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IMMUNOHISTOCHEMICAL EVALUATION OF β DEFENSINS ON INDUCED PERIODONTITIS IN RATS

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ABSTRACT

Background: The health of the gingival tissue has an important effect on the general health. Defensins are proteins found in humans as well as other vertebrates. They have antibacterial, antiviral and antifungal functions.

Objectives: The purpose of the present study was to assess expression of beta defensin on the gingival epithelium in rats.

Methods and materials: A 30 adult Sprague-Dawley male rats were randomly divided into 3equal groups (n=10), 5 rats for experimental study and other fives as control within each group. Ligatures were placed in the inferior frontal group which acted as a gingival irritant. Scarifications were done after 3 days, one week and two weeks alternative. The levels of defensins were tested in the gingival tissue of all rats.

Results: the expression of β defensin 2 showed that there is a statistically significant difference in immunolocalization of Defensin-2 percentage between the 1st group (after 3 days) and control one ($P \leq 0.05$), while there is a highly statistical difference when comparing both the 2nd (after 1 week) and third group (after 2 weeks) versus control group ($P \leq 0.01$).

Conclusions: From the present work, it may assume that, the rat model may be a good system for experimental analysis of the innate immune response to the bacteria in the oral cavity, as well as the potential role of β -defensins in the host response to colonization.

KEYWORDS: β defensin, Periodontitis, Rats.

INTRODUCTION

The gingiva is pink and extends to the alveolar mucosa. It may be keratinized or non-keratinized and adapted to resist the friction. The gingival epithelium is a stratified squamous epithelium which protects the deeper tissues of the periodontium. According to location and composition, the gingival epithelium can be divided into; oral, sulcular, and junctional epithelia. It was considered a strong and passive barrier against the passage of bacteria that

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is present in the mouth [1]. Nowadays, it has been identified that the epithelium is not only a mechanical barrier butt also it may play a role in the progression of the inflammatory destruction of periodontal tissues by secreting cytokines. Furthermore, a new group of antimicrobial peptides were identified in gingival cells. These antimicrobial peptides may be contributed to the innate host defense, and are called defensins [1, 2].

The defensins have broad spectrum antimicrobial activities against most pathogens [3-5]. In general, most of the periodontal diseases results in the destruction of the tooth supporting tissues [6, 7]. There are three types of defensins; the α-defensins are constitutively expressed in human neutrophils [3, 8]. They can inhibit most bacteria and some species of fungi and viruses [7]. These defensins phagocytize the microorganisms by neutrophils and macrophages [9]. The β-defensins were distributed in the respiratory, digestive, and genitourinary systems. They can kill several bacteria, and Candida albicans. Additionally, β-Defensin-3 even has bactericidal effect against multi-resistant Streptococcus. aureus and vancomycin-resistant Enterococcus [10, 11]. The third type of defensins has not been detected in humans but may be isolated from leukocytes from monkeys [12]. They may have antimicrobial activity against a great number of pathogens [13] and were found to have a protective effect l from pulmonary infection by a mouse adapted strain of SARS-coronavirus [14]. Recently, four different human β defensins have been found and functionally typified. However, the number of defensins has been suggested to be over 20 [6, 7]. The type that is expressed and secreted in the human oral cavity is known as hβD 1-3 [15].

According to "pore formation, theory, these antimicrobial peptides target the negatively charged bacterial membrane components, e.g. lipopolysaccharides and phospholipids. Then, they form transmembrane pores, disrupt the integrity of the cell, and finally lead to bacterial lysis [4]. However, these membrane disruptions are not universal, but may be vary depending on the type of defensin and bacteria [16]. Another mechanism has been reported, that defensins may kill bacteria by inhibiting bacterial cell wall synthesis by the interaction with some precursors such as lipid II [17].

