# *In vivo* efficacy of combination of colistin with fosfomycin or minocycline in a mouse model of multidrug-resistant *Acinetobacter baumannii* pneumonia

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# *In vivo* efficacy of combination of colistin with fosfomycin or minocycline in a mouse model of multidrug-resistant *Acinetobacter baumannii* pneumonia

Directed by Professor June Myung Kim

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I want to dedicate this paper to all of you with all of my heart.

Thanks everyone.

Nam Su Ku

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ABSTRACT

# *In vivo* efficacy of combination of colistin with fosfomycin or minocycline in a mouse model of multidrug-resistant *Acinetobacter baumannii* pneumonia

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**Background**: *Acinetobacter baumannii* is a well-documented multidrug-resistant (MDR) nosocomial pathogen. Unfortunately, the options for treating MDR *A. baumannii* infections are extremely limited. The administration of combination regimens has been proposed to achieve antibiotic synergy. Recently, fosfomycin and minocycline, two 'old' drugs, were newly introduced as a treatment option for MDR *A. baumannii* infection. Therefore, we investigated the efficacy of the combination of colistin with fosfomycin and minocycline, respectively, as therapeutic options in a mouse model of MDR *A. baumannii* pneumonia.

**Methods**: We examined carbapenem-resistant *A. baumannii* isolated from clinical specimens at Severance Hospital, Seoul, Korea. The efficacy of colistin, fosfomycin, minocycline, colistin with fosfomycin, and colistin with minocycline on the bacterial counts in lung tissue were investigated in a mouse model of pneumonia caused by MDR *A. baumannii*. Bactericidal activity was defined as a $\geq$3 $\log_{10}$ decrease compared with the initial inoculum. Synergy was

defined as a ≥2 $\log_{10}$ decrease caused by the combination compared with the most active single agent.

**Results**: *In vivo*, colistin with fosfomycin or minocycline significantly ($p$<0.05) reduced the bacterial load in the lungs compared with the controls at 24 and 48 h. In the combination groups, the bacterial loads differed significantly ($p$<0.05) from that with the more active antimicrobial alone. Moreover, the combination regimens of colistin with fosfomycin and colistin with minocycline showed bactericidal and synergistic effects compared with the more active antimicrobial alone at 24 and 48 h. No significant difference in survival was observed among the control and antibiotic-treated groups.

**Conclusion**: This study demonstrated the synergistic effects of combination regimens of colistin with fosfomycin and minocycline, respectively, as therapeutic options in pneumonia caused by MDR *A. baumannii*. Large clinical trials are needed to clarify the role of combination regimens with colistin and fosfomycin or minocycline in treating MDR *A. baumannii* pneumonia.

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Key words: multidrug-resistant (MDR), *Acinetobacter baumannii*, combination, colistin, fosfomycin, minocycline

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## I. INTRODUCTION

*Acinetobacter baumannii* is a well-documented, multidrug-resistant (MDR) nosocomial pathogen[1]. In the past, carbapenems have been recommended as the antibiotics of choice for treating *A. baumannii*. However, the increasing incidence of MDR strains has prompted the use of unconventional antibiotics, such as polymyxin, rifampicin, and tigecycline, for the treatment of infections caused by MDR isolates[2]. Nevertheless, MDR *A. baumannii* infections have a mortality rate of 25% associated with inappropriate antibiotic therapy[3].

Colistin has been used increasingly for the treatment of infections caused by MDR *A. baumannii*, despite its potential nephrotoxicity and neurotoxicity. It has shown excellent *in vitro* antibacterial activity against carbapenem-resistant *A. baumannii*[4]. However, rapid regrowth after colistin treatment, heteroresistance, and low plasma concentrations have raised questions about the efficacy of colistin monotherapy[5].

Consequently, colistin-based combination treatments have been proposed to attain antibiotic synergy. A systematic review revealed that colistin-based combinations with several other antibiotics showed synergy in many isolates of MDR *A. baumannii* and lowered mortality in some animal studies[6-11].

Recently, minocycline and fosfomycin, two 'old' drugs, were newly introduced as treatment options for MDR *A. baumannii* infection. Minocycline was the second most active (79.1% susceptible) agent, exceeded only by colistin *in vitro*[12]. Fosfomycin is a broad-spectrum antibiotic that is active against a wide range of Gram-positive and -negative bacteria[13]. Fosfomycin is an alternative drug for the treatment of drug-resistant bacterial infections, especially when combined with other antibiotics[14,15].

