EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 69, 241:259, JANUARY, 2023
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 02-04-2022 • Accept Date : 31-07-2022 • Available online: 1-1-2023 • DOI : 10.21608/edj.2022.130994.2055

THE EFFECT OF TETRACYCLINE LOADED ON NANOCHITOSAN IN THE TREATMENT OF INDUCED INFECTION WITH PORPHYROMONAS GINGIVALIS; (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

Rasha Mohamed Taha* *and* Rania Hanafi Mahmoud Said**

ABSTRACT

Background: The use of nanoparticles for medication delivery offers the possibility of avoiding the negative effects of systemic antibiotic dosing as well as antibiotic resistance in bacteria**.**

Aim of the study: the goal of this study was to see the efficiency of local administration of tetracycline loaded on nanochitosan in the treatment of the induced infection of the albino rats gingiva with *Porphyromonas gingivalis* through Immunohistochemical localization of Interleukin-1beta (IL-1β) as a proinflammatory cytokine.

Material and methods: Fifty adult male albino rats 150 - 180 grams body weight used in this investigation. The animals were observed for any body weight changes. The male albino rats were divided haphazardly into five groups as: **Group I** involved 10 rats; they served as normal negative control group. **Group II** involved 10 rats, they were infected once with *P.gingivalis* that injected into the interdental gingiva. **Group III** involved 10 rats, they were subjected to the same procedure as group II then to daily injection at the site of infection with diluted tetracycline powder. **Group IV** involved 10 rats they were subjected to the same procedure as group II then to daily injection of nano Chitosan at site of injection. **Group V** involved 10 rats they were subjected to the same procedure as group II then to daily injection of Tetracycline loaded on nano Chitosan at site of injection. After rats had been euthanized, the extraction and preparation of their ginigiva were carried out in order to examine histologically and immunohistochemically.

Results: The light microscopic results of group II, III and IV showed degeneration represented by swollen epithelial cells, collagen fibers dissociation of the connective tissue of lamina propria and areas of basement membrane discontinuation, while groups I and V showed almost normal histological picture of gingival tissue. Immunohistochemical results showed significant difference in Group II and III when compared to control. No significance difference appears in group V when compared to control (group I).

Conclusion: Tetracycline loaded on chitosan nanoparticle could represent a new trend to overcome the tetracycline resistance and to avoid the antibiotic adverse effects.

KEYWORDS: Tetracycline, nano particles, Porphyromonas Gingivalis, chitosan

* Associate Professor in Oral Biology Department, Faculty of Dentistry, Suez Canal University,
** Associate Professor in Oral Pathology Department, Faculty of Dentistry, Suez Canal University.

INTRODUCTION

Tetracyclines (TCs) are a family of antimicrobials with a broad spectrum of activity against a variety of Gram-negative bacteria, including A. actinomycetemcomitans. Because of their anti-inflammatory and anti-collagenase characteristics, they are important in the treatment of periodontitis[1]. TCs are antibiotics which decrease protein synthesis both in gramme positive and gramme negative bacteria through interfering with the aminoacyl-tRNA attachment towards the ribosomal acceptor(A) site [2].

In the twenty-first century, antimicrobial resistance (AMR) is a serious public health problem on a worldwide scale[3] . Drug resistance to tetracycline is common throughout bacteria of all types, including those that are Gram-positive or Gram-negative, and it can be affected by a range of mechanisms, including effluxing the drug before it achieves its target, avoiding the ribosomal binding site to prevent drug binding, as well as altering the cell envelope's permeability to prevent uptake [4]. The threat of antibiotic resistance comes from the fact that it has resulted in enormous human and economic losses. Every year, approximately 700,000 people die as a result of inappropriate antibiotic use, which leads to resistance to conventional therapy[5].

Porphyromonas gingivalis (P.gingivalis), a common pathogen found in oral microorganism communities, is the main pathogen responsible for periodontitis [6], an inflammatory condition that affect the supporting tissues of the tooth such can finally result in tooth loss[8]. Periodontitis is caused by *P.gingivalis*, which would be a Gram-negative oral anaerobe which has been detected in more over 500 species of bacteria in human subgingival plaque [7]. Keystone pathogen hypothesis states that *P.gingivalis* may cause chronic periodontitis at even minimal amounts by reshaping the bacteria in oral environment, which results in dysbiosis and disease [9].

There has been a great deal of work done on *P.gingivalis*, due to availability of mutant strains with well-characterized genomes [10]. There are numbers of virulence factors produced by *P.gingivalis* that can act independently or in conjunction with other mediators to induce inflammation[11]. Proinflammatory cytokines including interleukin-1 and interleukin-6 are increased in peripheral CD4+ T helper cells when *P.gingivalis* is present, which aids in the progression of severe periodontitis [12].

P.gingivalis's survival strategies and pathogenicity are heavily reliant on its varied virulence factors, which include structural components (heat shock proteins, fimbriae, lipopolysaccharide, and so on) and secretory components (gingipains and outer membrane vesicles)[4].

The use of nanoparticle (NP) treatments, nanostructured coating of indwelling and other medical devices, and nanodrug delivery systems is one of the promising options for controlling antibiotic resistance. Nanoparticles are materials with at least one dimension ranging from 1 to 100 nm[13]. When material is reduced to the nanoscale, the surface area to volume ratio increases, giving the resulting NPs very high flexibility, solubility, chemical reactivity, and diverse morphologies with different modes of action[14]. The development of nanoparticles for delivery of drugs offers the ability to prevent the harmful effects of systemic medication delivery as well as the injury caused by tumour removal, which has tremendous promise for future cancer treatments.[15]

Chitosan is a naturally occurring cationic polymer made up of units of β-1, 4 glucose amine and β-1, 4 N acetyl glucose amine. Its biocompatibility, biodegradability, and nontoxicity make it an excellent choice for a variety of biological applications, including protein carrier, medicine delivery, and wound healing[16].

Chitosan nanoparticles have a better antibacterial effect than chitosan because of the nanoparticle's

physiochemical characteristics. To better engage with the bacterium, polycationic chitosan nanoparticles with higher surface charge density interface with the polycation's target location, the bacterial cell's negatively charged surface. To achieve a quantum-size effect on bacteria, chitosan nanoparticles must be tightly adsorbed to the bacteria cells' surfaces. This will cause the bacteria cells' membranes to rupture, allowing intracellular components to leak out and eventually killing them[17]. Because of their numerous benefits, chitosan nanoparticles have been employed as medication delivery vehicles. First, because it is biocompatible and biodegradable, chitosan is a safe material. Second, it is made up of watersoluble polymers, which is good for drug delivery carriers since it allows for simple and mild production procedures. Chitosan nanoparticles are thus suited for a wide range of medicines, including macromolecules and labile compounds[18]. A third benefit is that the molecular weights of chitosan may be readily modified by coupling it with different ligands, making it easier to develop new products [19]. Fourth, chitosan promotes absorption by increasing the time in which the substrate is in touch with the cell membrane. In addition, their small size increases drug absorption via the cell membrane. Improved drug bioavailability was achieved by the combination of absorption enhancing effect and nano-sized particles [20]. As a fifth benefit, chitosan nanoparticles offer a wide range of distribution options, including non-invasive channels like the mucous membranes of the nasal, oral, and ocular cavities [21].

The rapid appearance of new resistance mechanisms has exacerbated the current state of AMR control and increased the issue's public health significance. Consequently, the present investigation study the effect of nanotechnology to overcome tetracycline resistance through the evaluation the efficacy of tetracycline loaded on chitosan nanoparticles in treatment of the induced infection of gingival tissue of Albino rats with *P.gingivalis* bacteria.

MATERIAL AND METHODS

The Suez Canal University's Faculty of Dentistry's Research Ethics Committee approved the present study with the number 356/2021. The Guidelines for Experimental Animal Studies were strictly followed in all experiments.

Sample selection and grouping

Fifty adult male albino rats 150 – 180 grams body weight used in this investigation. They were housed in rat cages and labelled with numerical numbers in well ventilated animal house of the Faculty of Dentistry, Suez Canal University, at temperature of 27-30°C, 12hours natural light and 12 hours darkness, with free access to tap water and dry rat pellets. The animals were observed for any body weight changes.

The male albino rats divided randomly into five groups as follows:

- Group I consisted of 10 rats; they served as normal negative control group.
- Group II (infected control) included a total of 10 rats While the rats were sedated with isoflurane aspiration anaesthesia, they were given an injection of 0.5 ml/kg of PBS with isolated colonies of P. gingivalis suspended in it and the turbidity of the inoculum was adjusted to 0.5 McFarland optical density which is equivalent to 1X108 CFU/mL through interdental gingiva, between the maxillary first and second molars on both sides, 50 µL syringe (Hamilton business, Reno, NV, USA) was attached to 1/2-inch with 30-gauge needles to provide injections (BD Franklin Lake, New Jersey, USA) 22. As infections by P.gingivalis easily occurred by contact with mucous membranes as well as accidental inoculation and transfer of bodily fluids. P. gingivalis can be transmitted to humans from animal bites or between humans via direct contact with human saliva, the injection was performed under certain rules of a laboratory scientific

expert at Egypt's National Research Centre to ensure the infection control instruction and decrease the infection hazards.

- **Group III (Tetracycline group)** consisted of 10 rats, they subjected to the same procedure as group II then to daily injection at the site of infection with diluted Tetracycline powder of 0.5ml/kg with concentration of 50 μg /mL of distilled water for fourteen days [23].
- **Group IV** consisted of 10 rats they subjected to the same procedure as group II then to daily injection Chitosan 0f 0.5ml/kg in concentration 50 μg / mL of distilled water at the site of infection for fourteen days [24]
- **Group V** consisted of 10 rats they subjected to the same procedure as group II then to daily injection of Tetracycline were loaded on Chitosan 50nm at the site of infection of 0.5ml/kg with a concentration of 140 μg/mL of distilled water for fourteen days [24].

