Single dose eradication of extensively drug resistant *Acinetobacter* spp. in a mouse model of burn infection by melittin antimicrobial peptide

Fatemeh Pashaei, Parvaneh Bevalian, Reza Akbari, Kamran Pooshang Bagheri

**ARTICLE INFO**

**Keywords:**
- Third degree burn infection
- *Acinetobacter* spp.
- Extensively drug-resistant
- Melittin
- Eradication
- Mouse model

**ABSTRACT**

Bacterial infections caused by antibiotic resistant bacteria are the leading cause of morbidity and mortality after burn injuries. This issue has driven the need for promising antimicrobial drugs to eradication of bacterial pathogens. Accordingly, we aimed to determine the therapeutic value of melittin, as a natural Antimicrobial peptide (AMP), in eradication of extensively drug-resistant (XDR) *Acinetobacter* spp. on a mouse model of third degree burn infection. Melittin killed all examined XDR isolates at 4 μg/mL up to 3 h. Melittin caused significant fluorescence release from XDR isolates at the minimum dose of 0.062 μg/mL. Vesicle formation on the membrane and squeezing of bacteria followed by cell lysis indicated the membranolytic effect of melittin. Melittin at 32 μg/mL completely eradicated the colonized XDR bacteria on infected burn mice during 2 h. No toxicity was observed on injured or healthy derma, as well as circulating Red Blood Cells (RBCs) in the examined mice. Potent promising antibacterial activity of melittin and the lack of toxicity at the therapeutic dose can clarify that melittin can be implemented as a topical drug lead in a preclinical trial of third degree burn infections.

1. Introduction

Mortality due to burn infections caused by antibiotic resistant bacteria are of significant value concerning to tracing for a new potent promising agent. Global statistics of death associated burn infections indicates a vital necessity to discovery or designing of new antimicrobial agents as well as implementation of already known antimicrobial peptides (AMPs) [1–4].

Reference to the report of American burn association in 2015, 486,000 persons burned and more than 16,000 deaths have been recorded [5]. In Europe, 2000 to 2900 cases per 10,000 people have being severely injured annually because of burn accident [6]. Estimates of the incidence of burn injuries in India are variable from 100,000 to 2 million per year, among them 100,000 cases were estimated to be fatal [7]. Burn is considered as the thirteenth cause of death in Iranian population [8]. In South Africa, about 200,000 people suffer from burn injury annually [9].

The burned wound area can easily be infected due to the release of nutrients from the necrotic and disrupted cells [1]. In addition to the extent and nature of thermal damage, the amount of bacteria entered into the wound is a critical parameter in the intensity of burn wound infection [10]. The loss of defensive barriers critically vanish cellular immune system that lead to colonization of bacteria on burn surface area [11].

DeLeon et al. estimated that 75% of the mortality following thermal injuries has been directly related to infections [12]. MDR strains are responsible for treatment failure in the burn wound infections that would be led to sepsis and death [13]. The most common bacteria that infect burn wounds are *Acinetobacter baumannii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* [1,14]. In a study in USA burn centers, among Gram-negative pathogens, *P. aeruginosa*, *A. baumannii* and *Enterococcus* spp. were the most common causes of burn infections respectively. In China, *A. baumannii*, *P. mirabilis*, and *P. aeruginosa* were the most common causes of burn infections respectively. In Europe, *P. aeruginosa* and *Escherichia coli* were common pathogens among gram negative bacteria as reported by Branski et al. [15].

Based on Bang et al. report, during a 9 year study, among 166 patients suffering from burn associated bloodstream infections, 23.5% of the patients were died [2,16]. In a case–control study of burn patients in America that acquired *A. baumannii* bloodstream infections demonstrated that infected patients had an overall mortality of 31% [4]. Based on conducting studies between 2000 and 2010 in Iran, on average,
about a quarter of the hospitalized patients had been died from burn infections [17–19]. In other study in USA, a total of 31 (13%) out of 247 patients with acute burn injuries acquired multi-resistant A. baumannii during the period between December 1998 and March 2000 [20]. Among 2684 samples that were cultured from 452 burn patients admitted to a burn center in Singapore between 2011 and 2013, 984 (36.7%) were A. baumannii [21]. In a study conducted in Turkey, among 169 burn infections A. baumannii with the incidence rate of 21% were the most common isolated bacteria [22].

A. baumannii is causative agent of urinary tract, bloodstream, lung, skin and soft tissue infections too [23–25]. The ability of A. baumannii to survive in harsh environmental conditions due to biofilm formation is also the principal reason for its high incidence rate in burn infections [26].

