Iranian Journal of Microbiology
Volume 13 Number 6 (December 2021) 824-831

ORIGINAL ARTICLE

Isolation and immunogenicity of extracted outer membrane vesicles from *Pseudomonas aeruginosa* under antibiotics treatment conditions

Mehdi Hadadi-Fishani[1], Shahin Najar-Peerayeh[1], Seyed Davar Siadat[2], Mohammad Sekhavati[3], Ashraf Mohabati Mobarez[1*]

[1]Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[2]Department of Mycobacteriology and Pulmonary Research, Pasteur Institute, Tehran, Iran
[3]Department of Aerobic Bacterial Research and Vaccine Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Received: January 2021**, Accepted:** August 2021

ABSTRACT

Background and Objectives: Different types of antibiotics have been indicated to enhance the secretion of OMVs from *Pseudomonas aeruginosa*. We aimed to investigate the effect of meropenem and amikacin antibiotics on inducing the secretion of OMVs and immunologic features in *P. aeruginosa*.

Materials and Methods: The OMVs were prepared from *P. aeruginosa* under hypervesiculation condition (treatment with amikacin and meropenem), and extraction was carried out by the sequential ultracentrifugation. Physicochemical features of extracted OMVs were evaluated by electron microscopy and SDS-PAGE. To quantify antibody synthesis and function after immunization with OMV, we used ELISA, serum bactericidal activity, and opsonophagocytosis. Production of cytokines from splenocytes of immunized mice was measured with ELISA.

Results: Specific-antibody IgG production, particularly IgG1 subclass, increased in mice primed with hypervesiculation-derived OMVs compared to normal condition-derived OMVs. Serum bactericidal activity and opsonophagocytosis of secreted antibody was enhanced in mice primed with hypervesiculation-derived OMVs. Investigation of cytokine production showed the upregulation of IL-8, IL-12, IL-17, and TNF-α and downregulation of IL-10.

Conclusion: Based on our findings, OMVs production can be increased by treating *P. aeruginosa* with amikacin and meropenem antibiotics. Moreover, hypervesiculation-derived OMV scan possibly activate the humoral and cellular immune response more than normal OMVs.

Keywords: Outer membrane vesicle; *Pseudomonas aeruginosa*; Hypervesiculation; Immunization

INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic Gram-negative pathogen that may give rise to dangerous nosocomial and community-acquired infections in the urinary tract, lower respiratory tract, and surgical or burn wounds (1, 2). Through vesiculation of the outer membrane, pathogenic Gram-negative bacteria secrete spherical particles, known as outer membrane vesicles (OMVs), with 20-200 nm in diameter. OMVs are generally composed of glycerol-phospholipids, lipopolysaccharide (LPS), and outer membrane proteins (OMPs), while the lumen of these vesicles contains periplasmic components such as DNA, RNA, and cytoplasmic proteins (3).

OMVs of *P. aeruginosa* (PA-OMVs) are comprised of several virulence factors, including phospholipase C, alkaline phosphatase, proelastase, hemolysin and

*Corresponding author: Ashraf Mohabati Mobarez, Ph.D, Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. Tel: +98-2182883862 Fax: +98-2182884545 Email: mmmobarez@modares.ac.ir

Pseudomonas quorum sensing molecules (4). Purified PA-OMVs have ability to stimulate alveolar epithelial cells and release IL-8 *in vitro*. They are also capable of generating inflammation in the lung, independent of live bacterial cell, through TLR2 and TLR4 molecules *in vivo* (5, 6). PA-OMVs are able to transport various enzymes and virulence factors into the host cells, which play a critical role in the pathogenesis of this infection (7). Different types of antibiotics have been indicated to enhance the secretion of OMVs from *P. aeruginosa* (8, 9).

This study aims to investigate the effect of meropenem and amikacin antibiotics on OMV secretion and to assess the OMVs features, including immunogenicity, protein concentration, size and cytokine profiles in PAO1.

MATERIALS AND METHODS

Bacterial strains and animal samples. *P. aeruginosa* PAO1 reference strain was acquired from the Collection of Standard Bacterial at the Pasteur Institute of Iran, Tehran. Female BALB/c mice (6-8 weeks) were also obtained from Pasteur Institute of Iran, Tehran. All the experimental procedures on animals were approved by the Ethical Committee of Tarbiat Modares University (Tehran, Iran; Ref: 52D/8165).