Rats of all groups left one week after the end of the treatment then., at the end of experiment, all rats were euthanized by cervical dislocation. At the end of the experiment, extraction and preparation of rats maxilla. Maxillae were fixed overnight in Neutral Buffered Formalin10%, then decalcified with 10% EDTA for 8 weekly exchanges. Processed tissues were embedded in paraffin. All samples were stained with hematoxylin and eosin for detection of any histological changes in the gingival tissue and with Interleukin-1 beta (Avidin Biotin peroxidase technique) mouse monoclonal antibody for detection of inflammatory response of tissues.

Immunohistochemical staining of (IL-1β) was carried as follows:

Ultravision mouse tissue detection system: Antimouse HRP/DAB which was brought together with primary antibody (mouse monoclonal antibody of **IL-1β**. The reagents in the kit constitute a label streptavidin-biotin immunoenzymatic antigen detection system. This technique involves the sequential incubation of the section with an unconjugated primary antibody specific to target antigen, a biotinylated secondary antibody that reacts with the primary antibody enzyme labelled streptavidin and DAB chromogen. Then, the immunostaining intensity of IL-1β were assessed using the J Image analyser computerized system to detect the early inflammatory response in gingiva.[25]

Bacterial Strain

P.gingivalis ATCC 33277 were delivered from Microbiologics' Company. 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 catalogue as follow:

Culturing *P.gingivalis*

Tryptone soy agar broth (TSB) was imported and purchased from TM Media, *P.gingivalis* grew on tryptone soy agar broth (TSB) with 5% sheep blood, (1 g/L) menadione, (1 g/L) hemin, and (5g/mL) erythromycin as needed. The strains were grown in an anaerobic chamber (Coy) 28 with a nitrogen, hydrogen, and carbon dioxide ratio of 85-55%, 10%, and 5%, respectively[26,28].

After 24 hours, cells were centrifuged from the growing medium and washed three times with sterilised water. The cells were then resuspended in phosphate buffer saline (PBS) buffer, centrifuged for 5 minutes at 12000 rpm, the supernatant should be discarded. The preceding procedure was performed numerous times before being resuspended in PBS buffer. The optical density of *P.gingivalis* bacteria growth was monitored at 600 nm (OD600) with a UNIKONXL spectrophotometer (Northstar Scientific Ltd.), and this was quantified using the McFarland test, in which all freshly generated bacteria suspensions were suspended at $1X10^8$ CFU/mL in accordance with the 0.5 McFarland test [27].

Preparation and characterization of chitosan nanoparticles

Chitosan nanoparticles and their loading with tetracycline were prepared and purchased from Nano Gate Company Cairo, Egypt with a characterization property for each product. Nanoparticles made from Chitosan are made using an ionotropic gelation technique under which the positive charge of amino groups of Chitosan is cross-linked with the negatively charged anions of tripolyphosphate (TPP). Firstly, chitosan powder has been dissolved in 1% v/v acetic acid solution (28.7 mL, 2.5 mg/mL) and stirred for 2hr to get homogenous solution and then, filtrated to remove the un-dissolved materials and other impurities. After that, TPP aqueous solution was added to a chitosan solution drop wise. After finishing addition of TPP the clear solution turned to turbid suspension. Then white suspension was sonicated for 30minutes, centrifuged at 15,000rpm with DH_2O and dried by freeze-dryer[29].

For the preparation of tetracycline encapsulated chitosan nanoparticles, tetracycline was dissolved in chitosan solution. Then, Tripolyphosphate was slowly added into the solution under magnetic stirring [30]. This was carried in the dark to avoid photodegradation of the tetracycline [31].

The spectrum analysis was performed to develop the nanoparticles at different reaction conditions using Fourier transform infrared spectroscopy (FTIR) for examination of nanochitosan spectra, resulted in 24 peaks. In addition, a Transmission Electron Microscope (TEM) JEM1200EX, JEOL 1010 was used to examine the size and shape of the produced nanoparticles. For TEM, a droplet (4 μL) of nanoparticle solution will be deposited on a simple carbon-coated copper plate.

RESULTS

- **Determination of the sample size**

According to Faul et al. (2007, University of Kiel, Germany), G*Power version 3.1.9.2 was used to calculate sample size; all copyrights were reserved (1992-2014). Using alpha and beta levels of 0.05, the effect size was 0.70, meaning that power was 95%; the estimated sample size (n) for all groups should be 50 samples (10 samples for each group)[32].

- **Body weight results**

For the weight changes during research study, one-way ANOVA was used to compare between the five groups at each time and between periods within each group. A post hoc test known as Duncan's was employed to evaluate the treatments' statistical significance. It is considered statistically significant to have a P-value of ≤0.05. SPSS software version 26.0 for Windows was used (Statistical Package for Social Science, IBM Corp, Armonk, NY).

The body weight findings of groups II, III, VI, and V showed no significant difference with the control group (group I) on day one, but beginning on day two and continuing until the end of the trial, groups II, III, and IV showed a significant difference with the control group. However, from day eight through the end of the experiment, group V exhibited no significant difference from the control group (table I, Histogram I).

Nanoparticles characterization:

The size and shape of particles were estimated and photographed by EM with the primary particle size ranges from 30-50nm, mostly with spherical shape which was verified through spectral signature identification at 430nm **(Fig.1).**

The FTIR spectrum of TC particles stabilized on chitosan nanoparticles (Tetra@ChNPs) which ended with 5 peaks found. The absorption bands at 3444.04 cm^{-1}, 2078.34cm^{-}1, 1634.77 cm^{-1}, and 678.54cm^{-1} as shown in (**Fig. 2).**

Examination with light microscope:

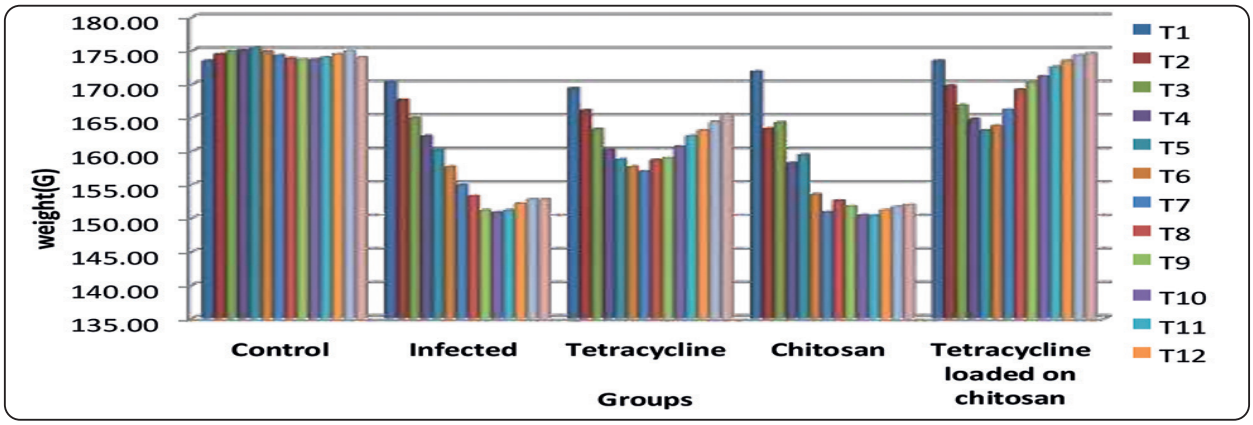
Group I animals (Control group)

The gingiva of control animals showed normal histological features of the surface epithelium and lamina propria. The surface epithelium was

TABLE (I) comparison of body weight between different groups at the same time

Period	Control	Infected	Tetracycline	Chitosan	Tetracycline loaded on chitosan
T1	173.40^{a} ±4.72	170.30^{a} ±5.70	169.30^{a} ±4.47	171.80^{a} ±4.80	173.40^{a} ±4.72
T2	174.40^{a} ±4.22	167.60^{b} ±5.23	166.00^{b} ±4.92	163.29^{b} ±3.74	169.70^{b} ±5.46
T3	174.80^{a} ±3.97	164.90^{b} ±5.20	163.20^{b} ±5.55	164.21^{b} ±5.27	166.80^{b} ±5.37
T4	175.00^{a} ±3.94	162.20^{b} ±5.45	160.10^{b} ±6.14	158.13^{b} ±6.38	164.70^{b} ±6.07
T5	175.40^{a} ± 3.81	160.10^{b} ±6.17	158.70^{b} ±7.04	159.41^{b} ±5.23	163.00^{b} ±5.10
T6	174.80^{a} ±4.39	157.60^{c} ±6.57	157.60^{c} ±7.73	153.49^{d} ±6.04	163.70^{b} ±6.93
T7	174.20^{a} ±4.39	154.90^{c} ±7.42	156.90^{c} ±7.58	150.82^{d} ±6.90	166.10^{b} ±6.76
T8	173.80^{a} ±5.01	153.20^{b} ±8.08	158.60^{b} ±7.50	152.52^{b} ±5.42	169.10^{a} ±6.79
T9	173.60^{a} ±4.48	151.10^{c} ±8.32	158.90^{b} ±7.56	151.68^{c} ±5.71	170.20^{a} ±5.39
T10	173.60^{a} ±4.17	150.80^{c} ±8.07	160.60^{b} ±6.52	150.38^{c} ±4.60	171.10^{a} ±4.93
T11	173.90^{a} ±4.48	151.10^{c} ±7.98	162.20^{b} ±5.53	150.26^{c} ±4.18	172.50^{a} ±4.74
T12	174.40^{a} ±4.38	152.10^{c} ±8.70	163.00^{b} ±4.81	151.10^{c} ±4.26	173.40^{a} ±4.72
T13	174.90^{a} ±3.98	152.70^{b} ±8.26	164.30^{b} ±4.24	151.60^{c} ±4.38	174.20^{a} ±4.02
T14	173.90^{a} ±3.90	152.70^{c} ±8.14	165.40^{b} ±4.60	151.90^{c} ±4.12	174.50^{a} ±3.47

A,b,c; different letters means significant difference between groups at the same Time at p(value)<0.05



Histogram I: Showed difference in body weight mean between different groups.

