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In vitro antimicrobial activity of alpha-melanocyte stimulating hormone against major human pathogen *Staphylococcus aureus*

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ABSTRACT

Alpha-melanocyte stimulating hormone (α-MSH) is an endogenous anti-inflammatory peptide reported to possess antimicrobial properties, however their role as antibacterial peptides is yet to be established. In the present study, we examined in vitro antibacterial activity of α-MSH against *S. aureus* strain ISP479C and several methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *S. aureus* strains. Antibacterial activity was examined by varying several parameters, viz., bacterial cell densities, growth phase, pH, salt concentration, and temperature. Antibacterial activity was also examined in complex biomatrices of rat whole blood, plasma and serum as well as in biofilm form of bacteria. Our results showed that α-MSH possessed significant and rapid antibacterial activity against all the studied strains including MRSA (84% strains were killed on exposure to 12 μM of α-MSH for 2 h). pH change from 7.4 to 4 increased α-MSH staphylocidal activity against ISP479C by 21%. Antibacterial activity of α-MSH was dependent on bacterial cell density and independent of growth phase. Moreover, antimicrobial activity was retained when α-MSH was placed into whole blood, plasma, and serum. Most importantly, α-MSH exhibited antibacterial activity against staphylococcal biofilms. Multiple membrane permeabilization assays suggested that membrane damage was, at least in part, a major mechanism of staphylocidal activity of α-MSH. Collectively the above findings suggest that α-MSH could be a promising candidate of a novel class of antimicrobial agents.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common causes of hospital- and community-acquired infections and the main threat to human health is the emergence of resistant *S. aureus*. The increasing resistance in staphylococci has created a need for the development of new antimicrobial agents and new targets for antibiotic therapy is currently of high priority in all over the world.

The best candidates among many possibilities are natural antimicrobial peptides which are abundant in nature and which have been conserved throughout evolution from bacteria to mammals as effective defense tools [48]. Most of these peptides are cationic, but they differ considerably in other characteristics such as size, structural motifs and presence of disulphide bonds [20,21]. They are typically produced by immune and barrier cells, neutrophil and epithelial cells [1,4,36]. They generally exert their antimicrobial activities through either formation of multimeric pores in the lipid bilayer of the cell membrane [27,28] or interaction with DNA or RNA after penetration into the cell [3,6,39]. Due to their overall positive charge, they bind preferentially to negatively charged bacterial membranes rather than to mammalian cell membranes that have neutral charge [26,32]. A very important feature of antimicrobial peptides is that they rarely induce bacterial resistance [48]. Therefore, these peptides are promising candidates for a new class of antibiotics.

One such antimicrobial peptide is alpha-melanocyte stimulating hormone (α-MSH), an endogenous linear tridecapeptide with potent anti-inflammatory effects [11,19,35]. The sequence of this neuropeptide is: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. It inhibits the activation of the transcription factor NF-κB and thus regulates several proinflammatory cytokines, stimulates the production of anti-inflammatory IL-10 and reduces upregulation of intercellular adhesion molecule 1 (ICAM-1) [2,30,37]. The widespread distribution of this peptide and its receptor in many barrier cells like keratinocytes, fibroblasts, melanocytes and in various immune cells including neutrophils,

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monocytes and macrophages suggests that it has a potential role in host defense [9,30,31]. A recent study suggests that both α-MSH and its C-terminal sequence (Lys-Pro-Val (α-MSH (11–13)) have rapid in vitro antimicrobial activity against a wide spread of organisms including, *S. aureus*, *Escherichia coli*, and *Candida albicans* [10,16,22]. It was shown that the antimicrobial activity of C-terminal analogue of α-MSH (Lys-Pro-$_D$-Val), which is a more potent anti-inflammatory peptide, did not differ from that of original C-terminal sequence (Lys-Pro-Val) [10]. Moreover, antibacterial activity of C-terminal analogue was not dependent on the presence of a cationic Lys residue but dependent on Pro and Val residue [10].

Studies have revealed that candidacidal effect of α-MSH might be mediated through the induction of cyclic adenosine monophosphate (cAMP) however its antibacterial mechanism is poorly understood. The purpose of this study was to examine the in vitro antibacterial activity of α-MSH against *S. aureus* including MSSA and MRSA in different micro-environmental conditions, e.g., bacterial cell densities, growth phase, pH, salt concentration, and temperature. Antibacterial activity was also examined in complex biomatrices of rat whole blood, plasma and serum. Study had also been extended to find the antimicrobial activity of α-MSH against *S. aureus* biofilms. In addition, the integrity of the *S. aureus* plasma membrane was investigated to delineate its bactericidal activity. Finally, the efficacy of the combination of this peptide with conventional antibiotic ampicillin was also evaluated.

2. Materials and methods

2.1. Antimicrobial peptides

α-Melanocyte stimulating hormone (α-MSH) and gramicidin D were purchased from Sigma–Aldrich (St. Louis, MO). The purity of α-MSH was 97% and the concentration of α-MSH was determined spectrophometrically (Cary 100 Bio/Varian), using molar extinction coefficient at 280 nm of $6.65 \times 10^3\ M^{-1}\ cm^{-1}$.

2.2. Microbial strains

A well-characterized methicillin sensitive *S. aureus* strain (ISP479C) was used in the study [39]. Nineteen *S. aureus* isolates (MSSA, 10; MRSA, 9) identified from soft tissue specimens obtained from patients admitted to the All India Institute of Medical Sciences, New Delhi, India, were also included in the study. The isolates were single-patient, non-duplicate strains. All staphylococci were identified by standard biochemical tests and confirmed as *S. aureus* by detection of *femB* gene using multiplex polymerase chain reaction (PCR) [23].

The isolates were then stored at −70 °C in 15% (v/v) glycerol until subcultured onto Brain heart infusion (BHI, Himedia Laboratories, India) agar plate for further analysis. Cultured cells were collected by centrifugation, washed with PBS buffer (10 mM Na–K phosphate buffer having 150 mM NaCl, pH 7.4) and adjusted to the desired final inoculum by spectrophotometry (OD_{600}) as appropriate. Amount of dead bacteria was estimated microscopically using LIVE/DEAD *Bac*Light Bacterial Viability Assay™ Kit (Invitrogen, USA).

2.3. Staphylocidal activities

Susceptibility to oxacillin was determined using cefoxitin (30 μg) disc diffusion method [12] and oxacillin (6 μg/ml) agar screening method. *mecA* gene multiplex PCR assay [23] was used for confirmation of methicillin resistance.