Determination of minimum inhibitory concentrations (MIC). The stocks of antimicrobial agents, meropenem and amikacin, were prepared as described earlier (10). Meropenem and amikacin were chosen to study their effects on OMVs production in *P. aeruginosa*. MIC for these two antibiotics was determined using the broth dilution method.

OMV production of *P. aeruginosa* under hypervesiculation conditions. In normal conditions, OMVs were produced from *P. aeruginosa* using a technique described elsewhere (11). Briefly, 30× the MIC concentration of each antibiotic was used for the induction of OMVs. After the first two hours, meropenem and amikacin were separately added to Luria-Bertani broth containing bacteria (*P. aeroginosa*) until the optical density (OD) of the bacteria reached 0.9-1.1. The OMVs were ultimately isolated using a standard method (12).

Composition quantification of OMVs. Following OMVs extraction, the stability and integrity of these vesicles were analyzed by transmission electron microscopy (TEM), as described previously (13). In summary, ultrasonication was employed to disperse the OMVs and attach to Formvar/carbon-coated nickel grids. The grids were then washed with PBS (0.01 M) supplemented with 0.5% bovine serum albumin (BSA-Sigma, USA) and 0.1% gelatin (PBG-Sigma, USA). Subsequently, the fixation of vesicles were carried out with 1% glutaraldehyde in PBS on grids and negatively stained with potassium phosphotungstate (pH 6.0). The grids were finally analyzed by TEM (Zeiss, Germany) at 80 kV.

Protein assay and proteomic analysis of the OMVs. The concentration of total protein in OMVs was determined using the Bradford method with bovine serum albumin as the standard (14). The protein composition of OMVs was investigated by SDS-PAGE in 12% gel using a Mini-protean II electrophoresis apparatus (Bio-Rad, USA). Protein standards (Bio-Rad) were used as molecular weight markers. Coomassie brilliant blue (Thermo Scientific, USA) was employed for gel staining subsequent to the electrophoresis.

Toxicity test. Three groups of BALB/c mice were used. On days zero or one, each of the animals were immunized intra-peritoneally with 200 µL (10 µg) of OMVs, *i.e*. both OMVs produced in normal conditions and those produced under antibiotic (amikacin and meropenem) stress conditions. The immunization of negative control mice was also performed with 200 µL of PBS. These mice were kept under completely sterile conditions in separate containers for one week, and their survival was monitored. The weight of mice and the number of their death in each group were recorded daily during a one-week observation period post OMV administration. The survival rate and weight loss was calculated for each group at end point.

Immunization. Immunization test was performed to investigate the immune response following OMV administration. To this end, each mouse received three times the intradermal injection of the same OMVs (200 µL) used in the toxicity test in two-week intervals. Mice were then sacrificed, and their serum samples were collected and kept at -80°C until the assessment of antigen-specific antibodies. PBS was

used as a control.

Detection of antigen-specific antibodies. To evaluate specific total sera IgG, IgG1, and IgG2a antibody response in mice immunized against PA-OMVs, indirect ELISA (enzyme-linked immunosorbent assay) was applied. Briefly, isolated OMVs from *P. aeruginosa*, under normal and hyper-vesiculation conditions, were coated on 96-well Maxisorp plates at a concentration of 5 µg/mL in PBS (pH 7.4) and incubated at 4°C overnight. The plates were washed three times with PBS (0.05% Tween 20) and blocked with PBS (1% BSA) at 37°C for 2 h. Mice sera was then added and incubated at 37°C for 90 min. After washing 5×, anti-mouse IgG conjugated with HRP (Sigma, USA) was added as a secondary antibody at 37°C for 90 min. Tetra-methyl-benzidine (TMB, Sigma) was used as a substrate to generate color for 30 min. Color generation was stopped with 100 µL of H_2SO (2N), and OD was read at 450 nm using an ELISA reader (Biotek, USA).

Cytokines measurements. Spleen tissues of the control and immunized mice with normal OMVs, OMVs-amikacin and OMVs-meropenem (5 mice/group) were isolated after 14 days. Spleen cells were isolated and its red blood cells (RBC) were treated by a lysis buffer. Subsequently, the splenocytes (2×10^6 cell/well) were cultured in 24-well plates supplemented with RPMI-1640 (Gibco, USA), fetal bovine serum (FBS), and penicillin-streptomycin (Gibco, USA). The cells were treated with tested OMVs (5 µg/mL) in wells and incubated in a humidified atmosphere (5% CO2) at 37°C for 72 h. The culture supernatants were aliquoted and kept at -80°C until use. The protein levels of IL-8, IL-10, IL-12, IL-17, and TNF-α were measured in the culture supernatants using a Mouse ELISA Kit (eBioscience, USA), according to the manufacturer's instructions.