In the last decade, colistin, carbapenems, and tigecycline resistance has significantly increased in A. baumannii bacteria [27,28]. The infections caused by MDR strains could be led to remarkable increasing in mortality or prolonged hospitalizations as well as increase in treatment costs [29]. Combination therapy has been also used for treatment of these infections that lead to prolonged patient survival although it cannot guarantee the complete eradication of bacteria [30]. Doripenem is a newly marketed carbapenem that currently used in Europe and North America but its efficiency is under question since doripenem resistant isolates have been recently reported [28,31].

Many reports of MDR A. baumannii have been documented in different geographical regions and territories like Europe, North America, Argentina, Brazil, China, Taiwan, Hong Kong, Japan, and South Pacific [32–41]. Beside intrinsic capability of A. baumannii to develop antibiotic resistance, excessive and inappropriate use of antibiotics led to increase in bacterial resistance. Concerning to many aforementioned unsolved problems, this period is called the end of the antibiotic era [29,42,43]. In this critical condition, AMPs would be new promising agents to overcome this threatening challenge [44].

Antimicrobial peptides are produced by multicellular organisms and act as a primary defense system against microbial infections. They also have the ability to regulate the immune system as immunomodulator molecules or act as an anticancer agent as well [45,46]. Antimicrobial peptides have a great therapeutic potential and may be a good alternative to antibiotics [47], or act as a good synergistic antibiotic as well [48–52]. In some cases, the AMPs are transferred themselves into the cytoplasm and interfere to replication, transcription, and translation [53].

During the past two decades, many AMPs have been discovered from natural resources including humans, amphibians, insects, plants and etc [54,55]. Among them, a series of drug candidates have been tested in different phases of clinical studies including lociexas™ against infections of diabetic foot ulcers, cefilavancin developed for skin bacterial infections (III) and pneumonia (I), NVB302 against Clostridium difficile infections, C16G2 for dental caries, MDL-63, 397 (Dalvance) for treatment of osteomyelitis and pneumonia, LTX-109 (Lytixar) for treatment of impetigo, (Lactoferrin) PXL01 for prevention of post-surgical adhesion, and LL-37 to enhance healing of venous leg ulcers [54].

The advantages of some AMPs are their wide range of activities and their high potency as well [49,56]. These issues are considerable advantage for their future application in human infections. Inversely, toxicity is the main disadvantage of antimicrobial peptides [57]. Many potent AMPs like Melittin, Thionin, and LL-37 are toxic so that their future clinical applications are questionable [58–60]. This issue is a hard challenge regarding application of AMPs as promising drugs. But based on our hypothesis, toxicity of antimicrobial peptides could not be a limitation factor in treatment of third degree burn infections since in this kind of injuries, all layers of skin are degenerated.

Here we showed that the treatment of burn infection with a toxic AMP would be an applicable strategy to eradication of bacteria. Among toxic antimicrobial peptides, melittin was selected as a good candidate to prove this hypothesis. Melittin is a toxic antimicrobial peptide from bee venom. It is a cationic hydrophobic peptide that consists of 26 amino acids [47].

Concerning to the major concern of global and local antibiotic resistance, this study was aimed to eradication of XDR A. baumannii infection in a mouse model of third degree burn infection by employing antimicrobial peptide, melittin.

2. Materials and methods

2.1. Reagents, media, bacterial isolates, and animals

Cefazidime, levofloxacin, doripenem, ciprofloxacin, doxycycline, cefepime, gentamycin, and piperacillin-tazobactam discs were purchased from MAST Company (UK). Antibiotic powders including doripenem and colistin were obtained from Sigma–Aldrich Chemie Co. (Germany). Muller hinton broth (MHB) and agar (MHA) were purchased from Merck (USA).

Fifty samples of Acinetobacter spp. were used from a previous study. These isolates had been collected from burn patients during the period between November 2016 and March 2017 from Shahid Motahari burn hospital, Tehran-Iran. According to the routine laboratory methods, the isolated bacteria characterized as Acinetobacter spp. in the hospital. The bacteria transferred to the laboratory, characterized as Acinetobacter spp. again by routine differential method for further confirmation, stored in storage media containing 30% glycerol, and maintained in −20 °C until use. The reason for collection of 50 bacteria was confirmation of the antibacterial value of the antimicrobial peptide, melittin, on many bacterial isolates. Acinetobacter baumannii ATCC 19,606 was used as standard strain in all antibacterial assays otherwise indicated.

