Research Paper:
AK2 Antibacterial Synthetic Peptide Can Potentiate Macrophage Responses

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ABSTRACT

Background: Emphasis on substitutional medications with the elevated bacterial resistance to current antibiotics is pivotal. We evaluated the antibacterial effect of AK2 by Minimum Bactericidal Concentration (MBC) and Minimum inhibitory Concentration (MIC) and its impact on macrophage responses in 17 strains of pathogenic bacteria. The gene expression of macrophage's cytokines was evaluated. Accordingly, the bioinformatic assessment predicted this peptide's physicochemical characteristics, behavior, and structures. The present study aimed to assess the antibacterial effect of AK2 peptides on Macrophage responses.

Materials and Methods: Cytotoxicity level was assessed by MTT assay on the HeLa cell line. The hemolytic activity of peptides on red blood cells was evaluated. The Griess assay was performed to assess the amount of macrophage nitric oxide production. The real-time PCR method measured the iNOS, IFN-γ, and TNF-α gene expression in isolated macrophages.

Results: Peptide concentrations (13-60 µg/mL) were observed as the MBC and MIC value results for various bacteria. No remarkable cytotoxicity was observed at 30 and 60 µg/ml concentrations after 24h. iNOS, IFN-γ, and TNF-α gene expression were upregulated. There was also a higher secretion of nitric oxide in 48 hour-culture of the cell line with peptide. Great antibacterial activity was observed in some bacterial strains, particularly B. melitensis.

Conclusion: AK2 peptides display suitable antibacterial activity with negligible toxicity for host cells. This peptide could also stimulate macrophage responses through nitric oxide production and gene expression in proinflammatory cytokines.

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1. Introduction

Bacterial infections are the leading reason for human disorders, including cholera, diphtheria, illness [1, 2].

Resistance to antibiotics can arise via molecular alterations, general resistance in some pathogenic bacteria [3]. Some mutations and arbitrary use of antibiotics can cause drug resistance [4]. All efforts should be conducted to improve a novel set of antibiotics via considered targets. There are natural and general antimicrobial molecules in plants and animals identified as primary resources of antibiotics, e.g., antibacterial peptides, including defensins and cathelicidin. Thus, Antibacterial Peptides (ABPs) are a section of the immunity reported between all organisms. There are critical eukaryotic variations compared to prokaryotic organisms that could display options for antibacterial elements. These elements are potent, broad-spectrum antibiotics that demonstrate capacity as new medications. Antibacterial components are displayed to combat pathogenic bacteria [5].

The characteristics of these antibiotics include robust cationic features (pI= 8.9-10.7), resistance (100oC for 10 minutes), and fewer deleterious complications than antibiotics, more considerations paid to these peptides for medical aims [6]. The amphipathic shapes of ABPs can react with both hydrophilic and hydrophobic membrane sites. The executive manner of the antibacterial peptides is often reported on the bacterial membrane. The synthetic peptide attracts to the cell membrane owing to the negative charge. Thereby, the penetration of peptides to the cytoplasm and then disruption is observed [7]. Notably, phospholipids with negative charges can counteract the bacterial membrane. Several outcomes were reported following the interaction between phospholipid and antibacterial peptides, including the destruction of bilayer totality, the dropping of the transmembrane gradients, and pore making. Despite the primary general sources of ABPs, their role and longevity can be extended via the presentation of multiple sequences and chemical alterations of peptide ingredients [8, 9]. Accordingly, various studies reported a specific effect of these ABPs on immune responses. As a critical component of the innate immune system, Macrophages can exert functional roles through produced cytokines, such as gamma-interferon, inducible nitric oxide synthetic, and TNF-α (tumor necrosis factor) in exposure to pathogenic bacteria [10].

AK2 has the ABPs properties applied for this aim. Thus, this study evaluated the effects of AK2 ABP on macrophage responses and 17 pathogenic bacteria (Table 1).