Opsonophagocytosis test. The activity of serum antibodies to *P. aeruginosa* opsonization was assessed as previously explained (15, 16). In brief, 100 µL of serum samples (dilutions: 1:4-1:64) were added to 100 µL of bacterial suspension (10^6 CFU/ml of *P. aeruginosa*) and incubated at 37°C for 30 min. This suspension was washed with PBS. Then 100 µL of isolated mouse macrophages (106/ml) and 100 µL of 10% rabbit serum as a complement source were added to the mixture and incubated at 37°C for 90 min. In the control wells, RPMI medium (Gibco, Germany) was used instead of antibody, macrophages, or complement. In the end, 100 µL of the mixture was cultured in Mueller-Hinton agar and incubated at 37°C for 18 h for colony count. The opsonic activity of the serum was calculated using this equation: [1-(CFU immune serum at 90 min/CFU of pre-immune serum at 90 min)] ×100.

Statistical analysis. GraphPad Prism software version 8 was used for statistical analyses. T-test and one-way and two-way analysis of variance (ANOVA) test was used to analyze statistically the data. $P \leq 0.05$ was considered as the statistical significant level.

RESULTS

MICs of the antibiotics and characterization of OMVs. The MIC of meropenem and amikacin for *P. aeruginosa* were 0.5 µg/mL and 4 µg/mL, respectively. After extraction and purification of the OMVs under normal and hypervesiculation conditions, OMVs were imaged using electron microscopy (Fig. 1A). The obtained OMVs contained multiple spherical vesicle and ranged between 50 and 150 nm in size. In the presence of amikacin (59.5 µg/mL) and meropenem (129.5 µg/mL),the protein content of hyper-vesiculation-derived OMVs increased relative to OMVs attained in the normal condition (38.5 µg/mL) Fig. 1C. To evaluate the protein components of the extracted PA-OMVs, the samples were subjected to SDS-PAGE, followed by Coomassie staining (Fig. 1B).

Antigen-specific humoral immune response. To investigate the antibody response induced by immunization with PA-OMVs and to determine the antigen-specific IgG antibodies by indirect ELISA, serum samples were collected before each immunization (on days 1, 14, and 28) and two weeks after the last immunization (day 42). For PA-OMVs-immunized and PBS-treated groups, there was no detectable antigen-specific IgG antibody before the first immunization. After the first and second boost immunization, high levels of IgG were observed in all mice primed with hypervesiculation-derived OMVs (amikacin OMVs and meropenem OMVs) on day 14 (1.03 ± 0.09 and 1.25 ± 0.08) and on day 28 (1.65 ± 0.09 and 1.9 ± 0.12), compared to the normal condition-derived OMVs (day 14 [0.71 ± 0.12] and day 28 [1.34

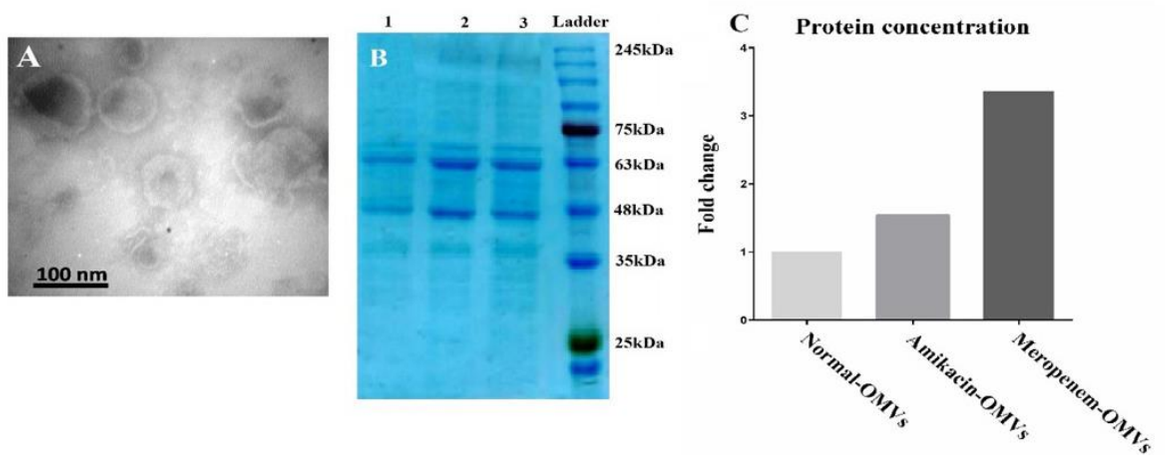

Fig. 1. Characterization of OMVs isolated from *P. aeruginosa*. The electron microscopic images of OMVs prepared from *P. aeruginosa*. The integrity and size of the OMVs was assayed by electron microscopy (A). Coomassie blue-stained SDS–PAGE of PA-OMVs (B). Hyper-vesiculation-derived OMVs protein content were measured by Bradford method and normalized to normal-OMVs (C).