However, to our literature search, little *in vivo* data is available on the use of colistin with minocycline or fosfomycin for the treatment of MDR *A. baumannii* infection, especially pneumonia. Therefore, we investigated the *in vivo* efficacy of combinations of colistin with minocycline and fosfomycin, respectively, as therapeutic options in a mouse model of MDR *A. baumannii* pneumonia.

## II. MATERIALS AND METHODS

### 1. Bacterial strains

We obtained carbapenem-resistant *A. baumannii*, which has an OXA-23 carbapenemase, isolated from clinical specimens of a patient with hematologic malignancy and pneumonia at Severance Hospital, Seoul, Korea.

### 2. Antibiotic susceptibility tests

Antibiotic susceptibility tests were performed using the agar dilution method and broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines and were used to determine the minimum inhibitory concentrations (MICs) against piperacillin/tazobactam, ceftazidime, imipenem, amikacin, ciprofloxacin, colistin, minocycline, and fosfomycin. A strain was considered resistant to carbapenems when the MIC against imipenem was ≥16 mg/L.

**3. Time–kill test**

For both agents and their combinations, time–kill tests were performed using sub-inhibitory concentration (0.5×MIC)[16]. Ten-fold dilutions were inoculated onto Mueller–Hinton agar and colonies were counted at 0, 4, 8, and 24 h. Bactericidal activity was defined as a ≥3 $\log_{10}$ decrease compared with the initial inoculums. Synergy was defined as a ≥2 $\log_{10}$ decrease with the combination, compared with most active single agent[17].

**4. Mouse model of MDR *A. baumannii* pneumonia**

The animal study was approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine (#2014-0275). Immunocompetent, specific pathogen–free, 6-week-old female mice weighing 18–20 g (C57BL/6N) were used. They were obtained from Orient Bio. Animals were rendered transiently neutropenic by injecting cyclophosphamide intraperitoneally (300 mg/kg body weight) in a volume of 0.2 mL 4 days before *A. baumann*ii inoculation in the lung. The mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Then, 1.2 mL/kg of a $5\times10^8$ colony-forming units (CFU)/mL bacterial suspension was inoculated through the nose using a syringe[18]. After being kept in a vertical position for 4 min, the mice were kept in a 30° decubitus position until regaining consciousness. Then, a necropsy was performed on one mouse each 4, 24, and 48 h after the nasal inoculation. The entire lung was removed in a sterile fashion and was evaluated pathologically, and the diagnosis of pneumonia was confirmed. Sterile lung specimens were fixed in 10% formalin and then immersed in paraffin wax. Specimens were prepared in 5-μm cross sections and were examined under a light microscope after hematoxylin/eosin staining.

**5. Study groups**

The mice with induced pneumonia were randomized into six groups of 15 mice each, comprising five treatment groups and a control group. Colistin was administered to the first group, fosfomycin to the second, minocycline to the third, the colistin/fosfomycin combination to the fourth, and the colistin/minocycline combination to the fifth. No antibacterial agent was used in the control group (Figure 1).

**6. Treatment protocol**

Treatments were initiated 4 h after nasal inoculation. The antibiotic agents were given by intraperitoneal injection, and the dosages were as follows: colistin, 20 mg/kg every 8 h[19]; fosfomycin, 100 mg/kg every 4 h[20]; and minocycline, 20 mg/kg every 12 h[21]. The colistin, fosfomycin, and minocycline were purchased from Sigma-Aldrich (Figure 1).

