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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Efficacy of antibiotic combinations in an experimental sepsis model with *Pseudomonas aeruginosa*

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Summary
This study aimed to compare the efficacy of fosfomycin, colistin, tobramycin and their dual combinations in an experimental sepsis model. After sepsis was established with a *Pseudomonas aeruginosa* isolate (P1), antibiotic-administered rats were divided into six groups: Fosfomycin, tobramycin, colistin and their dual combinations were administered by the intravenous or intraperitoneal route to the groups. The brain, heart, lung, liver, spleen and kidney tissues of rats were cultured to investigate bacterial translocation caused by P1. Given the antibiotics and their combinations, bacterial colony counts in liver tissues were decreased in colistin alone and colistin plus tobramycin groups compared with control group, but there were no significant differences. In addition, a non-statistical decrease was found in the spleen tissues of rats in the colistin plus tobramycin group. There was a >2 $\log_{10}$ CFU/ml decrease in the number of bacterial colonies in the kidney tissues of the rats in the fosfomycin group alone, but the decrease was not statistically significant. However, there was an increase in the number of bacterial colonies in the spleen and kidney samples in the group treated with colistin as monotherapy compared to the control group. The number of bacterial colonies in the spleen samples in fosfomycin plus tobramycin groups increased compared to the control group. Bacterial colony numbers in all tissue samples in the fosfomycin plus colistin group were found to be close to those in the control group. Colistin plus tobramycin combinations are effective against *P. aeruginosa* in experimental sepsis, and clinical success may be achieved. New in vivo studies demonstrating the ability of *P. aeruginosa* to biofilm formation in tissues other than the lung are warranted in future.

Keywords *Pseudomonas aeruginosa* · Experimental sepsis model · Fosfomycin · Colistin · Tobramycin

Introduction

Sepsis is a major healthcare problem, affecting more than 30 million people worldwide and causing high mortality and morbidity [1–3]. Many bacteria can cause sepsis, but *Pseudomonas aeruginosa* is one of the most serious bacteria because it easily develops resistance to antibiotics, causes difficult-to-treat infections and forms biofilms. *P. aeruginosa* can cause opportunistic infections, especially in immunocompromised cancer patients and patients with cystic fibrosis [4, 5]. Inability to develop new antibiotics against such bacteria requires more careful and effective use of existing antibiotics. Fosfomycin, colistin and tobramycin are among the treatment options for multidrug-resistant (MDR) Gram-negative bacterial infections. Using two agents from different classes is recommended for the empirical treatment of serious infections caused by *P. aeruginosa*. The reason for using two antibiotics together is to increase treatment effectiveness in *P. aeruginosa* strains, especially in the presence of high antibiotic resistance [6, 7]. The combined use of antibiotics aims to achieve a bactericidal effect at lower concentrations than the minimal inhibiton concentration (MIC). Bactericidal synergistic effects for different antibiotic combinations such as colistin + tobramycin, polymyxin + fosfomycin have been reported for *P. aeruginosa, Acinetobacter baumannii* and carbapenem-resistant *Klebsiella pneumoniae* in recent studies [7–9].

Time-kill assay is used to investigate the dynamics of synergy or antagonism for a combination of antimicrobial

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agents by determining the number of viable bacteria remaining over time after exposure to each agent and various combinations. Time-kill analysis data assess both the rate and extent of killing. Prior to performance of the time-kill assay, concentrations of individual agents and combinations most likely to result in synergism are planned based on a checkerboard synergism test data [10].

Many studies have reported that combination therapy is more advantageous than monotherapy and reduces mortality rates [11, 12]. Although conflicting results were reported for aminoglycosides combined with fosfomycin in checkerboard and time-kill assays in *P. aeruginosa* isolates in previous studies, it was recently reported that fosfomycin-colistin and fosfomycin-aminoglycoside combinations were synergistic in carbapenem-resistant (CR) *P. aeruginosa* strains [13].

Hospital infections are a serious healthcare problem in our country as well as all over the world. Although Turkiye is one of the countries with the high rates of antibiotic resistance, synergy tests, which determine the most effective combination options in the treatment of antibiotic-resistant infections, are not routinely applied in our country. This causes the true extent of the antibiotic resistance problem to be unknown and the ideal antibiotic combination options not to be determined.

