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EFFECT OF LOCAL NANOSILVER LOADED WITH TETRACYCLINE IN TREATMENT OF INDUCED INFECTION OF RAT BUCCAL MUCOSA WITH PORPHYROMONAS GINGIVALIS (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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KEYWORDS

Albino rat, Porphyromonas gingivalis, Proliferating Cell Nuclear Antigen, silver nanoparticles, tetracycline.

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ABSTRACT

Introduction: Porphyromonas gingivalis, a Gram-negative anaerobic bacterium found to be the major etiologic agent that contributes to periodontitis. It has been found to be the major etiologic agent that contributes to periodontitis and produces many factors that cause destruction to periodontal tissues in both ways directly and indirectly by modulating the host inflammatory response. Silver nanoparticles are considered as an effective antimicrobial agent and their ability to load various medication combinations onto silver nanoparticles provides them a highly antibacterial mechanism of action, to which bacteria have developed resistance. **Aim:** This study was designed to evaluate the effect of silver nanoparticles loaded with tetracycline in treatment of induced infection by Porphyromonas gingivalis of the rat's buccal mucosa through histological examination and immunohistochemical localization of Proliferating Cell Nuclear Antigen (PCNA). **Material and Methods:** Eighteen male albino rats were randomly divided into three groups: normal (negative), infected (positive) with induced P. gingivalis and treated group with nanosilver loaded with tetracycline for fourteen days. Samples were examined under the light microscope for histological and immunohistochemical evaluation through PCNA labeling index where the number of PCNA positive cells were divided by the total cell number in the basal cell layer was estimated and treated statistically. **Results:** Histological findings were represented by increase in the surface epithelium, numbers of clear cells throughout different layers of epithelium and great collagen dissociation at multiple areas in lamina propria of group II animals while group III treated with nanosilver loaded with tetracycline showed epithelium regained their normal histological structure with scattered inflammatory cells with almost normal structure of lamina propria. There was significant decrease in PCNA labeling index of the surface epithelium of the buccal mucosa of group II animals compared with normal group I as well as significant increase in PCNA labeling index of group III. **Conclusion:** Synergetic conjugation of nano-silver with antibiotic drug as tetracycline showed improvement in the histological picture and cellular proliferation rate of epithelial cells of rat's buccal mucosa.

INTRODUCTION

At least six billion bacteria, representing more than 700 species, survive in the human mouth [(1)]. Periodontitis is caused by Porphyromonas gingivalis (P. gingivalis), a Gram-negative oral anaerobe that plays a role in the aetiology of periodontitis, an inflammatory condition that

damages the tissues that support the tooth, eventually leading to tooth loss [2]. *P. gingivalis* rapidly adhere to the host cell surface followed by internalization via lipid rafts and incorporation of the bacterium into early phagosomes. *P. gingivalis* activate cellular autophagy to provide a replicative niche while declining apoptosis [3]. Tetracyclines (TC) being a large family of antibiotics were discovered in the form of chlortetracycline and oxytetracycline (first members of tetracycline group) as natural products [4]. Currently, as a consequence of their overuse, bacteria have developed TC resistance as opposed to the oldest compounds [5]. A study of interest examined the potential of silver nanoparticles (AgNPs) as drug-delivery carriers and vehicles to transport drug molecules to target areas and so improve therapeutic efficacy. They also showed antibacterial synergism with antibiotics [6,7,8]. These assumptions have been tested by several scientist who reported successful conjugation of tetracycline, vancomycin and the immunosuppressant azathioprine [9,10]. AgNPs are being used increasingly in wound dressings, catheters and various households' products due to their antimicrobial activity [11]. Antimicrobial agents are extremely vital in textile, medicine, water disinfection and food packaging. Therefore, the antimicrobial characteristics of inorganic nanoparticles add more potency to this important aspect, as compared to organic compounds, which are relatively toxic to the biological systems [12]. Multiple mechanisms of antibacterial action of AgNPs are considered, but most studies simplified to three primary mechanisms: 1- Adhesion of AgNPs onto the surface of cell wall and membrane.2- Penetration of AgNPs inside the cell and damaging of intracellular structures (mitochondria, vacuoles, and ribosomes), and biomolecules (protein, lipids, and DNA). 3- Generation of reactive oxygen species (ROS), leading to induced cellular toxicity, and oxidative stress [13-15]. The present investigation is to study the effect of local administration of silver nanoparticles loaded with tetracycline, antibiotic drug, in treatment of induced infection of the buccal mucosa of the rat with *Porphyromonas gingivalis* through histological examination using hematoxylin and eosin stained sections and immunohistochemical localization of Proliferating Cell Nuclear Antigen (PCNA) to assess the proliferating capacity of the cells at buccal mucosa. Conventional antibiotic therapeutic strategies against periodontal pathogens suffer from microbial resistance and problems maintaining a functional effective dose. Therefore, there is a need for antimicrobials that are effective against anaerobes as P.gingivalis and have minimal or no side effects. The present study should exaggerate the antibacterial synergic effect of nanosilver particles loaded with tetracycline on oral buccal mucosa, that might have a great periodontal and post-surgical impact.