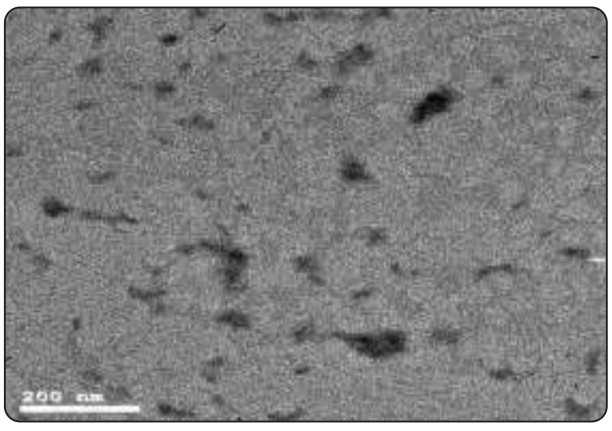


Fig. (1): Microscopic photo under TEM for nano-chitosan particles with average size 50 nm.

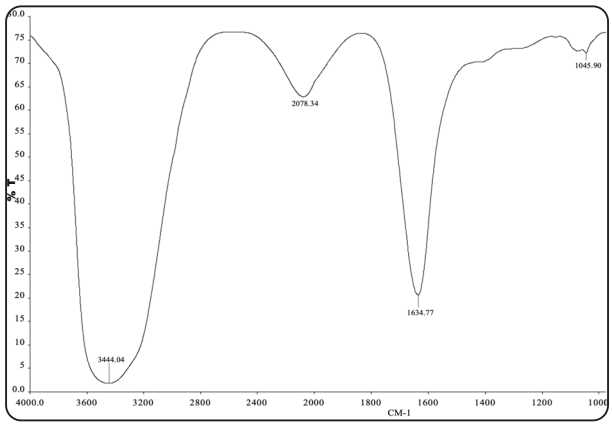


Fig. (2): FTIR spectra of Tetra@ChNPs by showing their wavelength (cm-1) against their transmittance (%).

keratinized stratified squamous type characterized by numerous folding towards the underlying connective tissue of lamina propria forming slender, long and irregular epithelial ridges. The epithelium was formed of four categories of cells, the basal cell layer formed of a single row of low columnar cells resting on the basement membrane, prickle cell layer formed of several rows of polyhydral cells with intercellular spaces and intercellular bridges giving them the spinous or prickly appearance. Then the granular cell containing basophilic keratohyaline granules, layer formed of 2-3 rows of large flattened granular cells and then the most superficial hornified layer with its eosinophillic amorphous appearance. The lamina propria has been made up of two indistinguishable layers, the papillary layer which created the connective tissue papillae and the reticular layer which had coarse collagen fibres, nerves, and massive blood vessels. The fibroblasts predominate among the many connective tissue cells located between all the collagen strands. Occasionally, persistent inflammatory cells have been found. **(Fig 3).**

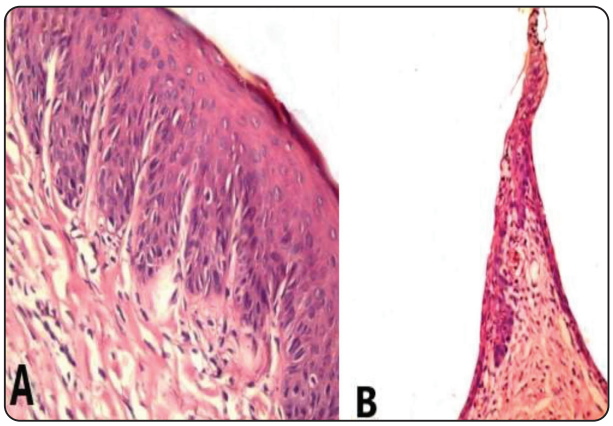


Fig. (3): Dental tissues from a control animal are shown in this photomicrograph with typical normal histological characteristics, (A) long, uneven, and multiple epithelial ridges; the lamina propria with the papillary/ reticular layers; and a normal appearance of the gingival epithelium. (B) normal dental papillae with normal epithelium and underlying connective tissue (H&E. orig. mag. X400).

Group II (infected group)

Gingiva of rats infected with *P.gingivalis* showed a dramatic change in histological appearance compared to controls, with variation in thickness of epithelium and swelling in some epithelial cells. Epithelium showed loss of characteristic pattern of epithelial ridges, where some of the epithelial cells became broad and flattened, sometimes partially or completely absent. Some areas of the epithelial cells, especially the stratum basal and suprabasal cells, showed marked increase in the number of clear cells. The underlying lamina propria revealed dissociation and degeneration of the collagen fibres. Areas of discontinuation of basement membrane were found. Dental papillae showed destruction in their surface epithelium with formation of microcystic lesion and dissociation of the underlying connective tissue **(Fig. 4).**

Group III (tetracycline group):

Gingiva of group III that was treated with diluted tetracycline powder showed marked atrophy of the surface epithelium, some areas showed swelling of epithelial cells. Areas of basement membrane discontinuation were recorded. Lamina propria showed collagen fibers dissociation and inflammatory cells infiltration. Dental papillae showed atrophied epithelium with cytoplasmic vacuolization and microcystic lesions and areas of connective tissue dissociation in lamina propria **(Fig. 5)**.

Group IV animals

The gingivae which were treated with Chitosan showed poor progress in the tissue as surface epithelium appeared atrophied with high numbers of clear cells than normal and abnormal pattern of epithelial ridges. Connective tissue showed partial regeneration. Dental papillae showed atrophied epithelium and dissociation of connective tissue **(Fig.6)**.

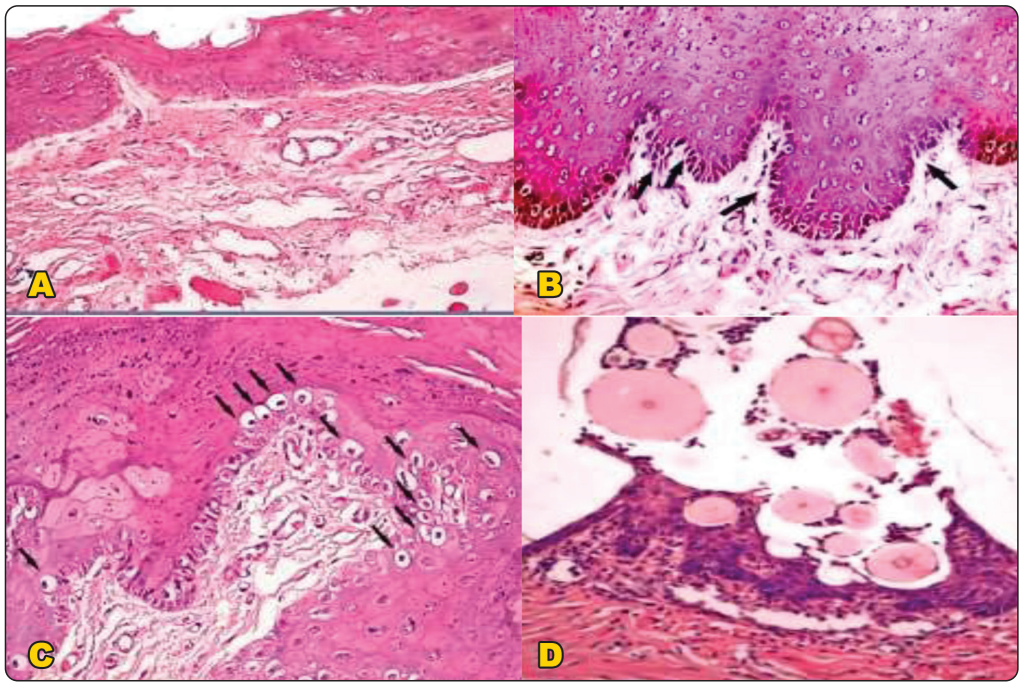


Fig. (4): Photomicrographs of group II showing (A) atrophied epithelium with abnormal pattern of epithelial ridges, dissociation of collagen fibers of the lamina propria. (B) Marked increase in number of clear cells in basal and suprabasal layers, areas of discontinuation of basement membrane. (C) marked swelling of cells in different epithelial layers, clear cells (arrows) in basal and parabasal cells and dissociation of underlying connective tissue. (D) destruction in the dental papillae with formation of microcystic lesion in the epithelial layers (H&E. orig. mag.X250, X400).

