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ORIGINAL ARTICLE

Modulation of Activity of Salivary Adrenomedullin by pH

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ABSTRACT

Background: Adrenomedullin is a multi-factorial peptide secreted by a range of human cells and is found in high levels in saliva. One potential role of Adrenomedullin is the maintenance of oral health through its antimicrobial activity.
Aim: To characterize how varying pH alters the antimicrobial effect of Adrenomedullin in short-term killing assays.
Study design: Experimental, Laboratory-based study.
Setting: Centre for Clinical and Diagnostic Sciences, Barts and The London, Queen Mary's School of Medicine and Dentistry, Queen Mary, University of London.
Method: Cultures of *Escherichia coli* and *Staphylococcus aureus* were used to demonstrate antimicrobial activity of Adrenomedullin at pH levels of 7.5, 6.5 and 5.5 by employing short-term bacterial killing assays.
Result: The results showed differences in the antimicrobial activity of salivary Adrenomedullin at different pH levels against the two different bacterial species used but were not significant.**Conclusion:** The results concluded that while Adrenomedullin has an antimicrobial effect, a greater range of pH and inclusion of specific oral microbes in the study will be helpful in investigating the hypothesis. However, it is likely that changes in pH in the oral cavity are important for the antimicrobial activity of salivary Adrenomedullin.
Keywords: Adrenomedullin (ADM), Minimum Inhibitory Concentration (MIC), Phosphate Buffered Saline (PBS)

INTRODUCTION

Adrenomedullin (ADM) is a 52 amino acid, multifunctional peptide produced by a wide variety of tissues and cells[1]. It was originally isolated from extracts of pheochromocytoma using elevated platelet cAMP activity as an indicator[2]. Previous studies have demonstrated the antimicrobial effect of ADM against a variety of normal skin, oral, gut and respiratory tract microflora[3,4,5,6]. The Minimum Inhibitory Concentration (MIC) of whole ADM against *E. coli* and the Oxford strain of *S. aureus*has been found to be 12.5µg/ml (2.07µmol l^{-1}) whereas ADM fragments have exhibited different ranges of MIC individually. The mechanism of antibacterial action of ADM is believed to be by cell lysis via channel formation in the bacterial cell membranes[7]. Kapas and colleagues have detected the presence of ADM in whole saliva and various ductal salivary secretions of normal adults as well as fromsupernatants of salivary ductal cell lines[4]. A study carried out by Gröschl and colleagues suggests an important role of salivary ADM in the maintenance of oral health where it was shown to be involved in oral cell proliferation and antibacterial defense[8]. Gene expression profiling followed by real-time polymerase chain reaction analysis has detected ADM up-regulation in carious pulpal tissue. It has also been demonstrated that the gingivalcrevicular fluid ADM levels are higher in chronic periodontitis than periodontally health tissues[9].*Porphyromonasgingivalis,* the major etiologic agent of chronic periodontitis, is killed by concentrations of Adrenomedullin above 500pmol/L[10]. ADM expression is elevated in response to polymicrobial attack and micro-environmental levels of ADM reach an effective antimicrobial peptide concentration[11]. Ultrastructural analyses have shown cell wall disruption of microbeswithin 30 minutes of treatment with ADMin *E. coli* and abnormal septum formation with no cell wall disruption was observed in *S. aureus*[6].

Dental caries is a microbial disease in which bacterial action on dietary fermentable carbohydrates produces acids which diffuse into the tooth causing demineralization of hydroxyapatite mineral. These acids diffuse through the plaque into the porous subsurface enamel dissociating to produce hydrogen ions thereby rapidly lowering the pH in the mouth[12]. The capacity of bacteria to produce acid in combination with growth at low pH is regarded as a virulence factor[13]. The onset and the rate of progression of dental caries are largely dependent on the action of dietary carbohydrates and microbes[14]. Additionally, it is also modulated by other factors such as the protective properties of saliva determined by the secretion rate, buffer capacity and antibacterial components[15]. The salivary pH normally varies from 6.0 to 7.5, with the most alkaline values obtained under stimulated flow rates[16]. In dental literature, the pH value that corresponds to the level of saturation is denoted as a fixed value of pH 5.5 and used as a threshold value for determination of demineralization of teeth in the oral cavity. The dependency of this value on the earlier mentioned determinants means that the critical pH in saliva is not constant, but a more dynamic variable varying around a mean pH of 5.5.

The aim of this study was to observe the antibacterial efficacy of human (whole) ADM against particular strains of *E. coli* and *S. aureus* at a range of pH given the dynamic nature of the pH of the mouth.

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MATERIAL AND METHODS

E. coli 'NCTC 9001' and *S. aureus* 'oxford strain' were first incubated overnight in culture medium, followed by four hour (short-term) killing assays under a range of pH values of 5.5, 6.5 and 7.5 in triplicates and subsequently grown on agar plates under favorable conditions for colony counting. This study included two basic experiments which were repeated 3 times each using two different bacterial species.