In vivo experiments were performed according to the regulations of the EU directive 2010/63/EU for animal experiments. All in vivo experiments were approved by ethical committee of the Pasteur Institute of Iran (authorization number: IR.PIL.REC.1396.12). All experiments were performed in triplicate and presented as mean ± Sd.

2.2. Peptide synthesis

Melittin was ordered to an external facility (Mimotops Co. Australia) in 98% purity. The peptide was synthesized using solid phase method. The purified peptide injected to RP-HPLC again to control the purity. The accuracy of synthetic peptide was controlled by mass spectrometry on a triple quad LC/MS instrument (Sciex API100 LC/MS instrument, Perkin Elmer Co., Norwalk, CT, USA). Briefly lyophilized purified peptide resuspended in 0.1% acetic acid and injected at 0.1 mL/min. The peptide ionized in positive mode and run at 140 V for 5 min.

2.3. In vitro antibacterial activity

2.3.1. Disc diffusion

Bacterial suspensions were prepared by spectrophotometry at 625 nm. Reference to the 0.5 McFarland standard, the optical density in the range of 0.08–0.1 is equivalent to 1.5 × 10⁸ CFU/mL. In this study, to increase the accuracy of quantification, OD of the suspension was considered at 0.09. Based on CLSI recommendation, disc diffusion assay was performed for cephazidime, levofloxacin, doripenem, ciprofloxacin, cefepime, doxycycline, gentamicin, and piperacillin-tazobactam [61,62].

2.3.2. Determination of MIC, MBC, MIC/MBC, MIC50/MIC90, and MBC50/MBC90

Bacterial suspensions were prepared as detailed above. Microdilution assay was performed based on CLSI recommendation [62] to determination of Minimum Inhibitory Concentration (MIC) of melittin. Briefly, melittin was serially diluted in MHB medium (ranging
8–0.0625 μg/mL) in a 96 well polystyrene microplate, the numbers of 1.5 × 10^5 bacteria were added to each well, and then incubated at 37 °C for 16–20 h. MIC for colistin and doripenem were determined in the same way as performed for the peptide. In order to determination of MBC for melittin, colistin, and doripenem, a sample volume was subcultured on MHA medium and the MBC defined as the concentration that killed all the examined bacteria.

According to results, the isolates were classified to MDR and XDR based on the definitions agreed by Magiorakos et al. [63]. The GM of MIC for melittin was determined and compared with that of colistin and doripenem using student’s t-test. The ratio of MIC/MBC, MIC50/MIC90, and MBC50/MBC90 were calculated ultimately.

2.4. Study of the mechanism of melittin on XDR isolates

Having a better view on antibacterial activity of melittin on XDR strains, inhibition and killing kinetics of melittin were evaluated as well as killing efficiency of the peptide based on dose and time dependency. Among 50 Acinetobacter spp. isolates, six XDR isolates were detected by antibacterial susceptibility tests. Since, XDR isolates are more dangerous than the others; those six XDR isolates were selected for further studies.

2.4.1. Inhibition kinetics assay (IKA)

This novel assay was designed to monitor the inhibition kinetics of melittin. Time dependency of melittin in growth inhibition of examined bacteria was evaluated by IKA at the dose of MIC. According to our experimental experience, at OD value of 0.065 that recorded by spectrophotometry at 625 nm, the wells have a turbidity that can be visualized by visual inspection. This OD value was used as a cutoff point of inhibition in inhibition kinetics of melittin.

To perform this assay, the bacterial suspensions of six XDR isolates and one standard strain (A. baumannii ATCC 19,606) were prepared as outlined above. Briefly, melittin was serially diluted (ranging 8–0.06 μg/mL) in a 96-well polystyrene microplate, the numbers of 1.5 × 10^5 bacteria were added to each well, and then incubated at 37 °C. In this assay, the growth of bacteria was measured by determination of OD of the suspension at 625 nm during 24 h by a microplate spectrophotometer (EPOCH, BioTek Co., USA). The ODs were recorded at the definite time points i.e. 0.5, 1, 2, 3, 4, 5, and 24 h after incubation.

2.4.2. Killing kinetics assay (KKA)

Killing efficiency of the peptide was evaluated in a series of doses at different time periods to determine the killing kinetics. This assay was simultaneously performed with IKA.

The examined doses were the same as IKA. Ten μL from each well was collected at the definite time points i.e. 0.5, 1, 3, 5, and 24 h, and cultured on MHA, and incubated at 37 °C. The numbers of all resultant colonies were recoded after 18–24 h.