To determine the staphylocidal activities of α-MSH, mid-logarithmic-phase *S. aureus* cells (10^3–10^6 cfu/ml) were exposed to peptide at various concentrations as described elsewhere [46]. At selected time points, aliquots were plated on BHI agar and were incubated at 37 °C for overnight. Survival of *S. aureus* was determined by quantitative counting of the colony and was expressed as mean percentage of survival vs. non-peptide-treated control (set at 100% survival). Each experiment was performed at least three times in triplicate independently.

2.3.1. Staphylocidal activities in blood matrices

The antimicrobial activities of α-MSH in rat whole blood, homologous plasma and serum fractions were assessed. Fresh rat blood was collected in presence of anticoagulant (0.75% (w/v) final concentration of sodium citrate) to prepare whole blood and plasma biomatrices. Plasma was then separated as supernatant after low centrifugation ($210 \times g$) of whole blood for 10 min [47]. To prepare serum, remaining rat blood was collected in absence of anticoagulant and allowed to clot at room temperature. The clot was removed with a sterile swab and the suspension was centrifuged at $300 \times g$ for 10 min to collect serum supernatant [47]. Staphylocidal activities were performed as discussed earlier, in whole blood (85%, v/v), plasma (85%, v/v) and serum (85% and 20%, v/v).

2.3.2. Staphylocidal activities of α-MSH in biofilm model

The membrane disrupting staphylocidal activities of α-MSH and gramicidin on sessile (biofilm) form of staphylococcus were assayed using the polystyrene biofilm model described by Heilman et al., with slight modifications [29]. Overnight grown cultures in trypticase soya broth (TSB, Himedia Laboratories, India) were centrifuged and re-suspended in saline. Bacterial suspension at 10^4 cfu/ml were subsequently dispensed equally (100 ml/well) in 96-well polystyrene plates, surface modified using corona discharge (Corning, USA). The plates were incubated for 1 h in a cell culture incubator at 37 °C, 80% relative humidity for surface adherence. Non-adherent cells were removed by an automated aspiration device and 100 μl of a 20-fold diluted TSB (in PBS) was added to each well. Plates were incubated for predetermined time periods (9–12 h) to allow similar concentration of sessile bacteria (approximately 10^8 cfu/ml) for each strain/plate. Similar concentration of planktonic bacteria was also dispensed in sterile microfuge tubes. The media was then replaced with 100 μl saline containing either α-MSH (1.2×10^{-5} M), or gramicidin (25 μg/ml) or control (saline blank). The plates were incubated for 2 h and supernatants replaced with chilled saline. Triplicate wells from each set were then subjected to (i) LIVE/DEAD *Bac*Light Bacterial Viability Assay™ Kit (Invitrogen, USA) (ii) serial dilution and plating on BHI agar plates.

2.3.3. Microscopic evaluation of bacterial (membrane) viability

Bacterial viability and membrane integrity was analyzed using the LIVE/DEAD *Bac*Light Bacterial Viability Assay™ Kit according to the protocol suggested by the manufacturer. Briefly, the supplied stains: SYTO 9 (for live cells) and propidium iodide (for dead cells) were diluted 1:100 and 1:200 respectively in deionized water and 5 μl from each set was added to each well. The plates were incubated for 15 min in dark at room temperature and supernatant from each well were aspirated off. The plates were fixed upside down on the stage of a Nikon upright fluorescence microscope model 80i equipped with water immersion objectives and individual wells (single field) viewed using 60× objective sequentially using fluorescence setting for FITC (green) and Cy-3 (red) filters respectively, followed by phase contrast and bright field settings. Images were acquired using an integrated cooled CCD digital camera (model Evolution VF, Media cybernatics, USA) and analyzed using image ProPlus version 5.0 software (Media cybernatics, USA).

2.4. Membrane permebilization

S. aureus membrane permeabilization following peptide exposure was measured using flow cytometry, fluorescence technique and two strategic fluorochromes: release of preloaded calcein and uptake of propidium iodide (PI). Both fluorochromes were purchased from Sigma–Aldrich (St. Louis, MO).

2.4.1. Release of calcein

Calcein acetoxymethylester (calcein AM) is a nonfluorescent derivative of calcein that is lipid soluble and therefore can readily diffuse across cell membranes of *S. aureus* to load the bacterial cell. Once within the cytoplasm of bacterial cells, calcein AM is hydrolyzed by cytoplasmic esterases, yielding the fluorescent derivative calcein (excitation and emission wavelengths of 494 nm and 517 nm, respectively). *S. aureus* cells were first loaded with calcein (2 μg/ml) as described elsewhere [33] and then calcein-loaded *S. aureus* cells were exposed to peptides. At the predetermined sample times, duplicate aliquots were removed. One sample was analyzed for the release of preloaded calcein via flow cytometry (BD FACS Calibur) as a measure of membrane permeabilization; second sample was used for the assessment of staphylocidal activity (see above). A total of 10,000 cells were acquired for each flow cytometry analysis. Cells at or above a threshold of 10 fluorescence units (FL1 units) were considered to have retained calcein, indicative of an intact cytoplasmic membrane; those cells exhibiting <10 FL1 units were interpreted to have lost calcein as a result of α-MSH induced membrane permeabilization. Gramicidin D (25 μg/ml) was used as positive control. Experiments were repeated at least three times independently on separate days. Differences in levels of membrane permeabilization were defined as the percentages of difference in cell fluorescence units between peptide-treated and control samples.

2.4.2. Uptake of propidium iodide

Propidium iodide is a membrane non-permeable dye. It does not give any fluorescence until it is bound to nucleic acid (DNA). When the bacterial cell membrane is rendered permeabilize, PI enters and interacts with cellular DNA and can be detect through spectrofluorimeter.

The PI uptake assay was performed according to the method described elsewhere [44]. *S. aureus* cells were grown in BHI broth to mid logarithmic-phase (OD_{600} = 0.5) and cells were harvested by centrifugation at 5000 rpm for 10 min, and washed once with PBS buffer pH 7.4. The cells turbidity were adjusted to OD_{600} = 0.5 in PBS buffer which correspond to 10^8 cfu/ml, Cells were diluted up to 10^3 cfu/ml using PBS buffer, and incubated with PI (2 μM) at 37 °C for 20 min. After incubating the cells with PI, cells were exposed to α-MSH (1.2×10^{-5} M) at 37 °C. Cells were also exposed with gramicidin D (25 μg/ml). Gramicidin-treated *S. aureus* cells were used as positive control (maximum PI-fluorescence intensity), since gramicidin kills the bacteria through membrane disruption [33]. PI fluorescence was measured at every 60 min intervals for 3 h at excitation and emission wavelength of 544 nm and 620 nm respectively using Shimadzu RF-5301 PC spectrofluorimeter.