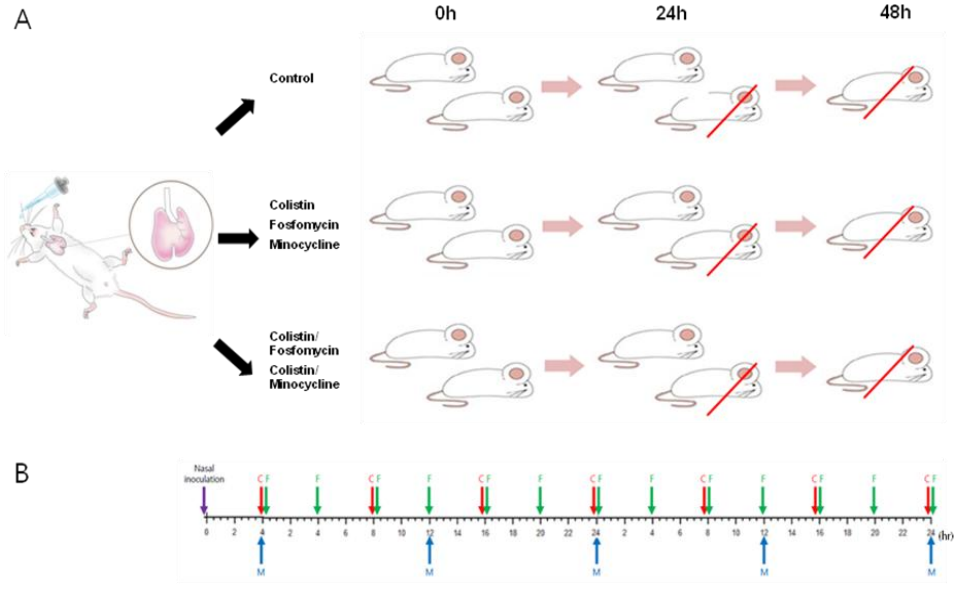


**Figure 1**. **Study scheme (A) and treatment protocol (B).** (A) The mice with induced pneumonia were randomized into six groups of 15 mice each, comprising five treatment groups (colistin, fosfomycin, minocycline, colistin/fosfomycin and

colistin/minocycline) and a control group. No antibacterial agent was used in the control group. (B) Treatments were initiated 4 h after nasal inoculation. The antibiotic agents were given by intraperitoneal injection, and the dosages were as follows: colistin, 20 mg/kg every 8 h; fosfomycin, 100 mg/kg every 4 h; and minocycline, 20 mg/kg every 12 h. (C, colistin; F, fosfomycin; M, minocycline)

**7. Effects on lung bacterial loads**

Bacteria in the lungs were counted 24 and 48 h after starting the antibiotic agents, in three mice at each time point. To eliminate any antibiotic carry-over effect, mice in the treatment groups were killed more than 3 h after the last antibiotic dose. For the quantitative bacteriological studies, the lungs were removed, weighed, and homogenized in 1 mL saline. Ten-fold dilutions were made, and 100-μL aliquots were placed on tryptic soy agar with 5% sheep blood plates for 24 h at 37℃. Once grown, colonies were counted for each dilution and each animal. The culture results are expressed as the mean ± standard deviation (SD) of the $\log_{10}$ CFU per gram of lung in each group at the two time points (24 and 48 h), and differences between pairs of groups were calculated as differences in $\log_{10}$=mean treated group – mean control group. Bactericidal activity was defined as a $\geq$3 $\log_{10}$ decrease compared with the initial inoculum at each time point. Synergy was defined as a $\geq$2 $\log_{10}$ decrease in killing with the combination compared with the most active single drug alone[22].

**8. Effects on survival**

The survival rates of all mice at 24 and 48 h were recorded and compared among the treatment and control groups.

**9. Statistical analysis**

All bacterial counts are presented as the mean ± SD. Student's *t*-test was used to analyze inter-group differences in the bacterial counts. To compare mortality between groups, Fisher's exact test was used. In all tests, differences were considered to be statistically significant when the *p*-value was $<0.05$.

## III. RESULTS

### 1. Antibiotic susceptibility tests

Table 1 shows the MICs for piperacillin/tazobactam, ceftazidime, imipenem, amikacin, ciprofloxacin, tigecycline, colistin, minocycline, and fosfomycin. The carbapenemase–producing *A. baumannii* was resistant to most antimicrobials, including imipenem.

**Table 1**. Antibiotic susceptibility of carbapenemase–producing *A. baumannii*

| β–lactamase | MIC(mg/L) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PIP/TAZ | CAZ | IMP | AMK | CIP | TIG | COL | MIN | FOS |
| OXA-23 | > 128 | 128 | 32 | > 128 | > 128 | 16 | 16 | 0.25 | 128 |

MIC, minimal inhibitory concentration; PIP/TAZ, piperacillin/tazobactam; CAZ, ceftazidime; IMP, imipenem; AMK, amikacin; CIP, ciprofloxacin; TIG, tigecycline; COL, colistin; MIN, minocycline; FOS, fosfomycin

### 2. Time-kill test

At 0.5×MIC, colistin, fosfomycin, and minocycline showed no bactericidal effect when tested singly. However, combinations of colistin with fosfomycin and colistin with minocycline showed bactericidal effects, with >3 $\log_{10}$ reductions in CFU at 4 and 8 h. Moreover, this reduction in CFU was maintained at 24 h. The combination regimens showed synergistic effects with ≥2 $\log_{10}$ decreases compared with the monotherapy regimens. The time–kill curves are shown in Figure 2.