2.4.3. Calcein release assay

To evaluate the effect of melittin on bacterial cell membrane, fluorescence release assay was performed by employing calcein-Am according to the protocol described by Akbari et al. [47]. Six XDR isolates and one standard strain (A. baumannii ATCC 19,606) were included in this assay.

Briefly, the bacteria were prepared as mentioned above and 1.5 × 10^5 cells from each isolate were incubated with calcein-AM (Calcein-Acetoxy Methyl Ester) (1 μL from 2 mg/mL solution) and incubated at 37 °C for 1 h.

Following dye uptake, calcein-AM (a non-fluorescent derivative of calcein) is cleaved by cytoplasmic esterases to form the fluorescent dye, calcein. The calcein can leak from the cells only if their membranes are damaged. The calcein leakage was monitored at 0, 0.5, 1, 2, 3, 4, 5, and 24 h after incubation with the peptide at excitation and emission at 496 and 517 nm, respectively. The calcein release was served as a marker for membrane damage and the trend of release was evaluated separately for each of isolates and compared together as well.

2.4.4. Morphological evaluation by field emission-scanning electron microscopy

One XDR isolate (as a sample among six XDR isolates) and one standard strain (A. baumannii ATCC 19,606) was selected to study the membranolytic effect of melittin on the examined bacteria.

The samples were prepared based on Memariani et al.’s protocol with slight modification [64]. The bacteria were cultured in MHB and incubated at 37 °C for 18–24 h. Bacterial cells were centrifuged at 7000 rpm for 5 min and washed three times with PBS (pH 7.4). A bacterial suspension equal to 10^6 CFU was incubated with a definite dose of melittin (XDR: 0.125 μg/mL; ATCC: 0.25 μg/mL) for 30 min and 1 h and centrifuged at 1180 g for 10 min. The pellet was gently mixed with PBS (pH 7.4) and centrifuged at 1180 g for 10 min. This step was repeated one more time. The soft pellet was fixed with 2.5% glutaraldehyde (100 μL) in 0.1 M PBS (pH 7.4) at room temperature for overnight in a dark chamber. After these steps, the fixed samples were centrifuged at 1180 g for 10 min and washed two times in distilled water. The pellet was resuspended in distilled water and placed on the FE-SEM slides and dehydrated in a series of graded ethanol sequentially (30%, 50%, 70%, 90%, and 100%). Finally, the dehydrated cells were allowed to dry under the room temperature. The prepared slides were coated with gold nanoparticles through an automatic sputter coater and visualized using a FE-SEM instrument (JSM-7610, JEOL Co., Japan and MIRA3, TESCAN Co., Czech).

2.5. Setting up the mouse model of third degree burn

The back surface area of the examined Balb/c mice (male, 25 g, 6–8 weeks old) was cleaned by ethanol (70%) and shaved, one day before examination. The mice were deeply anesthetized by 40 μL of ketamine-xylazine solution (at ratio of 90/10, Woerden Co., The Netherlands), and placed on a sterile place. Anesthesia was verified by showing no pain reflexes. The deep anesthetized mice were controlled during the experiments and if it was necessary, anesthetic solution was injected again.

To set up third degree burn in mice, different conditions, i.e. temperature, source of temperature, the time of heat transfer from applicator to skin, and the kind of metals in applicators, were examined to accomplish the best condition.

Homemade metal applicators consisted of pure iron with surface area of 0.785 cm² were used to burn the mice back area for 10 and 20 s. To selection of the source of temperature, at first, the applicators were heated in boiling water up to 100 °C. A gas flame was used as another source of temperature to heating the applicator up to 100 and 105 °C. The experiments were performed under sterile condition using a class II safety cabinet to avoid any kind of environmental contaminations.

The criteria to diagnosis of third degree burn in our study were three major following signs including alteration of skin color from white to brown, ruffling on the periphery of burn area, and skin roughness. Pathological study was conducted on skin biopsies to verify our visual inspection criteria.

2.6. In vivo antibacterial assay on mouse model of third degree burn

Antibacterial efficiency of melittin on one XDR Acinetobacter spp. isolate and one standard strain (A. baumannii ATCC 19,606) was evaluated in a mouse model of third degree burn infection. To reaching this goal, a mouse model of burn infection was developed on Balb/c mice.