This study aimed to compare the efficacy of fosfomycin, colistin, tobramycin monotherapies and their dual combinations in an experimental sepsis model induced by *P. aeruginosa* isolate. We believe that this experimental study will guide the selection of appropriate antibiotics for the treatment of MDR microorganisms and will contribute to the decrease of the morbidity and mortality of patients.

Material and methods

Bacterial strain

A *P. aeruginosa* strain (P1) isolated from nosocomial infection was used to construct an experimental sepsis model. The MIC results, C_{max} values, and breakpoint ranges of the strain were shown in Table 1.

Antibiotics and drugs

Colistimethate sodium (Sigma-Aldrich, USA), tobramycin (Sigma-Aldrich, USA) and fosfomycin (Sigma-Aldrich, USA) were used for in vitro studies. At the end of the study, ketamine (Pfizer, Turkey) was used to anesthetize the rats, and sodium pentobarbital (Sigma-Aldrich, USA) was used for euthanasia of the rats.

Table 1 C_{max} values, MIC results of the antibiotics and breakpoint ranges of the P1 strain

P. aeruginosa strain (P1)	FOS	COL	TOB	MEM	CIP
C_{max} (mg/L)	90	10	10	40	3.5
C_{max} breakpoint (mg/L)	132	2.3	4–6	15–20	3.2
MIC (mg/L)	64	8	1.5	16	0.125
MIC breakpoint (mg/L)	ECOFF: 256*	2–4	1–2	2–8	0.5–1

FOS fosfomycin, *COL* colistin, *TOB* tobramycin, *MEM* meropenem, *CIP* ciprofloxacin

*Since the CLSI guidelines do not have a breakpoint range for fosfomycin, the ECOFF value of EUCAST guidelines was used for this antibiotic. The ECOFF of 256 mg/L is not predictive of efficacy irrespective of whether the agent is used alone or in combination [16]. Infections caused by wild type *P. aeruginosa* isolates may be treated with combinations of fosfomycin[15]

In vitro studies

Firstly, antibiotic combinations to be studied in vivo were determined according to the MIC results of the antibiotics. The broth microdilution tests were performed according to CLSI guideline [14]. MICs of fosfomycin and its combinations were determined by the agar dilution method in the presence of 25 mg/L of glucose-6-phospate(Sigma-Aldrich, USA). Since the CLSI guidelines do not have a breakpoint range for fosfomycin, the epidemiological cut-off value (ECOFF) of European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were used for this antibiotic (Table 1). Also, there is no breakpoint range as *P. aeruginosa* is considered a poor target for systemic fosfomycin therapy [15, 16]. *P. aeruginosa* ATCC 27853 was used as standard strain (Table 1).

Time-kill assay

This assay was used to see how the process worked or changed during the treatment. Time-kill assay test was performed as previously recommended [10]. Antibiotics were tested alone and in combination with the other antibiotics in cation-adjusted Mueller Hinton Broth (Remel, USA). Bacterial cultures and antibiotic(s) were incubated at 37 °C in a shaking water bath (200 rpm) and viability assessed by the plate-count method at 0, 1, 2, 4, 6 and 24 h. A control without antibiotic was used in each assay. The ≥ 3 $\log_{10}$ CFU/mL decrease in bacterial counts of the antibiotic solution compared to the growth control was considered a bactericidal effect[10].

Although the in vitro 24-h dynamics of the interaction between bacteria and antibiotics was demonstrated with this methods, an in vivo model was designed to obtain results closer to clinical applications. For this purpose,

the interaction of antibiotics alone or in combination with the other antibiotics was investigated after inducing sepsis with *P. aeruginosa* in rats.

Experimental animals

Adult male Wistar rats weighing 240–400 g from Istanbul University, Aziz Sancar Institute of Experimental Medicine were used in this study. Rats were maintained in standard room conditions (20–22 °C, 12 h light, 12 h dark, 40–60% humidity) with two rats in each cage. Feed and water were provided ad libitum. All rats were followed for weight, nutrition and health status during the three-day experimental period. In any case of disease or ill condition such as rapid or very slow breathing, shallow or labored breathing, rapid weight loss, inclined posture, hypothermia or hyperthermia, diarrhea or constipation, weakness (difficulty or inability to get nutrition), inability to stand up and drowsiness (disliking physical activity, loss of mental alertness), rats were euthanized and excluded from the experiment.

At the end of all experiments, rats were sedated with ketamine (30 mg/kg) anesthesia and then euthanized by intraperitoneal administration of sodium pentobarbital (0.22 ml, 135 mg/kg).

