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Research Article

Canine Adipose Tissue Stem Cells Induced With Toll-Like Receptor Agonists Exhibit Antibacterial Activity Against Multi Drug Resistant Pathogens

Burak AKSU [1 (*)] Özgür YANILMAZ [2] Tunç AKKOÇ [1]

[1] Marmara University, School of Medicine, Department of Medical Microbiology, TR-34854 İstanbul - TÜRKİYE
[2] Marmara University Hospital, Medical Microbiology Department, TR-34899 İstanbul - TÜRKİYE
[3] Marmara University, School of Medicine Department of Immunology, TR-34854 İstanbul - TÜRKİYE

ORCIDs: B.A. 0000-0002-3439-9158; Ö.Y. 0000-0003-3847-7288; T.A. 0000-0001-9179-2805

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Abstract: Infections caused by antibiotic-resistant pathogens pose a major threat worldwide. There is an urgent need to develop effective strategies to solve this problem. The antibacterial activity of adult mesenchymal stem cells (MSCs) has recently been determined against various bacterial isolates. New approaches, such as Toll-like receptor activation, were used to enhance their antibacterial potency. This study examines the antibacterial activity of TLR agonist (TLR2/TLR1 and/or TLR2/TLR6) activated adipose-derived canine MSCs (AD-MSCs) on multi-drug resistant isolates including *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis,* and *Pseudomonas aeruginosa* regarding bacterial growth, and minimum inhibitory concentration (MIC) determination. Effects on bacterial morphology were assessed by electron microscopy. Our results showed that the AD-MSCs conditioned medium primed with different TLR agonists inhibited the growth of *E. coli* and *S. aureus*, but it had a decreased effect on *E. faecalis* and *P. aeruginosa*. Despite this, AD-MSCs conditioned medium prepared with the combination of TLR agonists exhibited antibacterial activity against all isolates. These findings were in parallel with MIC levels of conditioned media. We conclude that adipose-derived canine MSCs primed with TLR agonists (TLR2/TLR1 and TLR2/TLR6 combination) possess antimicrobial activity against multi-drug resistant isolates of *E. coli, S. aureus, E. faecalis* and *P. aeruginosa*. Further studies for testing in *in vivo* models are being planned to assess the potential application of AD-MSCs as an adjunct treatment modality for multi drug resistant infections.

Keywords: *Antibacterial agent, Gram negative bacteria, Gram positive bacteria, Mesenchymal stem cell, Multidrug resistance*

Toll-Benzeri Reseptör Agonistleri ile İndüklenen Köpek Adipoz Dokusu Kök Hücrelerinin Çoklu İlaca Dirençli Patojenlere Karşı Antibakteriyel Aktivitesi

Öz: Antibiyotik dirençli patojenlerin neden olduğu enfeksiyonlar dünya çapında büyük bir tehdit oluşturmaktadır. Bu sorunu çözmeye yönelik etkili stratejilerin geliştirilmesine acil gereksinim bulunmaktadır. Son dönemde, yetişkin mezenkimal kök hücrelerinin (MKH) çeşitli bakteri izolatlarına karşı antibakteriyel aktivite gösterdiği tespit edilmiştir ve antibakteriyel etkinliklerini artırmak için Toll benzeri reseptör aktivasyonu gibi yeni yaklaşımlar kullanılmıştır. Bu çalışmada, TLR agonisti (TLR2/TLR1 ve/veya TLR2/TLR6) ile aktive edilmiş adipoz türevli köpek MKH'lerin (AD-MKH) çok ilaca dirençli *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* ve *Pseudomonas aeruginosa* izolatları üzerindeki antibakteriyel etkisi, bakteri üremesi ve minimum inhibitör konsantrasyon (MİK) tayini ile belirlenmiştir. Bakteri morfolojisi üzerindeki etkiler elektron mikroskobu ile değerlendirilmiştir. Sonuçlarımız, farklı TLR agonistleri ile uyarılmış AD-MKH'lerden elde edilen şartlandırılmış besiyerinin *E. coli* ve *S. aureus* üremesini inhibe ettiğini, ancak *E. faecalis* ve *P. aeruginosa* üzerinde sınırlı bir etkiye sahip olduğunu gösterdi. Buna karşın, TLR agonistlerinin kombinasyonu ile hazırlanan AD-MKH şartlandırılmış besiyeri tüm izolatlar üzerinde antibakteriyel aktivite sergiledi. Bu bulgular, şartlandırılmış besiyerinin MİK seviyeleri ile paralellik göstermiştir. TLR agonistleri (TLR2/TLR1 ve TLR2/TLR6 kombinasyonu) ile uyarılmış adipoz türevli köpek MKH'lerinin, çoklu ilaca dirençli *E. coli, S. aureus, E. faecalis* ve *P. aeruginosa* izolatlarına karşı antibakteriyel aktiviteye sahip olduğu sonucuna vardık. AD-MKH'lerin çoklu ilaca dirençli enfeksiyonlar için ek bir tedavi yöntemi uygulaması olarak potansiyelini değerlendirmeye yönelik *in vivo* modellerde ileri çalışmalar planlanmaktadır.