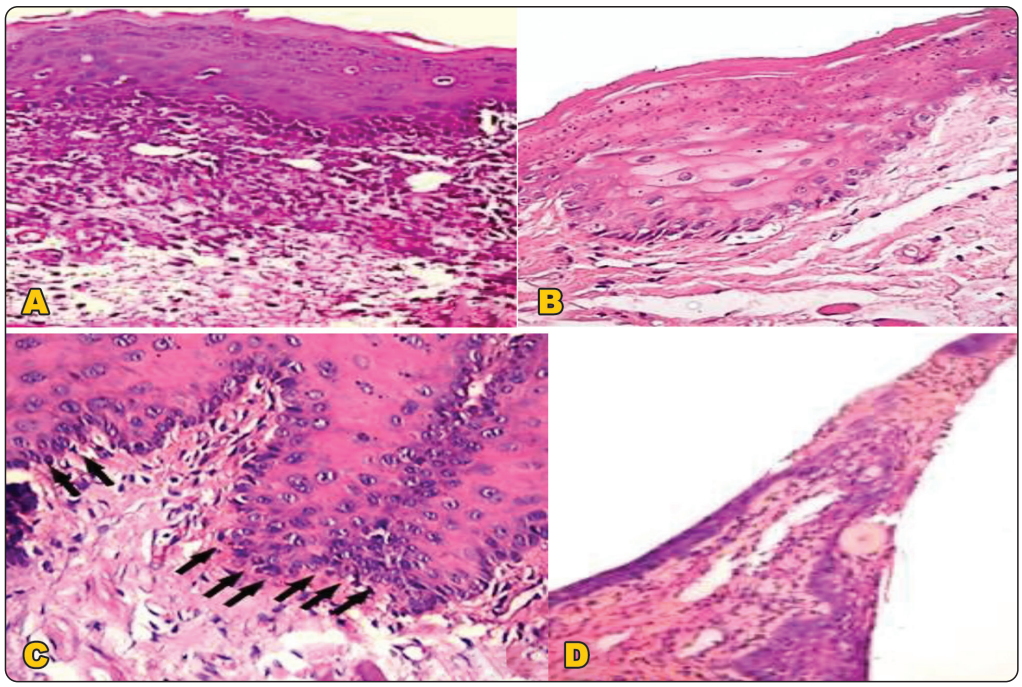


Fig. (5): Photomicrographs of group III showing (A) atrophied epithelium with cytoplasmic vacuolization, lamina propria with collagen fibers dissociation and inflammatory cell infiltration (B) surface epithelium with swelling of some epithelial cells disassociation of collagen fibers of lamina propria. (C) basement membrane with areas of discontinuation (arrows). (D) dental papillae showing atrophied epithelium with cytoplasmic vacuoles and microcystic lesion connective tissue showing areas of dissociation (H&E. orig. mag.X250, X400).

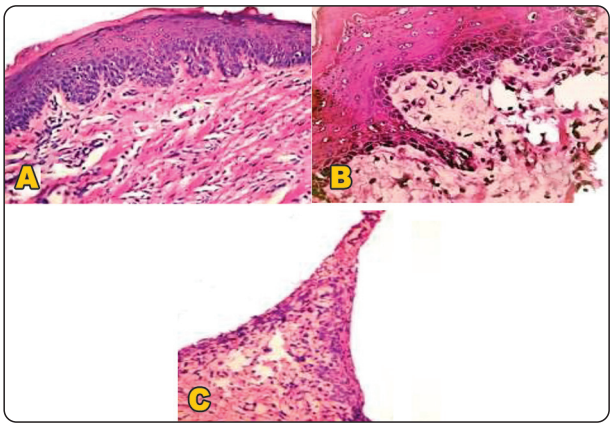


Fig. (6): A photomicrograph of group IV showing(A) atrophied epithelial surface of gingiva. (B) atrophied epithelium of gingiva with abnormal epithelia pattern, areas of connective tissue dissociation. (C) dental papillae with atrophied epithelium and dissociation of connective tissue with scattered inflammatory cells (H&E. orig. mag. X250, X400).

Group V animals:

The gingivae of that group were treated with tetracycline loaded on chitosan. The epithelium regained its histological structure with normal epithelial thickness and ridges pattern were with identified basal, prickle, granular and cornified layers, the epithelial ridges nearly regained their regular pattern. The epithelium- connective tissue interface was intact. Newly formed collagen fibers were found in lamina propria surrounding blood capillaries, diminish nerve fibers and inflammatory cells were observed (**Fig. 7**).

Immunohistochemical examination interleukin-1β

Control group showed negative immune reaction to interleukin-1β for epithelium and connective tissue. The infected group II showed strong positive reaction to the interleukin-1β in epithelium and connective tissue while tetracycline group showed moderate positive reaction to interleukin-1β especially in connective tissue. Moderate positive reaction in epithelium and connective tissue for group IV to IL1β. The group V treated with tetracycline loaded on chitosan showed mild positive reaction to interleukin-1β in epithelium and negative reaction in the connective tissue **(Fig. 8)**.

Statistical analysis

Normality testing was performed to ensure the samples were distributed normally, and the data was examined using Kruskal-Wallis analysis to have a comparison between 4 groups for at a P value ≤0. SPSS software (version 26.0, Statistical Package for Social Science, Armonk, NY: IBM Corp.) was utilised on a windows system.

Using Kruskal-Wallis analysis, the results of group II, III and IV showed marked significant difference from control group; however, group V showed no significant difference when compared with control group (**Table II, Histogram II).**

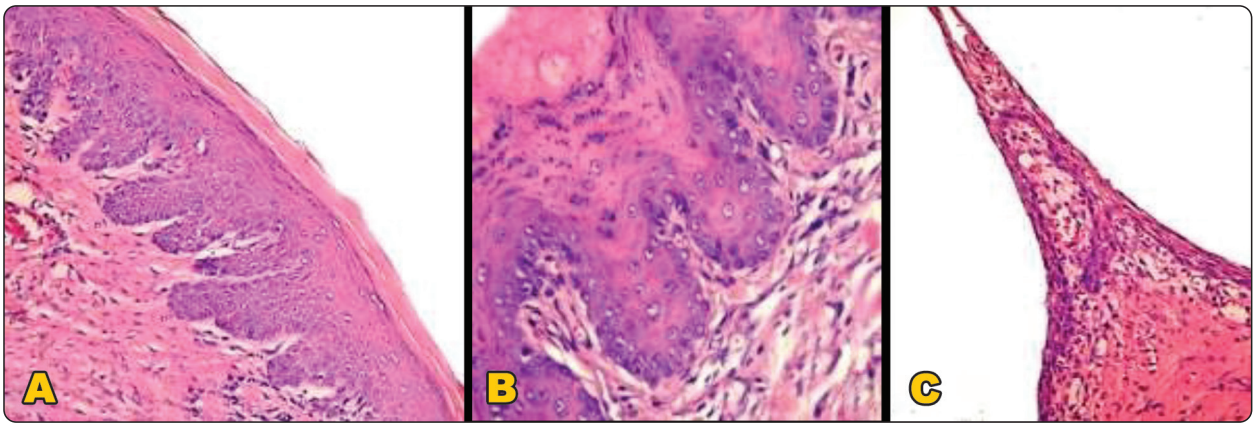


Fig. (7). A photomicrograph of group V animals showing (A&B) normal epithelium thickness with normal pattern of epithelial ridges, almost normal connective tissue of lamina propria with intact epithelial connective tissue interface. (C) dental papillae with almost normal surface epithelium and normal connective tissue (H&E. orig. mag. X250, X400).

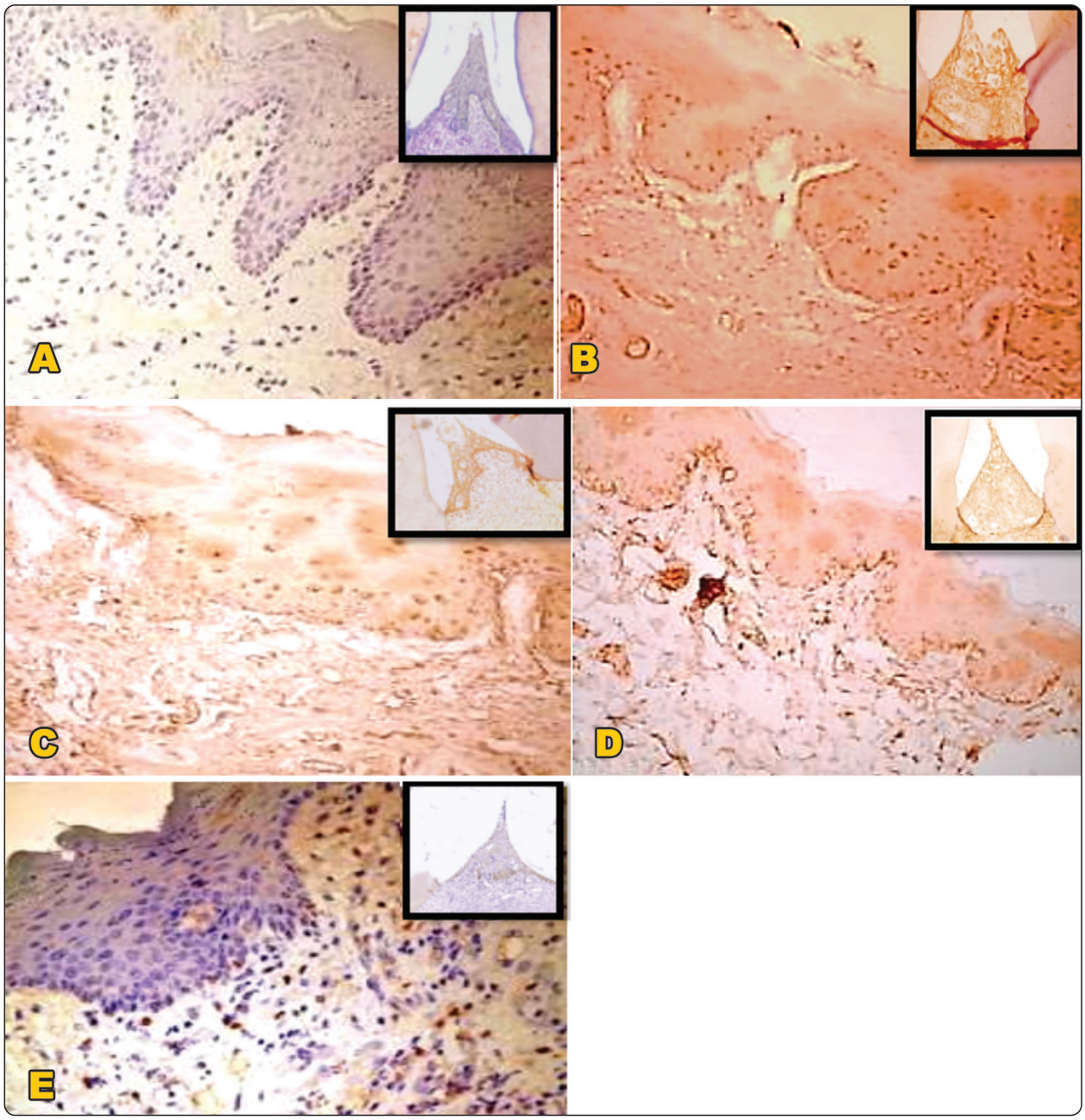
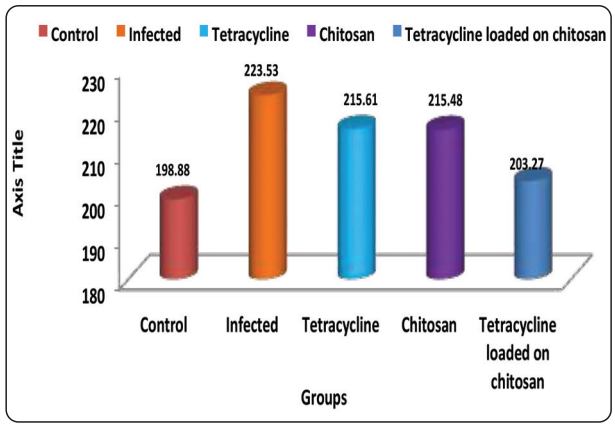


