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Original article

Adjunctive inhaled amikacin in infants with ventilator-associated pneumonia optimizes the complex antimicrobial therapy: pilot study

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Abstract. *Background and aim:* VAP remains the second leading cause of death among the patients with nosocomial infections and its incidence varies significantly from 5% to 60% reaching on average 10%. It is of crucial importance to develop novel treatment approaches and optimize the existing ones. Thus, the aim of this pilot study was to study the laboratory-microbiological effect of inhaled aminoglycosides in a complex treatment of patients with ventilator-associated pneumonia (VAP). *Methods*: To study the laboratory-microbiological effect of adjunctive inhaled aminoglycosides in the treatment of VAP, twenty enrolled patients were randomly subdivided into 2 groups (n=10). Amikacin was administered via a nebulizer starting from the first day of VAP manifestation. Inhalations were performed BID for 7 days via a nebulizer integrated into the breathing circuit. We assessed: cell membrane alterations in leukocytes, Annexin V/7-AAD staining for leukocytes, ROS detection assay for leukocytes. *Results:* Adjunctive administration of inhaled amikacin reduced the fluorescence intensity ratio more efficiently compared with the intravenous antimicrobial treatment with no aerosolized amikacin following both 48 h and 96 h of treatment. The amount of dead necrotic annexin V-negative, 7-AAD-positive leukocytes was significantly lower under the use of inhaled amikacin than at the beginning of treatment. *Conclusions* In this pilot study, we found that administration of aerosolized amikacin combined with the systemic antimicrobial therapy improves the clinical outcome of patients with VAP, effective early microbial decrease in the sputum, reduces reactive oxygen species generation in leukocytes and the degree of leukocyte apoptosis and necrosis, decreases VAP-mediated cell membrane alterations of circulating leukocytes.

Key words: aerosol therapy; antibiotics; amikacin; ventilator-associated pneumonia; apoptosis; oxidative stress

Introduction

Ventilator-associated pneumonia (VAP) is a nosocomial pneumonia that develops in patients after at least 48 h of mechanical ventilation (1,2). VAP remains the second leading cause of death among the patients with nosocomial infections and its incidence varies significantly from 5% to 60% reaching on average 10 %, while its total rate is over 13 per 1000 ventilator days (3,4,5). It has been reported that the list of VAP-associated microorganisms includes both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E.coli, P. aeruginosa*, and *K. pneumoniae*) (6). It is important to note that VAP is characterized by high

mortality rate in the intensive care units varying from 20% to over 50% (7), reaching on average 20% for early VAP and more than 30% for late VAP in European countries (8) and 13% in the United States (9). Due to some preventive measures, including minimization of mechanical ventilation exposure and promotion of early extubation, the mortality rate has been decreasing for years. However, appropriate antibiotic selection remains a huge challenge in the treatment of VAP. According to some estimates, approximately half of antibiotics administered to patients in the intensive care unit are prescribed for the therapy of VAP (10). Since the selection of appropriate antibiotic therapy remains a key determinant of the disease outcome and the fact that in up to 70% of cases the initial empirical therapy of VAP is ineffective (11), it is of crucial importance to develop novel treatment approaches and optimize the existing ones. One of such treatment strategies is to use nebulized antibiotics. There is accumulating evidence that inhaled antibiotics are effective as adjunctive therapeutic agents along with the systemic antibacterial treatment of patients with VAP associated with multidrug-resistant microorganisms (12,13,14). The major benefit of adjunctive inhaled antibiotics is to create high intra-pulmonary concentrations of antibiotics, which cannot be achieved via their systemic administration due to toxic effects (15) and poor penetration of intravenously administered antimicrobial agents, e.g. approximately 10-30% of aminoglycosides prescribed systemically can enter the pulmonary tissue leading to the insufficient local concentrations (16).

It has been demonstrated that nebulized antibiotic administration improves the clinical outcome and bacterial eradication, especially for resistant pathogens, without reducing the mortality risks (17,18).

The aim of this pilot study was to study the laboratory-microbiological effect of inhaled aminoglycosides in the complex treatment of patients with VAP as the preliminary study conducted before large-scale quantitative research in order to evaluate the potential for a future, full-scale project.

Materials and methods

Study design and patients

This pilot study involved infants hospitalized to the intensive care unit (ICU) of Vinnytsya Regional Childrens Clinical Hospital and lasted from February, 2019 to January, 2022. The department has 12 beds, 12 doctors and 32 nurses. The duty shift consists of 2 doctors and 3 nurses every day.

A total of 20 patients receiving mechanical ventilation for at least 48 hours were enrolled for the study. VAP was diagnosed based on the criteria of the US Centers for Disease Control and Prevention. According to these guidelines, diagnosis of VAP is confirmed by clinical manifestations, X-ray criteria, and microbiological investigation (19,20). A brief summary of patient selection is given in Figure 1.

The study was conducted in accordance with the Declaration of Helsinki and adhered to Good Clinical Practice guidelines and was approved of by the Ethics Committee of National Pirogov Memorial Medical University (Vinnytsya, Ukraine; minutes No 5 dated September 17, 2019). The research was carried out as part of a scientific research project named "The research of the biological properties of microorganisms classified by the World Health Organization to the list of "priority pathogens" that are the most threatening human health and the development of means of combating them" (No. 0117U006903). An informed written consent was obtained from patients' parents prior to participation in this study.

Inclusion criteria

The patients were enrolled for this research if they met the following inclusion criteria: clinically diagnosed VAP in children receiving mechanical ventilation for over 48 hours; clinical signs (acute onset; fever above 38.0 °C for 3 days or hypothermia below 36.0 °C; intoxication syndrome; sputum production during the endotracheal tube intubation; the 3rd degree of respiratory failure; auscultation revealed diminished respiration, crepitation or moist crackles; tachycardia or bradycardia; ultrasound showed hyperechogenicity of lung tissue or foci of infiltration); infiltrative focal or segmental changes in

