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Susceptibility of Clinical Isolates of *Staphylococcus aureus* to Alpha-Melanocyte Stimulating Hormone and Its *In Vivo* Efficacy

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Abstract

Antimicrobial Peptides (AMPs) are one of the most promising future strategies for defeating the antibiotic resistance. Alpha Melanocyte Stimulating Hormone (α-MSH) is a cationic neuropeptide and we have previously reported its strong *in vitro* antimicrobial activity against *Staphylococcus aureus*. However, the efficacy of this peptide needs to be tested against a large number of clinical *S. aureus* isolates to ascertain its antistaphylococcal potential. Moreover, *in vivo* efficacy of alpha-MSH need to be determined for establishing its biological significance. In the present study, we tested the susceptibility of a total of 100 clinical *S. aureus* isolates, including 43 MSSA and 57 MRSA towards α-MSH and found that 10 µg/ml of α-MSH killed 95% and 77% of MSSA and MRSA strains, respectively. To check if cross resistance existed among the α-MSH-non-susceptible strains, a susceptibility profiling test was done towards three other AMPs belonging to different sources. We chose gramicidin D, magainin 2 and human neutrophil peptide 1 for this purpose and it was observed that cross resistance was not present among the study strains. We further evaluated *in vivo* efficacy of α-MSH against *S. aureus* using mice intravenous and skin wound infection models. Results revealed that the therapeutic efficacy of α-MSH at 8 mg/kg is superior to that of oxacillin in animal infection model, leading to 100% survival of the *S. aureus* infected animals. The effectiveness of α-MSH both *in vitro* and *in vivo* models further strengthened the clinical potential of this peptide.

Keywords: *S. aureus*; α-MSH; Antimicrobial peptides; *In vivo*; Mice model

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Introduction

It is evident from the reports of the past few decades that increasing bacterial drug resistance has created an urgent need for new classes of antibiotics. Methicillin Resistant *S. aureus* (MRSA) remains one of the principal resistant bacterial pathogens causing a wide range of infections and diseases [1]. Antimicrobial Peptides (AMPs) seem to represent one of the most promising future strategies for defeating this threat. Despite the fact that most of the AMPs are active *in vitro*, many of them failed to show similar efficacy *in vivo* [2]. This is probably because of their low stability *in vivo* condition and mammalian cytotoxicity. This is the very reason why any AMP from vertebrate and invertebrate could not be qualified for clinical use even after 30 years of their discovery [3,4]. For example, the Human β Defensin1 (hBD1) is highly active *in vitro*, however, the presence of salt deactivates it [5]. Therefore, the validation of antibacterial potential of AMPs in animal model as well as evaluation of their cytotoxic effects on host cells is recommended in order to proceed further in developmental phase [6]. Besides bactericidal activity, AMPs have role in immunomodulation, immune cell migration and proliferation, which in turn helps in fast wound healing [7].

Our lab is engaged for the last ten years to understand the activity and mechanism of bactericidal action of alpha Melanocyte Stimulating Hormone (α-MSH). Of late, our group reported that α-MSH and its fragments exhibit antimicrobial activity against *S. aureus* by rapid membrane permeabilization and membrane depolarization [8-10]. Further, we showed that α-MSH-non-susceptible *S. aureus* strains had higher amount of cationic phospholipid and rigidity in their membrane [11]. We also reported that α-MSH had a synergistic effect with conventional antibiotics against MRSA and was least toxic to the mammalian cell line and it did not cause hemolysis of RBCs [9]. Recently the behavior of α-MSH was also studied in model membranes, where it was clearly evident that α-MSH has more affinity towards anionic membrane environment [12].