2. Materials and Methods

The anti-bacterial effect of the designed synthetic AK2 peptide (C89H122N18O15S0 molecular formula & 1599.08 molecular weight) was assessed in 17 strains of pathogenic bacteria and the macrophage responses. AK-KAWLFGLFFGLW is the sequence of AK2 peptides synthesized by the Biomatik Company (Kitchener, Ontario, Canada). The frequency of 97.76% resulted from High-Performance Liquid Chromatography (HPLC) techniques and mass spectroscopy to assess the purity level (qualitatively & quantitatively).

Pathogenic bacteria

In the present study, total bacterial strains were purchased from the Microbial Bank of Pasture Institute of Iran, which has brought bellow:

Bacillus cereus (ATCC 14579), *Salmonella enteritidis* (ATCC 13076), *Escherichia coli* (ATCC 25922), *Serratia marcescens* (ATCC 14756), *Acinetobacter baumannii* (ATCC 19606), *Proteus vulgaris* (ATCC 8427), *Staphylococcus aurous* (ATCC 25923), *Vibrio cholera* (ATCC 39315), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus epidermidis* (ATCC 14990), *Nocardia brasiliensis* (ATCC 19247), *Klebsiella pneumonia* (ATCC BAA-1705), *Shigella sonnei* (ATCC 9290), *Brucella melitensis* (ATCC 23457), *Brucella abortus* (ATCC 448), *Enterobacter cloacae* (ATCC 23355), and *Enterococcus faecalis* (ATCC 29212).

The primary interpretation of the sequence of ABPs applied online by Anti-bacterial Peptide Database (http://aps.unmc.edu/AP/prediction/prediction_main.php) and ProtParam (http://www.expasy.org/tools/protparam.html). Other characteristics, such as hydrophobic moments, hydrophobicity, charge at usual pH, and the amphipathicity of idealized helices assessed using producing helical wheel diagrams, were certified by the server online (Heliquest) (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py). PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) was employed to predict the secondary structure. The server of AGADIR was applied to calculate peptide sequences (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html) [6].

Assessing MBC and MIC

The broth microdilution method was applied to evaluate the Minimal Inhibitory Concentration (MIC). Various concentrations at 3-200 mg/l with 1.6×107 CFU/mL of bacteria in the plates were stored for 15 h at 37 °C. Hence, the plates were measured by an ELISA reader at a wavelength of 540 nm (Table 2).

IC50 level

HeLa cells were treated in RPMI1640 medium (Gibco) containing 10% (v/v) fetal bovine serum (Biowest) in the flask and then incubated in 5% CO_2 and 95% at 37 °C.

The cells were incubated by trypsin-EDTA and adjusted 10^5 cells/mL, and 100µL of cell suspension was added to the plates. Therefore, the cells were kept in an incubator. AK2 peptide was placed in determining concentrations (1.6-100 µg/mL). The given supernatant was removed and treated with MTT solution (Mabtek, Sweden). The upper solution was removed, and 100 µL DMSO was dispensed to each well.

Hemolysis assessment

We assessed the hemolytic activity of AK2 peptides on red blood cells. The 20% of incubation solution was performed for 15 min at 37 °C. The dilution of the solution was performed to 10% using various concentrations of AK2 peptide. The supernatant absorption was read at 415nm of wavelength.

Nitric oxide production

The J774 cell line was obtained from the cell bank of the Razi Institute of Iran. The culture of cells was conducted in the RPMI-1640 medium. Then, the cells were detached (EDTA-trypsin; 0.25%) and centrifuged for 6 min at 450 g. Next, the cells were adjusted to $1×10^5$ cells/mL in RPMI-1640 medium. Then, 120µl of the suspension was added to the wells and treated with various concentrations of AK2 peptide (3-200 µg/L) and placed in a moisten incubator at 37 °C with a 5% CO_2 atmosphere. After 24, 48, and 96 h, the supernatant of the cells was gathered, and the nitric oxide production was evaluated by Griess assay quantitatively [5]. Briefly, macrophages ($1×10^5$) were cultured in the presence of Fetal bovine serum (FBS, Gibco, USA). Subsequently, the collection and analysis of supernatants were performed. The determination of oxidation product nitrite (NO) was measured quantitatively by Griess assay. Briefly, 50µL of cell supernatants were discarded and treated with an equal volume of Griess reagent (Sigma–Aldrich) at 25 °C temperature for 20 min. The absorbance was evaluated with a microplate reader at 540 nm. All samples were analyzed in duplicates.