Study design and animal experiments

This study was carried out following the ethical values specified in the Declaration of Helsinki and was approved by the Istanbul University Animal Experiments Local Ethics Committee (Ethical approval number: 2013/129).

Fifty-six rats were used in this study. Rats were divided into seven groups including one control group, three antibiotics alone and three antibiotic combination groups (see the box). Eight rats were included in each group (Box 1).

Box 1. Rats were divided into six groups of eight, one of which was the control group (the group not given antibiotics), as belows:

Group 1 (8 rats): Control (C) group: Isotonic sodium chloride (500 µl) was administered

Group 2 (8 rats): Fosfomycin (FOS) (500 µl) was administered (150 mg/kg/12 hours)

Group 3 (8 rats): Tobramycin (TOB) (500 µl) was administered (10 mg/kg/24 hours)

Group 4 (8 rats): Colistimethate sodium (COL) (500 µl) was administered (5mg/kg/day)

Group 5* (8 rats): Fosfomycin plus tobramycin (FOS+TOB) were administered

Group 6* (8 rats): Fosfomycin plus colistimethate sodium (FOS+COL) were administered

Group 7* (8 rats): Colistimethate sodium plus tobramycin (COL+TOB) were administered

*The dosages used in combinations were administered as the dosages given alone

For the bacterial inoculation, the tail veins of a total of 56 rats including study groups and control group were cleaned with iodine. Rats were inoculated using intravenous administration with 10^7 CFU/ml of viable P1 bacterial suspension. A sepsis model was induced by intravenous administering 0.2 ml of this suspension. Rats were followed for 72 h.

Blood cultures

The presence of experimental sepsis was confirmed by blood culture, which is the gold standard. Within the one hour after bacterial inoculation, blood samples obtained from rats were incubated in a blood culture device (Beckton Dickinson, USA). All blood culture bottles showed a positive signal in the first 24 h. Blood samples from blood culture bottles with positive signals were inoculated into 5% sheep blood agar and Mac Conkey agar. After an overnight incubation period, bacterial identification was performed using conventional methods, and/or automated identification kits (API ID 32E, bioMérieux, France).

Administration of antibiotics and antibiotic groups

First dose of antibiotics was given three hours after bacterial inoculation to rats. Isotonic sodium chloride was administered to the control group, fosfomycin monotherapy to the second group, tobramycin monotherapy to the third group, colistin monotherapy to the fourth group, fosfomycin + tobramycin combination to the fifth group, fosfomycin + colistin to the sixth group, colistin + tobramycin to the seventh group by the intravenous or intraperitoneal

Table 2 The dosages of antibiotics used and amount of inoculum of the bacterial strain

Antibiotics/bacteria	Dosage	Volume	Route of administration	Dosages	Period	References
Fosfomycin sodium	150 mg/kg	500 µl	Intraperitoneal	2 doses/3 days (every 12 h)	24–48 h	[13]
Tobramycin	10 mg/kg	500 µl	Intraperitoneal	Single dose/3 days	24 h	[5]
Colistimethate sodium	5 mg/kg	500 µl	Intravenous	2 doses (every 12 h)	72 h	[41]
P. aeruginosa (P1)	10^7 CFU/ml	0.2 ml	Intravenous	Single dose	-	[41, 42]

route. The dosages of antibiotics used in study were given in Table 2.

The first dose of antibiotics was given three hours after bacterial inoculation. The antibiotics mentioned above were administered at appropriate doses and two times for 72 h. During these procedures, rats were observed for 72 h, every two hours for local inflammation, weight loss, vomiting, diarrhea and fever.

At the end of 72 h, all rats were sedated by intramuscular injection of ketamine (30 mg/kg) and then euthanized by intraperitoneal administration of sodium pentobarbital (135 mg/kg). The brain, heart, lung, liver, spleen and kidney tissues of rats were cultured to investigate bacterial translocation caused by *P. aeruginosa*. For this purpose, tissue samples were taken from the organs; each tissue piece was homogenized with steril distilled water (Heidolph Silent Crusher M, Schwabach, Germany) under sterile conditions, and then inoculated on tryptic soy agar, 5% sheep blood agar, MacConkey agar media, and incubated in an aerobic atmosphere at 37 °C for 18–24 h. At the end of the incubation period, bacteria with Gram-negative rod morphology were identified by conventional methods and/or automated identification kits (API ID 32E, bioMérieux, France). Colonies of growing bacteria in the brain, heart, lung, liver, spleen and kidney tissues were counted as CFUs per ml.