In this study, we extended our previous findings by investigating the bactericidal activity of

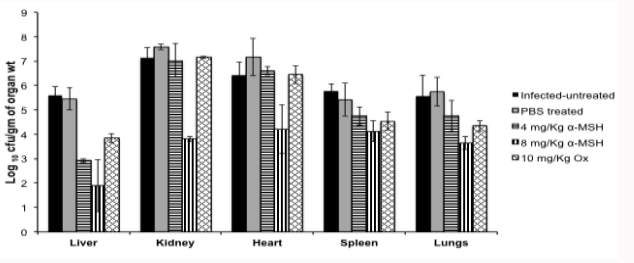

Figure 1: *In vivo* efficacy of α-MSH in mice model of *S. aureus* systemic infection. Bacterial culture (10^7 cfu) was injected *via* intravenous route, followed by peptide treatment and viable bacterial counting in liver, kidney, heart, spleen, and lungs. The bacterial counts of each group are representing the average counts of all animals sacrificed in that group (n = 4 as 4 animals were sacrificed in all groups except in α-MSH (8 mg/Kg) treated group where n = 5, as 5 animals were sacrificed). A significant difference in counts in α-MSH 8 mg/Kg ($p < 0.05$), compared to untreated infected control.

α-MSH against a wide range of *S. aureus* isolates. Firstly, we scanned 100 clinical isolates of *S. aureus* and tested their susceptibility towards methicillin. Based on their Minimum Inhibitory Concentration (MIC) values, we characterised them as Methicillin Sensitive *S. aureus* (MSSA) or Methicillin Resistant *S. aureus* (MRSA). MRSA strains were further categorised as low-MRSA, medium-MRSA and high-MRSA on the basis of their range of MIC values. We then checked the susceptibility of these strains towards α-MSH in order to determine whether *S. aureus*, including MRSA possesses resistance not only to antibiotics but also to host-derived AMPs. Depending on whether the strains were susceptible to α-MSH or not, we classified them into two groups, namely α-MSH-susceptible and α-MSH-non-susceptible. Further, we picked up a few strains from both categories and checked their susceptibility towards three other AMPs of different sources, namely magainin 2 (amphibian), gramicidin D (bacterial) and human neutrophil peptide-1 ((hNP 1) human). We tried to observe if there was any cross-resistance among these strains. In order to further evaluate the clinical relevance of α-MSH, we measured its antibacterial activity against *S. aureus* infected mice models. Here we demonstrated the therapeutic effectiveness of α-MSH both in systemic as well as wound mice models of *S. aureus* infection, with 100 percent survival of *S. aureus* infected animals after administration of α-MSH.

Materials and Methods

Bacterial strains

A total of 100 *S. aureus* strains were included in the study, collected and identified by Dr. Benu Dhawan of All India Institute of Medical Sciences, New Delhi, India. The clinical isolates were single-patient, non-duplicate strains. Methicillin sensitive *S. aureus* strain (ISP479C) and ATCC 29213 were used for *in vivo* studies. All the strains were cultured in Brain Heart Infusion (BHI) media (Himedia Laboratories, India).

Antibiotics and antimicrobial peptides

Oxacillin (Ox) (Sigma-Aldrich, St Louis, MO) was dissolved in sterile water and a stock concentration of 10 mg/ml was prepared. α-MSH, gramicidin D, magainin 2 and hNP 1 were purchased from Sigma-Aldrich (St. Louis, MO). Purity of all peptides was >97%. The concentration of peptides containing both Tryptophan (Trp) and Tyrosine (Tyr) was determined spectrophotometrically (Cary 100 Bio/Varian and UV-2450, UV-VIS spectrophotometer, Shimadzu), using a molar extinction coefficient (ε) at 280 nm of 6.65×10^3 $M^{-1}cm^{-1}$. The concentration of peptide containing only Trp was determined