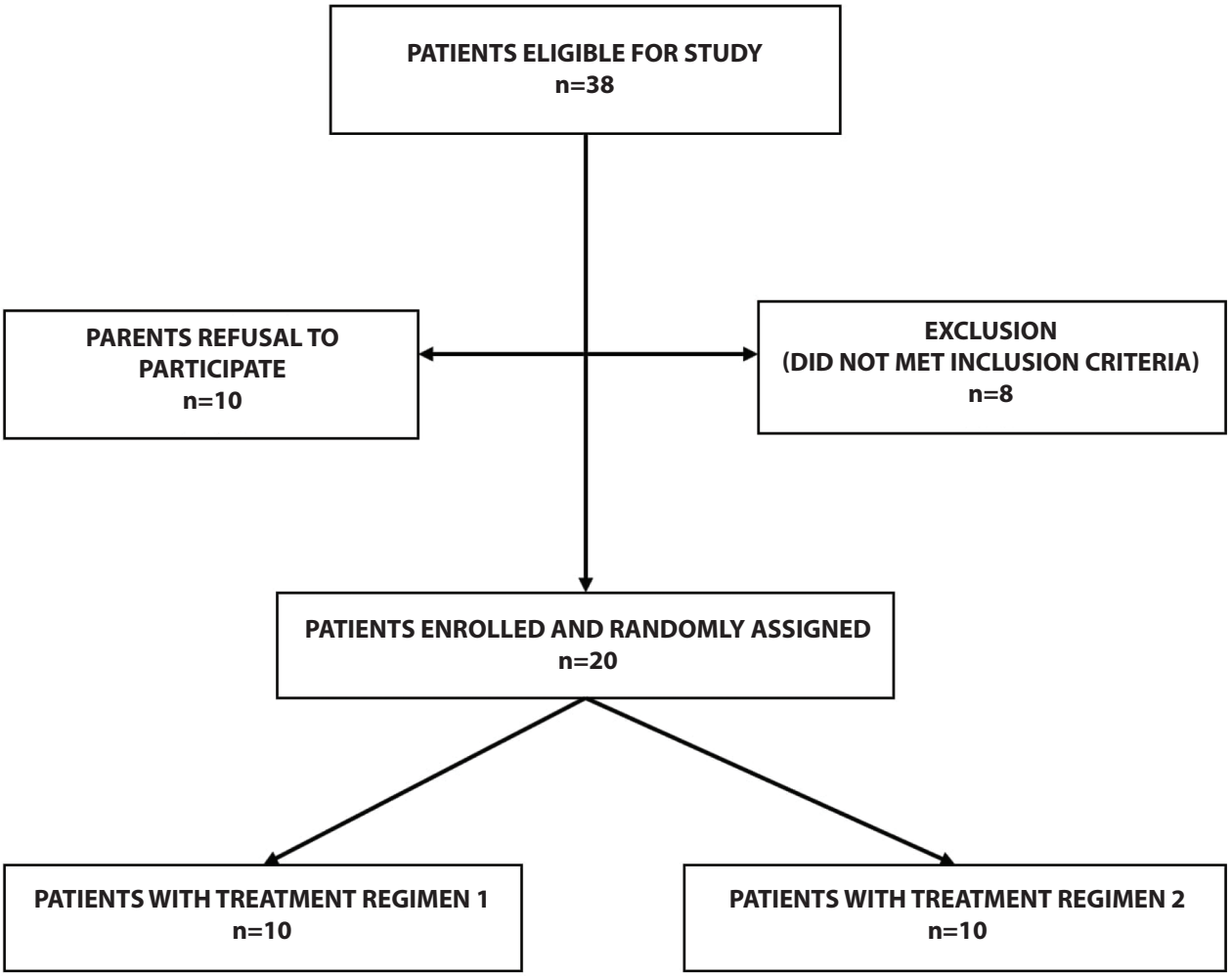

Figure 1. Flow-chart of patient selection.

the lungs were observed in chest radiography; laboratory tests demonstrated (leukocytosis with a shift of the formula to the left, or leukopenia, faster-than-normal ESR); adequate cooperation of a patient or his/her relatives with researchers; a written informed consent.

Among *exclusion criteria* there were: mechanical ventilation period less than 48 hours, the absence of clinical signs, ultrasound, X-ray and laboratory criteria exclusion of VAP, concomitant pathologies that are a contraindication to inhalation (cystic fibrosis and others).

Treatment regimens

Treatment of the patients from both groups included stabilization of hemodynamic parameters, including volemic, inotropic and vasopressor therapy, hemostasis, rheological blood parameters, acid-base and electrolyte balance, effective analgesia and sleep medication.

All patients received systemic de-escalating antibacterial therapy administered empirically at the beginning of treatment by means of intra venous (IV) administration. And the subsequent therapy correction in accordance with the results of antibiotic sensitivity tests was mandatory. Empirical antibacterial therapy corresponded to the generally accepted approaches to VAP treatment and followed the guidelines. The starting choice of an antibiotic was also based on the microbiological passport of the department. It represented the distribution of microorganisms obtained

from the nose, mouth, intubation tube and patients wounds. It was conducted every quarter. The choice of empiric antibiotics was based on the data of local microbiological monitoring of susceptibility of predominating pathogens in our ICU department. Treatment scheme is available in Table 1.

Among non pharmacological measures to prevent VAP in our ICU department, we use the following: avoiding intubation or re-intubation whenever possible; head of the bed elevation; hand hygiene; spontaneous breathing trials, and thromboembolic prophylaxis.

The patients with treatment regimen 1 (control group) were administered conventional antibacterial therapy received only systemic intravenous antibiotic regimen that included combination of cefoperazone-sulbactam (which was changed into meropenem 3 days later) and clindamycin (4 patients), aztreonam and clindamycin (3 patients), meropenem and vancomycin (3 patients).

Table 1. The list of antibiotics, recommended for the choice of empirical antimicrobial treatment of patients with VAP (received by means of intra-venous (IV) administration) in ICU of newborns in the clinic in accordance to the data of local microbiological passport, which patients.

Drug	**Administration**
Vancomycin	15 mg / kg IV every 8–12 hours
Linezolid	10 mg/ kg IV every 12 hours
Piperacillin with tazobactam	100 mg/ kg IV every 8 hours
Cefepime	50 mg / kg IV every 12 hours
Ceftazidime	50 mg / kg IV every 12 hours
Imipenem with cilastatin	10 mg / kg IV every 8 hours
Meropenem	10 mg / kg IV every 12 hours
Aztreonam	10 mg / kg IV every 8 hours
Ciprofloxacin	10 mg / kg IV every 12 hours
Levofloxacin	10 mg / kg IV every 24 hours
Amikacin	10 mg / kg IV every 12 hours
Gentamicin	5 mg / kg IV every 24 hours
Tobramycin	4 mg / kg IV every 24 hours
Colistin	25 thousands IU / kg IV every 8 hours
Polymyxin B	0.4 mg / kg IV every 12 hours

The patients with treatment regimen 2 (study group) received adjunctive nebulized amikacin (500 mg, twice a day) in addition to the empirically prescribed standard antibiotic therapy-intravenous meropenem (5 patients), cefoperazone-sulbactam (3 patients), aztreonam (2 patients). Amikacin was administered via a nebulizer starting from the first day of VAP manifestation. Inhalations were performed BID for 7 days via a nebulizer integrated into the breathing circuit.

The treatment effectiveness was assessed by evaluating clinical indicators, ventilation parameters, microbiological studies, eryptosis indices, ROS generation in leukocytes, analysis of leukocyte viability/apoptosis/necrosis and degree of leukocyte membrane alterations. In particular, the treatment effectiveness was determined by common clinical parameters (body temperature, breathing efficiency), laboratory indices (blood oxygen saturation, leukocyte count), instrumental methods (lung X-ray), and changes in respiratory parameters, including dynamic compliance (C dyn), positive end-expiratory pressure (PEEP), peak inspiratory pressure (PIP). The research was performed at the following stages: the beginning of artificial lung ventilation, after 48 h from the beginning of artificial lung ventilation, and after 96 h of artificial lung ventilation.