Statistical analysis

The results were analysed by SPSS 24.0 programme. The discontinuous data obtained from the study are presented as number and percentage, and continuous data are presented as median ± standard deviation (min–max). The conformity of the values obtained in the study to the normal distribution was analyzed with the Shapiro–Wilk test. Variables that do not conform to the normal distribution; Mann–Whitney *U* test between two groups and Kruskall Wallis test in groups with more than two. Multiple comparison tests were performed with Dunn's test. Statistical significance was accepted as two-tailed and $p < 0.05$.

Results

In vitro studies

Antibiotic combinations to be used in the treatment of rats were determined according to the C_{max} values and the results of time-kill curve assays (Table 1). MIC values of fosfomycin, colistin and tobramycin for standard strain (*P. aeruginosa* ATCC 27853) were detected as 4 mg/L, 1 mg/L and 0.5 mg/L, respectively. According to the broth microdilution method, P1 strain was resistant to colistin and meropenem with MICs of 8 µg/ml and 16 µg/ml, respectively. The strain was susceptible to tobramycin. At this time, neither EUCAST nor CLSI has set fosfomycin breakpoints for *P. aeruginosa*. However, EUCAST includes an ECOFF value (256 µg/ml) that distinguishes the wild-type (WT) population from the population harboring acquired resistance mechanisms [16]. Since the CLSI guidelines do not have a breakpoint range for fosfomycin, the ECOFF value of EUCAST guidelines was used for this antibiotic (Table 1). It has been reported that infections caused by wild-type *P. aeruginosa* isolates can be treated with fosfomycin combinations [15]. Consistent with this information, Diez-Aguilar et al. reported that no mutation was observed in the *glp*T gene sequence responsible for fosfomycin resistance in any of the isolates with MICs below the ECOFF value [17]. In studies on the subject, most of the *P. aeruginosa* isolates had a fosfomycin MIC of 64 µg/ml and fosfomycin showed fairly consistent activity against most isolates when using the susceptibility breakpoint of 64 µg/ml [18, 19]. MIC value of fosfomycin for P1 isolate was also found as 64 µg/ml in present study. Therefore, we decided to use fosfomycin and tobramycin alone or in combination with colistin in the treatment of rats with sepsis.

Establishment of experimental sepsis was confirmed by the growth of *P. aeruginosa* in the media cultivated from blood culture bottles that gave a positive signal in the first 24 h. No other bacterial growth or contamination was detected in blood culture.

Differences in bacterial colony counts of the rat groups treated with antibiotic/antibiotic combination compared to the control group were shown in Table 3 and Fig. 1. Comparing the combination of three antibiotics and three antibiotics, bacterial colony counts in liver samples were decreased in colistin alone and colistin plus tobramycin groups when compared with control group, but there were no significant differences. In addition, a non-statistical decrease was found in the spleen samples of rats in the COL + TOB groups.

Time-kill assay

The in vitro 24-h dynamics of the time-concentration interaction between bacteria and antibiotics was demonstrated by time-kill assay which standard method. Time kill assay results provide rational data showing the effectiveness of antibiotic combinations against microorganisms over a certain period of time. Since each antibiotic has different C_{max} values, the antibiotics to be used in the combination were decided according to the results of the time-kill assays (Table 1). Also, the results of the time-kill assay helped to determine the treatment doses and durations. Following detecting antibiotics with bactericidal activity, these antibiotics, alone or in combination, were inoculated into the rats to treat experimental sepsis.

Table 3 Differences in the rat groups treated with antibiotic/antibiotic combination compared to the control group according to bacterial counts

Groups	*Colony counts: Median ± Std Dev.(Min-Max) CFU/ml x10^{12}			
	Spleen	Lung	Liver	Kidney
Control	**32.2±135.5 (10-294)**	**10±37.5 (0.97-70)**	**10±24.3 (0.25-57)**	**5±4.93 (0.13-10)**
FOS	18.5±84.1 (11-184)	15.4±21.5 (0.024-43)	14±5.25 (4-15)	**0.009**±113.15 (0.0000005-196)
TOB	128±71.2 (21-156)	**88**±0 (88-88)	7.65±4.46 (5.3-15.5)	-
COL	**323**±523.8 (2.89-1210)	8.5±23.43 (0.235-51.9)	**0.7**±7.78 (0.012-16)	**198**±260.2 (14-382)
FOS+TOB	**381**±310.7 (207-875)	5.41±10.13 (0.005-23)	50±41.81 (0.3-102)	19.7±21.8 (1-44.6)
FOS+COL	38.4±456 (10-940)	10±0 (10-10)	2.27±123.7 (0.205-249)	10.5±6.36 (6-15)
COL+TOB	**2.46**±388.9 (0.3-788)	12.18±15.64 (0.000023-37)	**0.27**±233.8 (0.023-537.6)	-