MATERIALS AND METHODS

This study has been aproved from the ethical comitte in Faculty of Dentistery, Suez Canal University 52/2017. Eighteen adult male albino rats 150 - 180 gram body weight were used in this investigation. They were housed in rat cages and labeled with numerical numbers in well ventilated animal house of the Faculty of Dentistry, Suez Canal University. They were divided randomly into three groups as follows:

- **Group I** consisted of 6 rats, they served as normal negative control group.
- **Group II** (positive control) consisted of 6 rats, they were infected once by injecting the buccal mucosa of the vestibule opposite to upper first molar with 1×10^8 colony forming unit (CFU)/1mL of *Porphyromonas gingivalis* and left for fourteen days untreated [16].

- **Group III** consisted of 6 rats that were subjected to the same procedure as group II then to daily injection at the site of infection with 5g/1ml of Nanosilver 20nm loaded by Tetracycline with a concentration of 30g/1ml of distilled water for fourteen days [17].

Bacterial Strain P. gingivalis ATCC 33277 were imported and purchased from Microbiologics' Company. It was founded in 1971 and is headquartered in St. Cloud, Minnesota, USA.

Delyophilisation of *P. gingivalis* Delyophilisation and culturing *P. gingivalis* was done at National Research Center in Egypt under the supervision of Dr. Magdy Attia professor of microbiology at the agriculture department. All instructions were followed as recommended in the manual catalog bought with bacterial strain.

Preparation of silver nanoparticles Spherical silver nanoparticles and loaded tetracycline nanoparticles were prepared and purchased from Nano Gate Company Cairo, Egypt with a characterization properties for each product. According to Wei et al., the chemical reduction of silver nitrate (AgNO3) with sodium borohydride (NaBH4) and the use of chitosan biopolymer as a stabilising agent resulted in the effective synthesis of silver nanoparticles in an aqueous solution [18,19]. Tetracycline powder was dissolved in silver nitrate solution to get homogenous solution then, the reducing agent was added [7].

Characterization of silver nanoparticles through fourier transform infrared spectroscopy (FTIR) analysis FTIR spectrum of silver nanoparticles ended with 6 peaks found. The absorption bands at 3376 .91cm^{-1}, 2074.15cm-1, 1637.92cm-1, 1417.71cm-1, 1093.03cm-1 and 686.95cm-1 representing the overlapping of N-H group with OH as shown in fig. 1.

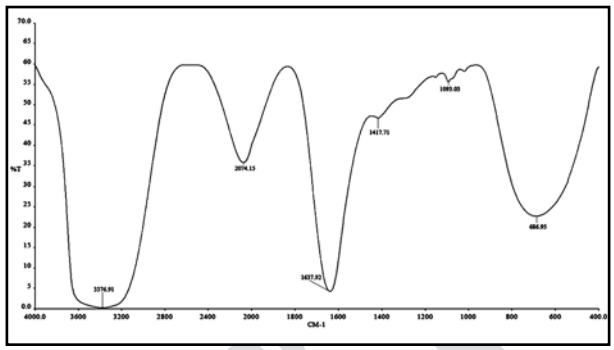

Fig. (1) FTIR spectra of silver nanoparticles stabilized in chitosan by showing their wavelength (cm-1) against their trasmittance (%).