Although, β-defensins are broad-spectrum antimicrobial peptides, however, their antibacterial effect is considerably salt dependent. This means that β-defensins show their highest activities with low ion concentrations. On other hand, the antibacterial activities were significantly impaired by the presence of some ions, such as Na, and Ca2 [18]. Among the three β-defensins found in gingiva, hβD- 3 has decreased sensitivity to salts due to its high positive charge [16]. Finally, the bacteria may be exerted resistance to the β-defensins by forming a capsule and modify their cell envelope molecules and cleaving defensins [19-21]. Also, other bacteria can protect themselves from β-defensins by inhibiting the defensin expression pathways. For instance, Treponema denticola inhibits the secretion of hβD-2 and hβD- 3 by suppressing the expression of tumor necrosis factor (TNF) [22, 24]. Hence, the ability of these periodontitis-associated organisms to overcome the β-defensin challenge seems to be associated with their virulence [25]. The aim of the present study was to assess expression and localization of β defensin-2 in the gingival epithelium in rats.

MATERIALS AND METHODS

A thirty male adult Sprague- Dawley rats were used in this study. The rats were obtained from Laboratory animal- Centre for Experimental Medicine, Faculty of Medicine. Al-Azhar University. They were housed for acclimatization, one week before the start of the experiment. All experimental animal protocols were performed according to regulations set by the Institutional Animal Care and Use Committee and were approved by Al-Azhar University. All animal procedures were also performed according to the Declaration of Helsinki and the guidelines

for the care and use of experimental animals established by the National Institutes of Health (NIH). The rats were divided into 3 equal groups, 10 rats each, 5 rats for experimental study and other fives as control within each group. Scarifications were done after 3 days, one week and two weeks alternatively.

The rats were placed on the operating table, which allowed open-mouth maintenance of the animals to facilitate access to the gingiva. Ligatures in "8" with 4/0 non-resorbable silk thread was placed. This ligature acted as a gingival irritant and developer of periodontal disease. After that the animals were kept in the same conditions. On completion of the experiment per each group the animals were anesthetized. The tissue sections were fixed in formalin10%. After complete fixation the samples were dehydrated, cleared and embedded in paraffin wax. The tissue sections were immune-stained using peroxidase-labelled streptavidin-biotin technique to detect rat beta defensin-2 using monoclonal anti-rat beta defensin. Sections of 4 μ thicknesses were cut, mounted, de-paraffinized and rehydrated. Then the Endogenous peroxidase activity was blocked.

After blotting off excess buffer, a universal staining kit was used (Peroxidase LSAB 2 System, Dako, code No K 0672). Tissue sections were treated with biotin for ten minutes, then rinsed and washed. Streptavidin was added for ten minutes then rinsed and washed. The slides were incubated for 5- 10 minutes, then washed in distilled water and counterstained using Mayer's Hematoxylin. The localization of βD-2 was detected by expression pattern. The quantitative image analysis was done by using BIOQUANT life science for windows XP version 8.00.20 MR copyright©2006 (BIOQUANT Image Analysis Corporation 5611 Ohio Avenue, Nashville, TN37209).

The data were collected, tabulated and statistically analyzed. We used one-way analysis of variance ANOVA for determination of the statistical significance of differences between mean values. A probability of ≤ 0.05 defined this significance.

RESULTS

Immunohistochemical evaluation of β-defensins

The immunohistochemical expression of βD was examined in all gingival tissue sections. It was expressed in the oral and the sulcular epithelium but not appeared in the junctional epithelium or the connective tissue. Mainly, it was confined to cytoplasm. The expression of βD-was found in the granular and spinous layers in the healthy control rats. (Fig.1 A). The expression of βD peptide in gingival epithelium after 3 day was mainly confined to the prickle cell and the granular layers (Fig.1B). But it was distributed in the stratum Basale in some sections. The localization of βD in gingival epithelium was appeared in the basal and spinous layers in rats with induced periodontitis after 1 week (Fig.1 C). With periodontitis progression, βD secretion was extends intensely in all layers as seen after 2 weeks (Fig.1D).