Anahtar sözcükler: *Antibakteriyel ajan, Çoklu ilaç direnci, Gram negatif bakteri, Gram pozitif bakteri, Mezenkimal kök hücre*

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(*) Corresponding author: Burak AKSU

Phone: +90 216 777 5640 Fax: +90 216 777 5501
E-mail: baksu@marmara.edu.tr

Introduction

Infections caused by antibiotic-resistant bacteria are a major global health problem and it is estimated that those infections result in nearly 5 million deaths each year [1]. Despite advances in healthcare-associated services, microorganisms evolve novel survival mechanisms and improve resistance through transferable genetic materials such as plasmids, integrons, and transposons [2]. The time required to develop a new antibacterial agent is between 10-15 years. On contrary, bacteria gain resistance rapidly. Authorities underline an urgent need to propose and develop alternative strategies against antibiotic-resistant bacterial pathogens, including multi-drug-resistant organisms. Bacteriophages, modified drugs, monoclonal antibodies, nanoparticles, anti-virulence agents, and antimicrobial peptides are among the novel therapies developing against antibiotic-resistant bacterial infections [3].

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have the potential to differentiate into osteogenic, chondrogenic, and adipogenic tissues. Moreover, MSCs exert anti-inflammatory, proliferative, and regenerative effects on tissue repair through angiogenesis, connective tissue formation, epithelialization, and production of inflammatory mediators [4]. All these features make them a promising therapeutic tool in regenerative medicine. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed the following criteria for MSCs definition; plastic surface adhesion, positive CD105, CD73 and CD90 expression, and negative surface markers, including CD45, CD34, CD14, or CD11b, differentiation to osteoblasts, adipocytes and chondroblasts *in vitro* [5]. MSCs can be isolated from different sources like bone marrow, adipose tissue, umbilical cord, placenta, dental follicles, etc.

Recent studies have revealed MSCs exert antimicrobial activity by directly secreting a range of antimicrobial peptides and indirectly triggering immune effector cells to call an innate immune response [6-9]. Antimicrobial peptide-based stem cell secretome is mainly induced by the activation of toll-like receptors (TLRs) found on the cellular surface. Adipose tissue and bone marrow-derived MSCs from humans and mice have been shown to express TLR 1 to 6 molecules [10].

Several studies have evaluated different TLR ligands to increase the antimicrobial and immunomodulatory properties of MSCs from different sources. In general, those studies used polyinosinic-polycytidylic acid (poly IC) and bacterial LPS to stimulate TLR-3 and TLR-4 receptors, respectively [11-13]. Activation of TLR receptors induces MSCs to secrete antimicrobial substances and also support innate immune cells for immunomodulation and clearance of pathogens [14]. In addition, studies have been performed for the antibacterial activity of TLR agonist (mainly with TLR3 ligand) stimulated canine adipose-derived MSCs against multi-drug resistant bacterial pathogens [9,15].

In this study, we aimed to examine the antibacterial effects of different TLR agonists (TLR2/TLR1 and/or TLR2/TLR6 agonists) primed canine adipose tissue mesenchymal stem cell (AD-MSC) conditioned media on antibiotic-resistant pathogenic isolates of *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis,* and *Pseudomonas aeruginosa*.

Material and Methods

Stem Cell Culture and TLR Agonist Priming

Canine adipose-derived mesenchymal stem cells (AD-MSCs) were purchased commercially (Generon Ltd, Slough, UK). Cells ($2x10^6$ cells) were maintained in Dulbecco's Modified Eagle Medium (DMEM, PAN-Biotech, Aidenbach, Germany) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin (PAN-Biotech) at 37°C in 5% CO_2. Subpassaging was performed at 80% confluency.

AD-MSCs at passage 3 and become 70-80% confluent were primed with TLR agonists. For priming, cells were seeded into 12-well tissue culture plates (10^5 cells/well) and incubated for 48 h in DMEM (PAN-Biotech) supplemented with 10% FBS and antibiotics at 37°C in 5% CO_2. Cells were washed twice with sterile phosphate-buffered saline (PBS) and fresh culture media without FBS and antibiotics were added. Synthetic TLR2/TLR1 agonist Pam3CSK4 (Invivogen, San Diego, CA, USA) and TLR2/TLR6 agonist Pam2CSK4 (Invivogen) were prepared in distilled water and used at 1.5 µg/mL final concentration for cell priming. Experiments were performed in triplicate.