Nebulized amikacin administration in the patients with VAP

Inhaled amikacin was administered in a combination with conventional antimicrobial treatment, provided that the patients had no contraindications. The tracheobronchial tree was cleaned prior to antibiotic inhalation. In case of high bronchospasm risks, bronchodilators were administered via a nebulizer before and after inhalation. Ventilation parameters were corrected prior to and immediately after inhaled amikacin administration (inspiratory pressure elevation to 30-35 cmH_2O), since prolongation of the inspiratory phase promoted better intra-pulmonary inhaled amikacin distribution.

A sterile nebulizer integrated into the respiratory circuit was pre-filled with amikacin (500 mg). During inhalation, amikacin solution was not mixed with any other drug. Inhalation was performed until the solution of amikacin in the nebulizer ran out. Two inhalations were carried out per day (500 mg of amikacin).

After the procedure, the nebulizer was removed from the respiratory circuit and disinfected in accordance with the manufacturer's recommendations to avoid its colonization with nosocomial microflora.

Microbiology testing

From patients with diagnosed VAP we received sputum on the 1st, 3rd and 5th day, which was collected from ventilated patients with the sterile catheter for cleansing of endotracheal tube during this the procedure. Sputum has been examined at certified bacteriological laboratory of Department of microbiology at National Pirogov Memorial Medical University, Vinnytsya, Vinnytsia, Ukraine. There was performed final identification of isolated microorganisms by standard methods with the determination of the species of pathogens and their general quantitative degree in the sputum (by the number of microorganisms in 1 ml of biological material, expressed in log CFU/ml).

Blood collection and preparation of cell suspensions

Blood was collected from patients with proved VAP, who had being received mechanical ventilation for 72 hours and longer. Specimens were received for the first time before the beginning of amikacin inhalation (main observation group) or correction of systemic antibiotic management of VAP according to the conventional protocols (comparison group), following 48 h and 96 h after the beginning of antimicrobial management of VAP in both groups of patients, respectively. For blood samples we used plastic tubes containing EDTA di-potassium salt (IMPROVACUTER, Guangzhou, China).

The freshly collected samples were used to obtain erythrocyte and leukocyte suspensions. To obtain erythrocyte suspensions, blood samples were washed twice with phosphate-buffered saline (PBS, pH 7.4) purchased from Becton Dickinson (USA). Thereafter, 2 μL of red blood cell mass was dissolved in 100 μL BD Pharmingen™ Annexin V Binding Buffer (BD Biosciences, USA) for determination of phosphatidylserine externalization or PBS for ROS detection.

To obtain leukocyte suspensions, blood aliquots of 100 μL were incubated for 15 min with 2 ml BD Pharm Lyse™ lysing solution purchased for Becton Dickinson (USA). After incubation, the suspensions were washed twice with PBS and resuspended in 100 μL annexin-binding buffer for Annexin V/7-aminoactinomycin D (7AAD) staining or PBS for H2DCFDA staining.

Fluorescent probe O1O used to assess the cell membrane alterations in leukocytes

The cells were stained with the fluorescent probe via addition of an aliquot of the probe stock solution in acetonitrile to the cell suspensions: a final probe concentration was ~5.10^{-6} mol/L and lipid-to-probe molar ratio was ~200:1. The cell suspensions were incubated with the probe at room temperature for 1 hour before fluorescence measurements. Fluorescence spectrometer "PerkinElmer FL8500" was used for the measurements of the probe emission in the range of 340-550 nm, with an increment of 0.1 nm. The other fluorescence acquisition parameters: the emission scan speed was 240 nm/min, the excitation wavelength was 330 nm, the excitation and emission slits were 5 nm.

Fluorescent probe O1O (2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole) was used in our research, because its fluorescence characteristics depend on the proton-donor ability and polarity of the probe environment (21,22), and, hence, depend upon the hydration of the microenvironment (23). Since the changes in membrane hydration are connected with the changes of the membrane lipid order (24), the probe can used to detect the lipid order.

The area of glycerol backbones of phospholipids closer to the center of the lipid bilayer, the area of carbonyl groups of phospholipids and the area of hydrocarbon chains of phospholipids near the area of the carbonyl groups of phospholipids are the regions of the probe O1O locates in lipid membrane (22). The location of the probe in phospholipid bilayers is shown in Figure 2.

In the excited elecronic state, the initial (or so-called "normal") form (N*) of probe O1O turns into phototautomer form (T*). The latter emits in significantly longer wavelengths than the initial form of the probe. The amount of the photoproduct (T*) depends on the probe microenvironment (22).

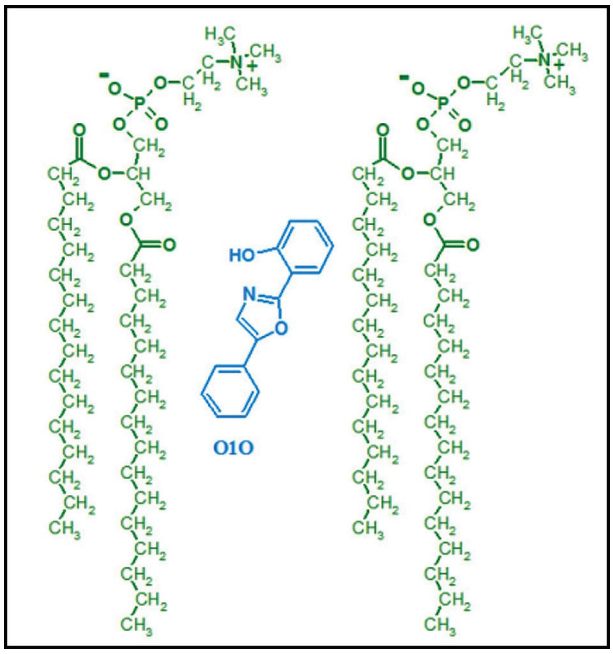

Figure 2. Localization and orientation of fluorescent probe O1O in the outer leaflet of the phospholipid membranes.

Because the probe has two-band fluorescence, the ratiometric measurements are possible: the phototautomer fluorescence intensity-to-the initial form fluorescence intensity ratio (I_{T^*}/I_{N^*}) can be used as a parameter to estimate the changes in chemical and physical properties of the microenvironment (e.g., with the increase in hydration of the media, the ratio I_{T^*}/I_{N^*} decreases (22)).

Annexin V/7-AAD staining for leukocytes

Leukocytes suspended in annexin-binding buffer were stained with 10 µl APC-Cy™7-labelled Mouse Anti-human antibodies to CD45 (BD Pharmingen™, USA), 5 µl FITC-labeled Annexin V and 10 µL 7-aminoactinomycin D (7-AAD, BD Pharmingen™, BD Biosciences, USA) and incubated for 15 minutes in the dark. Before the flow cytometric measurements, 400 µl annexin-binding buffer was added (25).