Macrophages isolation from peripheral blood

Three healthy individuals were taken blood samples of 15cc voluntary based on the Helsinki protocol. The collected samples were diluted with 1/2 PBS and dispensed on the Ficoll-Hypaque density gradient. In this step, the buffy coat layer was isolated and washed twice with cold PBS after 12 minutes of centrifugation at 2000 g at 25°C. Then, macrophages were stained by trypan blue and counted by an inverted microscope. The mononuclear cells were cultured. Furthermore, macrophages were isolated by discarding the suspended cells. Macrophages are attached mononuclear cells and were stimulated by 100 µg/mL of AK2 peptide. Macrophages incubated with PBS were considered as opposing control groups.

RNA isolation and cDNA synthesis

Macrophages were plated on a 24-well plate with $1×10^5$ cells per well and exposed 24 hours later to AK2 peptide. The mRNA levels of inducible Nitric Oxide Synthase (iNOS), IFN-γ, and TNF-α were evaluated in macrophages treated with AK2 peptide. However, before that, The RNA was isolated based on the manufacturer's protocol (Mabtech, Sweden), and cDNA was synthesized based on the protocol isolation of the Mabtech kit. Then, the gene expression was assessed by the Real-time PCR method. The real-time PCR assay was conducted by 10µL master mix (Yekta Tajhiz, Iran) 1µL of each forward. It reversed primer, 1 µL of cDNA template, 8 µL doubled distilled water in a total reaction volume of 20µL on a QIAGENE Real-time PCR system (Germany).

Hypoxanthine-guanine Phosphoribosyltransferase (HPRT) was used as a housekeeping gene.

The primers applied in this study are brought as follow: Nitric Oxide Synthase (iNOS) F: 5' CAGTATCACAACCTCAGCAAGC 3', R: 5' GTCAGTTGGTAGGTTCCTGTTG 3', Optimum Tm: 59 °C.

IFN-γ F: 5' GCATCCCAGTAATGGTTGTCC 3', R: 5' TTGGAAGCACCAGGCATG 3', Optimum Tm: 53 °C.

TNF-α F: ACAGCAGAGGACCAGCTAAGAG 3', R: 5' TTCGAGAAGATGATCTGACTGC 3', Optimum Tm: 58 °C.

Hypoxanthine-guanine Phosphoribosyltransferase (HPRT): F: 5' AAG CTT GCT GGT GAA AAG GA 3', R: 5' TTG CGC TCA TCT TAG GCT TT 3'. Optimum Tm: 57°C. Relative expression was normalized to HPRT and expressed as mRNA relative levels. The cycling conditions included a hot start at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Data analysis was performed by GraphPad Prism using Analysis of Variance (ANOVA). The target gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and HPRT was used as an internal control for normalization. The obtained data were expressed as Mean±Standard Deviations, and $P<0.05$ was considered significant.

3. Results

In silico study

The number of amino acids in this peptide equals 14 (n=14). Molecular weight is 1684.054 Da, and Theoretical pI is 10. Net charge, hydrophobicity, and AKKAWLFGLFFGLW peptide are +2 (charge) and 0.02 (H), respectively. The PROB score is normalized, and the Instability Index (II) measurement is calculated to be 35.01. The result of hemolysis activity showed 0.56, and the Instability Index (II) is calculated to be 35.01.

The Aliphatic Index (AI) score is 90.71, and the Grand average of hydropathicity is 0.264. Furthermore, the helical wheel diagram demonstrates that polar amino acids are placed on one side and hydrophobic amino acids on the other side (Figure 1).