Before starting antibacterial assay, the numbers of attached bacteria on burn area must be determined. In this case, a novel in vivo adhesion assay was developed to determine the real attached bacteria. After examining the potency of melittin, the tissue sections were
homogenized and cultured on MHB to control the bacterial eradication by melittin.

2.6.1. In vivo adhesion assay

Bacterial suspensions of the XDR isolate and standard strain were prepared in MHB as detailed above. A suspension (20 μL) containing 1.5 × 10^5 bacteria was inoculated on burn wound area, as detailed above, and incubated for 1 h. After that, the wound area was washed by MHB (20 μL) media three times. Then, 20 μL of MHB media was placed on the wound area and the surface was scratched softly by a sterile scalpel. This intervention was repeated three times. In each step, the collected fluid was transferred into MHB (1 mL). Twenty μL from this suspension was cultured on MHA and the resulting colonies were counted after 18–24 h. The numbers of attached bacteria in this group was used as control in antibacterial assay.

2.6.2. Antibacterial assay

At first, adhesion protocol was performed as detailed above. The burn area was washed by 20 μL MHB media three times. Then, different doses of melittin (i.e. 8, 16, and 32 μg/mL) were incubated on infected area for 2 h. The area was scratched, washed, and cultured on MHB as described before in adhesion assay. Colony count was performed after 18–24 h. Doripenem was used as control to compare its efficiency with melittin.

2.6.3. Eradication assay

To verify the complete eradication of bacteria, the burn tissue was removed and chopped to small segments by a scissors and transferred into MHB (1 mL). The suspension was vortexed for 30 s and 20 μL of this suspension was cultured on MHA for colony counting. To ensure the complete eradication of bacteria on burn area, the media containing segmented skin tissue was kept in incubator for 24 h. Clear appearance of the suspension after 24 h was indicated as reliable eradication.

2.7. Blood bacterial burdens and survival rate

To improve the rapid eradication of bacteria and disapproving any possibility of bacterial entrance to the blood stream, blood samples were taken each day and incubated on blood agar at 37 °C for 18–24 h. All the examined mice were monitored for one month in terms of survival rate.

2.8. In vivo toxicity assays

To ensure the safe topical application of melittin, its possible toxicity on burn skin area was evaluated by pathological study. To further confirmation regarding the safe application of melittin, the possible entrance of melittin into the mouse blood stream was evaluated using in vivo hemolysis assay.

2.8.1. Dermal toxicity of melittin

After induction of third degree burn, melittin at the doses of 16 and 32 μg/mL were inoculated on burn area for 2 h. Then, a tissue section was taken and fixed in buffered formalin (10%, pH 7), and after tissue processing stained with Hematoxylin and Eosin (H&E). Tissue sections of the third degree burn mice incubated with normal saline was considered as negative control. The sections in test groups were evaluated for any kind of alterations in comparison to negative control group. Melittin at 32 μg was incubated on normal shaved skin in control group to control the possible toxicity of melittin in case of unintentional exposures.

2.8.2. In vivo hemolysis assay

Blood samples were taken from test group (three mice) before and after incubation of melittin (32 μg/mL) on burn injuries. The same volume of blood samples were taken from negative control group (three mice). The blood samples centrifuged at 1180 g for 5 min and the absorbance of supernatants was read at 540 nm in a microplate spectrophotometer (EPOCH, BioTek Co., USA) [65].

3. Results

3.1. In vitro antibacterial activity

3.1.1. Determination of antibiotic resistance pattern

The examined strains were showed a wide verity of sensitivity patterns against cephtazidime, levofloxacine, doripenem, ciprofloxacine, cepemine, doxycycline, gentamicin, and pipracilin/tazobactam. Six strains were resistant to all of tested antibiotics and classified as XDR.

3.1.2. The frequency distribution of antibiotic resistance in Acinetobacter spp. isolates

The highest resistance was recorded against CAZ and CPM. The incidence rate of resistance against the examined antibiotics was ranked as CAZ > CPM > CIP > DOR > GM > PTZ > LEV > DXT. The frequency distribution of antibiotic resistance was showed in Fig. 1.

3.1.3. Determination of MIC, MBC, MIC/MBC, MIC50/MIC90, and MBC50/MBC90 for melittin, colistin, and doripenem in Acinetobacter spp. isolates

The highest frequency of melittin sensitive isolates was showed at 0.5 μg/mL. The frequency of sensitivity for melittin was ranged from 0.125 to 2 μg/mL. The highest frequency of resistance against inhibitory activity of doripenem and colistin were seen at 32 and greater than 32 μg/mL, respectively. The frequency of resistance against inhibitory activity of both doripenem and colistin were ranged from 2 to greater than 32 μg/mL (Fig. 2).