± 0.14]; P <0.0001), respectively. Moreover, 14 days after the last immunization, there were higher levels of IgG compared to days 14 and 28. For PBS-immunized group, no antigen-specific IgG antibody was found throughout the immunization process (Fig. 2A). The levels of the PA-OMVs-specific IgG subclasses, IgG1 and IgG2a, were evaluated in the serum samples at day 42 of immunization (Fig. 2B). However, the immunization of mice with hyper-vesiculation-derived OMVs (amikacin OMVs and meropenem OMVs) increased the titer of IgG1 (0.97 ± 0.18 and 1.04 ± 0.14) more than IgG2a (0.57 ± 0.12 and 0.56 ± 0.08) when compared to the normal condition-derived OMVs (0.62 ± 0.16 for IgG1 and 0.37 ± 0.04 for IgG2a with P=0.0008 and P=0.0001, respectively).

PA-OMVs-induced cytokine production. The cytokine profiles in the spleen cells of mice in each immunized group were determined two weeks after the last boost immunization by ELISA. As shown in Fig. 5, the elevated levels of IL-8, IL-12, IL-17, and TNF-α were observed in PA-OMVs-immunized group, but not in the PBS control group (P=0.0017, P=0.024, P=0.0001, and P=0.0001, respectively). In addition, IL-10 concentration reduced in PA-OMVs-treated group compared to PBS-treated one (P=0.0079; Fig. 5E). Besides, TNF-α production increased significantly in hyper-vesiculation condition (amikacin [7.9 ± 1.2-fold change; P=0.002] and meropenem [8.2 ± 1-fold change; P=0.001]) relative to the normal condition (5 ± 0.8-fold change). Amikacin and Meropenem induced OMVs (5.3 ± 1.7-fold change) could produce a significantly increased level of IL-17 compared to normal OMVs (2.9 ± 0.57 fold change; P= 0.02) Fig. 5C.

Opsonophagocytic killing activity. At serum dilution series from 1:2 to 1:64, the opsonic killing activity of mice immunized with hyper-vesiculation condition-derived PA-OMVs (amikacin OMVs and meropenem OMVs) was 84.2%-24.6% and 87%-28.6%, respectively, which was higher than mice immunized with OMV derived from the normal condition (Fig. 4). These events are also seen in serum bactericidal activity test (Fig. 3).

DISCUSSION

OMVs have a wide range of functions that can potentially affect bacterial survival and pathogenesis (11, 33). In this study, we aimed to compare normal OMVs with OMVs induced by *P. aeruginosa* in response to amikacin and meropenem antibiotics. In addition, we examined the OMVs features, including protein concentration (Bradford) and size, as well as immunogenicity and cytokine profiles.

In the current study, both antibiotics led to a significant upregulation in OMVs secretion, maybe due to the disturbed structure of cell wall or protein production compared to normal conditions. An earlier study has supported our finding and indicated the