*The > 1-$\log_{10}$ CFU/ml increases in bacterial colony count in post-mortem tissues are shown in blue, and > 1-$\log_{10}$ CFU/ml decreases are shown in green

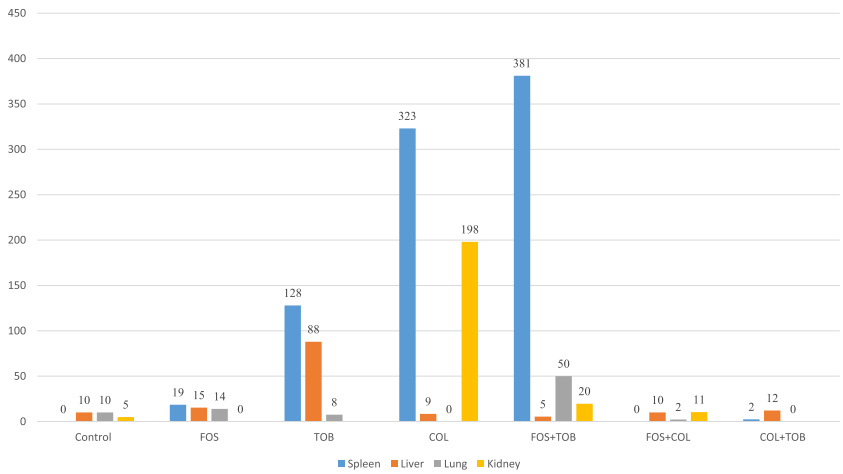


Fig. 1 Bacterial colony counts in tissue samples of the rat groups treated with antibiotics/antibiotic combinations compared to the control group (median × 10.^{12} CFU/ml)

In vivo studies

Rats were observed for local inflammation, weight loss, vomiting, diarrhea and fever every two hours. Classical signs of sepsis were observed in rats within 72 h, such as increased body temperature, increased pulse rate and malnutrition. Due to the design of the study, organ dysfunctions and systemic proinflammatory responses in rats could not

be confirmed biochemically, histochemically or by cytokine assays. It is also known that the rats in experimental sepsis models do not have similar clinical manifestations to that of human sepsis.

No bacterial growths were observed in post-mortem brain and heart tissues. Bacteria counts in liver, lung, spleen and kidney tissues are indicated in the Table 3.

Discussion

Broad-spectrum antibiotics is recommended in the treatment of sepsis caused by *P. aeruginosa*, but antibiotic options are limited due to the development of resistance. For this reason, the use of new antibiotics and antibiotic combinations has become widespread. Fosfomycin presents bactericidal activity against susceptible pathogens by blocking the early stage of bacterial cell wall synthesis. However the use of fosfomycin alone is not recommended due to the risk of developing resistance during treatment. Since the bactericidal effect of intravenous fosfomycin alone is eliminated in high inoculum in systemic infections caused by *P. aeruginosa*, a combination of fosfomycin with other antibiotics is required. The synergy of fosfomycin with other classes of antibiotics (especially aminoglycosides, polymyxin B) makes it possible to administer these antibiotics at lower and therefore less toxic doses [20].

The rates of resistance to aminoglycosides and carbapenems in *P. aeruginosa* isolated from blood cultures in Turkiye were 15.7 and 36.2%, respectively, according to ECDC data. The rate of MDR *P. aeruginosa* isolates was also found as 27.5% [21]. As known, the resistance rates against antibiotics are increasing gradually. According to the 2020 surveillance data of our hospital, both aminoglycoside and carbapenem resistance was found to be 33% in *P. aeruginosa* strains isolated from blood cultures [22]. Thus, alternative or combined treatments are needed in the treatment of sepsis caused by *P. aeruginosa.* The present study evaluated in vivo and in vitro efficacy of fosfomycin, colistin, tobramycin monotherapy, and fosfomycin + colistin, fosfomycin + tobramycin and colistin + tobramycin combination groups. In additon, the effects of colistin, fosfomycin and tobramycin alone and their dual combinations on bacterial colony counts in brain, heart, lung, liver, kidney and spleen tisues at 72 h by comparing with control group were evaluated.