Fig. (8): Photomicrograph showing (A) control group with negative reaction to interleukin 1β (B) strong positive reaction for both epithelium and connective tissue for group II to IL1β (C) moderate positive reaction in epithelium and connective tissue for group III to IL1β (D) moderate positive reaction in epithelium and connective tissue for group IV to IL1β (E) mild positive reaction to negative reaction of epithelium and connective tissue for group V to IL1β (Interleukin-1β antibody Orig.mag. X250, X400).

TABLE (II) Image

Groups	Image	Kruskal-Wallis Test	P-value 0.05
Control	198.88[d]±4.33		
Infected	223.53[a]±3.42		
Tetracycline	215.61[b]±3.32	42.32	0.000***
Chitosan	215.48[b]±3.00		
Tetracycline loaded on chitosan	203.27[c]±3.60		

a,b,c; means significant difference between groups at P value<0.05 using Kruskal-Wallis Test



Histogram II: Represents difference in the mean IL-1β optical density between the different groups.

DISCUSSION

Periodontitis is an inflammatory condition in teeth supporting structures like gingiva, periodontal ligament, the alveolar bone, and cementum. They are common in most human populations and may lead to teeth exfoliation in severe cases[33].

Periodontal diseases are classified by the American Academy of Periodontology (AAP) into two basic categories: gingivitis and periodontitis, depending on whether the periodontal tissue is envolved[34]. Gingivitis is described as inflammation of the gingival tissues caused by dental plaque accumulation. It is clinically described as tissue swelling, redness, and bleeding. In gingivitis, the periodontal ligament and alveolar bone are intact, that is to say the teeth attachments' are not affected[35]. Gingivitis can persist for long periods of time without affecting the periodontium unless subjected to local environment or the host has high susceptibility[36].

The gingival tissue was chosen in the present study as it is the first tissue affected by infection and if not treated the periodontal ligament and the alveolar bone could be involved causing periodontitis, also the gingival tissue showed high level of interleukein-β1 during inflammation[37] which is immunohistochemical marker used in the present study. The rout of administration of bacteria and antibiotic through the interdental papillae was selected as interdental papillae is more accessible for injection and is considered a representative portion of gingiva.

Organisms, particularly Gram-negative bacteria like *Porphyromonas gingivalis (P.gingivalis)*, have increasingly few therapeutic options. Recently, according to the ability of *P.gingivalis* strains to cause abscesses in a mouse model, they have been assorted into invasive or non-invasive. The invasive strain of *P.gingivalis* has been shown to have more pathogenic activity in vitro and in vivo than the non-invasive strain [38,39].

P.gingivalis induces apoptosis in human gingival epithelial cells (HGECs) via gingipains[40]. In a study performed by Stathopoulou et al. 2009, they found that the change in cell integrity and shape that leads to cell death is one of the most noticeable aspects of epithelial cell apoptosis. This morphological alteration is mostly caused by an abnormality in the actin cytoskeleton. The cleavage of actin by mammalian interleukin-1 beta-converting enzyme (ICE) -like proteases heralds the death of a cell, therefore mean actin is not a direct substrate for the ICE-like family of proteases but disable the cytoskeleton thus cause cell death[41].

The previous studies may explain the results of the present investigation as the infected group

by *P.gingivalis* bacteria showed variation of the gingival epithelial thickness as areas showed decrease in the thickness of the epithelium and other areas showed increase in their thickness with swelling of epithelial cells. Epithelium showed loss of characteristic pattern of epithelial ridges, where some of the epithelial cells became broad and flattened, sometimes partially or completely absent. Stathopoulou et al. 2009 observed changes in cell shape, integrity and detachment with *P.gingivalis* challenge in human gingival epithelial cell[41].

P.gingivalis has been shown to adhere to and infiltrate primary gingival epithelial cells (GEC) monolayers and multilayers[42]. The main fimbriae, a crucial virulence component, mediate *P.gingivalis* adhesion. Fimbrillin (FimA), a filamentous component subunit, interacts with of host tissue and other bacteria to enable interaction [43]. It has been discovered that the P.gingivalis fimbriae's epithelial cell cognate receptors via integrins β1[42]. Upon first attachment with *P.gingivalis*, an integrin-associated signal cascade is initiated. The microtubular structure and microfilaments of cellular actin facilitate bacterial entry. Through number of lipid elements, signals by the host cells lead to entry of *P.gingivalis*. Many virulence factors, such FimA, RgpA/B and Kgp, are decreased when P.gingivalis invades, whereas stress-related proteins, including heat shock and peroxidases proteins, are increased[44]. *P.gingivalis* require the previous changes to adapt to the intracellular environment[45].

Cell-cell interactions can be disrupted by *P.gingivalis*, resulting in decreased TER and increased FD-4 passage, which can compromise epithelial barrier function. *P.gingivalis* also had an impact on the distribution of two essential tight junction proteins (zonula occludens -1 and occludin)[46].

P.gingivalis can infiltrate several cell types, and data suggests that it employs diverse mechanisms to avoid lysosomes in epithelial and endothelial cells[47].

Tada et al. 2019 revealed that *P.gingivalis* may penetrate the basement membrane of gingival epithelial cells and reach connective tissues. Proteinase enzymes (gingipain) from *P. gingivalis* can destroy extracellular connective tissue adhesions.[48] That was encountered in our results which illustrated as discontinuation of basement membrane and disruption of epithelial cell layer.

A vast range of microorganisms can affect the buccal tissues of the mouth cavity by inflammation they cause. Microorganisms appear to change phagocytes by releasing anti-inflammatory signals that affect local tissue apoptosis, which in turn modulates the immune system's response to the infection[49] . The aberrant host response has been connected to *P. gingivalis* [50], that means in such complicated environment, inadequate control of the inflammatory response might result in chronic, hyper-inflammatory diseases. Local tissue damage is caused by a persistent hyper-inflammatory response make the Pattern Recognition Receptors (PRRs) get activated by the Pathogen-Associated Molecular Patterns (PAMPs). PRRs are found on epithelial, neutrophilic, macrophagic, and dendritic cells[51]. PAMP activation of PRRs triggers the innate response to microbial assault and produces adaptive immunity to eliminate infections[52,53]. The oral mucosa immunity contains aggregates of lymphoid follicles, and dendritic cells, as well as individual lymphocytes. Langerhans cells, macrophages, and mast cells are the most significant dendritic cells in the oral mucosa[54]. The ability of dendritic cells to trigger immunological responses was found to be related to their level of activation and maturity. Chemokines produced by activated dendritic cells have been shown to play a significant role in recruiting other immune cells, including natural killer cells, monocytes, and T cells. [55].

Thus, the presence of a local immunologic defence barrier in the oral mucosa is suggested by the constant link between immunocompetent cells and the epithelium of the oral mucosa which explain the persistence of high number of inflammatory non

keratinocytes through whole thickness of epithelium in the infected group II with *P.gingivalis*. Previous research clarified the *P.gingivalis's* significance in the breakdown of connective tissues and collagen fibres. *P.gingivalis* may infiltrate and reproduce in epithelial cells, allowing it to be transported into underlying epithelial cells and enter subepithelial connective tissue before the cell breaks off[56]. *P.gingivalis* is able to cause desquamation of oral mucosa lining which spreads the infection through taking the epithelial cells as carriers[57]. *P.gingivalis* is a pathogen that produces dipeptidyl aminopeptidase IV (DPPIV), which may operate as a virulence factor and contributes to connective tissue degradation[58]. The pathogenic role of *P.gingivalis* DPPIV in the advancement of periodontitis is thought to be caused by *P.gingivalis* infiltrating connective tissue, mostly via a paracellular pathway, then attaching to the ECM via the interaction of fibronectin and DPPIV which colonise together. In reaction to the bacterium, many pro-inflammatory mediators are produced, resulting in the mobilisation of inflammatory cells (though not necessarily in great numbers)[59]. Furthermore, *P.gingivalis* produces a considerable quantity of proteases, such as gingipains and DPPIV, which cause MMPs to be activated. MMPs, together with gingipains and DPPIV generated by this bacterium, subsequently degrade the ECM proteins. Furthermore, DPPIV reduces the mobilisation and attachment of fibroblasts to ECM components during the healing process from inflammation by inhibiting cell adhesion to fibronectin[60].