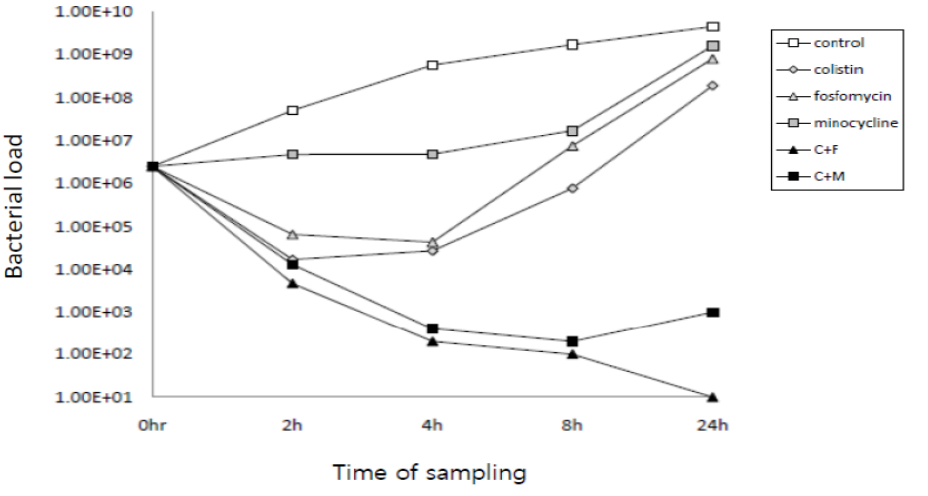


**Figure 2**. **Time–kill curves**. Colistin/fosfomycin and colistin/minocycline combinations showed bactericidal effects, with >3 log $_{10}$ reductions in CFU at 4, 8, and 24 h. The combination regimens showed synergistic effects, with ≥2 $\log_{10}$ decreases in CFU compared with the monotherapy regimens (0.5 x MIC).
C, colistin; F, fosfomycin; M, minocycline

### 3. Mouse model of MDR *A. baumannii* pneumonia

Figure 3 shows the lung pathology at 4, 24, and 48 h after nasal inoculation. Four hours after inoculation, no significant lesion was present in the alveoli, bronchioles, or bronchi, except for minimal edema in the alveolar cavity. At 24 h after nasal inoculation, moderate numbers of neutrophils and macrophages had infiltrated the alveoli, with moderate edema, and bacterial colonization was observed in the alveolar cavities. At 48 h after nasal inoculation, large numbers of macrophages and neutrophils had infiltrated the alveoli.

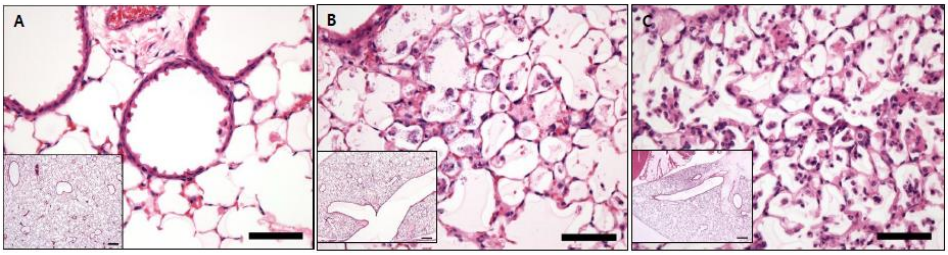


**Figure 3. Histopathology of the lung tissues of mice at 4 (A), 24 (B), and 48 h (C) after inoculation with *A. baumannii* (H&E; bar=100 μm)**. (A) At 4 h after inoculation, no significant lesion was present in the alveoli, bronchioles, or bronchi, except for minimal edema in the alveolar cavity. (B) At 24 h after inoculation, moderate numbers of neutrophils and macrophages had infiltrated the alveoli; moderate edema was present and bacterial colonization was observed in the alveolar cavities. (C) At 48 h after inoculation, large numbers of macrophages and neutrophils had infiltrated the alveoli.