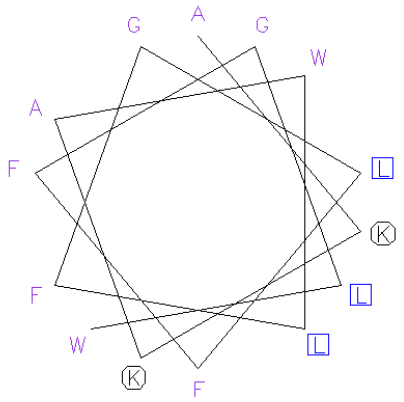

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Figure 1. The diagram of the Helical wheel of AK2 peptide

MBC and MIC assay

The Minimum Bacterial Concentration (MBC) and Minimum Inhibitory Concentration (MIC) were evaluated on seventeen pathogenic bacteria. The coolected results indicated a suppressor level between 12.5-40 µg/mL against antibiotic-resistant pathogenic bacteria. The lowest MIC and MBC were 12.5 µg/ml for *B. melitensis* and then 25 µg/mL for S. aureus.

Table 1. AK2 physicochemical properties

PROB Score	Boman Index (kcal/mol)	3D Structure	Method	Length	II	GRAVY	H	Aliphatic Index	Charge	pI	Mol wt
0.56	-1.62	Helix	NMR	14	35.01	0.929	-0.05	97.86	2	10	1684.054

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II: The Instability Index; GRAVY: Grand Average of Hydropathy; Mol wt: Molecular Weight; H: Hydrophobicity.

Table 2. MBC and MIC values of AK2 peptide

Bacteria	*S. enteritidis*	*B. cereus*	*E.coli*	*K. pneumonia*	*S. marcescens*	*A. baumannii*	*P. vulgaris*	*V. cholera*	*P. aerugi-nosa*
MIC (µg)	30	30	30	30	40	40	40	40	40
MBC (µg)	60	60	60	60	-	-	-	-	60

Bacteria	*S. aureus*	*S. epidermidis*	*N. brasiliensis*	*Sh. Sonei*	*B. melitensis*	*E. faecalis*	*E. cloacae*	*B. abortus*
MIC (µg)	25	30	40	30	12.5	30	25	30
MBC (µg)	50	60	-	60	25	60	50	60

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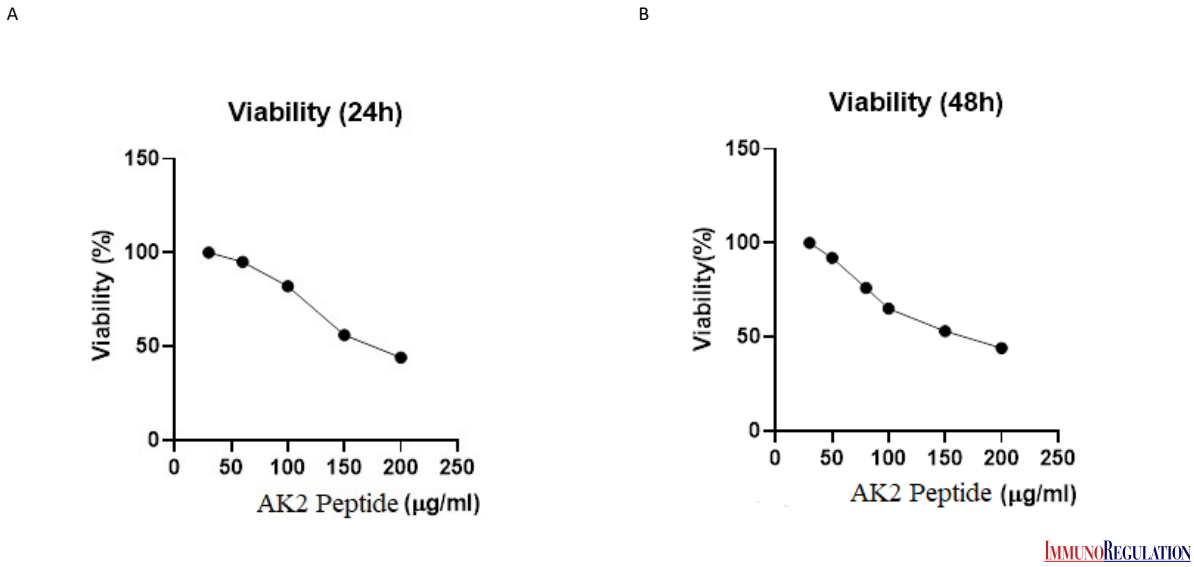

Figure 2. Cytotoxicity level was assessed by MTT assay on HeLa cells

A, B: The cell was cultured for 24 and 48h. A. There was no remarkable cytotoxicity at 30 µg/mL and 60 µg/mL concentrations after 24h (P=0.36). The cell viability was 82% after 24h (P=0.78). B. There was no remarkable cytotoxicity at 30 µg/mL and 50 µg/mL (P=0.09). The cell viability was 76% after 48h (P=0.22).