The FTIR spectrum of TC particles stabilized on silver nanoparticles (Tetra@ AgNPs) which ended with 5 peaks found. The absorption bands at 3436.92 cm^{-1}, 2072.04 cm^{-1}, 1638.20 cm^{-1},1095.41 cm^{-1} and 679.64 cm^{-1}as shown in fig.2.

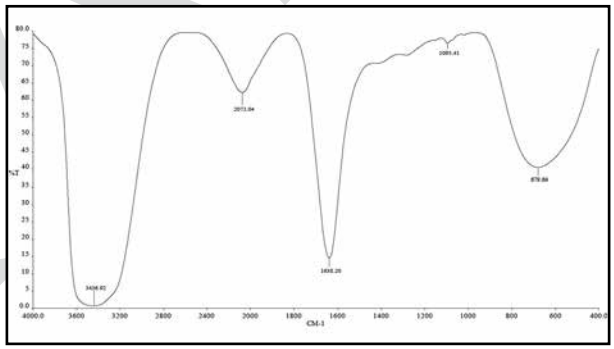

Fig. (2) FTIR spectra of Tetra@ AgNPs by showing their wavelength (cm-1) against their trasmittance (%).

At the end of experiment, all rats were sacrificed by cervical dislocation. Samples were taken from the buccal mucosa at the site of infection of the rats of different groups. Samples were fixed in 10% Neutral Buffered Formalin (NBF) then embedded in paraffin and sectioned to obtain 4-6 micrometer sections ready to be stained with hematoxylin and eosin. Immunohistochemical examination: Four micron thick sections of buccal mucosa were mounted on optiplus positive charged slides to be stained for immunohistochemical localization of PCNA.

Statistical analysis:

All collected data for eighteen slides were calculated, tabulated and statistically analyzed. A normality test was done to check normal distribution for data by Kolmogorov-Smirnov, and then the Kruskal Wallis test was used to compare the differences between groups with Mann Whitney U test to detect pair-wise comparison each group to another as $P<0.05$ was considered to be statistically significant. All statistical analysis was performed using the computer program SPSS software for windows version 26.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp).

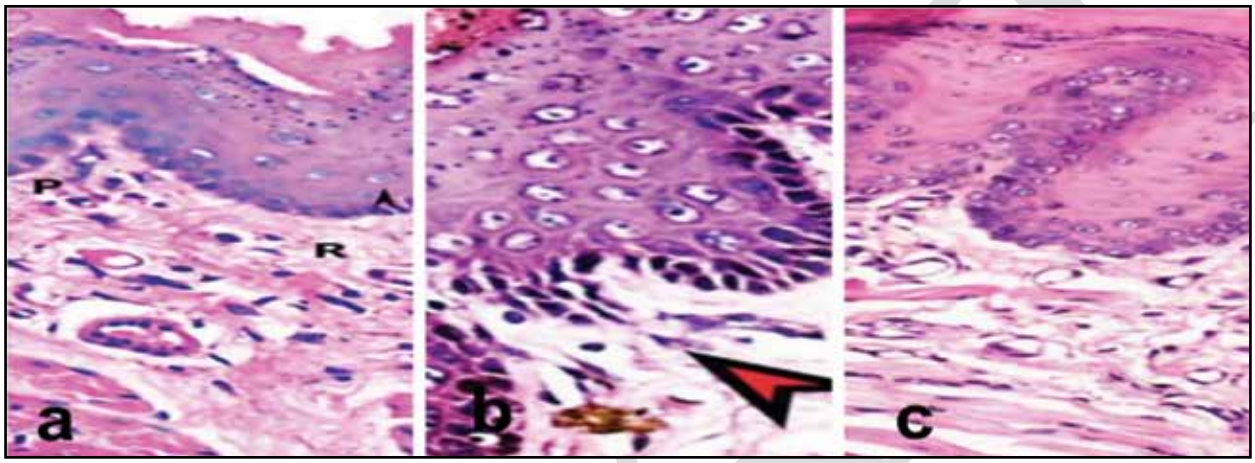

Fig. (3) (a) A photomicrograph of group I normal buccal mucosa showing epithelium with clear cell (arrows) and papillary (P) and reticular layer (R) of lamina propria with few inflammatory cells and bundles of muscles in a cross section (M). (HX&E. orig. mag. 200), (b) showed group II buccal mucosa clear cells scattered along epithelial layers with signs of apoptosis. Areas of discontinuation of basement membrane (red arrows) and dissociation in connective tissue of lamina propria were found. (HX&E. orig. mag. 400) and (c) group VI buccal mucosa showing almost normal appearance of epithelium and lamina propria. Less clear cells through different layers of epithelium was found (HX&E. orig. mag. 400)