ROS detection assay for leukocytes

The leukocyte suspensions prepared from freshly collected blood of the patients with VAT were incubated with PBS containing 10 µM H2DCFDA (Invitrogen™, USA) (26). The H2DCFDA working solution was prepared from its 10 mM stock solution in DMSO. Furthermore, 10 µL APC-Cy™7-labelled Mouse Anti-human antibodies to CD45 and 10 µL 7-aminoactinomycin D were added. The former is a superficially located pan-leukocyte marker, while the latter is a vital dye used to distinguish between viable and dead cells (27). The cells were incubated for 30 min under protection from light. Prior to fluorescence measurements, 400 µL PBS was added.

Data acquisition and post-acquisition analysis

Flow cytometry data were acquired by a BD FACSCanto™ II Cell Analyzer (BD Biosciences, USA). Fluorescence of Annexin V-FITC and H2DCFDA-derived DCF was detected by excitation at 488 nm and emission at 525 nm. To detect the fluorescence of 7-AAD and APC-Cy™ 7-labelled antibodies to CD45, excitation was at 488 and 633 nm, while emission was at 670 and 780 nm, respectively.

To analyze flow cytometry data, BD FACSDiva™ software (Becton Dickinson, USA) and FlowJo™ (v10, BD Biosciences, USA) software packages were used. The gating strategy is shown in Figure 3.

Statistical analysis

Two independent groups of variables were compared by performing the U Mann-Whitney test using GraphPad Prism 5 (GraphPad Software Inc., La Jolly, CA, USA) software. If more than two variables were compared, the Kruskal-Wallis and Dunn's tests were used. Numerical values in Tables are presented as Me [25th; 75th percentile]. The difference between variables was considered statistically significant if p values did not exceed 0.05.

Results

The age of patients varied from 1 to 12 months with the mean age of 5 ± 2.3 months. According to the gender distribution, 55 % of patients enrolled for the study were males, while females accounted for 45 %.

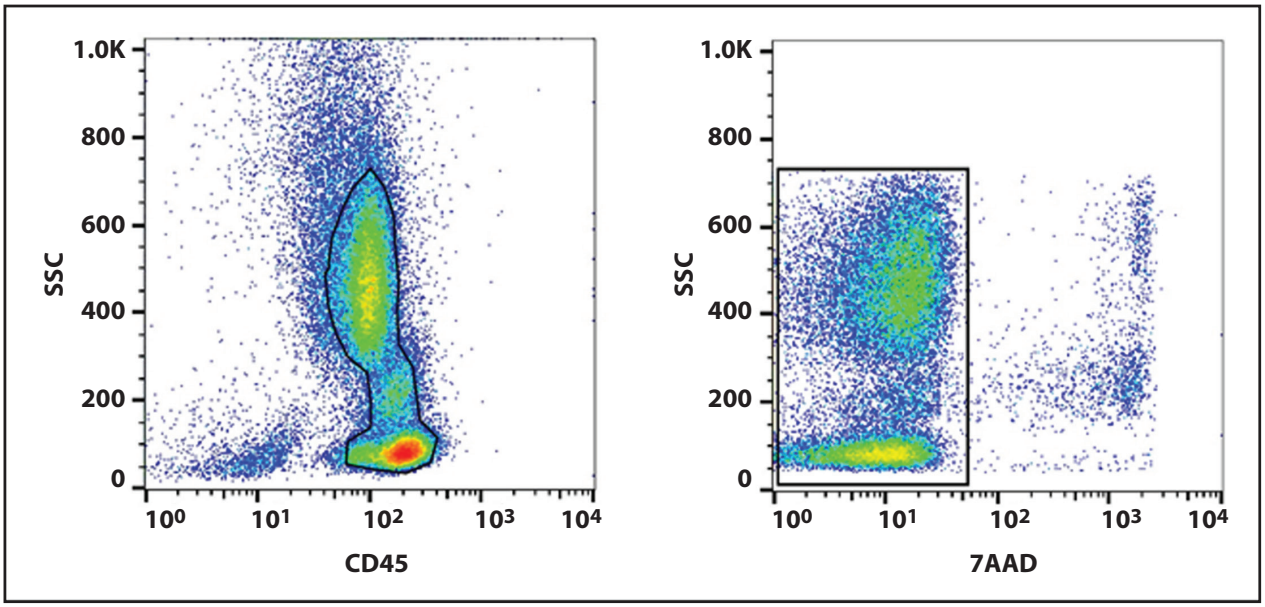

Figure 3. The subsequent double gating to identify the subpopulations of CD45-positive cells (left panel) and viable 7-aminoactinomycin D-negative CD45-positive cells (right panel).

No statistically significant differences in age/sex distribution between groups were observed.

Since VAP had been diagnosed, all patients in the main study group received inhalation of amikacin in combination with intravenous meropenem (5 patients), cefoperazone-sulbactam (3 patients), aztreonam (2 patients). In control group patients received only systemic intravenous antibiotic regimen that included combination of cefoperazone-sulbactam (which was changed into meropenem 3 days later) and clindamycin (4 patients), aztreonam and clindamycin (3 patients), meropenem and vankomycin (3 patients).

Clinical manifestations

A study of lung function in the patients with mechanical ventilation showed that no changes in C_{dyn} values were observed in study. Meanwhile, a gradual decrease in Cdyn values was observed in the control group. A 2-fold increase in resistance and a significant increase in PIP values (22–23 cm H_2O) were found in the control group. In this group, the maximum changes on the graph screen were observed, accompanied by a prolongation of the purulent-inflammatory process in the lungs, compared to the patients administered nebulized amikacin in the early period of VAP (Table 2).

The analysis of hemodynamic parameters showed that the heart rate (HR) increased by 3% on the 3rd day with probability ($p<0.05$) and the frequency returned to the previous value only on the fifth day. The mean arterial pressure (MAP) decreased by 12% on the 3rd day, and by 9% on the fifth day with probability ($p<0.05$) and returned to the previous value only on the 7th day in the control group. In the study group HR and MAP practically did not change (Table 3).

By comparing the data, we found that the average duration of mechanical ventilation was ± 7 days in the control group and ± 6 days in study group, which was 15% less than in the control group with probability ($p<0.05$). The duration of stay in the ICU department for 1 day was lower in the study group (Table 4).

Microbiological testing of the sputum samples, received from endotracheal tubes on the 1st day of the study, demonstrated no significant difference in the values of the general quantity of microorganisms in the main (log (7.04±0.22) CFU/ml) and control (log (7.02 ± 0.24) CFU/ml) groups ($p>0.05$). The most frequently found microorganisms were strains of

Table 2. The characteristics of lung function in the patients with VAP during mechanical ventilation and different antimicrobial regimes.