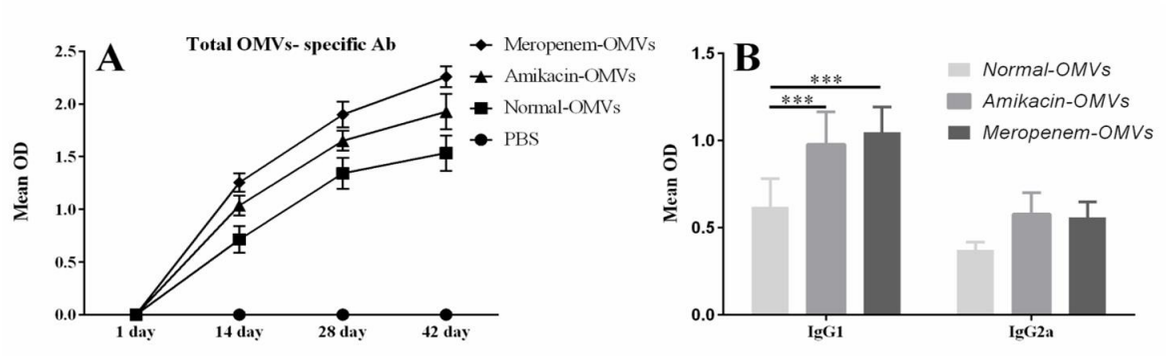

Fig. 2. Total antigen specific IgG antibodies elicited against PA-OMVs. High levels of OMVs-specific IgG were detected in all mice primed with hyper-vesiculation derived OMVs (Amikacin-OMVs and Meropenem-OMVs) compared to normal condition derived OMVs (A). The level of PA-OMVs specific IgG1 and IgG2a was evaluated at day 42 in serum samples. The level of anti-OMVs IgG1 and IgG2a subclasses were increased in mice primed with hyper-vesiculation derived OMVs (Amikacin-OMVs and Meropenem-OMVs) compared to normal condition derived OMVs (B). The data are presented as Mean±SD. *** p<0.001.

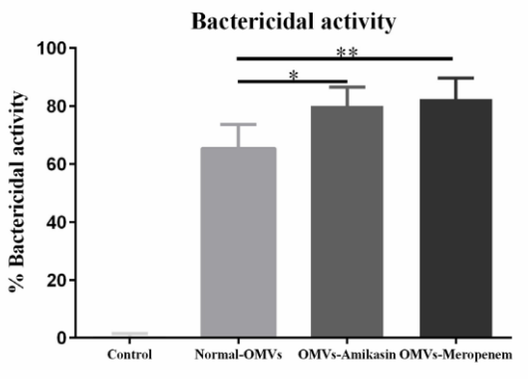

Fig. 3. The serum bactericidal activity of immunized mice with PA-OMVs derived in different condition. The serum bactericidal activity of each group against *P. aeruginosa* was measured two weeks after the final immunization. Error bars represent the Means ± SD. * p< 0.05, ** p< 0.01.

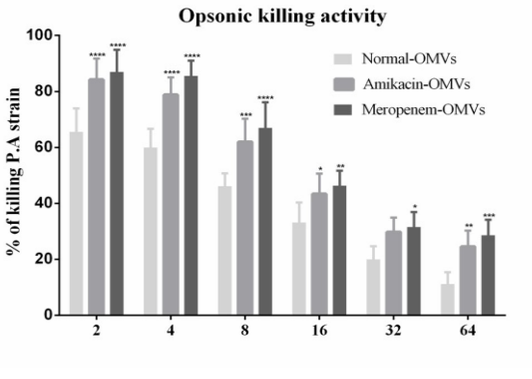

Fig. 4. Upregulation of the opsonic killing activity of serum in immunized mice with PA-OMVs against *P. aeruginosa.* The opsonic killing activity of several dilutions series of serum were measured in immunized mice with PA-OMVs against *P. aeruginosa.* Error bars represent the mean ± SD. *p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

important effects of stress, such as antibiotics, in the upregulation of OMVs production (17). Elevated PA-OMVs production in our research work is in agreement with parallel results demonstrated for meropenem in *P. aeruginosa* (18), for imipenem in *Stenotrophomonas maltophilia* (19), and for polymyxin B in non-enterohemorrhagic *Escherichia coli* (EHEC) (20) and *P. aeruginosa* (21). It has also been reported that ciprofloxacin, meropenem, fosfomycin, and polymyxin B maximize the production of OMVs in *E. coli* O104:H4 and O157:H7 (22). Another study has suggested that the treatment of the clinical strains of *Acinetobacter baumannii* with imipenem could upregulate OMVs production and alter OMVs protein components and increased pathogenicity for cultured cells (23). In addition, an investigation of human tear fluid and lysozyme component of tear fluid on *P. aeruginosa* strain PAO1 has demonstrated that OMVs secretion intensely increases compared to controls (PBS) (24). Meanwhile, antigen-specific antibodies have been demonstrated to be essential for FliC- and OprF-medicated protection (25).