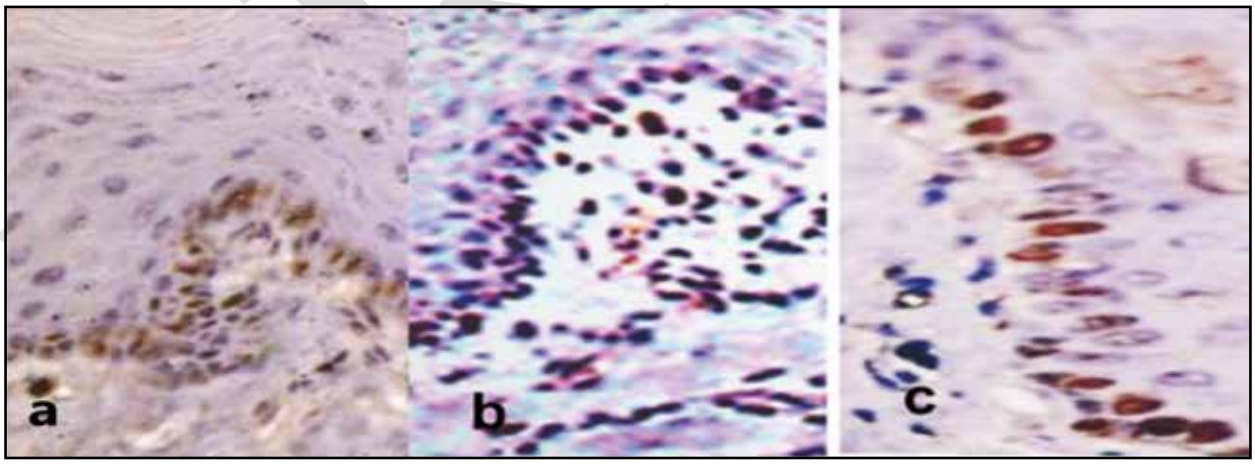

Fig. 4:(a) A photomicrograph of normal group I buccal mucosa showing moderately positive staining reactivity of the basal and supra-basal cells to PCNA.(orig. mag. 200), (b) group II buccal mucosa showing negative staining reactivity of the basal and supra-basal cells to PCNA.(orig. mag. 200) and (c) group III buccal mucosa showing moderate positive staining reactivity of the basal and supra-basal cells to PCNA.(orig. mag. 200)

RESULTS

Histological findings were detected under the light microscope in group II and represented by increase in the surface epithelium. Numbers of clear cells were increased throughout different layers of epithelium while in lamina propria, collagen destruction was distinct while blood vessels became dilated and exhibit increased permeability, and a lot of inflammatory cell infiltration was found. Group III that was treated with TC loaded on AgNPs, showed regeneration of epithelium as their normal histological structure with identified basal, prickle, granular and cornified layers with less clear cells were found. Also, lamina propria showed well defined collagen fibers with almost no dissociation in the connective tissue (fig.3). **Statistic results:** The immunohistochemical localization of the proliferating cell nuclear antigen using PCNA mouse monoclonal antibody revealed that there is a significant difference between the studied groups for the mean Intensity trait at a p-value ≤ 0.05. Group III animals treated with nanosilve loaded by tetracycline gave the highest value than others as shown in table 1.