Susceptibility to fosfomycin has been reported as 61–80% in MDR *P. aeruginosa* isolates isolated from bloodstream infections [23, 24]. P1 strain used in present study was found to be resistant to colistin and meropenem (Table 1). The ECOFF of 256 mg/L is not predictive of efficacy irrespective of whether the agent is used alone or in combination. However, as above mentioned, infections caused by wild type *P. aeruginosa* isolates may be treated with combinations of fosfomycin [15]. In the present study, when fosfomycin was used as monotherapy, an increase in bacterial counts in post-mortem lung and liver tissue samples and a decrease in spleen and kidney tissue samples were detected. The increase in bacterial colony counts may be due to the regrowth of resistant mutants. As is known, some of the bacterial populations are killed at concentrations lower than the MIC in concentration-dependent antibiotics, but some populations remain alive and continue to reproduce. A study has reported that fosfomycin monotherapy provided effective killing but rapid regrowth for fosfomycin-susceptible isolates. It has also been reported that for the susceptible isolates, the combination of fosfomycin with colistin or tobramycin increased bacterial killing compared to monotherapy, but did not prevent regrowth of resistant mutants [25]. When fosfomycin was used in combination with tobramycin, increased bacterial counts were observed in all tissue samples compared to control, except lung tissue. This effect may be due to tobramycin, not fosfomycin, because when tobramycin alone was used, increased bacterial colony counts were observed in all tissue samples (Table 3, Fig. 1). Although an decrease or increase in bacterial colony counts was observed in rats after fosfomycin treatment alone and in combination with tobramycin/colistin, these ones were not statistically significant ($p > 0.05$).

Time-kill assay results provide rational data showing the effectiveness of antibiotic combinations against microorganisms over a certain period of time. These data are especially important in terms of establishing effective antibacterial activity in clinical conditions such as lung infections, where bacteria develop resistance to antibiotics by forming biofilms. Time-kill assays are the most ideal methods to determine the necessary antibiotic combinations and concentrations in infections caused by biofilm-forming bacteria, where the MIC values against bacteria are approximately 1000 times higher. Therefore, these tests contribute greatly to the successful treatment of resistant bacterial infections, especially in intensive care units.

Regional antimicrobial resistance data were also taken into account when deciding on the antibiotics to be used in combinations in the study. Resistance to ciprofloxacin (CIP) is quite high in *P. aeruginosa* isolates in Turkiye [26, 27]. Moreover, clinical failure has been observed even in isolates that appear susceptible in antibiotic susceptibility tests. Although the MIC value of ciprofloxacin against the P1 isolate used in our study was low, we did not prefer to use quinolones in combinations because there was treatment failure in CIP-sensitive *P. aeruginosa* isolates in our hospital. Also we wanted to reflect the results of our study to clinical practice. As known, if an isolate is resistant to colistin, it is not preferred to use colistin alone in clinical therapy. That is why we did not choose to use colistin for the combinations.

Synergies between fosfomycin and colistin (22%) or carbapenems (40%) have been reported using checkerboard and time-killing analyses in CR *P. aeruginosa* strains [28]. It has been reported that synergistic combinations are observed more commonly when the antibiotics used for the combination are tobramycin (65%) or fosfomycin (57%) in MDR *P. aeruginosa* isolates. [29]. While synergy was reported with fosfomycin + carbapenem against 50% to 70% of MDR *P. aeruginosa* isolates, synergy with colistin was reported in 13% of the strains [30].

Bacterial growth of *P. aeruginosa* is not expected in post-mortem brain tissue in the experimental sepsis model. The blood–brain barrier (BBB) provides protection against pathogen invasion as well as provides a selective filter for the tightly regulated exchange of water, solutes, nutrients or compounds between the blood and the central nervous system (CNS). A small number of bacteria can cross the BBB and cause acute meningitis. These bacteria include *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Escherichia coli*, *group B Streptococcus* and *Listeria monocytogenes*. Less common bacteria responsible for acute meningitis include non-typhoidal *Salmonella*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Klebsiella* spp. and *Streptococcus suis* [31]. That is, in fact, *P. aeruginosa* is not one of the bacteria that can cross the BBB. However, *P. aeruginosa* neurosurgical meningitis is a rare condition usually associated with intraventricular catheters. Thus, no bacterial growth was detected in the brain tissue in any antibiotic/antibiotic combination any groups in our study, as expected.