Stem Cell Viability and Characterization After Priming

Canine AD-MSCs were verified that priming conditions did not reduce cell viability as detected by trypan blue staining. Briefly, cells from priming experiments were suspended in PBS and 0.1 mL cell suspension stained with 0.1 mL of 0.4% trypan blue solution (Merck KGaA, Darmstadt, Germany). Cells were counted on the Thoma cell counting chamber under a light microscope.

AD-MSCs were also characterized by flow cytometry analyses for the presence or absence of stem cell-specific surface markers. Cells were examined with the following monoclonal antibodies (BD Biosciences, San José, CA, USA) for flow cytometric immunophenotyping; CD73 APC (Allophycocyanin), CD90 FITC (fluorescein isothio-

cyanate), CD105 PerCP-Cy 5.5 (phycoerythrin-cyanine 5.5). A conjugated monoclonal antibody cocktail (BD Biosciences) containing CD34/ CD45/ HLA-DR/ CD11b PE (phycoerythrin) was used for negative markers. All experiments were performed with a FACSCalibur flow cytometer (BD Biosciences) equipped with the CellQuestTM software (BD Biosciences).

Preperation of Conditioned Media

Conditioned media was collected on 3rd day from AD-MSCs primed with each of TLR2/TLR1 or TLR2/TLR6 agonists or their combination in final concentrations at 1.5 µg/mL and 0.75 + 0.75 µg/mL, respectively. Samples were centrifuged for 5 min at 400g to remove cellular debris, then filtered through a 0.22 µm membrane filter and concentrated by freeze drying process (CoolSafe; LaboGene, Allerod, Denmark). Media from control wells without priming were also collected and processed. Samples were frozen at -80°C until further use in subsequent experiments.

Bacterial Strains and Antibiotic Susceptibility Determination

Bacterial strains including *S.aureus, E.coli, E.faecalis,* and *P.aeruginosa* were obtained from the culture collection of Marmara University Microbiology Laboratory. All isolates were grown on 5% sheep blood agar (bioMerieux, Marcy l' Etoile, France) at 37°C overnight in a bacteriological incubator. Microbiological identification of isolates was confirmed with MALDI-TOF Mass Spectrometry (Vitek MS; bioMerieux). Isolates were tested for antibiotic susceptibility with the disk diffusion method and results were evaluated by using breakpoint values from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical & Laboratory Standards Institute (CLSI) interpretation criteria [16,17].

Bacterial Growth Assay

Assessment of inhibition of bacterial growth by AD-MSCs conditioned medium was performed by spectrophotometric measurement. All bacterial isolates were grown at 37°C overnight in 5 ml tryptic soy broth (TSB) medium (Oxoid Ltd, Basingstoke, UK). Then the culture was diluted into fresh TSB medium to obtain 10^7 cfu/mL (approximately OD600=0.05) by using a densitometer (DEN-1; Biosan, Riga, Latvia). The resulting bacterial suspension was 1/10 diluted in AD-MSCs conditioned medium and transferred into a 96 well-plate for incubation at 37°C for 6 h. Bacteria with fresh cell culture medium was used as the positive control and medium alone was used as the negative control. Optical density measurements for each bacterial isolate were obtained by using a spectrophotometer (Synergy H1; BioTek Instruments, Winooski, VT, USA). Bacterial counts were quantitated by inoculating serial dilutions of samples taken from culture supernatants.

Assessment of Antibacterial Activity

The antibacterial activity of AD-MSCs conditioned media was tested by broth microdilution susceptibility test according to EUCAST [18]. Briefly, overnight cultures of bacterial isolates were diluted with 5 mL of Mueller Hinton broth (Oxoid Ltd, Basingstoke, UK) to obtain 10^6 cfu/ml. AD-MSCs conditioned media prepared with TLR agonists was added as 0.1 mL into a first well and diluted twofold in 8 wells of 96-well microplates. An equal volume (0.1 mL) of bacterial suspension was transferred to all wells containing conditioned media. Microplates were incubated at 37°C for 18 h. Results were evaluated visually and the highest dilution without visible bacterial growth was determined as minimum inhibitory concentration.

Electron Microscopy

Scanning electron microscopy (SEM) was used to examine the effect of AD-MSCs conditioned media prepared with TLR agonists on *E. coli* as a bacterial model. The bacterial cells (10^8 cells/mL) were incubated in a conditioned medium (0.5 mL) primed with Pam3CSK4/ Pam2CSK4 mixture (0.75 µg/mL for each) at 37°C for 4 h. After incubation, the suspension was centrifuged at 3000 rpm for 5 min and the pellet was washed twice with sterile PBS (0.05 M, pH: 7.4). Pellet was smeared on glass coverslips, and fixed with 2.5% glutaraldehyde for 2 h at ambient temperature. Samples were washed with PBS, then dehydrated with stepwise ethanol treatment. After drying at room temperature, coverslips were coated with gold and palladium in a sputter coater (Emitech SC7620; Quorum Technologies, Lewes, UK). Treated bacterial cells and untreated cells as control were subsequently analyzed with SEM (Evo MA 10; Carl Zeiss Microscopy GmbH, Jena, Germany).