Table (1) *Illustrates the mean and standard deviation of PCNA labeling index of the surface epithelium of the buccal mucosa of the different groups.*

Table I Mean intensity for PCNA

Groups	Mean	SD
Group I	125.10^{d}	6.89
Group II	88.89^{f}	4.39
Group VI	163.64^{a}	3.22
P-values <0.05	0.00**	

***$^{\&a,b,c}$; means significant difference between groups using Kruskal Wallis at P-values <0.05*

Immunohistochemical results: The infected group II animals by P. gingivalis showed low significant PCNA labeling index which may be due to a possible delay in the rate of turnover of the oral mucosa of the rats. PCNA labeling index of the surface epithelium of specimens of the rats of group III revealed significant increase of PCNA labeling index compared with their controls (fig.4).

DISCUSSION

P. gingivalis can spread through the upper layers of the buccal epithelium and can enter the foundation membrane into connective tissues, according to Tada et al. The exteracellular matrix and tight junction components can be degraded by P. gingivalis gingipain proteases, breaking the physical barriers produced by extracellular connective tissue and cellular adhesion [20,21], that was encountered in our results in group II which illustrated discontinuation of basement membrane and disruption of epithelial cell layer. Intercellular and intracellular invasion by P. gingivalis indicated apoptotic cells, our results supported those of Andrian et al., 2004 who proved that P. gingivalis adheres to, invades and penetrates through multilayers of epithelial cells [22]. Supporting this hypothesis Bhattacharya et al. discovered that keratinocytes exposed to P. gingivalis showed a 75–80% drop in the number of proliferating PCNA positive cells relative to cells in group III came in agreement with the study of Buszewski et al. [7,23] showed that AgNPs functionalized with tetracycline gives better antimicrobial activity, their studies suggest that AgNPs exert antimicrobial activity and proved that functionalization with tetracycline augments this effect. Histological evidence in group II indicates that local tissue apoptosis drives the regulation of immune-inflammatory reactions, which occur in response to microbes producing anti-inflammatory signals affecting phagocytes at the site of infection [24]. Defective control of the inflammatory response in this complex microenvironment can lead to the chronic, hyper-inflammatory diseases, as *Porphyromonas gingivalis* has been associated

with inducing and propagating this aberrant host response [25] which explain the increaese in clear cells within epithelium of buccal mocosa. Degeneration distortion and areas of dissociation of collagen bundles were obviously detected. Blood vessels became dilated and exhibit increased permeability.

In group III treated with tetracycline loaded on silver nanoparticles, the epithelium regained their normal histological structure with identified basal, prickle, granular and cornified layers and the lamina propria and submucosa showed few scattered inflammatory cells. Buccal mucosa of group III showed moderate positive staining reactivity of the basal and supra-basal cells to PCNA that was supported by the study of Ojeda-Martínez et al., who found a full-thickness re-epithelialization in wounds of rats treated with AgNPs dressing loaded by tetracycline [26]. This finding ensures that the rate of proliferation of the cells was markedly high in group III specimens. A study by Gurunathan et al., aimed to investigate antibacterial and anti-biofilm activities of silver nanoparticles alone and in combination with conventional antibiotics against various human pathogenic bacteria which is very close to the goals of the present study. They found that the potential additive or synergistic antibacterial effect of combining antibiotics with AgNPs was evaluated using the disc diffusion method. All six antibiotics tested (ampicillin, chloramphenicol, erythromycin, gentamicin, tetracycline, and vancomycin) showed significant ($p<0.05$) antibacterial effects against both Gram-negative and Gram-positive bacteria. The activities of all the antibiotics were increased in combination with AgNPs in all the test bacterial strains compared to control or AgNPs alone. The same study showed that CFU assay showed that sublethal concentrations of antibiotics or AgNPs alone had a killing effect of approximately 10% to 15%. However, combinations of antibiotics with AgNPs resulted in over an 80% decrease in CFUs compared to controls AgNPs alone [27]. PCNA labeling index of the surface epithelium of specimens of group III revealed significant increase of PCNA labeling index compared with their controls. This result was supported by those of Ojeda-Martínez et al., who found a full-thickness re-epithelialization in wounds of rats treated with AgNPs dressing loaded by tetracycline [26].

CONCLUSION

Based on the results of the present investigation, it can be concluded that, the effect of AgNPs loaded by tetracycline on activating proliferation & maturation of keratinocytes and non kratinocytes of buccal tissue was clear and observed in addition to organization of collagen fibers of lamina propria, thus enhancing the integrity of the buccal tissue and increasing in the labeling index for PCNA.

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