2.5. Statistical analysis

All killing experiments were performed in triplicate and repeated in three independent experiments in different days and were plotted as mean ± SD. Rest of the assays were performed as three independent experiments on different days and were plotted as mean ± SD. Statistical analysis (multiple comparison among data sets) was performed using one-way ANOVA using Minitab™ [10]. A p-value ≤0.05 was considered significant. Linear regression analysis was performed to compare the relationship between % of calcein leakage and % of killing by using Microsoft Excel software's statistical program.

3. Results

3.1. Staphylocidal activities

To examine the staphylocidal activity of α-MSH, killing assay was performed in PBS buffer (10 mM Na–K phosphate buffer having 150 mM NaCl, pH 7.4). Three *S. aureus* strains (10^3 cfu/ml) ISP479C, 510MSSA and 508MRSA were treated with broad range of concentration of α-MSH (1.2×10^{-9} M to 1.2×10^{-5} M) for 60 min and 120 min respectively. Percentage of survival vs. control was presented in Fig. 1a–c. α-MSH exhibited time and concentration dependent killing for all the strains. For example, for ISP479C strain, 1.2×10^{-8} M α-MSH killed 15.5 ± 6.0% and 21.9 ± 4.0% cells in 1 h and 2 h respectively whereas 1.2×10^{-5} M α-MSH exhibited 49.2 ± 5.0% and 62.0 ± 7.0% killing in 1 and 2 h respectively (p = 0.001 for 1 h and $p < 0.001$ for 2 h respectively). With respect to 510MSSA and 508MRSA strains killing by α-MSH was extremely efficient. On incubation with 3×10^{-6} M α-MSH for 1 h, the reduction in %survival compared to control was 92.6 ± 4.2 ($p < 0.001$) for 510MSSA strain and 85.5 ± 12.1 ($p < 0.001$) for 508MRSA strain respectively. Proportion of dead bacteria for all non-peptide-treated control *S. aureus* cells was less than 5% (Fig. 7c).

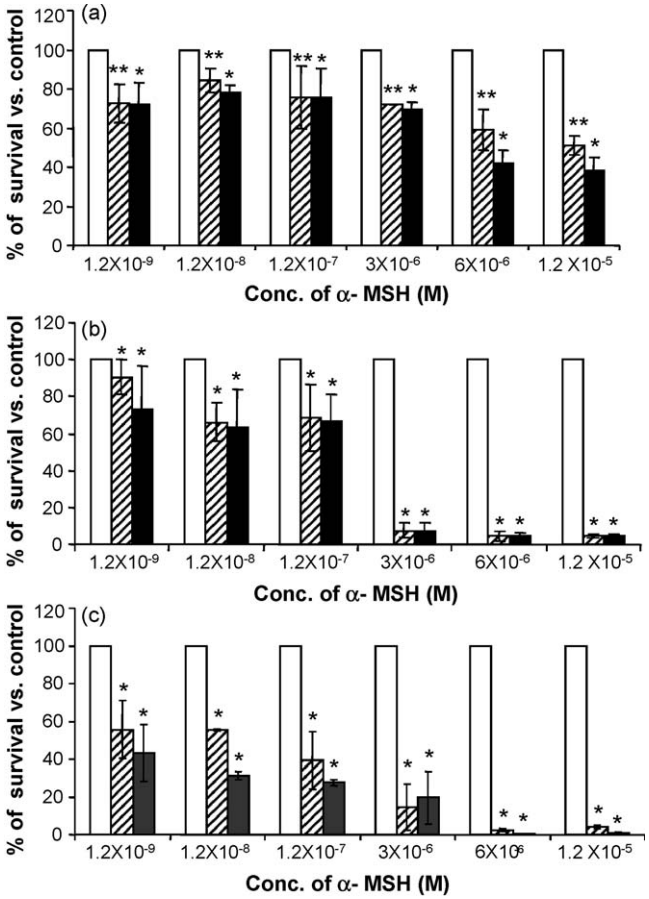


Fig. 1. Killing activity of α-MSH against three different logarithmic phase *S. aureus* cells after 60 min and 120 min of administration. (a) ISP479C, (b) 510MSSA and (c) 508MRSA. These data represent the means (±SD) of three independent experiments. Symbols: 0 min (open); 60 min (dashed); 120 min (filled). $^{*}p < 0.001$, $^{**}p = 0.001$, compared to PBS control.