Bacterial killing assays

Bacterial Growth Assays

Test Stock: A stock of 4 x Phosphate Buffered Saline (PBS) solution was made while maintaining ratio of PBS to distilled water at 1:3 and autoclaved. Different stocks of PBSsolutions were made at pH 7.5, 6.5, 5.5 using a pH meter.

Culture Medium: Culture medium used was Tryptone Soy Broth (TSB). A stock of TSB was prepared by mixing 30g of TBS powder in 1L of distilled water which was then autoclaved and stored at room temperature.

Culture plates: Agar was prepared by mixing 40g of powder in 1L of purified water, autoclaved and subsequently immersed into a

water bath at 55˚C to prevent setting. Culture plates were poured in a laminar flow hood and were stored in a cold room at 4˚C upon setting.

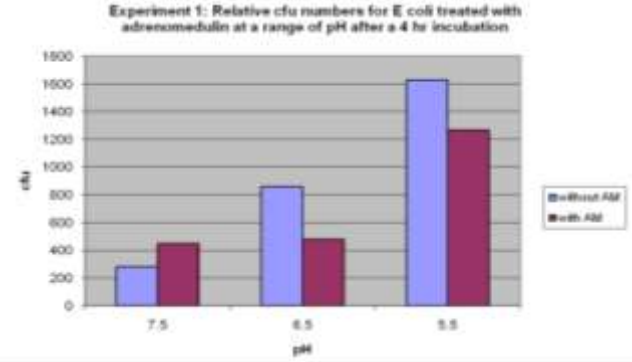
Bacterial Inoculum: 10ml of TSB was inoculated with bacterial strains employing the standard the standard protocol using wire loop and placed in an incubator shaker to allow overnight growth of bacteria at 37˚C.

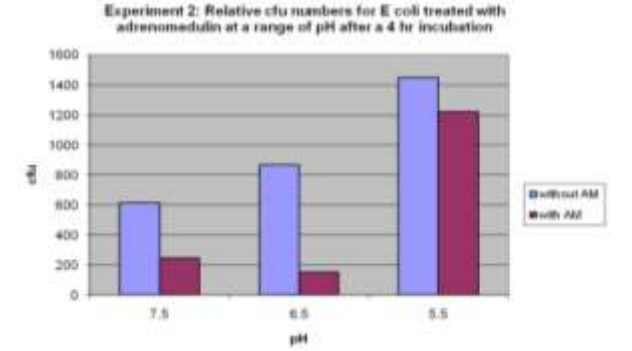
Serial Dilution and Control: Serial dilutions were done for PBS at pH 7.5, 6.5 and 5.5 with and without ADM up to $10^{-3}$and also for control which was 10µl of PBS at pH 7.5 with addition of 10µl of inoculum. The control was not subjected to the 4 hour killing assay in incubator shaker

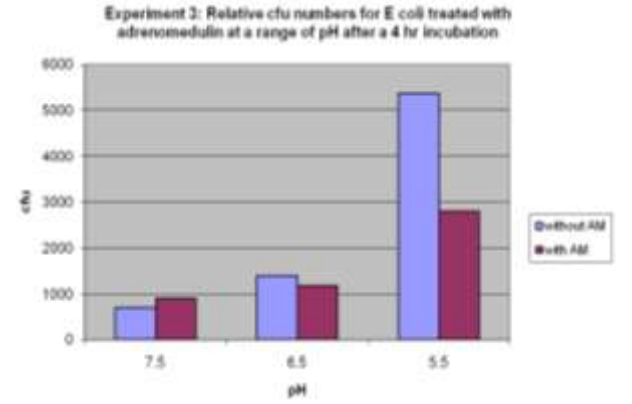
RESULTS

Killing Assays

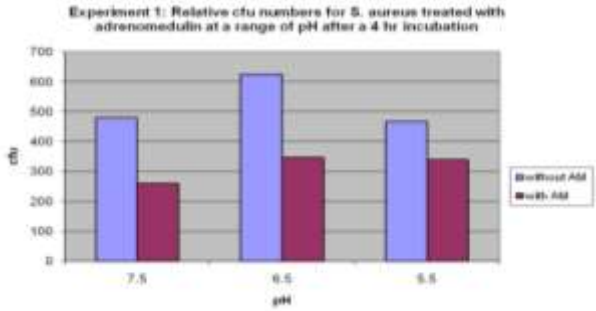
***E.coli*:** The experiments carried out in triplicates consistently showed decline in CFU in the presence of ADM indicating its antimicrobial action. There was a consistent downward trend of CFU at increased pH suggesting that this micro-organism favors lower pH for growth.