### 4. Effects on lung bacterial loads

Table 2 and Figure 4 show the lung bacterial loads in each group. Colistin, fosfomycin, and minocycline significantly ($p$<0.05) reduced the bacterial loads in the lungs, compared with the controls, at 24 and 48 h. At 24 h after starting the antibiotic agents, fosfomycin and minocycline showed bactericidal effects, but colistin did not. However, colistin in combination with fosfomycin and minocycline, respectively, significantly ($p$<0.05) reduced the bacterial load in the lungs compared with the controls at 24 and 48 h. In the combination groups, significant ($p$<0.05) differences were noted in the bacterial loads compared with the more active antimicrobial alone. Moreover, the combination regimens all showed bactericidal and synergistic effects at 24 and 48 h compared with the more active antimicrobial alone. The combination of colistin with fosfomycin significantly ($p$<0.05) reduced the bacterial load in the lungs at 48 h compared with colistin with minocycline.

**Table 2**. Therapeutic effects on the lung bacterial loads at 24 and 48 h after starting the antibiotic agents

| Antibiotic regimen | 24 h | 48 h |
|---|---|---|
| Control | 12.24±0.44 | 12.99±0.22 |
| COL | 9.65±0.43[a] | 8.73±0.34[a] |
| FOS | 7.68±0.47[a] | 6.68±1.02[a] |
| MIN | 9.26±0.19[a] | 7.73±1.33[a] |
| COL+FOS | 5.63±0.26[a,b] | 3.46±0.42[a,b,c] |
| COL+MIN | 6.07±1.04[a,b] | 5.16±0.83[a,b] |

COL, colistin; MIN, minocycline; FOS, fosfomycin

The lung bacterial loads are expressed as the mean ± standard deviation of $\log_{10}$ CFU per gram of lung at 24 and 48 h.

[a] Significant difference in bacterial load compared with the control group ($p$<0.05).

[b] Significant difference in bacterial load compared with the more active antibiotic alone ($p$<0.05).

[c] Significant difference in bacterial load compared with the other combination ($p$<0.05).

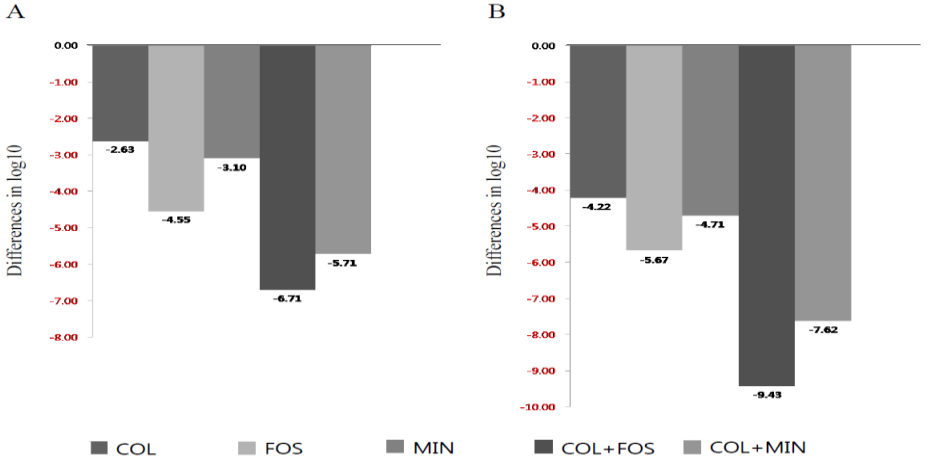


**Figure 4**. ***In vivo*** **efficacy of monotherapy and combination regimens on the lung bacterial load at 24 (A) and 48 (B) h after starting the antibiotic agents.** The colistin/fosfomycin and colistin/minocycline combination regimens showed bactericidal and synergistic effects at 24 and 48 h, compared with the more active antimicrobial alone. (Differences in $\log_{10}$=mean treated group – mean control group)

COL, colistin; MIN, minocycline; FOS, fosfomycin

**5. Effects on survival**

Table 3 shows the mortality rates of the mice. The mortality rate was 33.3% (4/12) in the untreated control group at 48 h. No significant difference in survival was observed among the control and antibiotic-treated groups.