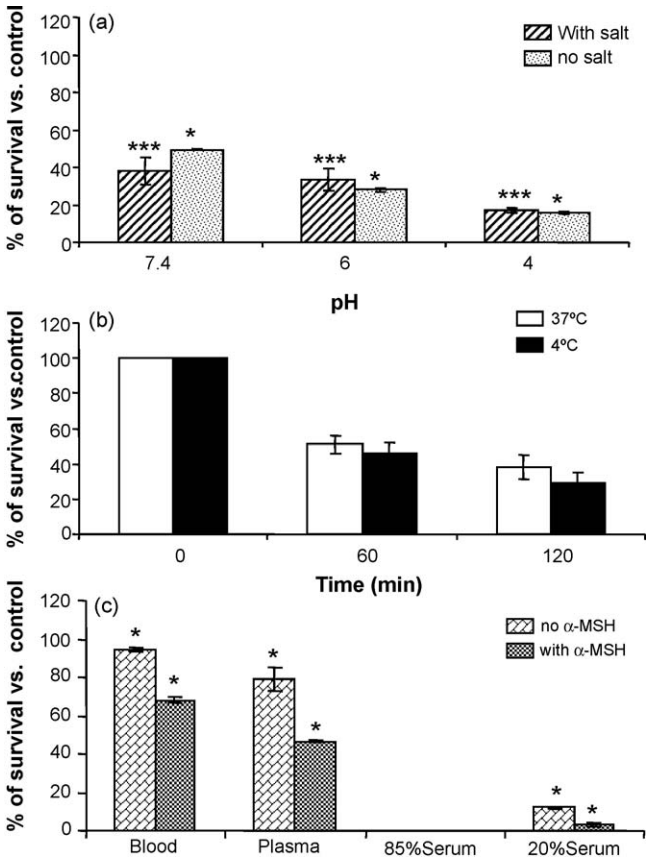


Fig. 2. Killing activity of α-MSH against ISP479C strain in different microenvironment. (a) Logarithmic phase *S. aureus* cells were exposed to 1.2×10^{-5} M α-MSH for 2 h at different pH in presence and absence of 150 mM NaCl; $^{***}p = 0.05$ comparing data at different pH in presence of salt, $^{*}p < 0.001$ comparing data at different pH in absence of salt. (b) Logarithmic phase *S. aureus* cells were exposed to 1.2×10^{-5} M α-MSH for 2 h at different temperature (4 °C and 37 °C); (c) logarithmic phase *S. aureus* cells were exposed to either 1.2×10^{-5} M α-MSH or PBS buffer for 2 h in presence of rat whole blood (85%, v/v), plasma (85%, v/v) and serum (85% and 20%, v/v). These data represent the means (±SD) of three independent experiments; $^{*}p < 0.001$ comparing data of blood vs. plasma vs. serum.

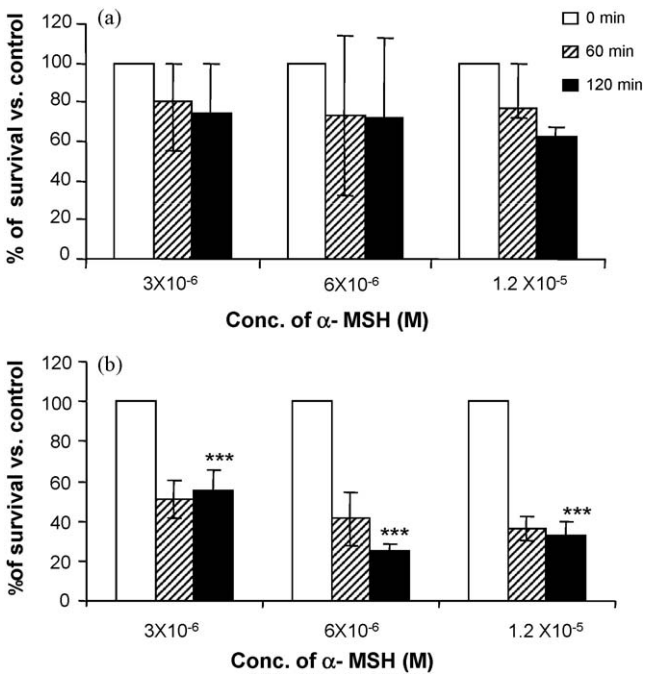


Fig. 3. (a) Killing activity of α-MSH against logarithmic phase *S. aureus* ISP479C cells having bacterial density 10^6 cfu/ml, $p = 0.023$ comparing data of bacterial density 10^6 cfu/ml vs. 10^3 cfu/ml. (b) Killing efficacy of stationary phase *S. aureus* ISP479C cells by different concentration of α-MSH. These data represent the means (±SD) of three independent experiments. Symbols: 0 min (open); 60 min (dashed); 120 min (filled). $^{***}p = 0.002$ compared to PBS control.

3.2. Staphylocidal activities in different microenvironment

To analyze whether different microenvironment affects the susceptibility of *S. aureus* cells towards α-MSH, antimicrobial assay was performed, by varying pH, salt concentration and temperature of the PBS buffer. Moreover, the influence of various blood components on the antimicrobial activity of α-MSH was assessed. Fig. 2a shows the influence of pH on staphylocidal activity of α-MSH in presence and absence of salt. The data suggested that bactericidal activity of α-MSH was pH dependent and increased with decrease in pH (e.g., on incubation with 1.2×10^{-5} M α-MSH for 2 h, %survival of ISP479C cells were 38 ± 7% at pH 7.4 and 17.2 ± 1.1% at pH 4 respectively. This difference was statistically significant ($p = 0.05$, comparing pH 7.4 vs. pH 6 vs. pH 4). There was no considerable change in killing activity on removing the salt from phosphate buffer. To see the effect of temperature on α-MSH activity against *S. aureus*, killing assays were performed at 37 °C and 4 °C (Fig. 2b). The viability of *S. aureus* was not affected at 4 °C. There was no significant change in bactericidal activity of α-MSH on reducing the temperature from 37 °C to 4 °C. Fig. 2c is showing the antimicrobial activity of α-MSH in complex fluid biomatrices of whole blood, plasma and serum. There was no decrease in *S. aureus* viability in whole blood cells (85%). In contrast, 85% plasma, 85% serum and 20% serum alone decreased *S. aureus* viability by 78%, 100% and 88% respectively, indicating intrinsic staphylocidal activities of plasma and serum (Fig. 2c). On incubation with 1.2×10^{-5} M α-MSH for 2 h in whole blood, plasma and 20% serum, there was further reduction in viability of *S. aureus* cells (survival was 68 ± 1.5%, 46.5 ± 0.5% and 3.1 ± 0.71% in whole blood, plasma and 20% serum respectively, in presence of α-MSH; $p < 0.001$ comparing data of blood vs. plasma vs. serum; Fig. 2c).

3.3. Staphylocidal activity by varying bacterial cell density and growth phase

Initial antimicrobial assay was performed with lower *S. aureus* cell density (ISP479C, 10^3 cfu/ml, Fig. 1a). In order to investigate whether the antibacterial effect of α-MSH was influenced by bacterial cell number, higher no. of ISP479C *S. aureus* cells (10^6 cfu/ml) were incubated for 1 h and 2 h with different concentration of α-MSH in PBS buffer, pH 7.4 (Fig. 3a). As can be seen from Fig. 3a, bactericidal effect of α-MSH decreased when higher numbers of bacterial cells were used. Thus, it was observed that there was 62.0 ± 7% killing when 10^3 cfu/ml *S. aureus* cells were incubated with 1.2×10^{-5} M of α-MSH for 2 h (Fig. 1a), however 38 ± 5% inhibition was observed when using 10^6 cfu/ml *S. aureus* cells (Fig. 3a). This difference reached statistical significance ($p = 0.023$ comparing data of bacterial density 10^6 cfu/ml vs. 10^3 cfu/ml).