Statistical Analysis

Statistical analyses were performed by using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The data were analysed with an independent *t*-test to test the significance of the effect of conditioned media from AD-MSCs on bacterial growth. The data have been normalized against non-treated control and are expressed as means±standard error of the mean (SEM). The level of significance was considered as $P<0.05$.

Results

Cell viability and stem cell characterization were analyzed on the third day after adding TLR agonists to cell cultures. Canine AD-MSCs were observed as 70-80% confluency and determined as adherent cells on the culture flask surface with spindle shape presentation under

microscopical examination. Cell viability was tested with trypan blue dye exclusion assay and by 72 h, ranging from 84% to 96%. Cell viability rates were not significantly different between any of the TLR agonist-primed AD-MSCs groups *(Fig. 1)*. Canine AD-MSCs characterization was performed with flow cytometry analyses for surface markers. It resulted that all the AD-MSCs groups were positive for CD73 (99.6%), CD90 (99.8%), and CD105 (94.9%), but negative for CD35, CD45, CD11b, and HLA-DR (1.1%) *(Fig. 1-A,B,C,D)*. These data confirm that TLR-primed AD-MSCs can retain their characteristics similarly to MSCs.

Selected Gram-positive and Gram-negative bacterial strains were tested against to appropriate antibiotics suggested by EUCAST [16]. *Staphylococcus aureus* isolate was found to be cefoxitin and oxacillin resistant and identified as methicillin-resistant *S.aureus* (MRSA). *E. coli* isolate was detected as an extended-spectrum β-lactamases (ESBL) producer according to a double disk synergy test performed with ceftazidime, ceftazidime-clavulanic acid, and cefotaxime, cefotaxime-clavulanic acid disks. *Enterococcus faecalis* isolate was found to be vancomycin resistant. *Pseudomonas aeruginosa* isolate was detected as resistant against imipenem, meropenem, and doripenem, so-identified it as carbapenem-resistant. All antibiotics were tested with the disk diffusion method and respective inhibition zones were measured. Zone of inhibition (ZOI) measurements and antibiotic susceptibility profiles of study isolates evaluated according to EUCAST and CLSI are shown in *Table 1* [16,17].

Conditioned medium samples obtained from Pam3CSK4 (TLR2/TLR1 agonist), Pam2CSK4 (TLR2/TLR6 agonist), or the agonist combination (Pam3CSK4 and Pam2CSK4) activated canine AD-MSCs were tested for growth