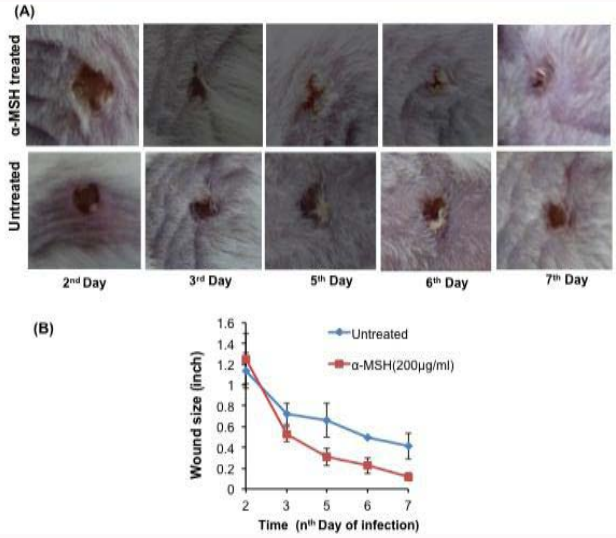

Figure 2: Wound healing activity of α-MSH in mice model of wound infected with *S. aureus*. (A) Pictures of the wounds untreated and α-MSH treated mice on 2nd, 3rd, 5th, 6th and 7th day of infection. (B) Wound sizes measured and presented as mean ± SD of mice (n=3) from untreated and α-MS.

using $\varepsilon_{Trp} = 5.7 \times 10^3$ $M^{-1}cm^{-1}$ at 280 nm and concentration of peptide containing only Tyr was determined using $\varepsilon_{Tyr} = 1.4 \times 10^3$ $M^{-1}cm^{-1}$ at 280 nm. Peptides were dissolved in sterile deionized water or DMSO and stored at 4°C.

Minimum inhibitory concentration (MIC) assay

Resistance to methicillin was determined using the Clinical and Laboratory Standards Institute approved broth microdilution method (CLSI, 2009). Antimicrobial susceptibility breakpoints were defined according to CLSI guidelines (CLSI, 2009). MIC of α-MSH and other AMPs could not be determined as their antibacterial activity is compromised in presence of MHB medium [13,14].

Antibacterial activities of the AMPs by killing assay

To determine the bactericidal activities of the study AMPs, *S. aureus* was grown and mid-logarithmic-phase cells were taken. Cells were then washed with potassium Phosphate Buffer Saline (PBS) and adjusted to $OD_{600nm} = 0.5$ and then dilutions were prepared in the same buffer of desired cell density (103 cfu/ml to 108 cfu/ml). Since the presence of salt mitigates the activity of hNP 1, potassium Phosphate Buffer (PB) without salt was used [15]. Cells were then exposed to different concentrations of the peptides in the presence of buffer at 37°C for 1 hour. Next, 15 µl aliquots from each concentration was taken out and spotted onto BHI agar plate in triplicates and the plate was incubated overnight at 37°C. Percentage of *S. aureus* clinical strains susceptible to AMPs was measured by calculating % survival *vs.* control as described earlier [10]. Since there are no standard susceptibility breakpoints for AMPs unlike antibiotics, we considered strains showing < 50% survival as susceptible and > 50% survival as non-susceptible at study concentration [11].

In vivo activity of α-MSH against *S. aureus* intravenous (IV) infection

S. aureus ISP479C strain was cultured up to mid-log phase and cells were adjusted to $OD_{600nm} = 0.5$ (108 CFU/ml) in PBS buffer. Next, 32 mice (swiss albino female mice, 6 weeks to 8 weeks old) were intravenously (IV) injected with 10^7 CFU of bacterial suspension for three days continuously at regular interval of 24 hours. After 24 hours

Table 1: Characterization of clinical isolates of *S. aureus* as MSSA and MRSA by their MIC for oxacillin (similar to methicillin) using broth microdilution method.