Similarly, infective endocarditis (IE) due to *P. aeruginosa* is rare and accounts for only about 3% of all patients with IE, and more than 90% of patients with IE caused by *P. aeruginosa* are intravenous drug users [32]. Since the rats used in our study did not represent such a population, the probability of developing IE was very low. Thus, as expected, bacterial growth was not found in post-mortem heart tissues in any group.

Zusman et al. in a meta-analysis in which they compiled 22 studies reported that polymyxin monotherapy was associated with higher mortality than the polymyxin + fosfomycin combination in CR *A. baumannii* and *P. aeruginosa* infection [9]. Similarly, a > 1-log10 CFU/ml increase in bacterial counts in post-mortem both spleen and kidney tissue samples was detected when colistin was used as monotherapy in the present study. The increase in the number of colonies in spleen, liver and kidney samples may be due to the disappearance of the susceptible population due to treatment, but the increase in the resistant population. Bacterial colony counts increased in spleen samples in the tobramycin plus fosfomycin group, too. The reason for this increase may be the emergence of mutations resulting in unresponsiveness to antibiotics due to selective antibiotic pressure or the ability of *P. aeruginosa* to form biofilm. Biofilm formation may have prevented antibiotics from taking effect. As known, antibiotic doses should be used about 1000 times as much in treatment of infections caused by biofilm-forming bacteria. However, it should be noted that *P. aeruginosa* can also form a biofilm in the tissues of rats in the control group. Thus, in vivo studies demonstrating the ability of *P. aeruginosa* to biofilm formation in tissues other than the lung are required.

Kidney

Fosfomycin is eliminated almost entirely through the kidneys. The pharmacokinetics of fosfomycin in critically ill patients vary due to differences in renal functions of patients [13, 20]. Similarly, tobramycin and colistin are also are excreted in the urine. No bacterial growth was observed in the kidney tissue of any rat in the tobramycin alone and colistin + tobramycin groups in our study. There was a > 2-$\log_{10}$ CFU/ml decrease in the number of bacterial colonies in the kidney tissues of fosfomycin alone group compared to the control group, but it was not statistically significant ($p > 0.05$). Although there was an increase in bacterial colony numbers in the kidney tissues of the colistin alone and colistin + fosfomycin group, no statistically significance was detect ($p = 0.465$).

Lung

Matzi et al. reported that intravenously administered fosfomycin exhibits good penetration into infected lung tissue [33]. In the present study, the number of bacterial colonies in the post mortem lung tissues in the all groups was the similar to the control group except tobramycin group (Table 3, Fig. 1). Bacterial growth was observed in the lung tissue of only a rat in the tobramycin group, but the increase in the number of bacterial colonies was not statistically significant compared to the control group ($p = 0.695$) (Table 3, Fig. 1). The use of colistin in the clinical presentation is limited due to side effects such as nephrotoxicity, difficulty in determining the optimal dose and reaching therapeutic levels, and the possibility of resistance development [34]. Colistin is often administered by inhalation and/or the parenteral route for the treatment of respiratory infections caused by MDR *P. aeruginosa* [35]. When fosfomycin is used in combination with other antibiotics to treat pneumonia and other respiratory tract infections, treatment success has been reported to be 80–85% [13]. However, bacterial colony counts in the lung tissue of rats treated with colistin and its combinations and fosfomycin and its combinations were similar to those in the control group in the present study. Unlike this result, in an experimental pneumonia model, it was reported that the FOS + TOB combination showed rapid bactericidal effect and bacterial death was concentration-dependent. It was

found that tobramycin alone was more active than fosfomycin alone and FOS + TOB combination for *P. aeruginosa* [36]. As known, fosfomycin increases tobramycin uptake, thereby increasing inhibition of protein synthesis, ultimately leading to bacterial death [37]. In another study, it has been reported that fosfomycin-tobramycin combination has synergistic effect and bactericidal activity in susceptible isolates and those with low tobramycin resistance; therefore, it can be an alternative for *P. aeruginosa*-related infections [17].