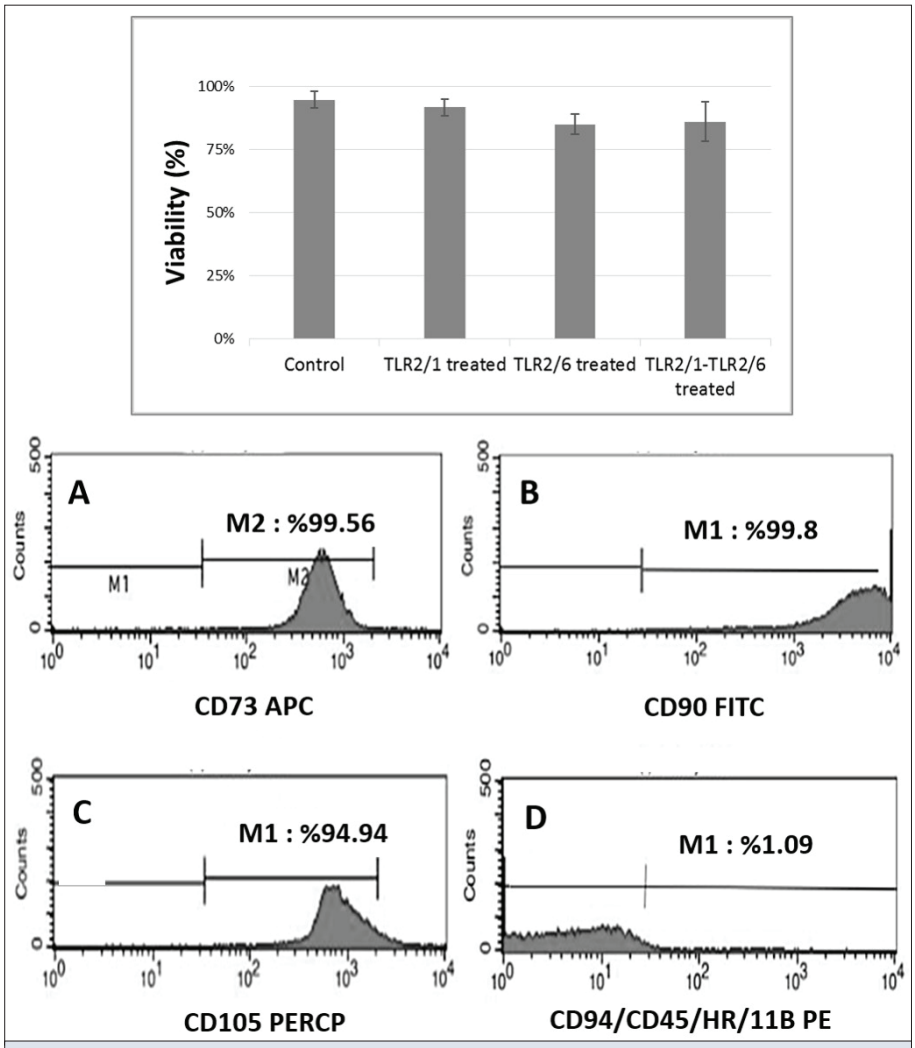

Fig 1. Cell viability rates and flow cytometric stem cell characterization of TLR agonist primed canine AD-MSCs. No significant difference was found in cell viability between any of the TLR-agonist primed AD-MSCs groups (P>0.05). Canine AD-MSCs analysed with flow cytometry for positive markers (A-C) and negative markers (D)

Table 1. *Antibiotic susceptibility profiles and resistance phenotypes of study isolates*

Bacterial Isolate	Antibiotic	Antibiotic Susceptibility[a]			Resistance Phenotype
		Zone of Inhibition (mm)	EUCAST Interpretation (breakpoint)	CLSI Interpretation (breakpoint)	
S. aureus	Cefoxitin	15	R (<22)	N/A	MRSA
	Penicillin	12	R (<26)	R (<28)	
	Erythromycin	11	R (<21)	R (<13)	
	Tetracycline	10	R (<22)	R (<14)	
	Fusidic acid	12	R (<24)	N/A	
	Mupirocin	8	R (<13)	N/A	
	Clindamycin	26	S (>22)	S (>21)	
	Linezolid	25	S (>21)	S (>21)	
	Tigecycline	24	S (>19)	N/A	
	Nitrofurantoin	19	S (>13)	S (>17)	
	TMP-SMX	18	S (>17)	S (>16)	
E. coli	Ampicillin	11	R (<14)	R (<13)	ESBL(+)
	Cefuroxime	11	R (<19)	R (<14)	
	Cefoxitin	12	R (<19)	R (<14)	
	Ceftazidime	14	R (<19)	R (<17)	
	Ceftriaxone	12	R (<22)	R (<19)	
	Cefazolin	16	R (<20)	R (<14)	
	Gentamicin	10	R (<17)	R (<12)	
	Ciprofloxacin	18	R (<22)	R (<21)	
	TMP-SMX[b]	9	R (<11)	R (<10)	
	Tigecycline	14	R (<18)	N/A	
	Amoxicillin/Clavulanate	22	S (>16)	S (>18)	
	Piperacillin/Tazobactam	26	S (>20)	S (>21)	
	Ertapenem	29	S (>25)	S (>22)	
	Meropenem	25	S (>22)	S (>23)	
	Amikacin	23	S (>18)	S (>17)	
	Nitrofurantoin	18	S (>11)	S (>17)	
E. faecalis	Ampicillin	6	R (<8)	R (<16)	VRE
	Gentamicin	7	N/A	N/A	
	Streptomycin	11	N/A	N/A	
	Vancomycin	10	R (<12)	R (<14)	
	Nitrofurantoin	11	R (<15)	R (<14)	
	Ciprofloxacin	9	R (<15)	R (<15)	
	Levofloxacin	10	R (<15)	R (<13)	
	Teicoplanin	21	S (>16)	S (>14)	
	Linezolide	25	S (>20)	S (>23)	
P. aeruginosa	Piperacillin/Tazobactam	13	R (<18)	R (<14)	Carbapenem resistant
	Ceftazidime	12	R (<17)	R (<14)	
	Imipenem	11	R (<20)	R (<15)	
	Meropenem	12	R (<14)	R (<15)	
	Doripenem	12	R (<22)	R (<15)	
	Cefepime	11	R (<21)	R (<14)	
	Ciprofloxacin	16	R (<26)	R (<18)	
	Levofloxacin	16	R (<18)	IM (15-21)	
	Ceftazidime- Avibactam	13	R (<17)	R (<20)	
	Amikacin	25	S (>15)	S (>17)	
	Tobramycin	23	S (>18)	S (>15)	