Tetracyclines have numerous qualities that make them ideal as antibiotic medications, such as effectiveness against Gram-positive and -negative organisms, demonstrated clinical safety, tolerability, and the ability to be administered via intravenous (IV) injections or oral formulations for most classes[61]. Tetracycline's antibacterial properties, like those of all antibiotic classes, are vulnerable to both class-specific and intrinsic antibiotic-resistance mechanisms[62].

Findings of tetracycline group of present study showed no improvement in the infected gingival tissues by *P.gingivalis* as the surface epithelium showed atrophy and formation of cytoplasmic vacuolization with areas of breakdown of epithelial-connective tissue interface. Also, the lamina propria showed destruction of the collagen fibers. Arredondo et al. 2021, suggested that although minimal use of tetracycline lately, a quite resistance against it appeared in subgingival bacteria that may be due to previous mutations of genes[63]. If the host recognizes that homeostasis has been disrupted by infection, it may initiate both immunological and tissue healing processes [63]. Tetracycline antibiotics are a class of medicines that can activate disease tolerance mechanisms[64]; Antigen presentation by Langerhans cells has also been shown to result in T-cell tolerance or deletion, which suggests a possible immunoregulatory role[65]. Subset of mouse dermal non-keratinocytes which express CD301b (also identified as macrophage galactose-type C-type lectin 2 (MGL2)) that drag induction of T-Helper cell responses by Interferon-Regulatory Factor-4 (IRF4) which help in tissue regeneration by improving granulation tissue formation and speeding up renewed epithelium[66].

The regulating role of epithelial non-keratinocytes led to enhanced toll-like receptor (TLR) signalling, which was associated with skin inflammation[67]. Myeloid non-keratinocytes in inflamed mouse skin may aid in the differentiation of the invading monocytoid precursors into activated non-keratinocytes which produce pro-inflammatory mediators (like IL-12, TNF, and IL-23) and trigger TH1-dependent and TH17-dependent immunological responses[68]. This explains why the number of non-keratinocytes (clear cells) increased in our study.

A wide variety of microorganisms, including bacteria, algae, fungus, and yeasts have been tested in vitro and in vivo using nanochitosan, including interactions with liquids, and composites and films [69]. Regardless to the antibacterial effect of

nanochitosan, the results of group IV in present study that treated with nanochitosan showed poor progress in the infected gingival tissue which may suggest that low doses of nanochitosan have weak antibacterial effect.

New and modified antibiotics have been created to get over the bacterial resistance. However, it is a slow and infrequently successful procedure, hence innovative nano-sized materials have been developed to efficiently transport these antibiotics to targeted tissues without bacterial resistance[70]. This was the idea of promoting antibacterial finishing chemicals that were added to textiles, as they are manufactured from nano-sized antobiotics which overcome the infection of textiles with germs causing unfavourable changes in odour and colour[71].

The term "nanoantibiotics" or "nanobactericides" refers to nanoparticles that have antimicrobial or antibacterial activity under their own, while the term "nanocarriers" refers to nanoparticle-based delivery methods, liposomes, a first nanotechnology that is used for such a purpose[72]. Dendrimers, polymeric nanoparticles are common metallic nanoparticles which are used to improve the pharmacokinetic/ pharmacodynamic optimization techniques exist for antibiotics, antibiotics. including adsorptive, dispersive, encapsulating, and nanocarrier encapsulation[73].

Previously, nanocarriers were employed to transport medications in a target-specific manner, raising the concentration of the drug at the desired location[74]. This can also lessen the drug's undesired interaction in healthy tissue, resulting in a lower overall harmful effect. They can also shield the encapsulating active agent from degradation or inactivation in a biological system. Biocompatible, biodegradable, non-immunogenic, and not fundamentally hazardous nanocarriers are required[75]. Several options for utilising nanoparticles as antimicrobials and for antibiotic administration have recently been reviewed[78].

There are several advantages to using nanocarriers in this regard including enhanced biodistribution, greater drug solubility and bioavailability dose reduction. Drug cotransport and extended systemic circulation are controlled in long term and in targeted drug delivery methods. Aside from the fact that nanocarriers may help reduce side effects and increase therapeutic effectiveness or restore clinical efficacy they can also allow drug modified releasing to accomplish the aforementioned clinical objectives. They have attracted many researchers who are currently examining different elements of their antimicrobial activities as antimicrobial nanomaterials as well as carriers of traditional antibiotics to fully understand the advantages against the limits[76].

Because Chitosan possesses several -NH 2 groups in its backbone, it is easily chemically and physically bonded to create nanoparticles in acidic environments[77]. One of the main advantages of using physical cross-linking is that it avoids harmful materials and reduces negative effects to enhance biocompatibility[78]. Physical cross-linking is created by combining the positive charges of chitosan with the negative charges of multivalent ions[79].

There are many amino groups on the surface of Chitosan nanoparticles because they contain the same functional groups as chitosan, but their nanostructured form gives them a larger surface area[80,81].

Divakar et al. 2018 conducted a study to increase the activity of chitosan by converting it into nanoparticles and conjugate it with TC to assess the capacity of chitosan nanoparticles to suppress dental infections such as *Streptococcus mutans* (S. mutans) and *P.gingivalis*. The study found that chitosan and TC-chitosan had more activity against P.gingivalis strains than the standard medication employed in the experiment because their minimum inhibitory concentrations (MICs). Theauther added that use of TC-chitosan nanoparticles on *S. mutans* and *P. gingivalis* resulted in a considerable drop in MIC and an increase in the zone of inhibition for

both control and test organisms[82]. Also, according to Bavariya et al., 2014 chitosan causes a mild inflammatory response, followed by fibroblast proliferation, granulation tissue development, and tissue remodelling[83].

Andrew et al. 2018, conducted a triple comparative study between chitosan hydrogel-based tetracycline (0.5 percent, 0.7 percent, and 1 percent) and chitosan hydrogel without tetracycline and dissolved tetracycline powder to assess the in-vitro cytotoxicity of chitosan hydrogel-based tetracycline on fibroblasts. The mean cell viability of fibroblasts grown and treated with chitosan hydrogel-based tetracycline was found to be the highest[84], that comes in co-ordination with the present study. In Group V (the gingiva was treated by tetracycline loaded on chitosan) has more privileges on group III which was treated with diluted tetracycline and also proves our findings in group V showed a low cytotoxic effect on fibroblast cells.

In the present study (**Histogram I**), loss of body weights of rat models was noticed in groups II, III, and IV that may be explained as rats in these groups lost their appetite and ability to feed because of the inflammation of treated gingivae. Meanwhile, it was obvious that rats of group V were in good health and gained more weights.

All sections of this study were incubated with mouse monoclonal antibody for immunohistochemical localization of interleukin-1β. Group II, III and IV sections showed strongly positive reaction at all strata of epithelium and more intense at superfacial layers besides basal ones. Scattered positive reaction was found in the lamina propria especially in the reticular layers.

Despite belonging to the same cytokine superfamily, *P.gingivalis* culture supernatants can variably regulate IL-1b and IL-18 expression in monocytic cells. IL-1b expression is induced early but transiently, whereas IL-18 induction is delayed but subsequent expression is stable throughout time. Both cytokines regulate *P.gingivalis* virulence factors in distinct ways, and this is important. Gingipain inhibition shows that gingipain activity is involved in some of the IL-1b induction via *P.gingivalis* culture supernatants, with LPS accounting for only the remainder [85].

Castro et al. 2011 approved our immunohistochemical results of group III animals treated with tetracycline powder, which revealed a strong positive reaction, as they evaluated the effectiveness of doxycycline and tetracycline to modulate serum levels of IL-6, IL-1, and TNF, as well as cytokine receptor/receptor antagonist TNFR1 and IL-1RA. The use of tetracycline or doxycycline reduced cytokine levels significantly. Similarly, serum IL-1 and TNF values were elevated at baseline and increased somewhat in untreated patients. Both medications caused a considerable increase in IL-1 levels in patients by day 3. However, when compared to the control group, intragroup analysis revealed a substantial increase post-treatment[86].

Examination of sections of group V treated with local administration of tetracycline loaded on chitosan, revealed weakly positive reaction to IL-1β as localized patches of staining through epithelium and more frequent at superficial layers rarely at basal ones besides some diffuse stains especially at superficial layers. Similar staining reactivity to IL-1β was found in the lamina propria, which was demonstrated as weakly positive staining reaction in the connective tissue fibers, cells and blood vessels, furthermore it was very limited to localized areas.

These findings in group V support the concept that nano TC's anti-inflammatory characteristics can reduce the activity of polymorphonuclear cells by inhibiting prostaglandin E2 synthesis through phospholipase A2[87]. Nano TC may reduce neutrophil-mediated tissue injury by blocking their migration and degranulation, as well as potentially decreasing oxygen radical synthesis[88].

CONCLUSION

Tetracycline loaded on chitosan nanoparticle could represent a new trend to overcome the tetracycline resistance and to avoid the antibiotic adverse effects.