Cytotoxicity assay

The toxicity function of AK2 on HeLa cells was studied with the MTT method. The dose-dependent findings of the technique were brought after 24 and 48h (Figure 2-A, B). There was no remarkable cytotoxicity at 30 µg/mL and 60 µg/mL concentrations after 24h (P=0.36). The rare cytotoxicity of this peptide was recorded at 100 µg/ml concentration. The cell viability percentage was 82 after 24h (P=0.78). No remarkable cytotoxicity was observed at 30 µg/mL and 50 µg/mL concentrations after 48h (P=0.09). Low cytotoxicity was observed at 80 µg/mL concentration, cell viability being 76% after 48h (P=0.22).

Hemolysis activity on red blood cells

The effect of Hemolytic of AK2 peptide on human erythrocytes was assessed. The hemolytic activity result displays slight hemolysis (2.4%) at this peptide's 100 µg/mL concentration (P=0.96). However, the MICs evaluated of AK2 peptides are lower than the hemolysis dose.

Nitric oxide secretion

Macrophages (J774 murine cell line) were applied to assess the secretion of nitric oxide. The production of nitric oxide was evaluated with multiple concentrations of peptide (0-100 µg/mL) during three incubation times (24, 48,

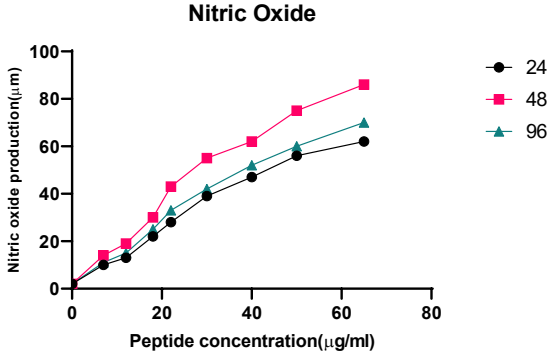

Figure 3. Griess method was performed to determine nitric oxide production at multiple concentrations of AK2 after 24, 48, and 96h

Results demonstrate maximum nitric oxide production in 48 h culture of the cell line with peptide (P=0.43). P<0.05 was considered significant.

and 96 hours) (Figure 3). Findings demonstrate the highest nitric oxide production after 48 h incubation time (P=0.43).

Gene expression

iNOS

The Result of iNOS expression in macrophages treated with 60 µg/mL AK2 (MQ+AK2) suggested a significant elevation (3.2 folds), compared to the control group (P<0.0001). Moreover, treatment with 60 µg/mL AK2 indicated a remarkable increase in iNOS expression versus the Macrophage group (P<0.0001) (Figure 4).

TNF-α

The result of TNF-α demonstrated a remarkable increment (2.6 folds) in the MQ+AK2 (60 µg/mL) group, compared to the PBS control group (P<0.0001). Besides, TNF-α mRNA expression revealed a significant increase in MQ+AK2 (60 µg/mL) versus the macrophage group (P<0.0001) (Figure 5).

IFN-γ

The result of the IFN-γ mRNA level showed 2-fold increments in the MQ+AK2 (60 µg/mL) group versus the control group (P<0.0001). Additionally, there was a significant elevation in the MQ+AK2 (60 µg/mL) group vs. the PBS control group (P<0.0001) (Figure 6).

4. Discussion

ABPs with specific properties can be desirable in incubating drug-resistant micro-organisms [11, 12]. In this study, AK2 peptide with AKKAWLFGLFFGLW sequence was assessed to evaluate its antibacterial function against 17 strains of antibiotic-resistant bacteria. Accordingly, we attempted to know what effect this peptide could have on the macrophage response.

