4. SUMMARY
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The increasing resistance of *S. aureus* against existing antibiotics has created a need for the development of novel antibacterial agents (Levy & Marshall, 2004; Sakoulas et al, 2012). Therefore, the new targets for antibiotic therapy are currently of high priority in all over the world (Li, et al, 2010; Rice, 2008; Verma et al, 2012). Of note, recently the host peptides having immunomodulatory action like chemokines and brain peptides i.e., substance P, NK-1 are being considered of high importance for the cure of bacterial infections (Brogden 2005; Catania et al, 2006; Yount & Yeaman, 2012). Some of these immunomodulatory host defense peptides (HDPs) including neuropeptides have already been reported to show direct antibacterial activity in in vitro conditions (Su et al, 2010; Lundy et al, 2008). It is argued that the peptide combined with direct antibacterial and immunomodulatory activity could be better antibacterial therapeutics than the peptide showing only antibacterial activities (Yount & Yeaman, 2012). The most probable reason for that is their interaction with host immune system, such HDP-immune interaction can potentiate killing of microbes by activating and subsequent recruitment of immune cells to the site of infection (Choi et al, 2012). One such host peptide is α-melanocyte stimulating hormone (α-MSH), it is a neuropeptide secreted in brain (Eberle et al 1988; Lipton & Catania, 1997; Rousseau et al, 2007). Apart from its endocrine role, α-MSH has also been known for its outstanding pharmaceutical properties including antipyretic, anti-inflammatory (Catania & Lipton, 1993). Its role in host immunomodulation is fundamental to its observed pharmaceutical properties (Catania et al, 2004) and these immunomodulatory action of α-MSH enable it to help recover brain from neurodegenerative disorders and cures many inflammatory diseases of peripheral tissues (Catania et al, 2010; Bertolini et al, 2009). Besides, the widespread distribution of this peptide and its receptors in many barrier cells like keratinocytes, fibroblasts, and immune cells including neutrophils, monocytes and macrophages suggests that α-MSH has potential role in host defense (Catania et al, 2006; Hill et al, 2006; Bertolini et al, 2009). Early studies had revealed the direct in vitro antibacterial activity of α-MSH and its analogues against *S. aureus*, *E. coli* and *C. albicans* (Charnley et al, 2008; Cutuli et al, 2000; Greico et al, 2003). However, the antibacterial activity and mechanism of α-MSH against *S. aureus* was not well reported. Therefore, the purpose of this study was to characterize the antibacterial activity and to decipher the mode of action of α-MSH against *S. aureus*.
In first section of this study, we began with an aim to explore the invitro antistaph potential of α-MSH against both MSSA and MRSA. Later on we elucidated the influence of several microenvironmental parameters like pH, ionic composition, cell density and time of incubation on the antibacterial activity. Activity of α-MSH was also examined against highly pathogenic and untreatable form of S. aureus i.e., biofilm (Singh et al, 2010; Madhuri et al, 2009). We also investigated the role of amino acid sequence in the staphylocidal mechanism of α-MSH. Our results showed that α-MSH and its C-terminal containing truncates i.e., α-MSH(11-13) and α-MSH(6-13) killed >90% of both MSSA and MRSA cells in the micromolar range and 50% of these cells in nanomolar range. The C-terminal tripeptide of α-MSH retained the antistaph activity of the entire peptide. Moreover, the C-terminal amino acids (K_{11}P_{12}V_{13}) of α-MSH were requisite in the staphylocidal activity of this peptide as removing this region had considerably diminished the staphylocidal activity (Singh & Mukhopadhyay, 2011). However, the peptide containing the N-terminal region, α-MSH(1-5) was found to be ineffective against S. aureus. It was interesting to note that the planktonic as well as biofilm form of MRSA strains were highly sensitive to α-MSH. The staphylocidal activity was dependent upon concentration of peptide, pH and cell density (Madhuri et al, 2009). Increasing inoculums size resulted in reduced killing, whereas decrease in pH of buffer from 7.4 to 4 increased the staphylocidal effect by 21%, this might be due to increase in overall cationic charge of peptide (Carneiro et al, 2003). The antibacterial activity of α-MSH and its C-terminal fragments was neither affected by presence of NaCl nor even by the divalent cations such as Ca^{++} and Mg^{++} at their physiological concentration.