**Table 3**. Mortality rates in the control and antibiotic-treated groups

| Antibiotic regimen | 24 h | 48 h |
|---|---|---|
| Control | 0/15 (0%) | 4/12 (33.3%) |
| COL | 0/15 (0%) | 0/12 (0%) |
| FOS | 0/15 (0%) | 0/12 (0%) |
| MIN | 0/15 (0%) | 0/12 (0%) |
| COL+FOS | 0/15 (0%) | 0/12 (0%) |
| COL+MIN | 0/15 (0%) | 0/12 (0%) |

COL, colistin; MIN, minocycline; FOS, fosfomycin

## IV. DISCUSSION

This study demonstrated the synergistic effects of regimens combining colistin with minocycline and fosfomycin, respectively, on pneumonia caused by MDR *A. baumannii*.

The treatment of *A. baumannii* infection is a major problem in the nosocomial setting[23]. In serious infections, such as pneumonia, the initial use of an appropriate antibiotic therapy is very important[6]. In addition to colistin, some potential treatment regimens have emerged, although none has been tested thoroughly. Carbapenem with sulbactam demonstrated a synergistic effect in MDR *A. baumannii* strains[6,7]. Although the study population was small and heterogeneous and no control group was used, a clinical trial of rifampicin in combination with colistin and imipenem, respectively, yielded promising results[8,9]. Tigecycline also showed good bacteriostatic activity against carbapenem-resistant *A. baumannii in vitro*[6,10]. However, the synergistic activity

of many antibiotics found *in vitro* may not correlate well with *in vivo* outcomes[24]. Nevertheless, some specific antibiotic combinations have shown increased *in vivo* efficacy against MDR isolates[22].

Recently, fosfomycin and minocycline were introduced as treatment options for MDR *A. baumannii* infection[12,13]. In our time–kill study, colistin with fosfomycin and minocycline, respectively, showed bactericidal and synergistic effects in the treatment of MDR *A. baumannii* at 8 and 24 h. A previous study using time–kill tests documented synergistic and bactericidal effects of minocycline and colistin in 92% of the strains tested at 24 h[2]. In another study using the E-test, the fractional inhibitory concentration indices (FICIs) for combinations of polymyxin B and minocycline were generally ≤0.5 or >0.5–1.0, suggesting that the two drugs have a synergistic or additive effect[25]. In the same study, most FICIs for polymyxin B and fosfomycin were within the ranges of 0.5–1.0 and 1.0–4.0, suggesting that the effects of polymyxin B and fosfomycin were additive or independent[25]. In comparison, another study of combination therapy against *A. baumannii* found that colistin combined with fosfomycin was more effective than colistin monotherapy in 83.3% (24 h) and 66.7% (48 h) of MDR strains[22].

We also found that colistin with minocycline and colistin with fosfomycin showed bactericidal and synergistic effects 24 and 48 h after nasal inoculation *in vivo*, in accordance with previous studies. Yang *et al*.[26] reported that minocycline in combination with colistin had *in vivo* synergistic efficacy against MDR A. *baumannii* pneumonia. Bowers *et al*.[27] showed that minocycline combined with polymyxin B further decreased the bacterial lung load at 24 h, compared with monotherapy. Sirijatuphat *et al*.[28] reported that colistin with fosfomycin showed a synergistic effect against carbapenem-resistant *A. baumannii*. They also recently conducted a preliminary clinical study , which showed that patients with MDR *A. baumannii* infection given a combination of colistin and fosfomycin had significantly better

microbiological responses with trends toward more favorable treatment outcomes and lower mortality compared with those treated with colistin alone[29].

Interestingly, our *in vivo* results showed that the combination of colistin with fosfomycin significantly reduced the bacterial load in the lungs, compared with monotherapy and colistin with minocycline, at 48 h. Fosfomycin has a higher MIC against *A. baumannii*. For an antibiotic to be effective clinically, it must achieve concentrations in the interstitial fluid that exceed the MICs for the pathogens[30,31]. One study showed that fosfomycin achieved antimicrobially effective concentrations in infected lung tissue[32]. Also, without pharmacokinetics (PKs) of fosfomycin in this study, we used it every 4h according to other study. These frequent injections of fosfomycin might attain more effective concentrations in infected lung tissue than minocycline. Moreover, in combination therapy, fosfomycin was injected with colistin simultaneously, but minocycline was not. This difference explains the greater effectiveness of colistin with fosfomycin relative to colistin with minocycline at 48 h.