Our data exhibited that immunization with PA-OMVs significantly elevates IgG levels in all mice primed

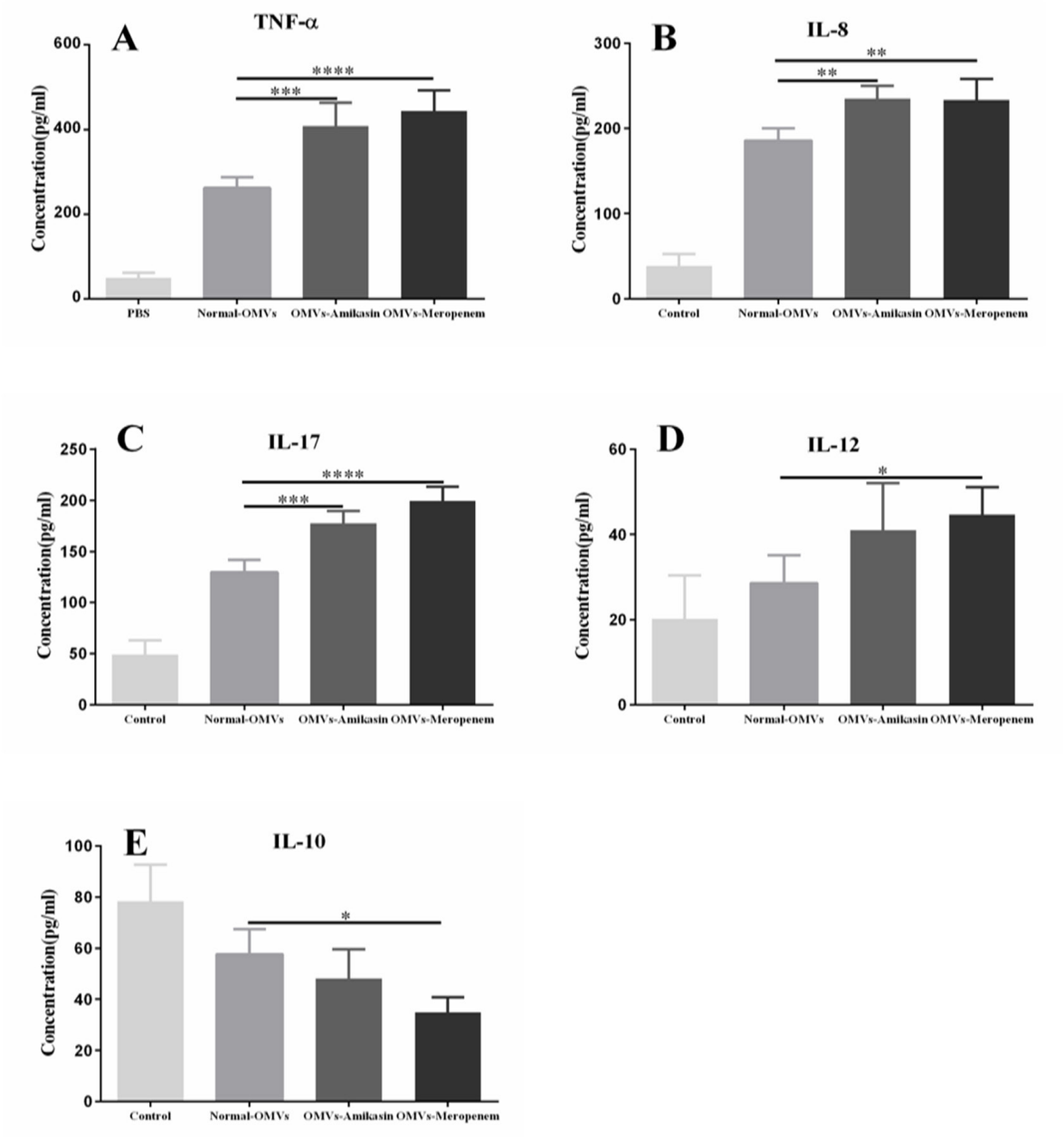

Fig. 5. The cytokines production by spleen cells of immunized mice. The concentrations of IL-8, IL-12, IL-17, IL-10 and TNF-α in supernatant of splenocytes of control and immunized mice groups measured with indirect ELISA. The IL-8, IL-12, IL-17 and TNF-α production from splenocytes were increased in immunized groups compared to control (5 mice/group). The IL-10 cytokine concentration was reduced in immunized groups against control (5 mice/group). Bars represent mean ± SD. *p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

with hyper-vesiculation-derived OMVs (amikacin OMVs and meropenem OMVs) compared to the normal condition-derived OMVs. The opsonic activity of antibodies elicited from mice immunized with hyper-vesiculation condition-derived PA-OMVs significantly increased in all dilution series (P<0.0001) in comparison to OMV groups derived from the normal condition. Based on the data presented here in hyper-vesiculation condition-derived PA-OMVs showed robust immunogenicity when used as antigen, and one boost immunization is sufficient to induce a high level of the humoral immune response.