Image analysis of βD-2 immunohistochemical staining

Image analysis for βBD-2 immunohistochemical staining was occurred by using computerized image analysis software. The quantitative evaluation of βD-2 staining was determined by the proportion of total positive-stained areas to total epithelial area in the form of percentage (%) within all gingival tissue sample sections.

The statistical comparison between the percentage (%) immunolocalization areas of βD-2 in different groups revealed that there is a statistically significant difference in immunolocalization of βBD-2 percentage between the first group (after 3 days) and control one ($P \leq 0.05$), while there is a highly statistical difference when comparing both the second (after 1 week) and third group (after 2 weeks) versus control group ($P \leq 0.01$) Table (1).

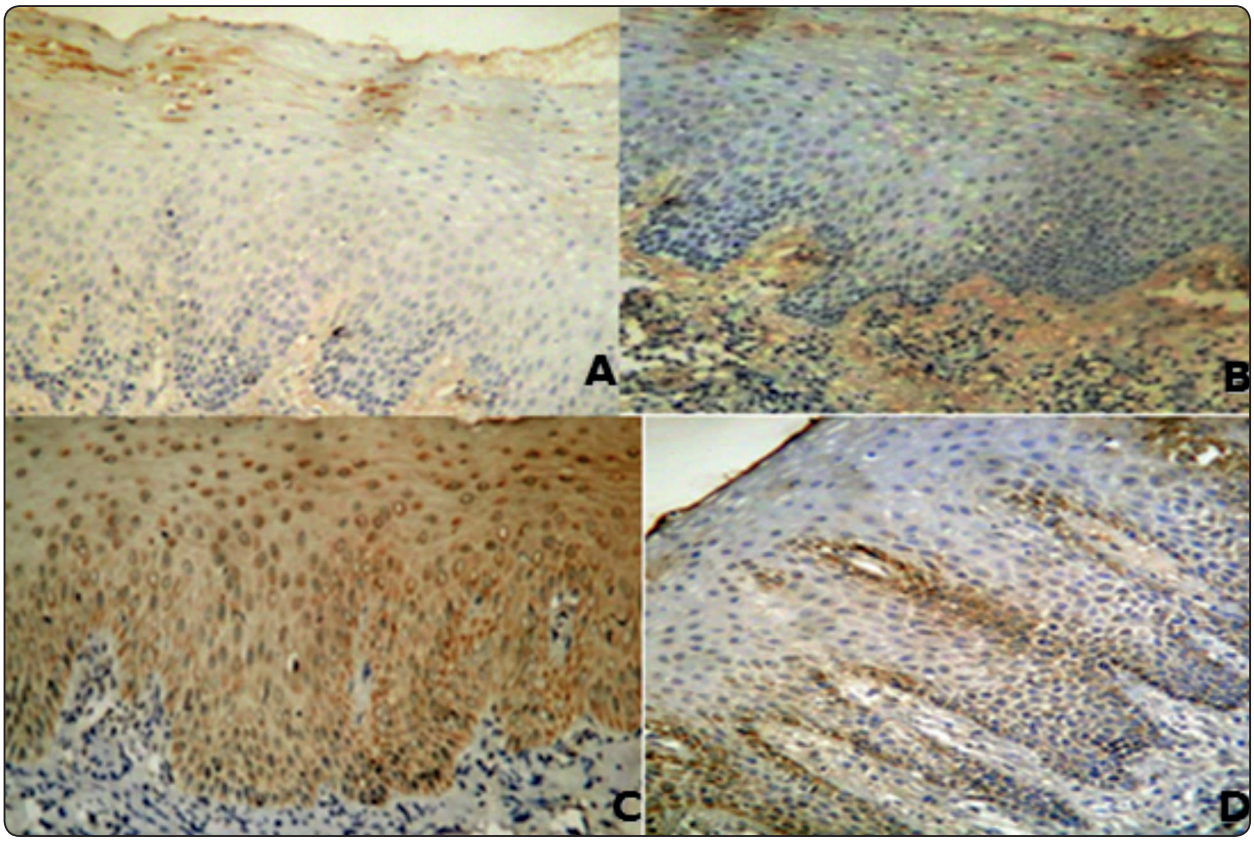

Fig. (1) Photomicrograph showing the re-localization assessment of β-defensins in gingiva before and after induced periodontitis; (A) control group.(B) after 3days of periodontitis induction (C) after 1 week of periodontitis induction (D) after 2 weeks of periodontitis induction . (Original magnification X 400)