In order to determine whether α-MSH had bactericidal activity against stationary phase organisms, ISP479C cells were allowed to grow for 18 h and then subjected to increasing concentrations of α-MSH in PBS buffer, pH 7.4. As it can be observed from Fig. 3b that there was no significant difference of antimicrobial activity of α-MSH for stationary phase cells in comparison to exponentially growing *S. aureus* cells when higher concentration of peptide used.

3.4. Studies with MRSA and MSSA strains

Bactericidal activities of α-MSH against 10 MSSA and 9 MRSA strains isolated from soft tissue specimen were evaluated. MSSA

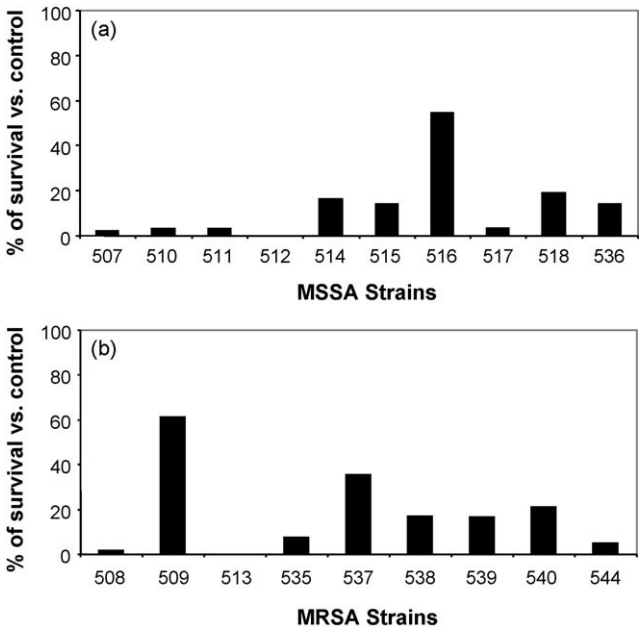


Fig. 4. (a) Killing efficacy of α-MSH (1.2×10^{-5} M) against clinically isolated different logarithmic *S. aureus* cells after 2 h of administration. (a) MSSA and (b) MRSA.

was defined as a strain with an oxacillin MIC of ≤2 mg/l and MRSA was defined as a strain with an oxacillin MIC of >2 mg/l [23]. For the cefoxitin disc diffusion test, strain with an inhibition zone diameter ≤19 mm were scored as methicillin-resistant and ≥20 mm as methiciliin-susceptibile [23]. All strains were uniformly susceptible to vancomycin (30 μg), teicoplanin (30 μg), and linezolid. All MRSA strains were considered resistant to amoxicillin/clavulanic acid (augmentin), imipenem, and piperacillin/tazobactam as per CLSI interpretative criteria [12].

Fig. 4a and b is showing time-kill results of α-MSH for MSSA and MRSA strains. α-MSH killed both type of strains efficiently (84% strains was killed on exposure of 1.2×10^{-5} M α-MSH for 2 h) and it did not differ with MRSA and MSSA strains in significant way (in case of MSSA strains, ∼10% had survival rate of >20% and ∼20% MRSA strains had >20% survival rate).

3.5. Membrane permeabilization

3.5.1. Calcein leakage assay

The ability of α-MSH to permeabilize the staphylococcal membrane was analyzed by the release of preloaded calcein via flow cytometry. Gramicidin D was used as positive control as it is one of the well-known pore forming peptides [33]. 25 μg/ml gramicidin D was used to ensure 100% killing as well as complete membrane pore formation. As can be seen from Fig. 5a, exposure of *S. aureus* cells with gramicidin and α-MSH caused substantial membrane permeabilization. Fig. 5b shows the membrane permeabilization using different concentrations of α-MSH. 1.2×10^{-8} M α-MSH exposure for 2 h caused 25% of calcein leakage whereas for 6×10^{-6} M α-MSH 91% calcein leakage was observed. There was no loss of calcein from control cells over 2 h of experimental period.

To further characterize the relationship between membrane permeabilization and staphylocidal activity of the peptide, regression analysis were performed comparing these parameters (Fig. 5c). A significant positive correlation exists between killing activity and membrane damage ($r^2 = 0.97$).

3.5.2. Propidium iodide uptake assay

To further confirm the membrane damage, α-MSH treated *S. aureus* strain was examined by PI uptake assay. Fig. 5d shows the %increase in PI-fluorescence intensity with time on incubation of *S. aureus* cells with α-MSH and gramicidin D. As it can be seen from Fig. 5d, there were 29% and 35% increase in PI-fluorescence intensity after 1 h and 3 h incubation of *S. aureus* cells with 1.2×10^{-5} M α-MSH respectively, compared to 100% increase in case of gramicidin D.

3.6. Synergism with ampicillin

Recent reports have shown the synergistic effect of antimicrobial peptides when combined with conventional antibiotics [17]. Antimicrobial activity of α-MSH against *S. aureus* was evaluated in combination with ampicillin. Ampicillin exhibited MIC_{50} of 0.06 μg/ml for 10^3 cfu/ml ISP479C. As can be seen from Fig. 6, 94 ± 7% and 39.5 ± 12.7% killing was observed for 4×10^{-6} M and 8×10^{-6} M ampicillin respectively, whereas no killing was observed when lower concentration of ampicillin (10^{-8} M to 10^{-7} M) was used. 1.2×10^{-5} M α-MSH killed 62 ± 7% of *S. aureus* cells in 2 h. On using 1.2×10^{-5} M α-MSH in combination with different concentration of ampicillin particularly in lower concentrations, ∼6–7% increase in killing was observed.

3.7. Studies with biofilm

In an array of three different bacterial strains tested, biofilm formation on polystyrene plates, resulted in sessile, irregularly shaped bacterial forms enmeshed in extra cellular polymeric substance (EPS) that strongly stained against Congo Red stain (data not shown). As can be seen from figures non-peptide treated control *S. aureus* planktonic (Fig. 7c) and sessile form (Fig. 8c) appeared predominantly green (demonstrating live cells), the peptide treated cultures (Figs. 7a and b, 8a and b) appeared substantially red (indicating dead cells). However, relatively higher live (green) cells in biofilms than planktonic cells indicated that biofilm formation conferred higher resistance both to gramicidin and α-MSH compared to planktonic bacteria. The degree of susceptibility to α-MSH exhibited variation between the planktonic vs. sessile forms; not all bacterial biofilms exhibited the same degree of resistance against α-MSH (data not shown). Extensive studies on the 508MRSA showed that the biofilm form exhibited ∼30% higher survival against gramicidin compared to their planktonic counterpart (Figs. 7b and 8b). Parallel biofilm sets exhibited 12% higher survival against α-MSH than their planktonic counterparts (Figs. 7a and 8a). In all cases, the biofilm or planktonic cells in the control sets (without exposure to gramicidin or α-MSH) exhibited predominantly live (green) cells confirming, that neither the planktonic to sessile transformation nor the assay methods generated membrane damage or death (Figs. 7c and 8c respectively). The results were further confirmed by plating and cfu based counting assays (data not shown).