Spleen

Bacterial growth was detected in spleen samples of three (43%) of seven rats in the tobramycin group. For all antibiotic/antibiotic combinations, except FOS and COL + TOB groups, bacterial colony counts in spleen tissues increased compared to the control group. A > 1-$\log_{10}$ CFU/ml decrease in COL + TOB group was important, but it was not statistically significant ($p = 0.437$) (Table 3, Fig. 1). For the treatment of infections caused by MDR bacteria, the use of intravenous fosfomycin in combination with other antibiotics is recommended because of its excellent in vitro bactericidal activity against a wide range of microorganisms. However, more studies indicate that the optimal dose of fosfomycin for the treatment of difficult-to-treat infections has not yet been defined [38].

Liver

In the present study, bacterial growth was detected in liver samples of four (57%) of seven rats in the tobramycin group. Bacterial colony counts in the liver tissue were found close to the control group in the groups in which fosfomycin and tobramycin were given as monotherapy. Although some antibiotics are ineffective when used alone, they are known to be synergistic in combination. For example, although cefepime and amikacin were inactive in intensive care patients infected with *P. aeruginosa* resistant to all antibiotics except colistin, but their combinations were found to be synergistic in vitro [11]. In this study, a > 1-log10 CFU/ml decrease was observed in the number of bacterial colonies in the liver tissue in the colistin alone group and the COL + TOB group compared to the control group, but no statistical significance was found ($p > 0.05$). It has been reported that the susceptibility rate to fosfomycin varies between 50 and 90% among MDR *P. aeruginosa* isolates, and synergy with fosfomycin was observed in 53.5% of the isolates when combined with beta-lactams, aminoglycosides and quinolones [11].

In summary, comparing the antibiotic and antibiotic combinations, bacterial colony counts in liver samples of rats in the colistin alone and colistin plus tobramycin groups decreased by > 1-$\log_{10}$ CFU/ml compared to the control group, but there was no significant difference. In addition, a non-statistical significant > 1-$\log_{10}$ CFU/ml decrease was found in the spleen samples of rats in COL + TOB group. Although there was a > 2-$\log_{10}$ CFU/ml decrease in the number of bacterial colonies in kidney tissues of rats in the fosfomycin group compared to the control group, it was not statistically significant ($p > 0.05$).

The most important limitation of in vivo sepsis studies on antimicrobial activity is that they are not compatible with the results of clinical studies in humans. Experimental animal models of sepsis often lead to misleading findings. There are inconsistencies between testing with antibiotics in animal models of sepsis and results from clinical studies in humans for several reasons. For example, the gene expression profile in human sepsis is inconsistent with that in mice. Also, the recovery time, that is, the time required to restore normal gene expression levels, is as short as hours/days in mice and as short as months in humans. Also, many species, such as mice, are highly resistant to certain bacteria. Bacterial lipopolysaccharides (LPS) have toxic effects, but humans are extremely sensitive to LPS. In addition, the animals preclinical experiments are usually young and have no comorbidity. In contrast, patients with sepsis are often older and have underlying comorbidity, both of which are strong predictors of sepsis susceptibility and outcome. The Cecal Ligation Puncture Procedure (CLP) has been accepted as the gold standard of experimental sepsis model, which has contributed significantly to our understanding of many pathophysiological and immunological features of sepsis. At present, however, results from preclinical studies of new therapeutic agents for sepsis should be viewed with some degree of skepticism. Thus, data from experiments using animal models can never be used directly to solve the problem of sepsis in humans [39, 40].

Conclusion

There was a decrease in the number of bacterial colonies in the kidney tissues of the rats in the fosfomycin group alone and in the liver tissues of the rats in the colistin alone group. In addition, a decrease in the number of bacteria was observed in the spleen and liver tissues in the COL + TOB group. Bacterial colony numbers in all tissue samples in the FOS + COL group were found to be close to those in the control group. Therefore, COL + TOB combinations are effective against *P. aeruginosa* in experimental sepsis and clinical success may be achieved. New in vivo studies demonstrating the ability of *P. aeruginosa* to biofilm formation in tissues other than the lung are warranted in future.

Author contribution ZA: conceptualization, methodology, in vivo studies, reviewing, supervision and editing. NS: methodology, in vivo studies. LO: ınvestigation, writing, data curation, visualization reviewing, supervision and editing. OB: conceptualization, methodology, in vivo studies, reviewing, supervision and editing. HI: statistical analyses. OO: conceptualization, methodology, reviewing, supervision and editing.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval This study was carried out following the ethical values specified in the Declaration of Helsinki and was approved by the Istanbul University Animal Experiments Local Ethics Committee (Ethical Approval Number: 2013/129).

Conflict of interest The authors declare no competing interests.

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