The second section of the study emphasized upon the mode of antistaph action of α-MSH and its active fragments. Permeabilization and depolarization assay results suggested that membrane damage was, at least in part, a major mechanism of staphylocidal activity of α-MSH. Similar to the parent peptide, α-MSH(6-13), α-MSH(11-13) also depolarized and permeabilized Staphylococcus cells (~70-80% cells were depolarized and lysed after 2 hrs peptide exposure in micromolar concentration) (Singh & Mukhopadhyay, 2011; Madhuri et al, 2009). Our SEM and TEM studies clearly indicated severe staphylococcal envelope perturbations including leakage of cell content and loss of cell walls by all three α-MSH based peptides. Furthermore, the penetration of α-MSH based peptides inside the S. aureus cell was investigated by fluorescence microscopy.
Our data also suggests that membrane depolarization occurs prior to membrane permeabilization. As observed, 30 min and 60 min incubation of *S. aureus* cells with α-MSH and either of its C-terminal fragments could induce only up to 10% membrane leakage and 40% membrane depolarization, whereas >90% killing was obtained by all three peptides within 15 minutes. Comparison of the time kinetics of killing and those of membrane permeabilization and depolarization suggests membrane perturbation as a secondary event following the lethal hit of α-MSH based peptides rather than a major cause of staphylocidal activity. This prompted us to speculate the existence of other target site of α-MSH due to the observed gap between membrane damage and killing activity of α-MSH. Interestingly, the inhibition of bacterial DNA and protein synthesis was identified as a result of α-MSH exposure which was also confirmed by the microarray results of α-MSH exposed cells. The cluster of genes related to DNA replication, protein synthesis and transportation pathways were majorly downregulated in α-MSH treated cells, whereas genes involved in glycolysis and amino acid degradation were found upregulated.

Recent research suggests that combining host defense peptides and conventional antibiotics could provide an effective solution against resistant bugs (Gordon et al, 2010; Naghmouchi et al, 2012). Therefore, in the third section of this study we explored the synergistic potential of α-MSH with five conventional antibiotics belonging to four functionally different classes through time kill method. The supplementation of α-MSH resulted in ≥4 fold reduction in bactericidal concentration of gentamicin, ciprofloxacin and tetracycline. This synergistic relation was explained by mechanistic analogy between α-MSH and antibiotics along with increase in antibiotic uptake through α-MSH mediated membrane permeabilization (Sakoulas et al, 2012).

Fourth section of the study focused to evaluate ex-vivo and in vivo activity of α-MSH. Our ex-vivo results showed that the antistaphylococcal activity was retained when α-MSH was placed into whole blood, plasma, and serum. The in vivo antibacterial efficacy of α-MSH was evaluated in mice intravenous staph infection model by quantitative as well as histological analysis. Interestingly, the in vivo results revealed that α-MSH was very active in animal infection model. The bacterial counts in all the target organs of α-MSH treated animals reduced in dose dependent manner. α-MSH (8mg/Kg/day) treated group had shown ≥3 log reduction in kidney bacterial count and ≥2 log reduction in heart, liver, spleen and lungs.
Additionally, the rapid healing of wound was observed in case of the α-MSH treated *S. aureus* infected wounds of mice.

Moreover, α-MSH and its C-terminal fragments α-MSH(6-13) and α-MSH(11-13) had demonstrated the marginal hemolytic and cytotoxic effects in in vitro assay.

In conclusion, α-MSH and its C-terminal fragments, α-MSH(6-13) and tripeptide α-MSH(11-13) exhibited rapid and strong antibacterial activities against both MSSA and MRSA strains. Like other host defense antimicrobial peptides, the α-MSH based peptides caused membrane depolarization followed by membrane permeabilization and the membrane damaging action of full-length α-MSH and its C-terminal fragments were equivalent. Importantly, N-terminal fragments of α-MSH, i.e, α-MSH(1-5) did not contribute in antistaphylococcal activity of α-MSH. It is also important to mention that the minimum sequence required for anti-inflammatory activity of α-MSH is the C-terminal tripeptide i.e., α-MSH(11-13). Therefore, the C-terminal fragments of α-MSH having both anti-inflammatory and antibacterial properties could emerge as excellent antibacterial agents in the treatment of staphylococcal infection. Furthermore, the synergistic effect of α-MSH with gentamicin, ciprofloxacin and tetracycline and its in vivo efficacy in mice infection model and most importantly its antibacterial activity in biofilms as demonstrated in this study suggests its therapeutic potential to combat bacterial infections. Of note, α-MSH peptides have been found to have very little or no toxicity in vitro in the present study or in preclinical studies (Gatti et al, 2006).

Thus, α-MSH and its C-terminal tripeptide with its low toxicity and combined antipyretic, anti-inflammatory and in vivo antimicrobial properties could emerge as an excellent therapeutic agent against resistant pathogens including *S. aureus*.