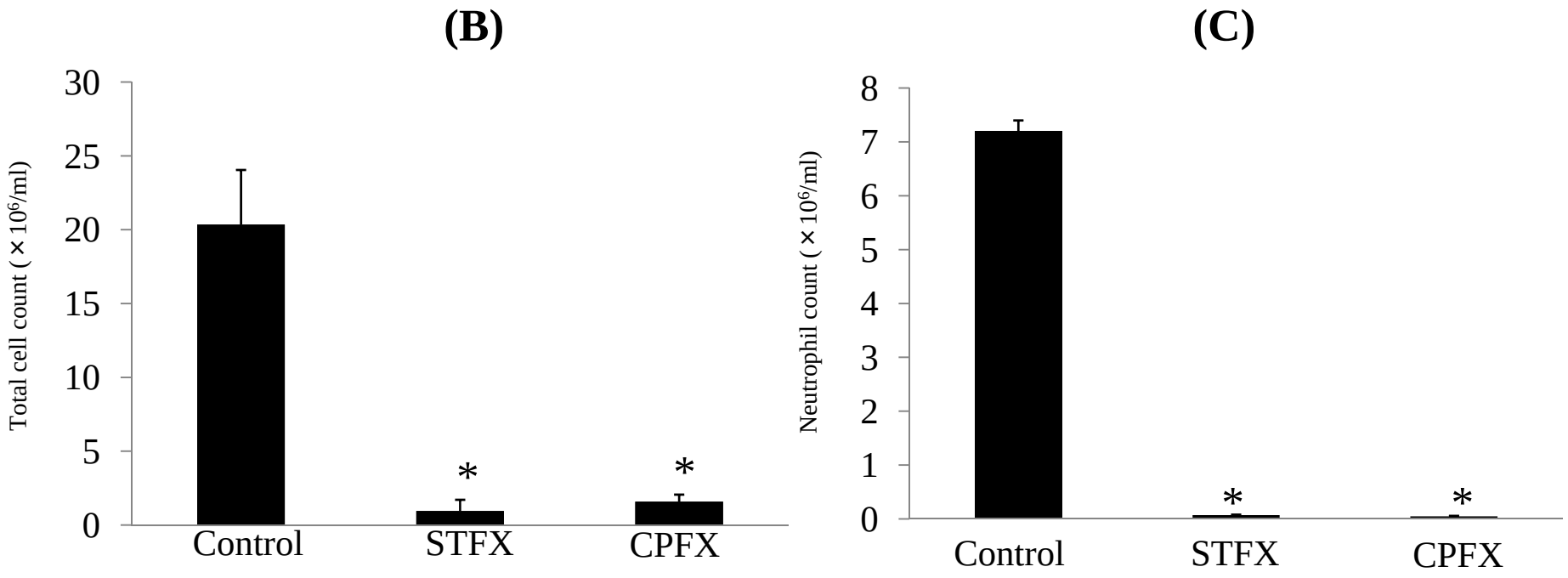
(B)
Total cell count (× 10⁶/ml)
30
25
20
15
10
5
0
*
*
Control
STFX
CPFX
(C)
Neutrophil count (× 10⁶/ml)
8
7
6
5
4
3
2
1
0
*
*
Control
STFX
CPFX

Figure 4

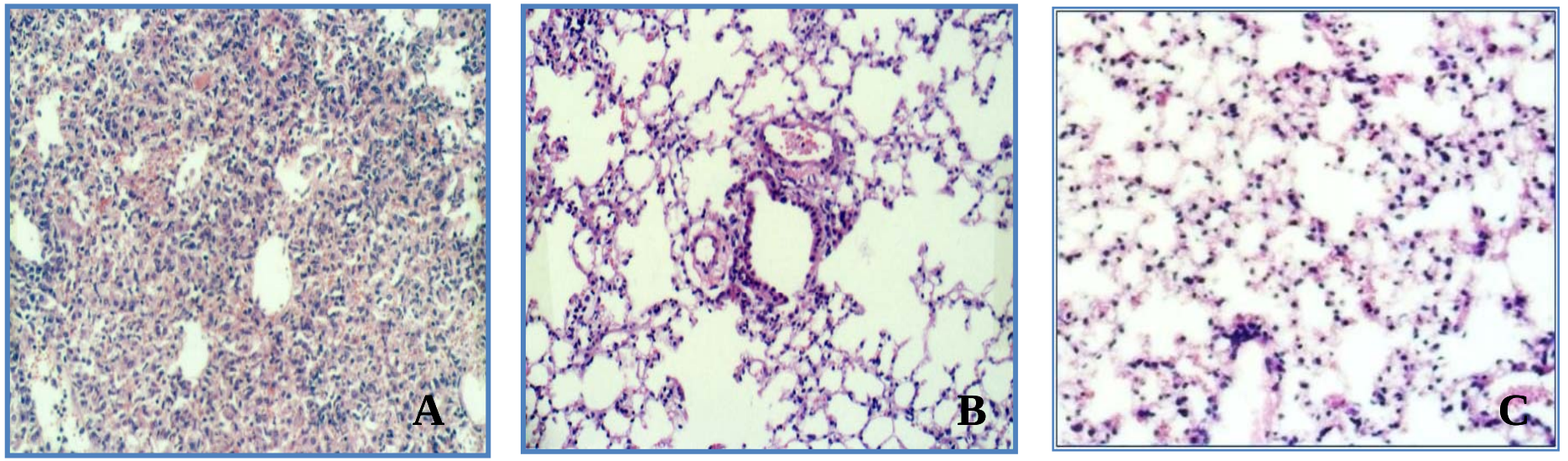

Figure 5