4. Discussion

α-Melanocyte stimulating hormone (α-MSH) a thirteen amino-acid long neuropeptide is well known for its anti-inflammatory and melanogenic property [19,40,41]. It is secreted mainly by pituitary gland but it is also present in various extrapituitary cells such as keratinocytes, neutrophils, and melanocytes [19]. The presence of this peptide in barrier organs supports its involvement in innate immune system and recently it has been reported that α-MSH possess antimicrobial activity [10,16,22]. Therefore, the objective of the present study was to examine in detail its antibacterial

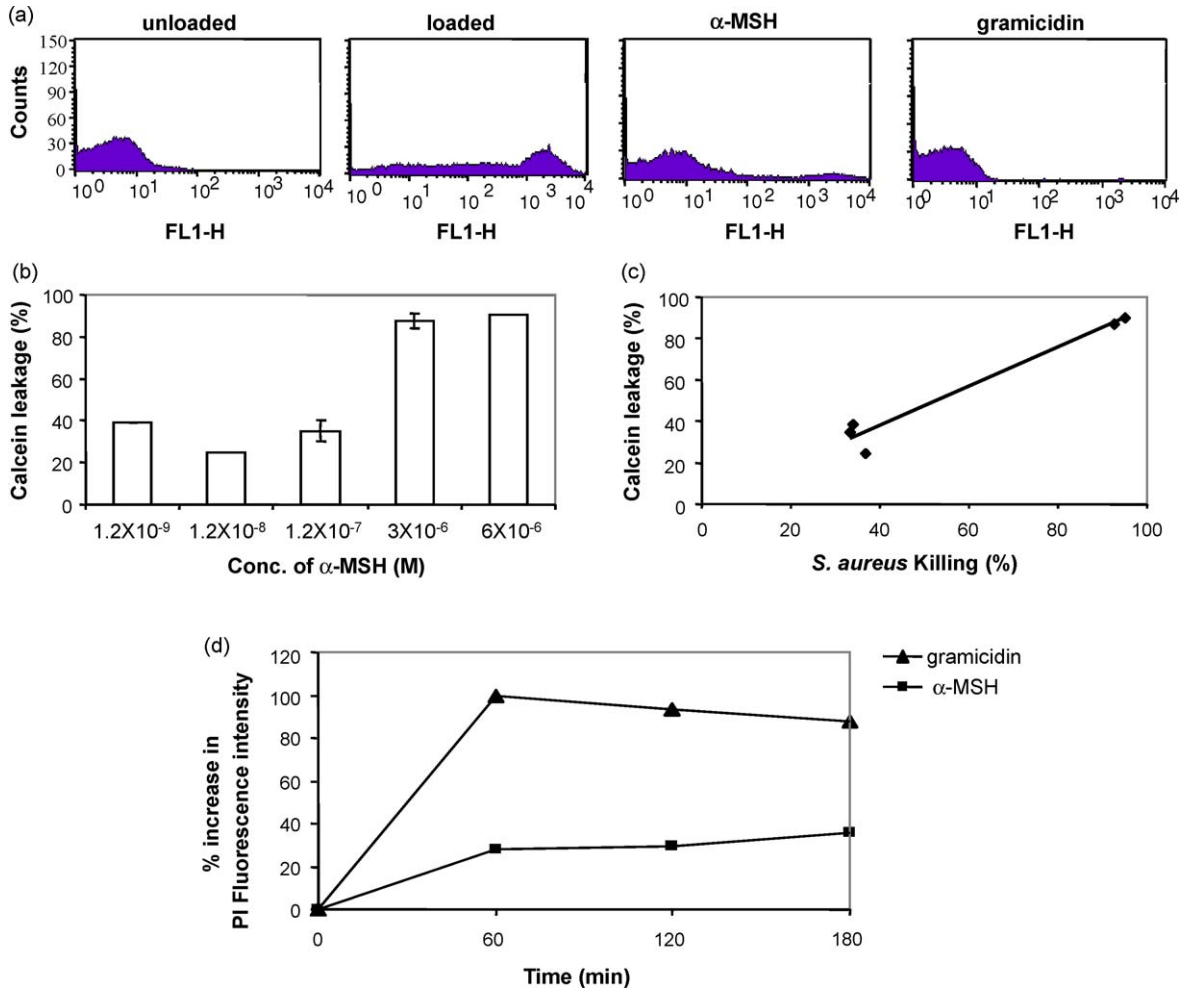


Fig. 5. Membrane permeabilization of *S. aureus* by α-MSH. (a) Calcein leakage assay: logarithmic *S. aureus* 510MSSA was labeled with calcein and analyzed for membrane permeabilization after incubation with 1.2×10^{-5} M α-MSH and 25 μg/ml gramicidin D. A total of 10,000 cells were acquired for each flow cytometry analysis. Cells at or above a threshold of 10 fluorescence units (FL1 units) were considered to have retained calcein, indicative of an intact cytoplasmic membrane; those cells exhibiting <10 FL1 units were interpreted to have lost calcein as a result of α-MSH induced membrane permeabilization. Histograms (left to right) are showing *S. aureus* cells unloaded and loaded with calcein and loaded cells treated with α-MSH and gramicidin respectively. (b) Membrane permeabilization of *S. aureus* is shown by calcein leakage (%) by different concentration of α-MSH. These data represent the means (±SD) of two independent experiments. (c) Correlation analysis of the relationship between membrane permeabilization (%calcein leakage) and *S. aureus* killing (%) by α-MSH. Linear regression was determined and correlation coefficient ($r^2 = 0.97$) was calculated. (d) PI-uptake assay: %increase in PI-fluorescence intensity of 510MSSA *S. aureus* cells on incubation with 1.2×10^{-5} M of α-MSH and 25 μg/ml of gramicidin D.

activity in different micro-environmental conditions. Whether α-MSH operates like other natural antimicrobial agents or not, was also investigated.