Other studies have reported that intact vesicles provoke an extremely superior inflammatory response rather than response to purified LPS. Therefore, both proteins and LPS participated in specific vesicle cytokine responses (26, 27).

IgG subtypes analysis showed that the immunization of mice with amikacin and meropenem derived OMVs could elevate the titer of IgG1 more than IgG2a, as compared to normal condition-derived OMVs. Therefore, these findings propose that immunization with PA-OMVs in three groups could elicit both the Th1 and Th2 arms of the immune response. However, immunization of mice with hyper-vesiculation-derived OMVs significantly upregulated IgG1 levels and could induce more Th1 response compared to normal OMVs.

In the present study, by the measurement of inflammatory and anti-inflammatory cytokines production, we observed that T-cell response enhanced against OMVs. Our findings demonstrated significantly higher levels of IL-8, IL-12, IL-17, and TNF-α production, but decreased level of IL-10, in mice immunized with hypervesiculation condition-derived PA-OMVs compared to normal OMVs.

A number of studies have described how vesicles activate the innate inflammatory response. *Helicobacter pylori*- and *P. aeruginosa* derived OMVs have been reported to induce IL-8 production (28-31), and OMVs from *Salmonella enterica* have been shown to stimulate dendritic cells to secrete IL-12 and TNF-α (32). This inflammation is linked with the local upregulation of several cytokines and chemokines, including CCL2, CXCL1, and TNF-α, IL-1β, IFN-γ, and IL-6 (33). The analysis of antibodies and cytokines indicated the induction of a mixed humoral and cellular immune response against PA-OMVs immunization. However, it was not clear what type of immune response could be efficient in clearing the infection.

In summary, the treatment of the *P. aeruginosa* with amikacin and meropenem antibiotics augments OMVs production. Moreover, hypervesiculation condition-induced OMVs with the two antibiotics could potentially activate humoral and cellular immune response more than normal condition-induced OMVs.

ACKNOWLEDGEMENTS

This project was supported by Faculty of Medical Sciences, Tarbiat Modares University.

REFERENCES

1. Esmaeili D, Daymad SF, Neshani A, Rashki S, Marzhoseyni Z, Khaledi A. Alerting prevalence of MBLs producing *Pseudomonas aeruginosa* isolates. *Gene Rep* 2019;16:100460.
2. Döring G, Pier GB. Vaccines and immunotherapy against *Pseudomonas aeruginosa*. *Vaccine* 2008;26:1011-1024.
3. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol* 2010;64:163-184.
4. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 2005;437:422-425.
5. Bauman SJ, Kuehn MJ. Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response. *Microbes Infect* 2006;8:2400-2408.
6. Bauman SJ, Kuehn MJ. *Pseudomonas aeruginosa* vesicles associate with and are internalized by human lung epithelial cells. *BMC Microbiol* 2009;9:26.
7. Bomberger JM, MacEachran DP, Coutermarsh BA,Ye S, O'Toole GA, Stanton BA. Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog* 2009;5(4):e1000382.
8. Sinha AK, Possoz C, Leach DRF. The roles of bacterial DNA double-strand break repair proteins in chromosomal DNA replication. *FEMS Microbiol Rev* 2020;44:351-368.
9. Heidarzadeh S, Enayati Kaliji Y, Pourpaknia R, Mohammadzadeh A, Ghazali-Bina M, Saburi E, et al. A meta-analysis of the prevalence of class 1 integron and correlation with antibiotic resistance in *Pseudomonas aeruginosa* recovered from Iranian burn patients. *J Burn Care Res* 2019;40:972-978.
10. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001;48 Suppl 1:5-16.
11. Delbaz SA, Siadat SD, Aghasadeghi MR, Piryaie M, Najar Peerayeh SH, Mousavi SF,et al. Biological and immunological evaluation of *Neisseria meningitidis* serogroup A outer membrane vesicle as vaccine candidates. *Jundishapur J Microbiol* 2013;6(4):e5007.
12. Malekan M, Siadat SD, Aghasadeghi M, Shahrokhi N, Afrough P, Behrouzi Ava, et al. Evaluation of protective immunity responses against pneumococcal PhtD and its C-terminal in combination with outer-membrane vesicles as adjuvants. *J Med Microbiol* 2020;69:465-