	No. of isolates with MIC (µg/ml)						
Isolate	0.25	0.5	1	2	Apr-32	64-512	≥1024
MSSA(43)	9	25	7	2			
MRSA(57)							
L-MRSA					7		
M-MRSA						46	
H-MRSA							4

of the 3rd bacterial injection dose, level of infection in different organ homogenates of 2 out of 32 infected animals was enumerated by viable bacterial counting [16,17]. The remaining 30 animals were divided randomly into five different groups as follows - (i) Untreated (n = 10), (ii) PBS treated/placebo (n = 5), (iii) 4 mg/kg α-MSH treated (n = 5), (iv) 8 mg/kg α-MSH treated (n = 5), (v) 10 mg/kg oxacillin treated (n = 5). In addition to these groups, a group of uninfected animals (n = 5) was included to rule out previous infection of animals with *S. aureus* and served as negative control for the infection. A-MSH treatment of the infected animals began after 24 hours of the last infection through IV route once daily up to 5 days. Animals were observed daily for any physical abnormalities and mortality. After 24 hours of the last drug injection, the animals were sacrificed by cervical dislocation, and the target organs were excised aseptically, weighed and 20% homogenates were prepared in PBS buffer using tissue homogenizer. Aliquots were then diluted in PBS buffer and plated on Mannitol Salt Agar (MSA) plate. After overnight incubation at 37°C, viable colonies of bacteria were counted and log10 of cfu/gm of organ weight were enumerated. This experiment was repeated on two independent occasions. The JNU-Institutional Animal Ethics Committee (JNU-IAEC) approved use of animals for this work.

Wound healing activity of α-MSH in *S. aureus* skin infection model

S. aureus is most frequently implicated in Skin and Soft Tissue Infections (SSTIs), causing a delay in wound healing. In order to mimic the SSTI, a skin *S. aureus* infection mice model was developed as described elsewhere [18]. In brief, a ~ 3 cm^2 area on the back of swiss/albino female mice (6 weeks to 8 weeks old) was shaved by razor and was sterilized using ethanol. Shaved animal was anaesthetized by injecting a mixture of ketamine (0.5 mg/animal) and xylazine (0.25 mg/animal). Thereafter, a single punch biopsy was performed using biopsy puncher, leaving a wound of approximately 5 mm diameter. After 30min of creating the wound, a 100 µl (107 cfu) suspension of *S. aureus* in PBS buffer was inoculated directly onto the wound (referred as Day1). A total six animals were infected and divided randomly in two groups; (i) untreated control and (ii) treated with 200 µg/ml of α-MSH. Treatment with α-MSH was begun after 24 hours of inoculation of bacteria into the animals of treated group (i.e., on Day 2) and was continued for 5 days at regular intervals of 24 hours. The reduction in average wound size was measured from the digitally captured images using Adobe Photoshop V.5.

Statistical analysis

Significance difference in staphylococcal counts among different treatment groups was compared by one-way analysis of variance using Prism 7.

Results and Discussions

Characterization of clinical isolates of *S. aureus* as MSSA and MRSA

In an attempt to investigate the prevalence of methicillin resistance in hospital environment, we included 100 non-duplicate clinical *S. aureus* strains isolated from different patients admitted to AIIMS, India. As illustrated in Table 1, out of the 100 clinical strains, 43 showed oxacillin MIC in the susceptible range (< 2 µg/ml), hence were classified as MSSA; and the rest isolates with MIC ≥ 2 µg/ml were classified as MRSA. Among the MSSA, 9, 25, 7 and 2 number of strains showed MIC value of 0.25, 0.5, 1 and 2 µg/ml respectively. We further divided the MRSA strains into 3 categories on the basis of their level of resistance- L-MRSA, M-MRSA and H-MRSA with MIC ranges of 4-32, 64-512 and ≥1024 µg/ml, respectively. As shown in Table 1, 7 were L-MRSA, 46 were M-MRSA and 4 were H-MRSA. Thus it was seen that maximum number of MRSA belonged to the M-MRSA category. Similar findings of MRSA in hospital settings from India and other developing countries have been reported in previous studies [19-22]. Therefore time and again, the emergence of strains of pathogenic bacteria with resistance to commonly used antibiotics in all regions of the world constitutes a serious threat to public health and has necessitated a search for novel types of antimicrobial agents to which the microorganisms have not been exposed [23,24].