The highest frequency of melittin sensitive stains was showed at 1 μg/mL. The frequency of sensitivity for melittin was ranged from 0.125 to 4 μg/mL.

The frequency of resistance against bactericidal activity of doripenem and colistin were ranged between ‘1 to greater than 32 μg/mL’ and ‘4 to greater than 32 μg/mL’, respectively (Fig. 3). The GM (geometric mean) of MIC for melittin, colistin, and doripenem were 0.66, 17.11, and 20.6, respectively. In comparison, there was a significance difference between GM of MIC for melittin with that of colistin and doripenem (p value < 0.05).

According to the results gathered from all antimicrobial assays, 88 and 12% of the isolates were classified as MDR and XDR, respectively. MIC/MBC, MIC50/MIC90, MBC50/MBC90 were determined for melittin, colistin, and doripenem and demonstrated in Fig. 4.
3.2. Inhibition and killing kinetic assay

Kinetic of the growth inhibition of XDR isolates and ATCC strain by melittin showed that OD of the suspensions were similar to negative control in all time points. The results indicated the complete growth inhibition conducted by melittin. This inhibition sustained until 24 h.

The KKA showed that all isolates were killed by melittin at MBC dose during 30 min in exception of XDR1, XDR2, and standard strain (A. baumannii ATCC 19,606) that they were eradicated after 3 h (Fig. 5).

3.3. Calcein release assay

Calcein release activity of melittin at the doses of 8 μg/mL were measured on six XDR isolates and one standard strain (A. baumannii ATCC 19,606) by fluorometry. According to the results, the significant calcein release was seen at 8 μg/mL of melittin against abovementioned bacterial isolates or ATCC strain in comparison to other examined doses during 24 h. XDR3 had maximum release comparing to other strains (Fig. 6).

3.4. Field emission-scanning electron microscopy

Analysis of the effects of melittin on standard strains of A. baumannii (ATCC 19,606) at the concentration of 0.25 μg/mL showed squeezed, vesiculated, and degraded donut shape bacteria after 30 min incubation. Sixty min incubation of melittin at 0.125 μg/mL (MIC dose) on standard strain showed more effective events like donut shape cells, disruption, and large central pore in bacteria. Melittin at MIC dose induced aggregation, cell lysis, membrane disruption, and vesicle on XDR Acinetobacter spp. isolate during 30 min of incubation. After 60 min, vesiculated cells, free vesicles, and cell lysis, were showed (Fig. 7).
3.5. Setting up third degree burn model

After performing different conditions to optimization of third degree burn induction, the temperature of 105 °C and incubation time of 10 s were the conditions in which all three layers of derma and surface layer of fat tissue were burnt (Fig. 8b) comparing to normal control group (Fig. 8a).

3.6. In vivo activity of melittin on burn infection in balb/c mice model

3.6.1. Adhesion assay

The burn wounds were infected with one XDR Acinetobacter spp. isolate or one standard strain (A. baumannii ATCC 19,606). The numbers of attached bacteria were counted after 2h. The results showed that 179,200 bacteria from XDR isolate and 144,000 bacteria from standard strain existed on the burn area, respectively. These numbers were considered as a fixed value in our calculations to determine the true numbers of remaining bacteria after incubation with different
3.6.2. Antibacterial and eradication assay

Melittin at the amounts of 8 and 16 μg/mL were able to kill ‘94.65 and 93.3%’ of XDR isolate and standard strain (A. baumannii ATCC 19,606) whereas the amount of 32 μg/mL eradicated all of the attached bacteria. The remaining tissue was incubated in MHB and the clarity of the medium after 24 h confirmed the complete eradication of bacteria (Fig. 9). Linear regression analyses showed the linear trend of killing by melittin on XDR isolate and standard strain, respectively ($R^2_{XDR} = 0.82$, $R^2_{ATCC} = 0.92$).

3.6.3. Blood bacterial burdens and survival rate

Blood culture was taken each day from the examined mice. No bacteria were seen. All the examined mice were monitored for one month in terms of survival rate. All the examined mice were alive after one month.

3.7. In vivo toxicity assays

3.7.1. Dermal toxicity

No toxicity was observed in melittin treated normal group (Fig. 10A). Comparison of melittin untreated burn group with melittin treated burn group indicated that melittin induced no further toxicity on burn area (Fig. 10B and C). Melittin at the dose of 32 μg was incubated on the shaved skin in normal mice. No toxicity was found after 2, 24, 48, and 72 h in comparison to untreated normal skin (Fig. 10 D1-D4).