Indicator	The 1st day	The 3rd day	The 5th day	The 7th day
The patients with treatment regimen 1, n = 10				
C $_{dyn}$ (ml/cm H_2O)	1,81 ± 0,2	0,52 ± 0,1*	1,24 ± 0,1*	2,01 ± 0,2
PIP (cm H_2O)	14,4 ± 1,8	19,6 ± 2,4*	17,6 ± 1,9	14,0 ± 2,0
PEEP (cm H_2O)	5,0 ± 0,06	7,0 ± 0,04*	5,0 ± 0,08*	5,0 ± 0,08
FiO_2 (%)	40 ± 1	45 ± 1	40 ± 1	30 ± 1
Raw смH_2O/L/s	1,2± 0,2	2,4± 0,3	1,5± 0,2	1,1± 0,2
The patients with treatment regimen 2, n = 10				
C $_{dyn}$ (ml/cm H_2O)	2,02 ± 0,1	2,03 ± 0,2	2,02 ± 0,3	2,02 ± 0,2
PIP (cm H_2O)	14,9 ± 2,0	14,2 ± 1,8	15,4 ± 2,3	14,0 ± 2,2
PEEP (cm H_2O)	5,1 ± 0,04	5,2 ± 0,04	5,6 ± 0,04	5,2 ± 0,08
FiO_2 (%)	30 ± 1	40 ± 1	25 ± 1	20 ± 1
Raw смH_2O/L/s	1,2± 0,3	1,5± 0,3	1,4± 0,2	1,0± 0,2

Note: * p<0.05 when comparing patients of two groups.

Table 3. Hemodynamic changes in patients with VAP during amikacin inhalation and standard systemic antimicrobial treatment.

Indicators	Beginning of VAP	The 3rd day	The 5th day	The 7th day
The patients with treatment regimen 1, n =10				
Heart rate (beats/min)	145 ± 5*	150 ± 5*	140 ± 5*	130 ± 5
MAP (mmHg)	45 ± 6*	40 ± 6*	42 ± 7	45 ± 5
SpO_2 (%)	93 ± 5*	92 ± 5*	93 ± 5*	94 ± 5
The patients with treatment regimen 2, n =10				
Heart rate (beats/min)	145 ± 5*	140 ± 5*	135 ± 5*	125 ± 5
MAP (mmHg)	45 ± 6*	44 ± 6*	46 ± 5	46 ± 5
SpO_2 (%)	94 ± 5*	93 ± 5*	94 ± 5*	95 ± 5

Note: * p<0.05 when comparing patients of two groups.

Klebsiella pneumoniae (28%), *Pseudomonas aeruginosa* (18%), *Acinetobacter baumannii* (18%) and *Staphylococcus aureus* (18%) (Table 5).

In the main group we found the decrease of microbial load in the sputum form the endotracheal tubes on the 3rd day of antimicrobial therapy (log (3.59±0.32) CFU/ml), that was significantly lower than in control one ((log (5.49±0.27) CFU/ml) (p<0.001). The same tendency was proved on the 5th day of antibiotic management of patients with VAP. Though, microbial quantity in the sputum was much lower than on the 3rd day and also the difference was found between both groups. In the main group the sputum was contaminated in 70% of patients but the number of microorganisms was lower 10^4 ((log (1.25±0.39) CFU/ml). And in the control group its rate was about (log (2.64±0.43) CFU/ml). Received data proved the significant effectiveness of local inhalation administration of aminoglycoside antibiotic in situ of the infectious process (p<0.05).

Characteristics of leukocyte cell membranes

The results of fluorescence measurements of the spectra of probe O1O bound to the leukocytes of the patients are presented in Figure 4.

For better comparison the presented spectra were normalized to the intensity of the initial form of the probe. The samples were prepared from blood collected directly prior the beginning of treatment (panel a), after 48 h (panel b) and 96 h (panel c). In the two-band fluorescence spectra of the probe, the low-intensity short-wavelength fluorescence band (max. ~ 370 nm) is the band of to the normal form of the probe, while the highly intensive long-wavelength band (max. ~ 477 nm) belongs to the fluorescence of the phototautomer form (Figure 4).

The time-dependent changes in the fluorescence spectra of the probe were detected in case of treatment regimen two only. The changes in the spectra were reflected by a statistically valid decrease in the fluorescence intensity ratios I_{T^*}/I_{N^*} after 96 h (Table 6).

Moreover, adjunctive administration of inhaled amikacin reduced the fluorescence intensity ratio more efficiently compared with the intravenous antimicrobial treatment with no aerosolized amikacin following both 48 h and 96 h of treatment (Table 6).

ROS generation in leukocytes

The highest ROS generation in circulating leukocytes obtained from the patients with VAP was

Table 4. Peculiarities of patients, being ventilated in both groups (colomn 1 – the patients with treatment regimen 1; column 2 – The patients with treatment regimen 2).

Indicators	The patients with treatment regimen 1, n =10	The patients with treatment regimen 2, n =10
Duration of mechanical ventilation (days)	7	6
Intubation time (sec)	12	11
Reintubation	13	12
Duration of stay in ICU (days)	8	7

Table 5. The qualitative microbiological characteristics of the sputum of patients with VAP, receiving different antimicrobial regimes.

The patients with treatment regimen 1, n = 10						
	The 1st day (n=10*)		**The 3rd day (n=11*)**		**The 5th day (n=4*)**	
Microrganisms	**Number**	**Percent**	**Number**	**Percent**	**Number**	**Percent**
Klebsiella pneumoniae	3	28%	4	30%	1	20%
Pseudomonas aeruginosa	2	18%	3	23%	1	20%
Staphylococcus aureus	2	18%	2	15%	1	10%
Acinetobacter baumannii	2	18%	2	15%	1	20%
Other	2	18%	2	15%	1	30%
The patients with treatment regimen 2, n = 10						
	The 1st day (n=11*)		**The 3rd day (n=13*)**		**The 5th day (n=5*)**	
Microrganisms	**Number**	**Percent**	**Number**	**Percent**	**Number**	**Percent**
Klebsiella pneumoniae	3	30%	3	28%	1	20%
Pseudomonas aeruginosa	2	20%	2	18%	1	20%
Staphylococcus aureus	2	20%	2	18%	1	20%
Acinetobacter baumannii	2	20%	2	18%	1	20%
Other	1	10%	2	18%	0	0%

*- General No. of isolated microbes from specimens.