Several interesting results emerged from our studies. α-MSH showed significant antibacterial activity against *S. aureus* strains including both MSSA and MRSA. This is in good agreement with the earlier report by Cutuli et al. [16]. The antibacterial effect observed was dependent upon bacterial number. When 10^6 cfu/ml *S. aureus* cells were exposed to 1.2×10^{-5} M α-MSH for 2 h, the inhibition was 38 ± 5% (Fig. 3a), whereas for 10^3 cfu/ml *S. aureus* cells killing was 64 ± 4% (Fig. 1a). This is in agreement with the recent report by Charnley et al. [10]. Furthermore, change in growth phase of *S. aureus* did not show any influence on its susceptibility towards α-MSH (Fig. 3b). This peptide showed same degree of killing for stationary phase cells as well as in the case of exponentially growing cells.

In the present study, α-MSH activity did not significantly change on lowering the temperature from 37 °C to 4 °C (Fig. 2b). This finding is in contrast to the activity of other antimicrobial peptides such as human neutrophil peptide-1 and thrombin induced platelet membrane protein, which showed maximum activity near physiological temperature [34].

α-MSH bactericidal activity was pH dependent with maximum activity observed at pH 4 (Fig. 2a). This might be due to increase in net positive charge of peptide. α-MSH contains Histidine (His) residue at sixth position, and His has tendency to protonate in acidic condition and in absence of salt [7]. As a consequence of protonation, His acquires a positive charge [7], which leads to net

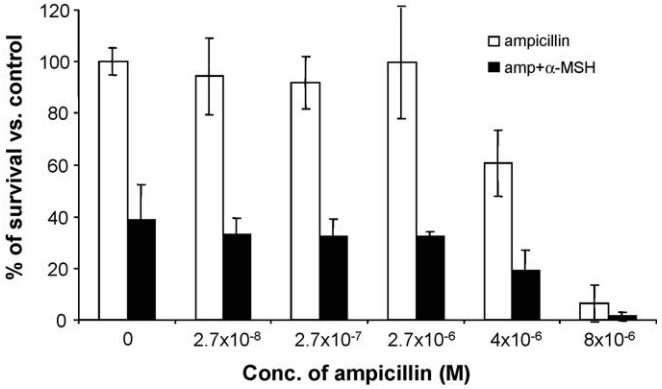
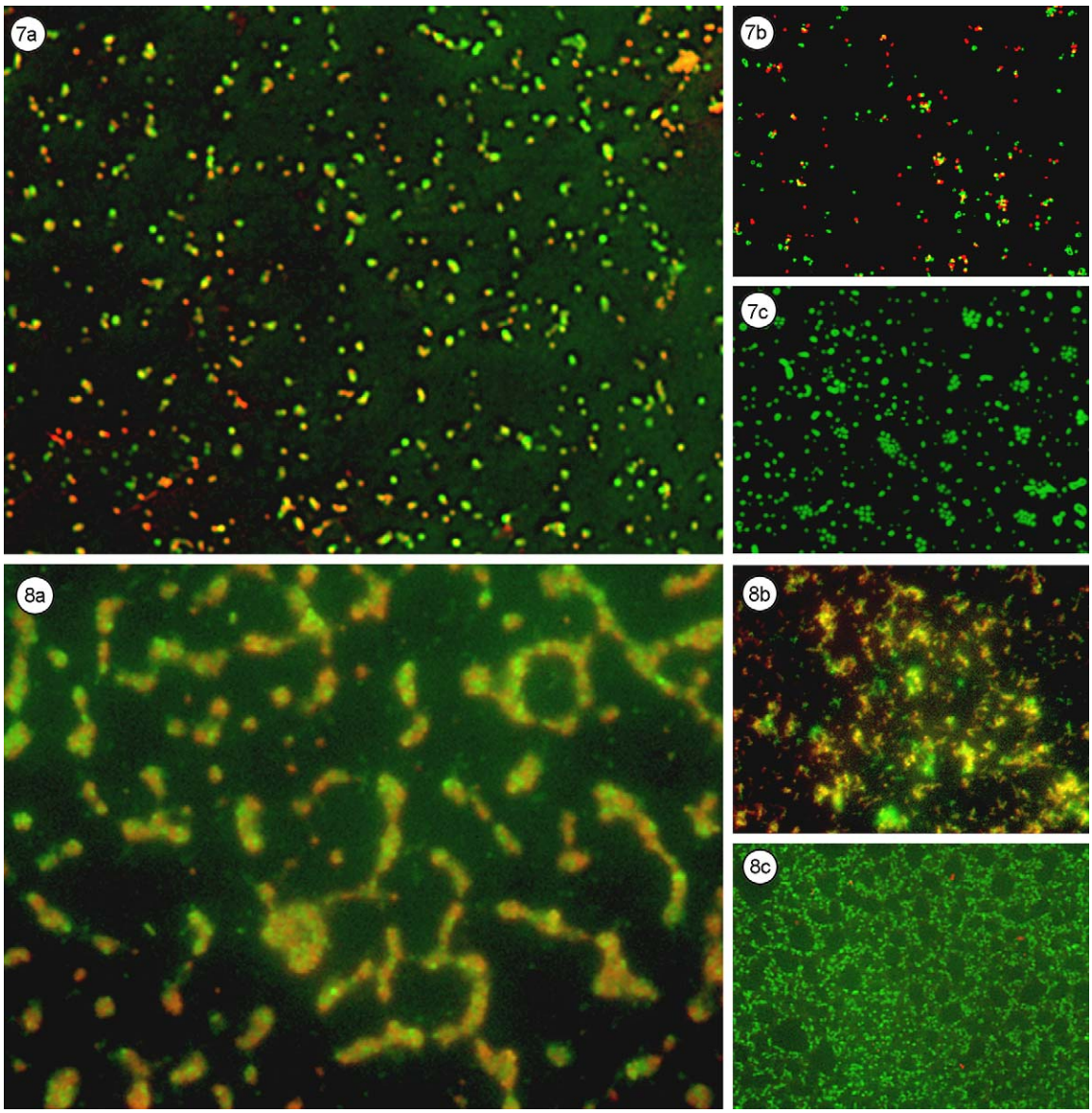


Fig. 6. Killing activity of different concentrations of ampicillin against *S. aureus* ISP479C cells in absence (open) and in presence (filled) of 1.2×10^{-5} M α-MSH after 120 min incubation. These data represent the means (±SD) of three independent experiments.