The findings of MBC and MIC were 13-60 µg/mL. E. cloacae and S. aureus showed the susceptibility to a 25 µg/ml concentration; however, *Sh. Sonei*, *S. epidermidis*, *P. aeruginosa*, *E. faecalis*, *E. coli*, *B. cereus*, *K. pneumonia*, and *B. abortus* were suppressed at 30 µg/mL of concentration. Additionally, the best MIC results belonged to B. melitensis. The challenges of drug resistance in studied bacteria, particularly B. melitensis, may suppress the medications. It can be a suitable option to control these infections.

Furthermore, the inhibition and killing effect of AK2 peptide was demonstrated on B. melitensis (12.5 µg/mL & 25 µg/mL, respectively) [13]. Moreover, several studies reported that A. baumannii and P. aeruginosa could exhibit some resistance to medications by β-lactamase gene and efflux manner [14, 15]. The collected findings indicated the susceptibility of studied bacteria in exposure to AK2 peptides, especially brucellosis. As per mul-

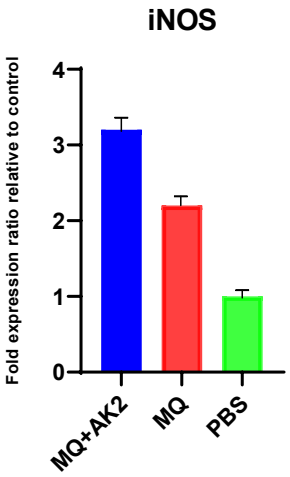

Figure 4. iNOS gene expression

Fold change of iNOS gene expression was evaluated using AK2 antimicrobial peptide. The mRNA level of iNOS increased 3.2 folds in MQ+AK2 groups compared to the PBS control group. They were normalized to HPRT as the housekeeping gene.

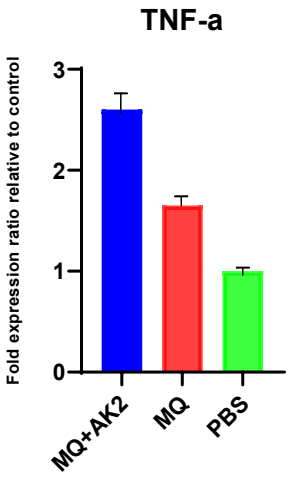

Figure 5. TNF-α gene expression

Fold change in TNF-α gene expression was evaluated using AK2 antimicrobial peptide. The mRNA level of TNF-α increased 2.6 folds in MQ+AK2 groups compared to the PBS control group. It was normalized to HPRT as a housekeeping gene.

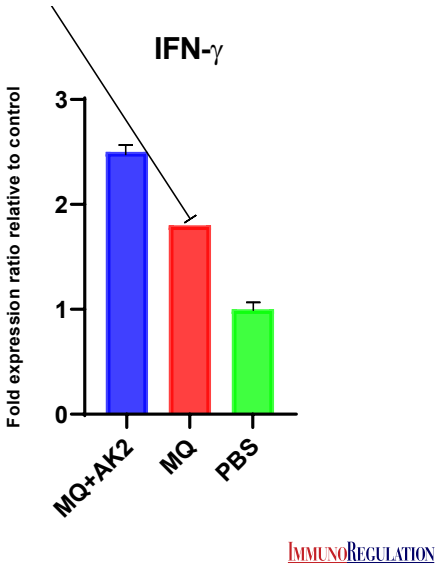

Figure 6. IFN-γ gene expression

Fold change in IFN-γ gene expression was evaluated using AK2 antimicrobial peptide. The mRNA level of IFN-γ increased 2 folds in MQ+AK2 groups, compared to the PBS control group. It was normalized to HPRT as a housekeeping gene.

tiple similar reports, the cationic anti-bacterial peptides have suppressor effects on infectious diseases [7, 16-19].