Susceptibility of clinical MSSA and MRSA isolates to α-MSH

Although effective new types of antibiotics against MRSA have been introduced or are in clinical trials, the situation regarding new treatment options for infections due to multidrug-resistant pathogens remains a serious matter of concern [22]. Therefore, we evaluated

Table 3: Comparison of bactericidal activity of other AMPs against α-MSH-susceptible (AMS-S 1-5) and α-MSH-non-susceptible (AMSH-NS 1-5) S. aureus clinical strains. Cells were exposed to the peptide for 1 h and susceptibility was determined by calculating % survival *vs* control (untreated).

Strain serial no.	Gramicidin D	Magainin 2	hNP 1
AMSH-S-1	S	S	NS
AMSH-S-2	S	NS	S
AMSH-S-3	S	S	NS
AMSH-S-4	S	S	S
AMSH-S-5	S	S	S
AMSH-NS-1	S	S	S
AMSH-NS-2	S	S	S
AMSH-NS-3	S	S	S
AMSH-NS-4	S	S	NS
AMSH-NS-5	S	S	S

H treated groups.

Table 2: Percentage of *S. aureus* clinical strains susceptible to different concentrations of α-MSH. Results are presented as means ± SDs of three independent runs.

S. aureus	Percentage of strains susceptible to different concentration of µ-MSH on exposure for 1 hour		
	5 µg/ml	10 µg/ml	20 µg/ml
MSSA	88% ± 1.03	95% ± 0.37	84% ± 3.16
MRSA	74% ± 0.74	77% ± 2.22	75% ± 0.81

the antibacterial efficacy of an immunomodulatory neuropeptide, α-MSH. Interestingly, α-MSH has been found highly promising in our previous *in vitro* experiments where we investigated the antistaph potential of α-MSH including standard MRSA strain [10]. Here, the staphylocidal activity of α-MSH was further validated using a large number of *S. aureus* isolates including both MSSA and MRSA. Mid-logarithmic phase bacterial cells (10^3 cfu/ml) were treated with three different concentrations of α-MSH (5 μg/ml, 10 μg/ml and 20 μg/ml) for 1 hour. As we could observe from Table 2, α-MSH exhibited a substantial staphylocidal activity at all concentrations against both MSSA and MRSA strains within 1 hour of treatment. 10 μg/ml of α-MSH proved to be the most effective dose, killing 95% and 77% of MSSA and MRSA strains, respectively. In our previous study also, we reported 6 μM α-MSH (equivalent to 10 μg/ml) to be a potent concentration, killing substantially both MSSA and MRSA within an hour of exposure [11]. Therefore, we considered strains showing < 50% survival as α-MSH susceptible and ≥ 50% survival as α-MSH-non-susceptible at 10 μg/ml of α-MSH. This finding is in concordance with our previous studies and other reports as well, showing antibacterial activity of the cationic neuropeptide against *S. aureus*.

Sensitivity profiling of α-MSH-susceptible and α-MSH-non-susceptible clinical isolates of *S. aureus* to other AMPs