TABLE (1): Statistical comparisons of the mean value of percentage for BD-2 positive immunohistochemical localization in all test groups versus control one

	Mean ± SD	P-value
After 3days Vs Control	Control (4.37± 1.82) After 3days (5.14 ± 2.07)	0.0416*
After 1week Vs Control	Control (4.37± 1.82) After 1week (5.68 ± 2.24)	0.0085**
After 2weeks Vs Control	Control (4.37± 1.82) After 2weeks (6.09 ± 2.19)	0.0024**

(P≤0.05) *: significant. (P≤0.01) **: highly significant.

DISCUSSION

The innate immunity, utilizes a broad-spectrum antibacterial peptides named defensins. The defensins, which have been identified in humans and rodents, are divided into three structural. All defensins identified to kill or inactivate a spectrum of bacteria, fungi, and some viruses. [26] The mechanism by which defensins kill or inactivate bacteria is not clear. However, the antibacterial activity of defensins is generally attributed to their ability to disrupt membrane integrity and function, which leads to the microbial lysis. The positively charged defensins interact with negatively charged components of microbial membranes in Gram-ve bacteria, polyteichoic acid in Gram +ve bacteria and phospholipids. [27] They are commonly referred to as natural endogenous antibiotics. Also, these

molecules seem to function far beyond that of simple antimicrobial peptides, including immune-modulatory and anti-tumor activities. In addition, they seem to facilitate and amplify subsequent innate and adaptive immune responses, such as activation and degranulation of mast cells, interleukin and TNF productions. [28]

In the present work, the expression of β defensin 2, showed positive immunoreactions in the in oral and sulcular epithelia but not appeared in both junctional epithelium and connective tissue. This is in accordance with Dale BA, et al. 2001. who attributed that beta -defensins are not detected in the junctional epithelium, an area which is often affected with inflammation, but they are secreted and present in the oral and sulcular epithelium. *(1)* The reaction was detected mainly in the cytoplasm of the granular and spinous cells in healthy control rats as well as the basal and spinous cells in rats with induced periodontitis after 1 week and in the all epithelial cells after 3 weeks. This is in agreement with Lu Q et.al 2005 on their work which stated that, in the gingival tissues, the βD-3 was localized in the basal layers of the gingival epithelium. *(30)*

The β-defensins have been isolated from various tissues, such as skeletal muscles, esophagus, oral mucosa, intestine, and liver. [26] The periodontitis involving the degradation and destruction of periodontal supporting tissue of the teeth. Inflammation and innate immunity have, however, proven to be of major importance in periodontal disease processes. [29] The presence of these molecules may prove central to the gingival epithelial protective mechanisms against induced periodontitis. This study was designed to assess expression of β defensin 2 in gingival epithelium in rats.

The findings of this work could be supported by Oren et.al 2005, who reported that the βD-2 was expressed in the brain and liver. They concluded that the rat may be a useful model to investigate the function and contribution of defensins. [31] Additionally, Annika et.al 2008 suggested that BD1, which was initially believed to be specific epithelium- derived peptide, may be involved in local cardiac innate immune defense mechanisms. [32] Moreover, Kurland et.al 2006 revealed that, three β-defensins (RBD-1, -2 and -5) are expressed in normal rat gingival epithelium. [33]. Lastly, this study also concluded that, the rat model provides a good system for experimental analysis of the innate immune response to the bacteria in the oral cavity, as well as the potential role of β -defensins in the host response to colonization.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

RECOMMENDATIONS

Further comparative studies of multiple and longer periods for investigation are recommended.

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