According to the cytotoxicity findings obtained of AK2 peptide, we found that this peptide could not show remarkable cytotoxicity at 30 µg/mL and 60 µg/mL concentrations. There was a slight cytotoxicity effect at a 100 µg/mL dose. A percentage of 82 was observed about cell viability after 24h. No remarkable cytotoxicity was observed at 30 µg/mL doses and 50 µg/mL after 48h. There was 76% cell viability after 48h. in treatment with a dose of100µg/ml of AK2 peptide has been shown a few hemolysis effects (2.4%) on RBCs was. This concentration is also higher than MICs evaluated for 17 strains of pathogenic bacteria. While Zardini and their colleagues indicated HD50 at 100 µg/ml concentration and 3% lysis effect on erythrocytes [20]. Additionally, Moosazadeh Moghaddam et al. assessed the hemolytic activity of anti-microbial peptide (CM11) on red blood cells in the MIC range and showed 10% cytotoxicity at 64 mg/L concentration [21].

Mare and colleagues demonstrated L-118, E60, and RI-118, as three fabricated peptides can display leishmanicidal function against L. major and L. donovani [22]. Furthermore, Zardini et al. demonstrated that the anti-bacterial function of Mastoparan-s against microorganisms is effective. Moreover, the Minimum Inhibitory Concentration (MIC) levels of Mastoparan were 14.9-27.6. µg/mL for bacterial and 18.9-25.1 µg/mL for fungal pathogens [20]. The finding obtained from the MIC value of AK2 peptide compared to other MIC results in scientific research indicates a close range of MIC for AK2 versus mastoparan-S. Meanwhile, the MIC value of AK2 is upper than HHP1, CM11, di-WP2 antimicrobial peptides [20, 21, 23].

Accordingly, iNOS gene expression was increased after treating with AK2 antimicrobial peptide. The present study findings revealed that AK2 peptides could stimulate macrophage responses against the studied pathogenic bacteria. Some studies indicated that iNOS could be activated by IFN-γ cytokine and reinforced to eliminate infection [24]. Thereby, elevating iNOS gene expression can be considered in promoting innate immune responses by macrophages. J.Locht et al. evaluated iNOS gene expression in the J774 cell line, and they found that iNOS was increased after 24hrs treating with silver nano-material [25]. In this regard, the mRNA level of IFN-γ and TNF-α in macrophages treated with AK2 peptide were evaluated. Our results demonstrated considerable upregulation in gene expression of IFN-γ and TNF-α cytokines in the presence of AK2 peptide. We found that AK2 can stimulate macrophage responses to counteract against bacterial pathogenic through expressing pro-inflammatory cytokines. To our knowledge, IFN-γ cytokine can promote antigen presentation to T cells, enhance TNF-α and iNOS production, stimulate phagocytosis, and burst oxidative [26-28].

Furthermore, TNF-α is an inflammatory cytokine that is produced by macrophages. This cytokine can kill the microbial pathogenic via activating phagocytosis and burst oxidative by macrophages [29, 30]. Thereby, the AK2 peptide has suitable potency for stimulating macrophage responses. AK2 may affect anti-bacterial activities and macrophage responses due to aromatic and positive charge amino acids via functional cytokines, i.e., IFN [31].

The current scientific project revealed that the synthetic anti-bacterial peptide has a suitable capacity to affect pathogenic bacteria, and it could be applied in future in vivo studies. Furthermore, nitrite oxide is evaluated in macrophages to determine bactericidal capacity [32]. This research indicates the suitable capacity of the synthetic AK2 in the nitric oxide stimulation, which the highest secretion was recorded at 48 h post-incubation. Nitric oxide is a potent anti-microbial factor released by macrophages and removes pathogenic bacteria disregarding their strains [33, 34]. Park et al. evaluated thalidomide activity on nitric oxide secretion and reported that thalidomide could considerably suppress the secretion of nitric oxide [32]. We assessed the activity of small

cationic peptides on multiple pathogenic bacteria, and these results could demonstrate the bactericidal effects on pathogenic bacteria [35-37]

5. Conclusion

The present study demonstrated that short cationic AK2 peptide presents inhibitory and bactericidal activities against pathogenic bacteria, especially B. melitensis. Accordingly, AK2 peptides could potentiate macrophage responses through stimulating nitric oxide production and increasing mRNA levels of IFN-γ, TNF-α, and iNOS by macrophages.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflicts of interest

The authors declared no conflict of interest.

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