In order to observe if cross-resistance existed among the study strains, we checked the susceptibility of a few α-MSH-susceptible and -non-susceptible strains towards three other AMPs of different origins. For this, we picked up five strains each from α-MSH-susceptible (AMSH-S) and -non-susceptible group (AMSH-NS) and tested their cross-resistance towards magainin 2, gramicidin D and hNP 1. MIC of magainin 2 and gramicidin D against *S. aureus* ATCC 29213 were observed as 50 μg/ml and 4 μg/ml respectively. We chose 4 times the MIC value as test concentration i.e., 200 μg/ml for magainin 2 and 16 μg/ml for gramicidin D. Since MIC for hNP 1 could not be determined due to deactivation of its activity in the presence of conventional media, its test concentration was determined by killing assay. On incubating *S. aureus* ATCC 29213 with three different concentrations (5, 10 and 20 μg/ml) of hNP 1 for 1 hour, > 90% of the cells was killed. Increasing incubation time to 2 hours did not show any additional bactericidal effect (data not shown). We chose the minimum concentration i.e., 5 μg/ml hNP 1 to determine cross-resistance of the selected clinical strains. Mid-logarithmic cells (10^3 cfu/ml) were treated with each peptide for 1 hour in presence of buffer. Similar to α-MSH, we considered strains showing < 50% survival as susceptible and ≥ 50% survival as non-susceptible to AMPs. Table 3 illustrates the comparison of bactericidal activity of the study AMPs against the chosen *S. aureus* strains. As seen in Table 3, AMPs showed substantial killing activity against the clinical S. aureus strains within an hour of exposure irrespective of their α-MSH susceptibility. All the studied strains were susceptible to gramicidin D. Similarly all strains were susceptible to magainin 2 except AMSH-S-2. Two α -MSH susceptible strains (AMSH-S-1 and AMSH-S-3) and one α-MSH non-susceptible strain (AMSH-NS-4) were non-susceptible to hNP 1 (Table 3).

From the above findings we can infer that all the four AMPs belonging to various sources possessed substantial killing efficacy against clinical S. aureus strains. A strain non-susceptible to one AMP was susceptible to the other, which indicates that cross-resistance to AMPs was not present among the clinical *S. aureus* isolates studied here. These findings also justify the abundance of AMPs in nature to tackle a wide range of microorganisms having different susceptible profile.

α-MSH showed therapeutic efficacy in *S. aureus* systemic infection mice model

Previously, our group reported that α-MSH is least toxic to the mammalian cell line and it did not cause hemolysis of RBCs. α-MSH also has remarkable synergistic activity with ciprofloxacin and gentamicin [9]. To further comment on its clinical efficacy, we experimentally evaluated the antibacterial potential of α-MSH *in vivo* conditions using mice model of *S. aureus* systemic infection *via* IV route. The log10 cfu/gm of organ weight in various groups, including α-MSH treated and untreated is presented in Figure 1. We considered untreated, PBS treated animals as infection control groups and 10 mg/kg oxacillin treated animals were used as positive treatment control. The *in vivo* viable counting showed that the number of bacteria in α-MSH treated groups reduced in a dose dependent manner in all the target organs. For example, treatment with 8 mg/kg α-MSH caused 3.6 log and 3.3 log reduction of cfu in the liver and kidney, respectively ($p < 0.05$ compared to untreated), whereas treatment with 4 mg/kg α-MSH and 10 mg/kg oxacillin showed count reduction of 2.67 log and 1.4 log, respectively in liver. In contrast, there was less than 0.2 log reduction of kidney bacterial load by both 4 mg/kg α-MSH and 10 mg/kg oxacillin. Likewise, α-MSH at 8 mg/kg showed 2.2 log, 1.6 log and 1.9 log reduction of bacterial counts in heart, spleen and lungs, respectively. Notably, the decolonization of *S. aureus* in kidney, heart, spleen and liver of animals was higher in the case of α-MSH (8 mg/kg) treatment than that of oxacillin (10 mg/kg) administration. The better killing efficacy of α-MSH than oxacillin may be due to its immunomodulatory behavior *in vivo* condition [25]. Possibly it might be helping the host immunity to eradicate bacterial infection [26]. Interestingly, 100% post infection animal survival was found in α-MSH (8 mg/kg) treatment group, whereas 60% animals died in untreated infected control group (Table 4).

However, being an immunomodulatory neuropeptide, the systemic use of whole α-MSH might induce other unwanted endocrine and overt immunological downstream effects. Therefore, work is undergoing in our laboratory to test various α-MSH analogues devoid of melanocortin receptor region, i.e., HFRW with selectively increased efficacy against *S. aureus* membrane [10,27]. Very recently, we showed that the efficacy of α-MSH increases selectively against *S. aureus* membrane with increase in cationic charge [27].