477.
13. Deo P, Chow SH, Hay ID, Kleifeld O, Costin A, Elgass KD, et al. Outer membrane vesicles from N*eisseria gonorrhoeae* target PorB to mitochondria and induce apoptosis. *PLoS Pathog* 2018;14(3):e1006945.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
15. Pier GB, Coleman F, Grout M, Franklin M, Ohman DE. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect Immun* 2001;69:1895-1901.
16. Theilacker C, Coleman FT, Mueschenborn S, Llosa N, Grout M, Pier GB. Construction and characterization of a *Pseudomonas aeruginosa* mucoid exopolysaccharide-alginate conjugate vaccine. *Infect Immun* 2003;71:3875-3884.
17. El Solh AA, Alhajhusain A. Update on the treatment of *Pseudomonas aeruginosa* pneumonia. *J Antimicrob Chemother* 2009;64:229-238.
18. Siqueira VL, Cardoso RF, Caleffi-Ferracioli KR, Scodro RB, Fernandez MA, Fiorini A, et al. Structural changes and differentially expressed genes in *Pseudomonas aeruginosa* exposed to meropenem-ciprofloxacin combination. *Antimicrob Agents chemother* 2014;58:3957-3967.
19. Devos S, Van Oudenhove L, Stremersch S, Van Putte W, De Rycke R, Van Driessche G, et al. The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*. *Front Microbiol* 2015;6:298.
20. Manning AJ, Kuehn MJ. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiol* 2011;11:258.
21. MacDonald IA, Kuehn MJ. Stress-induced outer membrane vesicle production by *Pseudomonas aeruginosa*. *J Bacteriol* 2013;195:2971-2981.
22. Bauwens A, Kunsmann L, Karch H, Mellmann A, Bielaszewska M. Antibiotic-mediated modulations of outer membrane vesicles in enterohemorrhagic *Escherichia coli* O104: H4 and O157: H7. *Antimicrob Agents Chemother* 2017;61(9):e00937-17.
23. Yun SH, Park EC, Lee SY, Lee H, Choi CW, Yi YS, et al. Antibiotic treatment modulates protein components of cytotoxic outer membrane vesicles of multidrug-resistant clinical strain, *Acinetobacter baumannii* DU202. *Clin Proteomics* 2018;15:28.
24. Metruccio MM, Evans DJ, Gabriel MM, Kadurugamuwa JL, Fleiszig SM. *Pseudomonas aeruginosa* outer membrane vesicles triggered by human mucosal fluid and lysozyme can prime host tissue surfaces for bacterial adhesion. *Front Microbiol* 2016;7:871.
25. Zhang X, Yang F, Zou J, Wu W, Jing H, Gou Q, et al. Immunization with *Pseudomonas aeruginosa* outer membrane vesicles stimulates protective immunity in mice. *Vaccine* 2018;36:1047-1054.
26. Ellis TN, Leiman SA, Kuehn MJ. Naturally produced outer membrane vesicles from *Pseudomonas aeruginosa* elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect Immun* 2010;78:3822-3831.
27. Neshani A, Zare H, Akbari Eidgahi MR, Khaledi A, Ghazvini K. Epinecidin-1, a highly potent marine antimicrobial peptide with anticancer and immunomodulatory activities. *BMC Pharmacol Toxicol* 2019;20:33.
28. Mowat E, Paterson S, Fothergill JL, Wright EA, Ledson MJ, Walshaw MJ, et al. *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. *Am J Respir Crit Care Med* 2011;183:1674-1679.
29. Chung KF. Cytokines as targets in chronic obstructive pulmonary disease. *Curr Drug Targets* 2006;7:675-681.
30. Abadi AH, Mahdavi M, Khaledi A, Esmaeili SA, Esmaeili D, Sahebkar AH. Study of serum bactericidal and splenic activity of Total-OMP-CagA combination from *Brucella abortus* and *Helicobacter pylori* in BALB/c mouse model. *Microb Pathog* 2018;121:100-105.
31. Khaledi A, Khademi F, Esmaeili D, Esmaeili SA, Rostami H. The role of HPaA protein as candidate vaccine against *Helicobacter pylori*. *Der Pharma Chemica* 2016;8:235-237.
32. Murphy TF, Brauer AL, Eschberger K, Lobbins P, Grove L, Cai X, et al. *Pseudomonas aeruginosa* in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008;177:853-860.
33. Park KS, Lee J, Jang SC, Kim SR, Jong MH, Lötvall J, et al. Pulmonary inflammation induced by bacteria-free outer membrane vesicles from *Pseudomonas aeruginosa*. *Am J Respir Cell Mol Biol* 2013;49:637-645.