Antibiotic susceptibility was tested with disk diffusion method. ***EUCAST:*** *European Committee on Antimicrobial Susceptibility Testing;* ***CLSI:*** *Clinical & Laboratory Standards Institute;* ***TMP-SMX:*** *Trimethoprim-sulfamethoxazole;* ***MRSA:*** *Methicillin-resistant Staphylococcus aureus;* ***ESBL(+):*** *Extended spectrum beta-lactamase positive;* ***VRE:*** *Vancomycin-resistant Enterococci;* ***S:*** *Susceptible;* ***R:*** *Resistant;* ***IM:*** *Intermediate resistant;* ***N/A:*** *Not applicable*

inhibitory activity on bacterial isolates. Bacterial growth was interpreted by absorbance measurement of bacterial cultures at 600 nm and relatively compared to DMEM (negative control), which was accepted as 100%. Bacterial loads were confirmed with plating experiments. The most potent growth inhibition for all study isolates was obtained with agonist combination, followed by TLR2/TLR6 agonist Pam2CSK4. The growth of *E. coli* and *S. aureus* isolates was highly inhibited compared to *E. faecalis* and *P. aeruginosa*. *E.coli* was determined as the most susceptible isolate according to growth reduction rates *(Fig. 2)*.

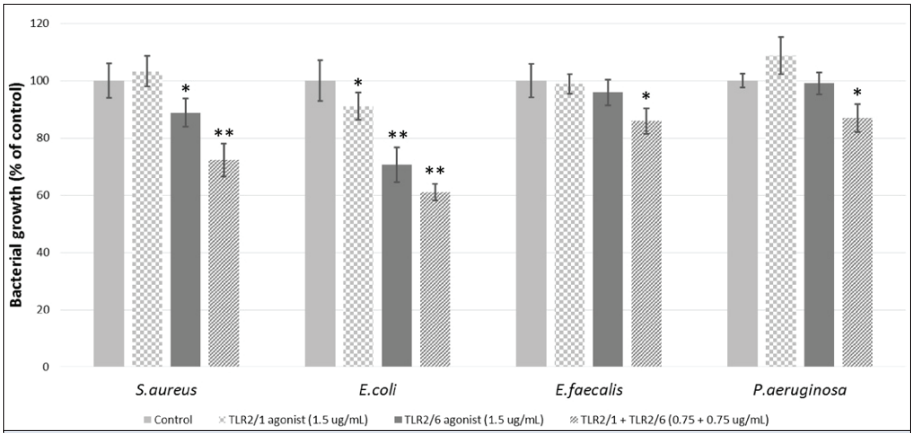

Fig 2. The activity of conditioned media from TLR agonist primed canine AD-MSCs on bacterial growth. Pam3CSK4 (TLR2/TLR1 agonist), Pam2CSK4 (TLR2/TLR6 agonist), and the agonist combination were tested for growth inhibitory activity on bacterial isolates. The data were expressed relative to bacterial growth in the control (non-conditioned medium). The data are shown as means±SEM. *P<0.5 and **P<0.05

The antibacterial activity of canine AD-MSCs conditioned media prepared with Pam3CSK4 (TLR2/TLR1 agonist), Pam2CSK4 (TLR2/TLR6 agonist), or the agonist combination (Pam3CSK4 and Pam2CSK4) was assessed in broth microdilution assay. Conditioned media was diluted in the range between 1/2 to 1/128 and inoculated with bacterial suspensions. TLR agonist combination (Pam3CSK4 and Pam2CSK4) primed conditioned media expressed solid antibacterial activity against *E. coli* and *S. aureus* isolates at 1/4 dilution and *E. faecalis* isolate at 1/2 dilution. However, it was active in *P. aeruginosa* only as undiluted. The undiluted AD-MSCs' conditioned medium primed with Pam2CSK4 (TLR2/TLR6 agonist) inhibited the growth of *E. coli* and *S. aureus* isolates, but it was not expressed antibacterial activity against *E. faecalis* and *P. aeruginosa*. On the other hand, Pam3CSK4 (TLR2/TLR1 agonist) conditioned medium was not shown antibacterial activity in any of the study isolates.

Scanning electron microscopy was used to observe the effects of TLR agonist combination (Pam3CSK4 and Pam2CSK4) primed canine AD-MSCs conditioned medium at 1/2 dilution on *E.coli* cell morphology. After 4 h of incubation, bacterial cells exposed to the conditioned medium showed unusual morphological changes, as observed by the perturbation of membrane and pore formation, in contrast, the bacterial cell in untreated control had a buxom shape and smooth cell surface (*Fig. 3*).