3.7.2. In vivo hemolysis assay

The results indicated no hemolysis in the collected plasma from melittin treated and untreated burn groups confirmed by spectrophotometric assay.

4. Discussion

Bacterial infections in burn patients are the major cause of mortality and morbidity yet [44]. During the recent years, some efforts led to presentations of a few AMPs as promising agents to combat local and systemic infections [44,48] but the treatment of bacterial burn infections is a major concern yet [1,10]. This issue is attributed to antibiotic resistance mechanisms as a defense reaction of bacteria to antibiotic pressure. From the advent of antibiotics till now, the issue of antibiotic resistance is a terrible term for the patients. Many documented testimonials confirm that some chemical based antibiotics have come to end due to distribution of multiple drug resistant bacteria [42,43].

In this vital condition, AMPs have opened a new horizon to eradication of MDR or XDR bacteria. In this regards, some pharmaceutical companies have introduced their novel AMPs drug leads to clinical trials but at the moment no AMP drug has been suggested to cure third degree burn infections yet [54].

Concerning to this vital necessity, here we represent melittin as a potent AMP to overcome the issue. In this regards, our study focused on evaluation of therapeutic value of melittin on eradication of MDR or
were ranged from 0.125 to 2 μg/mL at the concentration of 8 μg/mL. Melittin had a rapid inhibitory or killing kinetics. The results showed melittin in inhibition or killing the bacteria. Our results demonstrated that melittin has the greatest inhibitory activity than all those examined AMPs. This issue was also seen for bactericidal activity of melittin. Mishra et al. designed a series of 10 residues AMPs among which L10 had the greatest antibacterial activity against Acinetobacter baumannii isolates (5 cases) (ranging 1.95–7.95 μg/mL). This comparison demonstrated that melittin has about 3.9–15.6 fold more potent than L10 [67]. Comparing the inhibitory activity of D16 peptide derived from Lactoferrin with melittin showed that geometric mean of MIC for melittin (0.6 μg/mL) had about 9.6 fold greater than D16 (5.88 μg/mL) [68]. This issue indicated that melittin can affect an infected site more efficiently than an engineered peptide, in this case D16. Feng et al. showed that the engineered peptide derived from LL-37 and K530 had the greatest inhibitory activity on five MDR and one ATCC strains of A. baumannii at geometric mean of 12.8 μg/mL (ranging 8–16 μg/mL) in comparison to the parent peptides [69] whereas in our study, melittin inhibited the growth of 50 MDR and XDR Acinetobacter spp. isolates at GM of 0.6 μg/mL. This comparison demonstrated that melittin was about 21.3 fold more potent than K530. In other study performed by Conlon et al. some peptides derived from Brevinin-2 (B2-RP). Among them, D4K B2-RP had the greatest inhibitory activity at GM of 2.4 μg/mL (1.12 μg/mL) on MDR isolates of A. baumannii while melittin showed antibacterial activity about 1.86 fold greater than D4K B2-RP [70]. Comparing the geometric mean of melittin in our study with GM of 6 frog skin-derived AMPs including [G4K]XT-7 (4.8 μg/mL), [E4K] altysercin-1c (1.6 μg/mL), PGLa-AM1 (10.13 μg/mL), B2RP-Era (6.13 μg/mL), CPF-AM1 (9.2 μg/mL), and [D4K]B2RP (2.5 μg/mL) used in the study of Conlon et al. showed that the inhibitory activity of melittin was 8, 2.6, 16.8, 10.2, 15.3, and 4.1 fold more potent than the abovementioned AMPs, respectively [70]. Vila-Farres et al. examined the antibacterial activity of mastoparan and indolicidin on 27 A. baumannii clinical isolates and reported their MIC ranging 1–16 μg/mL and 4–32 μg/mL, respectively while the MIC for melittin on our isolates were ranged from 0.125 to 2 μg/mL. This comparison indicated that the melittin was 8 and 16–32 fold more potent than mastoparan and indolicidin, respectively. Vila-Farres did not obtained the complete removal of two examined A. baumannii even after 24 h in killing kinetic assay while melittin induced 100% removal of six XDR isolates up to 3 h [71].

To have a deep view on the events that lead to eradication of bacteria, IKA and KKA were performed to determine the velocity of melittin in inhibition or killing the bacteria. Our results demonstrated that melittin had a rapid inhibitory or killing kinetics. The results showed that almost all examined XDR bacteria were killed during 30 min and incubation for 3 h could guarantee the complete eradication.