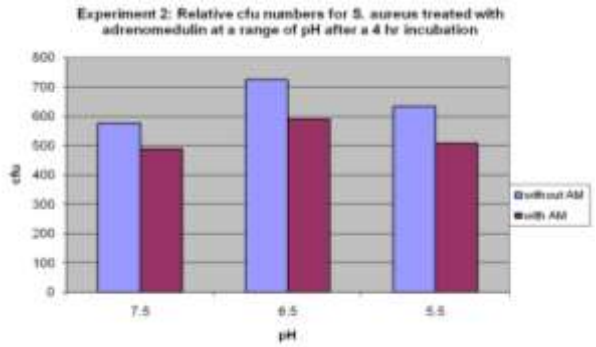


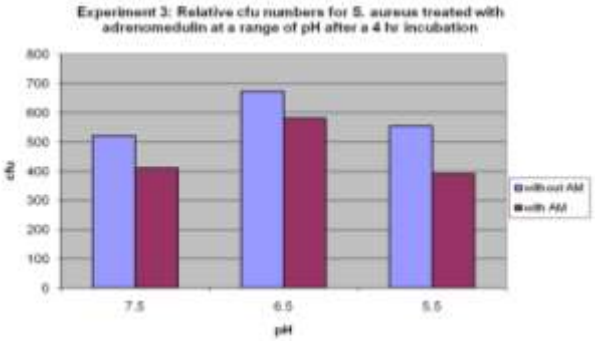




***S. aureus*:** The results of all 3 experiments indicated a decline in CFU in the presence of ADM at all 3 pH levels signifying antimicrobial activity.

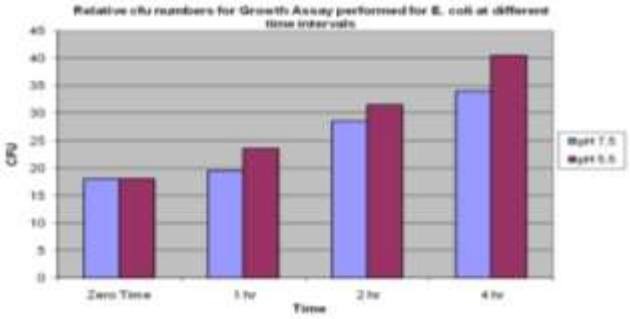




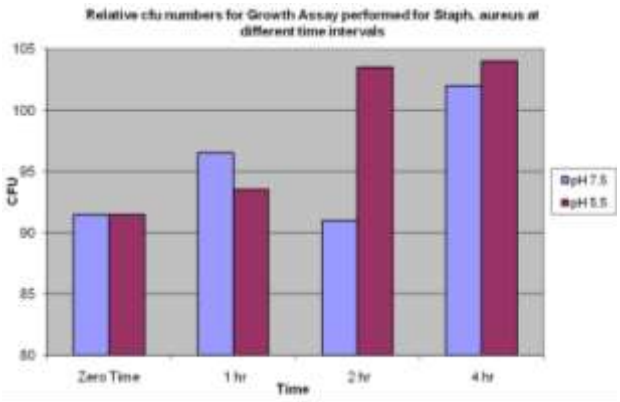


Growth Assays: These were carried out to gain an insight into the growth pattern of both bacterial species in relation to time and pH; and to compare the killing and growth assays for validation of findings obtained for the killing assays.

E. coli: Growth assays showed higher bacterial growth at pH 5.5 at all the time intervals thereby validating the findings of the killing assays.



S. aureus: Higher CFU numbers were observed with each time interval signifying bacterial growth. CFU numbers for the lower pH were more in comparison to the higher pH.CFU numbers were higher for *S. aureus* as compared to*E.coli* at the same Optical Density (OD).



Statistical Analysis: The results from different experiments would have been normalized according to the initial inoculum. Since this was not measured, the results therefore could not be expressed as the same way as the growth assays. Given that there seemed to be a pattern, we used a non-parametric test, (where the data does not fit a standard normal distribution) the Kruskal Wallis Test, to look for any significant association. No such association could be detected.

DISCUSSION

Our study further strengthens the findings of Allaker & colleagues[17] in 1999 and Groschl[8] in 2009 that ADM shows antimicrobial effects against Gram- positive and Gram-negative bacteria.

Bacterial growth inhibition depends largely on the concentration of the antimicrobial agent as well as the CFU of the bacteria used in experiments[18]. Growth inhibition assays could not be carried out in this study due to high cost of peptides. Reports in the literature suggest that electrostatic attraction between negatively charged bacterial cells and the positively charged ADM[19,20].

An interesting and evident finding in our study was that the CFU for *S. aureus* was noticeably higher than *E. coli.* Moreover, antimicrobial activity of ADM was not as efficient against *S. aureus* as against *E. coli.* It has been demonstrated that ADM has high affinity with *E. coli* lipopolysaccharide (LPS)[21] and therefore, it is expected that Gram-negative bacteria would be more susceptible to killing than Gram-positive bacteria[17]. A similar trend was observed in this study.

CONCLUSION

The results concluded that salivary ADM has a definitive antimicrobial effect against the test organisms and changes are seen with varying values of pH supporting our hypothesis. However, a greater range of pH and inclusion of other bacterial species such as *P. gingivalis* will prove to be helpful in investigating the hypothesis and to further unravel the modulation of salivary ADM by varying pH.

Conflict of Interest: Nil

Authors' Contribution: BS: Concept & Design of Study, Principal Author, **ER:** Manuscript writing, **NK:** Statistical analysis, **SH:** Revisiting Critically, **OA:** Quality insurer & Data Collection, **MBS:** Interpretation of results, Final Approval of version **RZ:** Referencing & Final Approval of version

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