Discussion

In this study, we investigated the antibacterial effect of TLR2/1 and TLR2/6 primed canine adipose tissue-

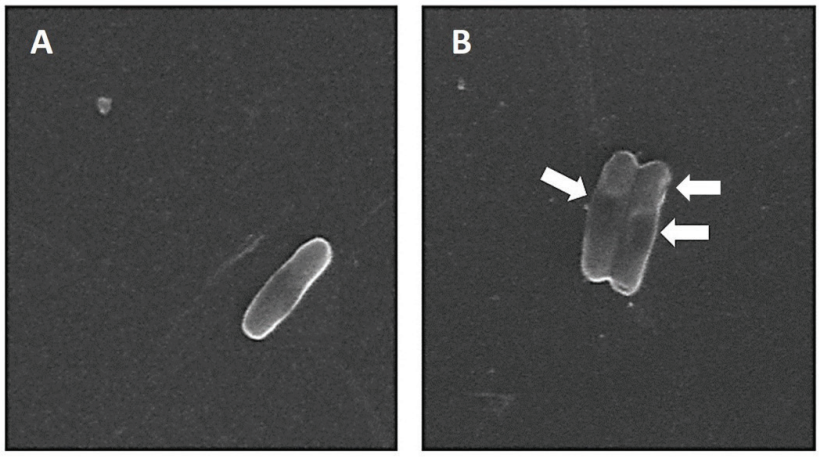

Fig 3. The effects of TLR agonist primed canine AD-MSCs conditioned medium on *E.coli* cell morphology were examined with SEM. A- Untreated control, B- Conditioned medium treated. Arrows indicate pore formation and membrane damage of bacterial cells

derived MSCs (AD-MSCs) on antibiotic-resistant bacterial pathogens. Priming with combined TLR agonists significantly increased the antibacterial activity of the AD-MSCs against multidrug resistant isolates of *S. aureus, E. coli*, *E. faecalis*, and *P. aeruginosa*.

Infections caused by multidrug-resistant (MDR) bacteria have progressively increased in recent years and exhibit a major health problem causing limited treatment options in humans and animals. One Health concept has been defined as the collaborative effort of multiple disciplines working locally, nationally, and globally to attain optimal health for people, animals, plants, and our environment. In this context, human, animal, and environmental health are interrelated and bound to each other; thus, MDR bacteria represent a significant threat to public health worldwide [19].

MDR pathogen-caused infections were accepted as epidemics in veterinary medicine, and indicate an important problem related to the transmission of these pathogens within veterinary environments [20]. Many published studies on this subject reported that the genera frequently associated with MDR infections in veterinary medicine contain Gram-positive and Gram-negative bacteria including methicillin-resistant staphylococci, vancomycin-resistant enterococci, and extended-spectrum beta lactamase (ESBL)-producing Enterobacteriaceae and *P.aeruginosa* [21-25]. Therefore, in this study, we selected four bacterial isolates; methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecalis*, extended-spectrum beta lactamase (ESBL) producing *E. coli* and carbapenem resistant *P.aeruginosa* which were identified as multidrug-resistant in antibiotic susceptibility testing *(Table 1)*.

With the growing problem of antibiotic resistance in veterinary pathogens, the demand for alternative treatment approaches results in several non-antibiotic strategies such as novel nanoparticles, phage therapies, and antimicrobial substances released from stromal cells [3,25]. In this scope, mesenchymal stem cells (MSCs) have been considered a reliable choice for scientists in the treatment of resistant bacteria [26].

Toll-like receptors (TLRs) for defending against micro-organisms are widely expressed by the immune system cells as well as other body cells such as epithelial cells, endothelial cells, fibroblasts, and MSCs. The expression levels of TLR1 through TLR6 are stable between the different types of MSCs, while the expression of TLR7, TLR8, TLR9, and TLR10 show variance. TLR2 alone senses bacterial peptidoglycan, in heterodimeric form with TLR1 (TLR2/TLR1) or TLR6 (TLR2/TLR6) binds to triacylated and diacylated lipopeptides, respectively [27].

Data from the literature have previously presented that pre-activation of MSCs with TLR agonists can enhance antimicrobial activity against different species of bacteria or bacterial biofilms through *in vitro* and *in vivo* conditions. In these studies, TLR activation is mainly produced by native bacteria itself or TLR3 and TLR4 ligands such as polyinosinic-polycytidylic acid (pIC) and Gram-negative bacterial lipopolysaccharide (LPS) [7,11,15,28,29]. In line with those studies, our results confirm that TLR activation is involved in MSCs' antibacterial activity.