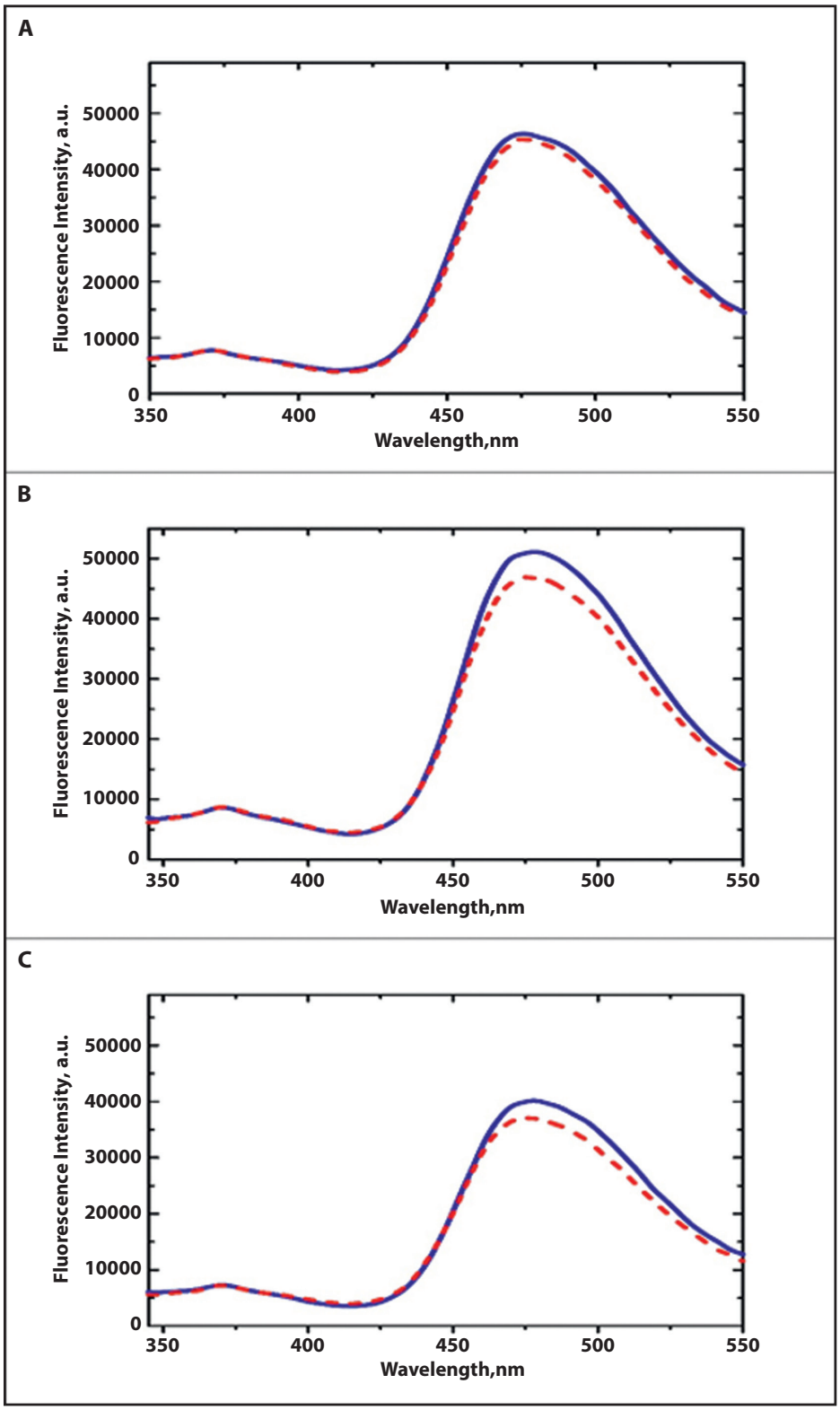

Figure 4. Representative fluorescence spectra of probe O1O in leukocyte suspensions of patients with ventilator-associated pneumonia at the beginning (panel a), after 48 h (panel b), and 96 h (panel c) of treatment duration: conventional systemic antimicrobial treatment (blue solid line) and systemic antibacterial treatment in combination with nebulized amikacin (red dashed line).

Table 6. Fluorescence of probe O1O in leukocyte suspensions and ROS generation degree in circulating leukocytes obtained from the patients with VAP treated using different treatment regimens.

	Fluorescence intensity ratio (I_{T^*}/I_{N^*}) of probe O1O			**Mean fluorescence intensity of dichlorofluorescein, a.u.**		
Index	**0 h**	**48 h**	**96 h**	**0 h**	**48 h**	**96 h**
VAP, treatment regimen 1, (n=10)	5.98 [5.72; 6.25]	5.83 [5.56; 6.01], $p_2 > 0.05$	5.47 [5.31; 5.59], $p_2 > 0.05$	8382 [7449; 10944]	6858 [4656; 7988], $p_2 > 0.05$	3456 [2518; 3865], $p_2 < 0.0001$
VAP, treatment regimen 2, (n=10)	5.79 [5.61; 6.03], $p_1 > 0.05$	5.31 [5.08; 5.69], $p_1 = 0.0191$; $p_2 > 0.05$	4.96 [4.64; 5.14], $p_1 = 0.0022$; $p_2 < 0.0001$	8359 [7716; 10978], $p_1 > 0.05$	4218 [3932; 4695], $p_1 = 0.0003$; $p_2 < 0.0001$	2303 [2089; 2901], $p_1 = 0.0113$; $p_2 < 0.0001$

Note: p_1 indicates the difference between the groups of patients treated with various therapy regimens, while p_2 shows the difference between the groups of patients treated in the same way after 0 h, 48 h and 96 h of treatment duration.

observed at the beginning of treatment (Table 6). After 48 h of therapy, a statistically significant reduction of intracellular levels was found in the patients with treatment regimen 2 compared with the beginning of therapy. After 96 h, both treatment regimens statistically significantly reduced MFI values of DCF suggesting a decrease in ROS production. Following 48 h and 96 h treatment, nebulized amikacin was more efficient in fighting oxidative stress in circulating leukocytes compared with the systemic antibacterial therapy (Figure 5).

Viability/apoptosis/necrosis of circulating leukocytes

Numerical data on annexin V/7-AAD combined staining are available in Table 7.

Viability of circulating leukocytes was revealed to be significantly higher after 96 h of treatment compared to the initial values under both treatment regimens. Moreover, when treatment regimen 2 was administered, the amount of viable annexin V-negative, 7-AAD-negative cells was significantly higher even after 48 h (Figure 6).

In addition, treatment regimen 2 increased cell viability more noticeably when compared to treatment regimen 1. Furthermore, patients with VAP treated with adjunctive nebulized amikacin had a significantly lower number of early apoptotic annexin V-negative, 7-AAD-negative leukocytes in comparison with the initial values. The comparison of effectiveness of therapy regimens revealed that treatment regimen 2 significantly reduced the amount of early apoptotic leukocytes compared to regimen 1 after 48 h. Both regimens had no impact on the percentage of late apoptotic / necrotic annexin V-positive, 7-AAD-postive leukocytes during the entire treatment duration. However, the amount of dead necrotic annexin V-negative, 7-AAD-positive leukocytes was significantly lower under the use of inhaled amikacin than at the beginning of treatment. Nevertheless, this parameter was not dependent on the treatment regimen.

Discussion

In this pilot study, the laboratory-microbiological effect of adjunctive nebulized amikacin in patients with VAP is analyzed. Our clinical data corroborate other studies, which support the idea that aerosolized antibiotics improve the clinical cure results (7, 28).

The risk of the development VAP is higher in children because of anatomical-physiological features, and ventilation without a cuff. In addition, the difference is explained by the features of the microflora of the upper respiratory tract-gram-negative bacteria dominate in adults and gram-positive bacteria in children, but mainly it depends on the local infectious spectrum of the ICU department.