RECOMMENDATION

It is recommended further studies on bigger samples with adding extra- positive control group to study the possible mechanical impact on tissues after injection with saline considering possible mechanical trauma.

REFERENCES

1. Goodson JM, Haffajee A, Socransky SS. "Periodontal therapy by local delivery of tetracycline." J Clin Periodontol. 1979;6:83–92
2. Connell SR, Tracz DM, Nierhaus KH, Taylor DE. "Ribosomal protection proteins and their mechanism of tetracycline resistance." Antimicrobial agents and chemotherapy 2003;47:3675-3681.
3. WHO Antimicrobial resistance: global report on surveillance. Geneva: World Health Organization. 2014.
4. Tenover F.C. and McGowan J.E. "Antimicrobial Resistance." International Encyclopedia of Public Health. 2008; 3:211–219.
5. Willyard, C. "The drug-resistant bacteria that pose the greatest health threats." Nature. 2017; 543:15-19.
6. Mei F. , Xie M., H. Xiaofei, Long Y, Lu X. , Wang X. and-Chen L. "Porphyromonas gingivalis and Its Systemic Impact: Current Status." Pathogens. 2020, 9: 944-976.
7. How KY, Song KP, Chan KG. "Porphyromonas gingivalis: an overview of periodontopathic pathogen below the gum line." Frontiers in microbiology. 2016; 7:53-58
8. Bodet C, Chandad F, Grenier D. "Pathogenic potential of Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia, the red bacterial complex associated with periodontitis." Pathologie-biologie. 2007; 55:154-162.
9. Olsen, I., Lambris, J. D., & Hajishengallis, G. "Porphyromonas gingivalis disturbs hostcommensal homeostasis by changing complement function." Journal of Oral Microbiology. 2017; 9(1), 1340085.
10. Hajishengallis, G., & Lamont, R. J. "Beyond the red complex and into more complexity: The polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology." Molecular oral microbiology. 2012; 27(6), 409e419.
11. Hajishengallis, G. "Immunomicrobial pathogenesis of periodontitis: Keystones, pathobionts, and host response." Trends in Immunology. 2014; 35(1), 3e11.
12. Khan I, Saeed K, Khan I. "Nanoparticles: Properties, applications and toxicities." Arab J Chem. 2017; 12:908–931
13. Gonzales JR, Groeger S, Johansson A, Meyle J. "T helper cells from aggressive periodontitis patients produce higher levels of interleukin-1 beta and interleukin-6 in interaction with Porphyromonas gingivalis." Clinical oral investigations. 2014; 18:1835-1843.
14. Simon-Deckers A, Loo S, Mayne-L'hermite M, Herlin-Boime N, Menguy N, Reynaud C. "Size, composition and shape-dependent toxicological of metal oxide nanoparticles and carbon nanotubes toward bacteria." Environ Sci Technol. 2009; 43:8423–8429
15. Bin He,Xin Sui,Bing Yu,Song Wang,Youqing Shen &Hailin Cong. "Recent advances in drug delivery systems for enhancing drug penetration into tumors." Drug Delivery. 2020; 27: 1474–1490.
16. Ibrahim H.M., Reda M.M., Klingner A. "Preparation and characterization of green carboxymethylchitosan (CMCS) – Polyvinyl alcohol (PVA) electrospun nanofibers containing gold nanoparticles (AuNPs) and its potential use as biomaterials." International Journal of Biological Macromolecules. 2020; 151:821 -829
17. Abdel-Fattah WI, Sallam ASM, Attawa N, Salama E, Maghraby AM,Ali GW. "Functionality, antibacterial efficiency and biocompatibility of nanosilver/chitosan/silk/phosphate scaffolds 1. Synthesis and optimization of nanosilver/chitosan matrices through gamma rays irradiation and their antibacterial activity." Materials Research Express, 2012. 1: 035-024.
18. Ragelle H, Riva R, Vandermeulen G, Naeye B, Pourcelle V, Le Duff CS, D'Haese C, Nysten B, Braeckmans K, De Smedt SC. "Chitosan nanoparticles for siRNA delivery: optimizing formulation to increase stability and efficiency." Journal of Controlled Release. 2014; 176 54-63.
19. Wang, Q.-S.; Wang, G.-F.; Zhou, J.; Gao, L.-N.; Cui, Y.-L. "Colon targeted oral drug delivery system based on alginate-chitosan microspheres loaded with icariin in the treatment of ulcerative colitis." Int. J. Pharm. 2016; 515:176–185.

20. Kim TH, Park IK, Nah JW, Choi YJ, Cho CS. "Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier." Biomaterials. 2004; 25 (17): 3783-3792.

21. Lu Y, Cheng D, Lu S, Huang F, Li G. "Preparation of quaternary ammonium salt of chitosan nanoparticles and their textile properties on Antheraea pernyi silk modification." Textile Research Journal. 2014; 84 (19): 2115-2124.

22. Hu Y, Li H, Zhang J, Zhang X, Xia X, Qiu C, Liao Y, Chen H, Song Z, Zhou W. eriodontitis Induced by P. gingivalis-LPS Is Associated With Neuroinflammation and Learning and Memory Impairment in Sprague-Dawley Rats. Frontiers in Neuroscience, 2020; 14: 658- 668.

23. Warren D. and Fraser V. "Infection control measures to limit antimicrobial resistance." Crit Care Med. 2001; 29(4):128-134

24. Zheng Y, Ke L, Lu Y, Zuo Q, Deng G, Wang H, Zeng X. Enhanced Healing and Antimicrobial Efficacy of Chitosan-g-Polyacrylamide in a Rat Model of Gingival Ulcers. Frontiers in Chemistry. 2020; 8: 273-289.

25. Wu Y, Fassihi R. "Stability of metronidazole, tetracycline HCl and famotidine alone and in combination." Int J Pharm. 2005; 290:1-13.

26. Wessman P, Håkansson S, Leifer K, Rubino S. "Formulations for freeze-drying of bacteria and their influence on cell survival." Journal of visualized experiments: JoVE. 2013.

27. TM Media. "SOYA PEPTONE DIGEST AGAR is used for enrichment and isolation of fastidious microorganism with or without blood." 2020.

28. Akca AE and Akca G. "The Comparative Evaluation of the Antimicrobial Effect of Propolis with Chlorhexidine against Oral Pathogens: An In Vitro Study." BioMed Research International. 2016; 2016:8-15.

29. McFarland J. "The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines." Journal of the American Medical Association 1907; 49:1176-1178.

30. Taha M, Elfeky S, Mohamed M, Amin R. "Production of WellDispersed Aqueous Cross-Linked Chitosan-Based Nanomaterials as Alternative Antimicrobial Approach." Journal of Inorganic and Organometallic Polymers and Materials. 2018; 28.

31. Lee B-S, Lee C-C, Wang Y-P, Chen H-J, Lai C-H, Hsieh W-L, et al. "Controlled-release of tetracycline and lovastatin by poly(D, Llactide-co-glycolide acid)-chitosan nanoparticles enhances periodontal regeneration in dogs." International journal of nanomedicine. 2016;11:285-297.

32. Ramos-Vara J, Miller M. "When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry—the red, brown, and blue technique." Veterinary pathology 2

33. Faul, F., Erdfelder, E., Lang, A.G., & Buchner, A. "G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences". Behavior Research Methods. 2007; 39:175191

34. Bostanci N., Belibasakis G. N. "Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen." FEMS Microbiol. Lett. 333 1–9. 10.1111/j.1574-6968; 2012; 2012.02579.x

35. Wiebe C. B., Putnins E. E. "The periodontal disease classification system of the American Academy of Periodontology–an update." J.Can. Dent. Assoc. 2000; 66: 594–597

36. Williams R. O., Feldmann M., Maini R. N. "Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis." Proc. Natl. Acad. Sci. U.S.A. 1992; 89 9784–9788.

37. Preiss DS, Meyle J. Interleukin-1 beta concentration of gingival crevicular fluid. J Periodontol. 1994 May;65(5): 423-8.

38. Offenbacher S. "Periodontal diseases: pathogenesis." Ann. Periodontol. 1996; 1:1-821.

39. Livermore DM. "Has the era of untreatable infections arrived?" Journal of Antimicrobial Chemotherapy. 2009; 64:i29-i36.

40. Baek KJ, Ji S, Kim YC, Choi YmVirulence. "Association of the invasion ability of Porphyromonas gingivalis with the severity of periodontitis. Virulence, 2015; 6(3):274-81

41. Stathopoulou, P.G., Galicia, J.C., Benakanakere, M.R., Garcia, C.A., Potempa, J., and Kinane, D.F. "Porphyromonas gingivalis induce apoptosis in human gingival epithelial cells through a gingipaindependent mechanism. BMC Microbiol." 2009; 9: 107-119

42. Nagata, S. Apoptosis by death factor. Cell. 1997; 88: 355–365.

43. Dickinson, B.C.; Moffatt, C.E.; Hagerty, D.; Whitmore, S.E.; A Brown, T.; Graves, D.T.; Lamont, R.J. "Interaction of oral bacteria with gingival epithelial cell multilayers." Mol. Oral Microbiol. 2011; 26: 210–220.

44. Nakagawa, I.; Amano, A.; Kuboniwa, M.; Nakamura, T.; Kawabata, S.; Hamada, S. "Functional Differences among FimA Variants of porphyromonas gingivalis and their effects on adhesion and invasion of human epitheilial cells." Infect. Immun. 2002; 70, 277–285

45. Tribble, G.; Lamont, R.J. "Bacterial invasion of epithelial cells and spreading in periodontal tissue." Periodontology. 2000; 2010: 52, 68–83,

46. Hanel, Alyssa N.; Herzog, Hannah M.; James, Michelle G.; Cuadra, Giancarlo A. "Effects of Oral Commensal Streptococci on Porphyromonas gingivalis Invasion into Oral Epithelial Cells." Dentistry Journal. 2020; 8(2), 39–53.