We used two approaches to examine the antibacterial activity of conditioned medium after TLR2/TLR1, and TLR2/TLR6 priming of canine adipose-derived MSCs, namely bacterial growth assay and minimal inhibitory concentration determination. Our data from *in vitro* bacterial growth study indicate that conditioned medium from TLR2/TLR1 primed AD-MSCs has only shown activity for *E. coli* with slightly decreased growth relative to control (8.9%). However, conditioned medium from TLR2/TLR6 primed cells reduced the growth of *S. aureus, E. coli,* and *E. faecalis* by 11.2%, 29.3%, and 4.1%, respectively. Moreover, conditioned medium primed with TLR2/TLR1 and TLR2/TLR6 combination expressed a significant growth inhibition on *S. aureus, E. coli*, *E. faecalis,* and *P. aeruginosa* isolates with a range of 27.7%, 38.9%, 14.1%, and 13.0%, respectively *(Fig. 2)*. We obtained concordant results with the MIC study; 1/4 dilution of conditioned medium prepared with TLR2/TLR1 and TLR2/TLR6 combination was detected as MIC against *E. coli* and *S. aureus* isolates. However, *E. faecalis* isolate had MIC at 1/2 dilution of the same conditioned medium; and undiluted conditioned medium expressed as MIC for *P. aeruginosa* isolate.

Pezzanite et al.[11] demonstrated equine MSCs stimulated with TLR-3, TLR-4, and NOD-like receptor (NLR) agonists produce bactericidal activity against multidrug-resistant *S.aureus* both in planktonic and biofilm forms. Compared to other ligands, activation with TLR-3 agonist (polyinosinic:polycytidylic acid, pIC) was most effective in triggering antibacterial activity and immunomodulatory cytokine production. In our study, two different TLR ligands were used solely and in combination for MSC stimulation. The antibacterial activity of stimulated MSCs' was most prominent with ligand combination followed by TLR2/TLR6 agonist, than TLR2/TLR1 compared to unstimulated control cells. Moreover, conditioned medium from MSCs primed with combined agonists exhibited lower minimal inhibitory concentration levels against pathogens.

Johnson et al.[9] reported the mouse and canine MSCs primed with TLR-3 ligand (pIC) reduced *S. aureus* bacterial count in mice with chronic infection and also in dogs with wound infections compared to antibiotics or MSCs alone. Interestingly, TLR3-activated canine MSCs were found to be ineffective for the enhancement of antibacterial activity and detected antibacterial effects

associated with constitutively secreted antimicrobial molecules from MSCs. This could be associated with the pathogens involved; our results notably demonstrated the antibacterial potential for TLR-activated MSCs' varied between bacterial pathogens, such as *E.coli* vs *P.aeruginosa* which showed the 3-fold difference in growth inhibition rates after treatment (38.9% vs 13.0%) *(Fig. 2)*.

In a recent study [15], the effectiveness of allogenic canine MSCs primed with TLR3 ligand was investigated for the treatment of naturally occurring drug-resistant infections in dogs. Results showed that TLR3 priming of canine MSCs caused enhancement of macrophage bactericidal activity and increased both MSCs' migration capacity and IL-8 secretion involved in innate immunity. The *in vivo* part of the study revealed an improved microbiological response against pathogens (methicillin-resistant *Staphylococcus pseudointermedius, Proteus mirabillis,* and *P. aeruginosa*) detected in half of the treated dogs. The authors concluded that TLR3 priming of canine MSCs caused an increase in indirect antimicrobial properties, especially which is much more important clinically. This statement suggests that TLR activation of MSCs can produce more effective treatment results in the clinical setting rather than in laboratory experiments.

We take into account that the differences between the results of studies are likely to be associated with methodological variations such as cell-culture techniques, TLR agonist concentration, exposure time, tested bacterial targets, the source and the number of exposed MSCs, etc.

Electron microscopy was employed to observe alterations of treated *E. coli* cells. The effects of conditioned medium prepared with TLR2/TLR1 and TLR2/TLR6 combination on *E. coli* morphology were similar to that obtained by cationic antimicrobial peptides [30]. Treated bacterial cells have shown perturbation of cell membranes and pore formation *(Fig. 3)*.

The results of this study demonstrate that TLR2/TLR1 and TLR2/TLR6 primed canine adipose-derived MSCs conditioned medium has antimicrobial activity against multidrug-resistant isolates of Gram-positive and Gram-negative bacteria. Moreover, compared with the single agonist stimulation, the conditioned medium prepared with the combination of TLR2/TLR1 and TLR2/TLR6 agonists was more potent with up to 4-fold decreased MICs against studied pathogens. These results should be evaluated by performing *in vivo* testing in clinical models of multidrug-resistant infections.

Availability of Data and Materials

The data that support the findings of this study are available on a reasonable request from the corresponding author (B. Aksu).

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Ethical Statement

This study does not need ethical approval because experiments did not involve any animals or humans. Animal cells were purchased from a commercial company.

Conflict of Interest

The authors declared that there is no conflict of interest

Author Contributions

BA and TA conceived and supervised this study. BA and OY completed the main experimental content. BA and OY collected and analyzed the data. BA and TA wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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