No significant difference in survival was observed among the control and antibiotic-treated groups. This finding might be due to the relatively short duration of follow-up in our study or the relatively low virulence of OXA-23 carbapenemase-producing *A. baumannii*. Consequently, further evaluations of mortality with longer follow-up and the virulence of OXA-23 carbapenemase-producing *A. baumannii* are needed.

Our study has some limitations. First, only one strain from a single center was used. However, we used an OXA-23 carbapenemase-producing *A. baumannii* isolated from clinical specimens obtained in our hospital. OXA-23 carbapenemase is the most common carbapenemase in Korea[33]. Therefore, our study was very meaningful in this regard. Second, we did not investigate the PKs of the drugs used in this study. However, we used dosages that have been used in other studies.

## V. CONCLUSION

We investigated the efficacy of colistin in combination with minocycline and fosfomycin, respectively, as therapeutic options in a mouse model of MDR *A. baumannii* pneumonia. We demonstrated the synergistic effects of regimens combining colistin with minocycline and fosfomycin, respectively, on pneumonia caused by MDR *A. baumannii*. Large clinical trials are needed to clarify the role of regimens combining colistin with fosfomycin or minocycline in treating MDR *A. baumannii* pneumonia.

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ABSTRACT (IN KOREAN)

# 다제내성 *Acinetobacter baumannii* 에 의한 폐렴 마우스 모델에서 colistin과 minocycline 또는 minocycline 병합요법의 생체내 효과

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**배경:** *Acinetobacter baumannii* 는 잘 알려진 다제내성 원내세균이다. 하지만 안타깝게도 다제내성 *Acinetobacter baumannii* 의 치료는 상당히 어렵고 그 치료제 또한 제한적이다. 이를 극복하기 위해 항생제의 길항작용을 이용하는 방식으로 항생제 병합요법이 제안되어 왔다. 최근 예전에 사용되었던 fosfomycin 과 minocycline 이 이러한 다제내성 *Acinetobacter baumannii* 감염의 치료제로 다시 고려하게 되었다. 따라서, 본 연구진은 다제내성 *Acinetobacter baumannii* 에 의한 폐렴 마우스 모델에서 치료제로서 colistin과 fosfomycin 또는 minocycline 병합요법이 효과가 있는지 알아보고자 하였다.

**방법:** 세브란스 병원에서 얻어진 임상 검체에서 분리된 carbapenem 내성 *Acinetobacter baumannii* 를 사용하였다. 다제내성 *Acinetobacter baumannii* 에 의한 폐렴 마우스 모델에서 colistin, fosfomycin, minocycline, colistin과 fosfomycin, colistin과

minocycline으로 치료한 후 24시간, 48시간 폐 조직의 세균수 및 생존율을 비교하였다. 살균력은 대조군에 비해 치료 후 세균수가 $3\log_{10}$ 이상 감소한 것으로 정의하였다. 길항력은 가장 효과가 있었던 단독 요법과 비교해서 세균수가 $2\log_{10}$ 이상 감소한 것으로 정의하였다.

**결과:** 생체내 실험에서 colistin과 fosfomycin, colistin 과 minocycline 병합요법은 치료 후 24시간, 48시간에 대조군에 비해 유의하게 폐 조직의 세균수가 감소하였다 ($p$<0.05). 병합요법은 가장 효과가 있었던 단독요법 보다도 더 유의하게 세균수가 감소하였다 ($p$<0.05). 또한, 병합요법은 치료 후 24시간, 48시간에 가장 효과가 있었던 단독요법과 비교해서 의미있는 살균력과 길항력을 보였다. 생존율은 대조군과 치료군에서 차이가 없었다.

**결론:** 이상의 결과를 종합하여 볼 때, 본 연구는 다제내성 *Acinetobacter baumannii* 에 의한 폐렴 마우스 모델에서 치료제로서 colistin과 fosfomycin, colistin과 minocycline 병합요법이 효과가 있었음을 보였다. 따라서, 다제내성 *Acinetobacter baumannii* 에 의한 폐렴을 치료하는데 있어서 colistin과 fosfomycin, colistin과 minocycline 병합요법의 효과를 좀더 명확히 보기 위한 대규모의 임상 연구가 필요하겠다.

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핵심되는 말: 다제내성 *Acinetobacter baumannii*, colistin, fosfomycin, minocycline, 병합요법