The effectiveness of inhaled amikacin in this study is judged by its ability to reduce the degree of apoptosis and necrosis of circulating leukocytes. Accelerated and delayed apoptosis or necrosis can contribute to dysregulation of inflammation in pneumonia. In particular,

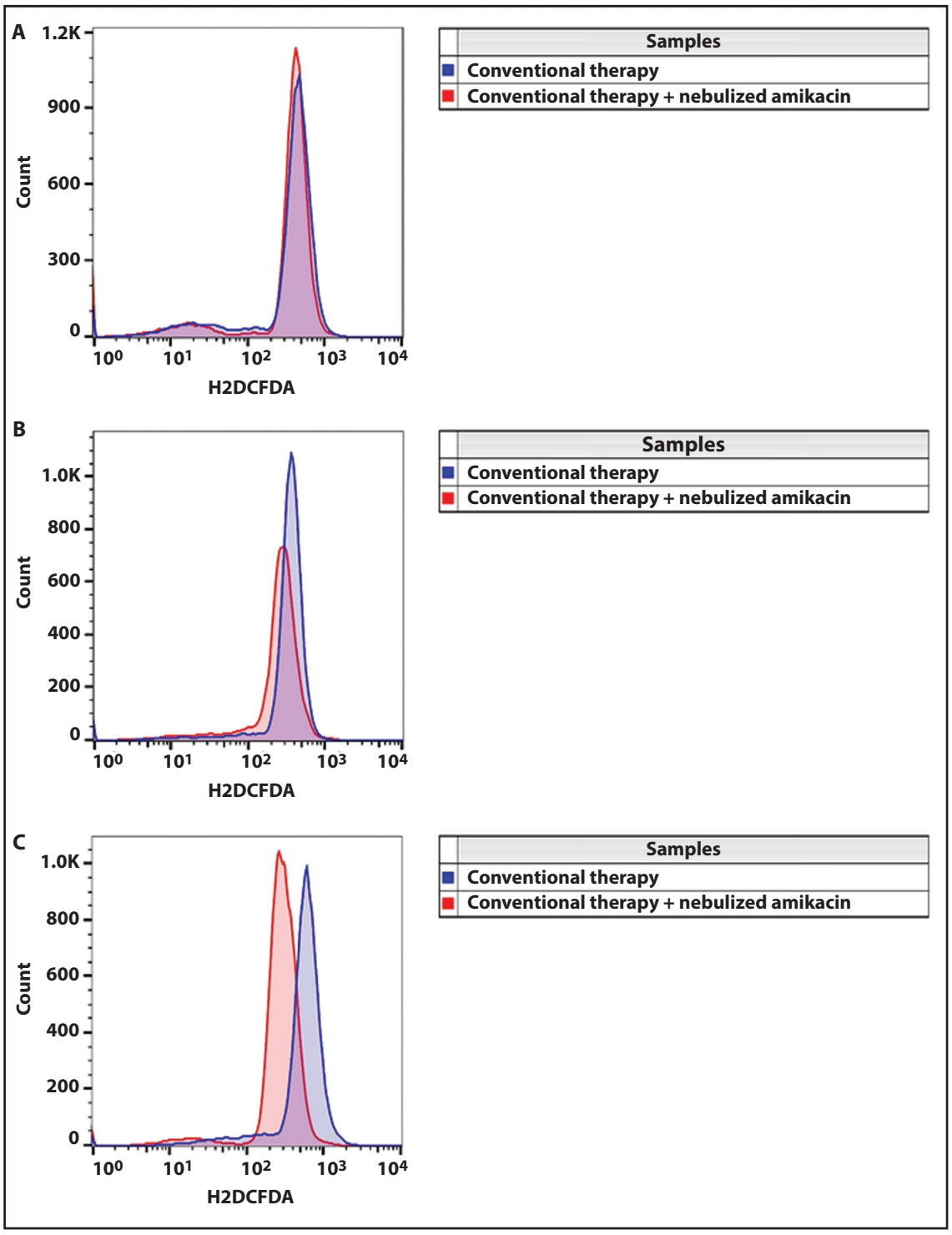

Figure 5. Representative histograms of dichlorofluorescein fluorescence in viable leukocytes obtained from the patients with ventilator-associated pneumonia treated with conventional systemic antibiotics and systemic antibacterial agents in combination with adjunctive inhaled amikacin. The samples were prepared from blood collected directly prior the beginning of treatment (panel a), after 48 h (panel b) and 96 h (panel c).

Table 7. Analysis of viability/apoptosis/necrosis of circulating leukocytes obtained from the patients with VAP treated using different treatment regimens.

Index Group of patients	Annexin-negative, 7-AAD-negative cells, %	Annexin-positive, 7-AAD-negative cells, %	Annexin-positive, 7-AAD-positive cells, %	Annexin-negative, 7-AAD-positive cells, %
VAT, treatment regimen 1, 0 h (n=10)	77.1 [71.0; 83.1]	9.1 [6.9; 12.6]	5.3 [1.3; 8.00]	5.7 [2.7; 16.2]
VAT, treatment regimen 2, 0 h (n=10)	79.3 [72.3; 83.5], $p_1 > 0.05$	8.8 [6.0; 12.7], $p_1 > 0.05$	5.8 [3.0; 8.6], $p_1 > 0.05$	5.4 [4.5; 8.6], $p_1 > 0.05$
VAT, treatment regimen 1, 48 h (n=10)	83.8 [81.0; 85.5], $p_2 > 0.05$	10.4 [9.6; 12.0], $p_2 > 0.05$	2.0 [0.9; 3.5], $p_2 > 0.05$	2.9 [1.9; 5.5], $p_2 > 0.05$
VAT, treatment regimen 2, 48 h (n=10)	88.8 [85.7; 90.2], $p_1 = 0.0050$; $p_2 < 0.0001$	5.2 [3.7; 7.3], $p_1 = 0.0010$; $p_2 > 0.05$	2.1 [1.7; 2.6], $p_1 > 0.05$; $p_2 > 0.05$	4.8 [3.2; 5.1], $p_1 > 0.05$; $p_2 > 0.05$
VAT, treatment regimen 1, 96 h (n=10)	87.6 [85.5; 91.1], $p_2 < 0.0001$	5.3 [3.9; 7.8], $p_2 > 0.05$	2.5 [1.4; 3.6], $p_2 > 0.05$	2.7 [1.8; 5.3], $p_2 > 0.05$
VAT, treatment regimen 2, 96 h (n=10)	91.3 [89.7; 92.3], $p_1 > 0.05$ $p_2 < 0.0001$	3.8 [2.5; 4.8], $p_1 > 0.05$; $p_2 < 0.0001$	2.0 [1.5; 3.8], $p_1 > 0.05$; $p_2 > 0.05$	1.9 [1.4; 2.8], $p_1 > 0.05$; $p_2 < 0.0001$

Note: p_1 indicates the difference between the groups of patients treated with various therapy regimens, while p_2 shows the difference between the groups of patients treated in the same way after 0 h, 48 h and 96 h of treatment duration.