47. Ben Lagha, A., Yang, Y., Trivedi, H. M., Masters, J. G., & Grenier, D. "A Dual Zinc plus Arginine formulation attenuates the pathogenic properties of Porphyromonas gingivalis and protects gingival keratinocyte barrier function in an in vitro model." Journal of Oral Microbiology. 2020; 12(1), 1798044.

48. Tada H, Nishioka T, Takase A, Numazaki K, Bando K, Matsushita K. "Porphyromonas gingivalis induces the production of interleukin-31 by human mast cells, resulting in dysfunction of the gingival epithelial barrier." Cellular microbiology. 2019;21:e12972.

49. Reyes L, Herrera D, Kozarov E, Roldán S, Progulske-Fox A. "Periodontal bacterial invasion and infection: contribution to atherosclerotic pathology." Journal of clinical periodontology. 2013;40:S30-S50

50. Gonzalez OA, Novak MJ, Kirakodu S, Stromberg AJ, Shen S, Orraca L, et al. "Effects of aging on apoptosis gene expression in oral mucosal tissues." Apoptosis. 2013; 18:249-259.

51. Socransky S, Haffajee A, Cugini M, Smith C, Kent Jr R. "Microbial complexes in subgingival plaque." Journal of clinical periodontology. 1998; 25:134-144.

52. Medzhitov R. "Toll-like receptors and innate immunity." Nature Reviews Immunology. 2001,1:135-145

53. Kawai T, Akira S. "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nature immunology. 2010,11:373.

54. Ricklin D, Hajishengallis G, Yang K, Lambris JD. "Complement: a key system for immune surveillance and homeostasis." Nature immunology. 2010,11:785-797.

55. Janeway CA. "The immune system in health and disease." http://www garlandscience.com 2001.

56. Cutler CW, Teng Y-TA. "Oral mucosal dendritic cells and periodontitis: many sides of the same coin with new twists." Periodontology. 2007;45:35-43.

57. Ji S., Choi Y., Choi Y. "Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis?" J. Periodontal Res. 2015;50: 570e585.

58. Groeger S., Doman E., Chakraborty T., Meyle J. "Effects of Porphyromonas gingivalis infection on human gingival epithelial barrier function in vitro." Eur. J. Oral Sci. 2010; 118, 582e589

59. Kumagai Y ,1 Yagishita H ,2 Yajima A,1 Okamoto T ,3 and Konishi K. "Molecular Mechanism for Connective Tissue Destruction by Dipeptidyl Aminopeptidase IV Produced by the Periodontal Pathogen Porphyromonas gingivalis." Infect Immun. 2005; 73(5): 2655–2664.

60. Kiyama M, Hayakawa M, Shiroza T, Nakamura S, Takeuchi A, Masamoto Y, Abiko Y. "Biochim Sequence analysis of the Porphyromonas gingivalis dipeptidyl peptidase IV gene." Biophys Acta. 1998; 1396(1):39-46

61. D'Ostroph AR, So TY. Treatment of pediatric Clostridium difficile infection: a review on treatment efficacy and economic value. Infect Drug Resist. 2017;10:365-375.

62. Gao P., Xu W., Ruan X., Qian Y., Xue G., and Jia H. "Long-Term impact of a tetracycline concentration gradient on the bacterial resistance in anaerobic-aerobic sequential bioreactors," Chemosphere J. 2018; 205:305-316

63. Arredondo A. Blanc V. , Mor C. , Nart J , and León R. "Tetracycline and multidrug resistance in the oral microbiota: differences between healthy subjects and patients with periodontitis in Spain." J Oral Microbiol. 2021; 13(1): 1847431

64. Grossman T. H. "Tetracycline Antibiotics and Resistance." Cold Spring Harb Perspect Med. 2016; 6(4): a025387

65. Colaço HG, Moita LF." Initiation of innate immune responses by surveillance of homeostasis perturbations." The FEBS journal. 2016;283:2448-2457.

66. Gokulan K, Cerniglia CE, Thomas C, Pineiro SA, Khare S. "Effects of residual levels of tetracycline on the barrier functions of human intestinal epithelial cells." Food and Chemical Toxicology. 2017;109:253-263.

67. Igyártó BZ, Kaplan DH. "Antigen presentation by Langerhans cells." Current opinion in immunology. 2013; 25:115-119.

68. Flutter B, Nestle FO. "What on "irf" is this gene 4? Irf4 transcriptionfactor-dependent dendritic cells are required

for T helper 2 cell responses in murine skin." Immunity. 2013;39:625-627.

69. Abram CL, Roberge GL, Pao LI, Neel BG, Lowell CA. "Distinct roles for neutrophils and dendritic cells in inflammation and autoimmunity in motheaten mice." Immunity. 2013;38:489-501

70. Wohn C, Ober-Blöbaum JL, Haak S, Pantelyushin S, Cheong C, Zahner SP. "Langerinneg conventional dendritic cells produce IL23 to drive psoriatic plaque formation in mice." Proceedings of the National Academy of Sciences. 2013;110:10723-10728.

71. Kong M, Chen XG, Xing K, Park HJ. "Antimicrobial properties of chitosan and mode of action: a state of the art review". Int J Food Microbiol. 2010;144:51–63.

72. Ibrahim H. M., Farid O. A., A. Samir A. and Mosaad R.M. "Preparation of chitosan antioxidant nanoparticles as drug delivery system for enhancing of anti -cancer drug." Key Engineering Materials. 2018; pp. 92 -97.

73. Farouk R., Youssef Y.A., Mousa A.A., Ibrahim H.M. Simultaneous dyeing and antibacterial finishing of nylon 6 fabric using reactive cationic dyes, World Applied Sciences Journal. 2013; 26(10):1280 1287

74. Bangham, A. D. "Surrogate cells or Trojan horses. The discovery of liposomes," BioEssays. 1995;n17(12): 1081–1088,

75. Vassallo A, Francesca M Immacolata S, Faraone, Milella L, "Nanoparticulate Antibiotic Systems as Antibacterial Agents and Antibiotic Delivery Platforms to Fight Infections", Journal of Nanomaterials. 2020; 2020:31-62.

76. Din FU, Aman W, Ullah I, Qureshi OS, Mustapha O, Shafique S, et al. "Effective use of nanocarriers as drug delivery systems for the treatment of selected tumors." Int J Nanomedicine. 2017;12:7291-7309.

77. Jahangirian H, Lemraski EG, Webster TJ, Rafiee-Moghaddam R, Abdollahi Y. "A review of drug delivery systems based on nanotechnology and green chemistry." green nanomedicine. Int J Nanomedicine. 2017;12:2957-2978.

78. Gupta A, Mumtaz S, Li C-H, Hussain I, Rotello VM. "Combatting antibiotic-resistant bacteria using nanomaterials." Chemical Society Reviews. 2019;48:415-427.

79. Ghadi A , Mahjoub S, Tabandeh F , and Talebnia . Synthesis and optimization of chitosan nanoparticles: Potential applications in nanomedicine and biomedical engineering. Caspian J Intern Med. 2014; Summer; 5(3): 156–161.

80. Naskar S., Kuotsu K, Sharma S. "Chitosan -based nanoparticles as drug delivery systems: a review on two decades of research, Journal of Drug Targeting." 2019; 27(4): 379 -393

81. Ekhlas A., El-Alfy A., Manal K., El-BisiGhada M., Hassan T., Ibrahim M. "Preparation of biocompatible chitosan nanoparticles loaded by tetracycline, gentamycin and ciprofloxacin as novel drug delivery system for improvement the antibacterial properties of cellulose based fabrics." International Journal of Biological Macromolecules. 2020; 161: 1247-1260

82. Divakar DD, Jastaniyah NT, Altamimi HG, Alnakhli YO, Alkheraif AA, Haleem S. "Enhanced antimicrobial activity of naturally derived bioactive molecule chitosan conjugated silver nanoparticle against dental implant pathogens." International journal of biological macromolecules. 2018;108:790-797.

83. Bavariya AJ, Andrew Norowski Jr P, Mark Anderson K, Adatrow PC, Garcia-Godoy F, Stein SH. "Evaluation of biocompatibility and degradation of chitosan nanofiber membrane crosslinked with genipin." Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2014;102:1084-1092.

84. Andrew A, Ervina I and Agusnar H. "In Vitro Chitosan Hydrogel Based Tetracycline Cytotoxicity Test on Fibroblast Viability." 11th International Dentistry Scientific Meeting (IDSM 2017): Atlantis Press. 2018;265-272.

85. Hamedi M.; Belibasakis G.N.; Cruchley A.T.; Rangarajan M.; Curtis M.A. and Bostanci N. "Porphyromonas gingivalis culture supernatants differentially regulate Interleukin-1β and Interleukin-18 in human monocytic cells Cytokine, 2009; 45(2):99–104.

86. Castro JEZ, Vado-Solis I, Perez-Osorio C, Fredeking TM. "Modulation of cytokine and cytokine receptor/antagonist by treatment with doxycycline and tetracycline in patients with dengue fever." Clin Dev Immunol. 2011; 2011:370872.

87. Fernandes LA, Martins TM, Almeida JMD, Nagata MJH, Theodoro LH, Garcia VG. "Experimental periodontal disease treatment by subgingival irrigation with tetracycline hydrochloride in rats." Journal of Applied Oral Science. 2010;18:635-640.

88. Gabler WL and Creamer HR. "Suppression of human neutrophil functions by tetracyclines." Journal of periodontal research. 1991;26:52-58.