Figs. 7 and 8. Epifluorescence Miscroscopy Assay for viability of planktonic and sessile *Staphylococcus* 508MRSA against α-MSH. Similar concentration of bacteria (10^8 cfu/ml) either in planktonic form or biofilms on polystyrene surfaces was exposed to 1.2×10^{-5} M α-MSH (experimental 7a and 8a); 25 μg/ml gramicidin (7b and 8b) or saline (negative control 7c and 8c) for 2 h at 37 °C. Subsequently bacterial sets were washed in saline and subjected to LIVE/DEAD *Bac*Light Bacterial Viability Assay™ (Invitrogen, USA) following manufacturer's instructions. Bacteria in red are indicative of dead or membrane damaged bacteria, whereas, green indicates live/healthy bacteria. The experiment was performed in triplicate on independent days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

increase in positive charge of α-MSH and thus, antimicrobial activity of the peptide may have got enhanced.

Whole blood itself did not exhibit antimicrobial activity against *S. aureus* whereas both plasma and serum alone exhibited innate staphylocidal activity. This is consistent with previous observations [34]. Staphylocidal activity of α-MSH in presence of either blood or plasma or serum was greater than any of the agents alone indicating that the peptide was not inactivated within these biomatrices. This is a significant observation indicating the potential of this peptide to be developed as a therapeutic agent in vivo.

The most important observation of this study was the good in vitro bactericidal activity against all *S. aureus* strains with little difference in susceptibility to MRSA and MSSA strains. This high bactericidal activity of α-MSH against MRSA strains may have tremendous therapeutic consequence.

Cationic antimicrobial peptides target the bacterial membrane through electrostatic interaction, with negatively charged membrane components, resulting in membrane pore formation and eventually cell death [27,48]. It is also possible that some antimicrobial peptides target different intracellular components such as DNA, RNA and proteins and membrane disruption may not be the only cause of cell death [5,15,43]. Previous reports suggested that candidacidal effect of α-MSH was mediated through the induction of cyclic adenosine monophosphate (cAMP). They have also reported candida cell death, which occurred prior to membrane damage and thus suggested that membrane disruption is perhaps the consequence rather that cause of the cell damage [16]. However, in other studies, replacement of positively charged amino acid Lys at position 11 with neutral Ala diminished anti-candidal activity of α-MSH (6–13) by 50% [25]. Recently Charnley et al. [10] showed that the cationic charge on the lysine residue was not required for the bactericidal activity of α-MSH (11–13). In the present study, both permeabilization assays (Fig. 5a–d), confirmed that direct correlation exists between staphylocidal activity and

membrane permeabilization. PI uptake assay and calcein leakage assay showed 30–70% membrane damage of *S. aureus* 510 MSSA on exposure of 1.2×10^{-5} M of α-MSH for 2 h in comparison to gramicidin D, well-known pore forming peptides. However, of note, similar exposure of α-MSH caused 95% killing of MSSA strain (Fig. 1b). Also, in our PI uptake assay, incorporation of PI was low compared to that of gramicidin and PI incorporation continued to rise well beyond 2 h. This raises the question about membrane permeabilization being the primary lethal mechanism for this peptide. Calcein leakage by α-MSH was comparable with that of gramicidin. Thus, it appears that membrane permeabilization may be sufficient to explain, at least in part, the cause of antimicrobial activity of α-MSH against *S. aureus*.

Several hypotheses exist regarding α-MSH antimicrobial mode of action [8,18,19,42]. It may act against microbes through interference in the yeast cell cycle upon binding, so far unknown, melanocortin-like receptor on the microbial surface [19,42]. It has been also reported to act through reducing the internalization of *S. aureus* in the human epithelia by downregulating the expression of HSP70 and integrins [18]. Though we have found that membrane permeabilization may be sufficient to explain, at least in part, the antistaphylococcal activity of α-MSH, co-existence of above factors for bacterial killing cannot be denied. Therefore, to determine the precise mechanism of microbicidal action of α-MSH, further detail investigation is required.

The present study also aimed to find whether using in combination with conventional antibiotic such as ampicillin, antimicrobial activity of α-MSH/ampicillin could be increased. An increase in bactericidal effect was observed when α-MSH was used in combination with different concentration of ampicillin particularly in lower concentration (Fig. 6). Fraction inhibitory concentration (FIC) studies for the two drugs in combination need to be performed to prove this synergistic effect of ampicillin and α-MSH. α-MSH adopts conventional amphipathic α-helical structures in membrane like environments, a feature commonly found in cationic peptides [38]. Moreover, our study indicates that it also acts as a membrane permeabilizer and this characteristic can promote maximal entry of hydrophobic substrates like ampicillin. Furthermore, it may be possible that increase in bactericidal effect on using peptide and antibiotic together is a combined effect of increased access to the intracellular target for antibiotics coupled with the secondary effects of the peptides themselves.

It is accepted that the sessile (biofilm) form of bacterial growth is frequently a true reflection of its natural growth patterns. Particularly in case of *Staphylococcus* the biofilm mode is widely reported to be the most potent pathogenic form encountered in arthroplasty related infections and others [13,14,45]. Such planktonic to biofilm transformation often confers enhanced recalcitrance to *Staphylococcus* against host defense mechanisms and antibiotics [13]. In this context, it is interesting to note, whereas we observed significant resistance to gramicidin in *Staphylococcus* biofilms, the latter did not offer the same degree of resistance against α-MSH (Figs. 7 and 8).

5. Conclusion

To conclude, α-MSH exhibited potent antimicrobial activity against both for MSSA and MRSA strains. Like other cationic antimicrobial peptides, it is first time revealed from our studies that membrane permeabilization at least in part, was responsible for its antimicrobial activity. The synergistic effect of α-MSH with ampicillin and its efficacy in complex fluid biomatrices and most importantly its antibacterial activity in staphylococcal biofilms as demonstrated in this study suggests its therapeutic potential to combat bacterial infections. Of note, α-MSH peptides have been found to have little or no toxicity in vitro or in preclinical studies [24]. Thus, α-MSH with its low toxicity and combined antipyretic, anti-inflammatory and antimicrobial properties could emerge as an excellent therapeutic agent against major human pathogens including *S. aureus*.

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