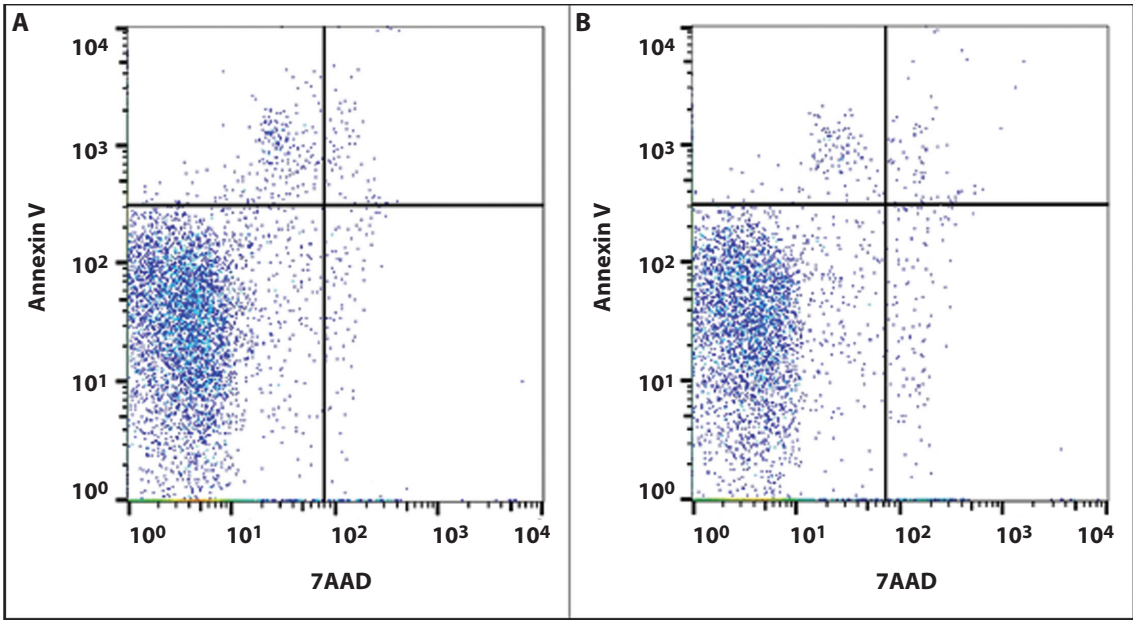

Figure 6. Representative annexin V / 7-aminoactinomycin D dotplots of CD45-expressing cells obtained from the blood of patients with ventilator-associated pneumonia following 48 h with conventional systemic antibiotics (panel a) and systemic antibacterial agents + adjunctive inhaled amikacin (panel b).

inducing apoptosis of host cells microorganisms can facilitate infection of other cells, since apoptotic cells are engulfed by macrophages (29). Necrosis is a strongly pro-inflammatory cell death mode, since membrane rupture in necrosis promotes the release of damage-associated molecular patterns (DAMPs) acting as ligands for pattern recognition receptors and activating the innate immune pathways (30,31). Thus, there is accumulating evidence that apoptosis markers of circulating leukocytes can be used in monitoring of inflammatory diseases (32). Our observations indicate that the administration of nebulized amikacin combined with the systemic antimicrobial agents in VAP increases the leukocyte viability, reduces apoptosis and less considerably diminishes leukocyte necrosis compared with the conventional therapy. These data supplement our findings on reduction of eryptosis degree and confirm a higher effectiveness of combined systemic and inhaled use of antibiotics. Both apoptosis and necrosis can be induced by excessive ROS (33, 34). ROS overgeneration promotes oxidative stress, which is typical for inflammatory diseases, including VAP (35). In this research, both treatment regimens effectively reduce oxidative stress in circulating leukocytes in a time-dependent manner. However, adjunctive inhaled amikacin accelerates the reduction of oxidative stress, which confirms its effectiveness.

Oxidative stress in cells is known to promote cell membrane alterations, including an increase in microviscosity and a decrease in fluidity, due to the preferential oxidation of polyunsaturated fatty acids by ROS and free radicals (36, 37). The I_{T^*}/I_{N^*} ratios of fluorescent probe O1O in leukocytes at the beginning of treatment is higher than usually observed in intact leukocytes (38, 39). The mentioned decrease in I_{T^*}/I_{N^*} ratios is indicative of the increase in the membrane hydration (21) in the area of the probe location, and, thus, points to the decrease in the membrane lipid order (22) of the rather polar region of the lipid membrane, i.e. an increase in microviscosity and reduction of fluidity. In contrast to the conventional therapy, adjunctive nebulized amikacin reduces VAP-associated leukocyte cell membrane alterations. These changes in membrane lipid order alter functions of leukocytes, including their motility, chemotactic capacities, and extravasation (40,41). Thus, our findings on the effects of inhaled amikacin suggest that it may improve the functional capacities of leukocytes in the patients with VAP.

Given together our data indicate that administration of adjunctive inhaled amikacin is an effective approach in the treatment of VAP. It provides clinical improvements, reduces the degree of eryptosis, diminishes oxidative stress in circulating erythrocytes and leukocytes, increases the viability of circulating leukocytes, decreases the intensity of their apoptosis and necrosis, and reduces VAP-mediated cell membrane alterations of circulating leukocytes. Our study supplements other researches in which the effectiveness of inhaled amikacin is postulated (42, 43). Thus, the efficacy of aerosolized antibiotics used in combination with intravenous antibiotic administration for the prevention and treatment of VAP is confirmed in several studies. However, it has not yet clear whether this therapy should be routinely prescribed to all patients infected with multidrug-resistant bacteria, or only to patients who do not respond to conventional systemic antibacterial treatment.

Nevertheless, some studies have demonstrated that inhaled antibiotics are ineffective in VAP (15, 44), which contradicts our findings. This inconsistency may be explained by different outcome criteria used in the studies. However, we believe that adjunctive inhaled antimicrobial therapy is a promising strategy to treat VAP whose efficiency should be confirmed in multicentered, large-sample-size clinical trials.

This study has some limitations, including small sample size and lack of control samples. In addition, the study is non-randomized and monocentric. Also, this study may have been related to trial design and execution. The data learned from these studies need to be incorporated in any future trials. So currently, routine use of adjunctive aerosolized therapy cannot be supported by available data, and this therapy is only recommended to assist in the eradication of highly resistant pathogens and to be used as salvage therapy for VAP patients failing systemic therapy.

Conclusions

In this pilot study, we found that administration of adjunctive aerosolized amikacin combined

with the systemic antimicrobial therapy improves the laboratory-microbiological indicators in patients with VAP, effective early microbial decrease in the sputum, reduces reactive oxygen species generation in leukocytes and erythrocytes and the degree of leukocyte apoptosis and necrosis, and decreases VAP-mediated cell membrane alterations of circulating leukocytes. Aerosolized amikacin adjunctive to intravenous conventional therapy is a promising strategy for VAP therapy.

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