Wound healing efficacy of α-MSH in *S. aureus* skin infection model

It is estimated that the Skin and Soft Tissue Infections (SSTIs) account for more than 14 million outpatients visit each year in the USA [28]. The colonization of SSTIs with *S. aureus* results in prolongation of wound healing. In addition to the direct anti-bacterial activities, the immunomodulatory action of α-MSH might be useful in polarizing immune response to clear the wounds.

In order to study the wound healing efficacy of α-MSH, we designed an experiment where a wound infected with S. aureus was created on the back of the mouse to reflect the clinical manifestation of *S. aureus* infection, followed by treatment with α-MSH (200 μg/ml) for 5 consecutive days. Closure of the lesions was measured on each day up to 7^{th} day of post infection (Figures 2A and 2B). Figure 2A shows the comparison of the lesion sizes between untreated and α-MSH treated groups on the indicated days- 2^{nd}, 3^{rd}, 5^{th}, 6^{th} and 7^{th} day of creation of infected wound. A visible decrease in wound size in

case of α-MSH treated group can be observed from the digital images of all days, particularly on the 7th day. The average lesion size was determined from all the three animals from both α-MSH treated and untreated groups at indicated time points and is presented in Figure 2B. A fast healing with α-MSH treatment was observed, with wound size reducing from 1.3 inch to 0.11 inch, while untreated infected wound size was 0.41 inch on the 7th day (Figure 2B).

As *S. aureus* is majorly involved in SSTIs [29], the observed wound-healing efficacy of α-MSH could be useful in topical treatment of *S. aureus* born SSTIs. Previously we reported the synergistic interaction of α-MSH with conventional antibiotics namely, gentamicin, ciprofloxacin and tetracycline against multi-drug resistant MRSA [9]. Therefore, the combination therapy involving α-MSH and conventional antibiotics might avoid the development of resistant SSTIs. The wound healing property of α-MSH has further broadened its therapeutic role and is in agreement with previously published articles those describing how immunomodulatory AMPs can induce cell proliferation/migration and enhances wound healing [7,30]. More precisely, a dimer of C-terminal KPV of α-MSH has been found anti-inflammatory in human neutrophils [31]. Whether the enhanced wound healing due to α-MSH administration was through attenuation of the pro-inflammatory cytokines remains to be elucidated. Of note, a derivative of α-MSH is already in Phase II clinical trial for prevention of post-surgical kidney injury after thoracic aortic aneurysm repair (ClinicalTrials.gov Identifier: NCT00903604).

Conclusion

S. aureus is the most common cause of hospital acquired infections and increasing reports of emergence of drug resistant *S. aureus* in hospitals as well as in the community settings are creating medical emergency [32,33]. With reporting of 57 MRSA out of 100 clinical *S. aureus* strains, we observed high prevalence of the superbug among the patients that are admitted to Indian hospitals for various reasons. Interestingly, this study showed that α-MSH killed the MSSA and MRSA isolates regardless of their sensitivity to oxacillin. Both MSSA and MRSA were susceptible to either α-MSH or other AMPs. This indicates a broad-spectrum anti-microbial coverage of host AMPs, suggesting them as prospective antimicrobials. Not only *in vitro*, but we also showed the *in vivo* anti-staphylococcal effect of α-MSH in animal models of S. aureus infection. α-MSH showed therapeutic efficacy in systemic infection model, as well as in wound infection model created by S. aueus infection. Perhaps, the immunomodulatory behavior of α-MSH is responsible for its better *in vivo* antibacterial efficacy than oxacillin. The experimental outcomes of our previous [8-10,27] and the present work suggest that α-MSH based antimicrobials are clinically suitable and might be developed as anti-MRSA agents.

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