Reference to the results obtained by calcein release assay, melittin at the concentration of 8 μg/mL induced the significant release of calcein from all XDR isolates and standard strain as well. The results indicated that melittin can affect the bacterial membrane and made the gradual release of calcein in a time dependent manner.

FE-SEM analysis indicated a time dependent activity of melittin on both examined bacteria. The existence of many free vesicles in the environment and also observation of large pores indicated that melittin molecules concentrate on the membrane. This accumulation phenomenon would be described by this fact that N-terminal and central regions of melittin are highly hydrophobic and after attachment of one melittin, the other one can bind via non-covalent hydrophobic bond easier than the previous one and vice versa. This accumulation effect would be synergistically assisted by other melittin molecules to increase the velocity of membrane disruption or pore formation. Comparing the results of KKA with observation of death in the examined bacteria after 60 min demonstrated a rapid critical effect of melittin on Acinetobacter spp. isolates.

In this study, third degree burn was successfully set up on a BALB/c mouse model. It should be pointed to this note that in spite of standard definition of third burn degree in which just three layers of derma are injured; our experiments showed that the surface layer of fatty tissue is injured too.

Since the number of bacteria after incubation on injured area was moderately increased, this fact indicated that primary colonized bacteria were multiplied during incubation. As some bacteria were seen in washing solution, this finding showed that all multiplied bacteria are not able to adhere to the injured surface. This fact may be due to the limited space to attachment of all bacteria. In burn centers or emergency ward of hospitals, washing and debridement are standard procedures after admission of the burn patients. Based on our results, washing is not able to remove all attached bacteria. In terms of debridement, according to our experience in scratching and washing of burn area, many bacteria removed but some of them remain intact. This insufficiency in removing all bacteria would be led to growth of remaining bacteria and subsequent infection as well. Thus, application of efficient antimicrobial peptides, in this case melittin, is suggested in washing solution to disinfection of burn area. This suggestion is being supported by rapid in vitro killing kinetics of melittin and also in vivo eradication of all colonized bacteria during 2 h.

According to the results obtained from in vivo activity and eradication assays, melittin at the dose of 32 μg/mL successfully eradicated all colonized bacteria on burn infected area with no in vivo toxicity on burn skin and circulating RBCs as well.

Ostorbazi et al. applied an antimicrobial peptide, A3-APO, intramuscularly, on mouse model of burn infection injected with A. baumannii BAA-1805 strain. They failed to eradicate the bacteria in their three implemented methods [72]. In comparison, melittin was able to fully eradicate the incubated XDR Acinetobacter spp. isolate just during 2 h after topical incubation. Oddo et al. showed that among their engineered peptides, BP214 had the best activity on colistin susceptible and –resistant A. baumannii isolates at 3.25 and 13.75 μg/mL, respectively. Our results indicated the superior MIC value of melittin in comparison to BP214 [73]. Saugar et al. claimed that the four cecropin A-melittin (CA-M) peptides had MICs ranging 1.1–2.5 μg/mL against all tested Acinetobacter baumannii strains whereas in our study the MIC of melittin against 50 clinical isolates collected from burn infections ranged from 0.125 to 2 μg/mL [74].

Comparing the microscopic demonstration of both melittin treated and untreated groups confirmed that melittin did not promote toxicity on burn injured derma. This issue demonstrated that a toxic peptide with potent antibacterial activity could be applied in a necrotic area to treatment of bacterial infections. Moreover, this suggestion could be supported by this fact that melittin did not make any toxicity on normal derma in the periphery of injured area in our experiments. Our pilot toxicity assay confirmed that melittin is not entered to blood circulation since no in vivo hemolysis was showed.

In conclusion, potent antibacterial activity and induction of no toxicity at the examined dose clarify that melittin can be suggested as a promising topical drug lead to eradicate the colonized MDR or XDR Acinetobacter spp. bacteria in the third degree burn infections.
Contributions

F.P and P.B performed all experiments and contributed in writing the manuscript. F.P and P.B contributed equally to this work. R.A contributed in in vitro assays. K.P.B contributed in experimental design and also in writing and revision of the manuscript. The idea for application of melittin in eradication of bacteria in third degree burn infections belongs to the corresponding author, K.P.B.

Conflicts of interest

The authors declare that they have no conflict of interest.

Funding

The present study was self-funded.

References


