# INTERNATIONAL JOURNAL
## OF
## PHARMACEUTICAL SCIENCES
### AND
### RESEARCH

# THE EFFECT OF DOXYCYCLINE HYDROCHLORIDE ON MMP-2 AND 9 IN EXPERIMENTALLY INDUCED PERIODONTITIS – AN ANIMAL STUDY

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**ABSTRACT:** The periodontal tissues undergo constant changes of remodelling when subject to bacterial infection and the resultant host response. The balance between enzymes secreted by the host such as matrix metalloproteinases (MMPs) and their antagonists tissue inhibitor of metalloproteinases (TIMPs) are major contributors to the progression of periodontal destruction. A sub-type of MMPs referred to gelatinases (MMP-2 and 9) are known to be responsible for the pathologic tissue degradation, with MMP-9 playing a potential role in periodontal pocket formation in periodontitis. The use of anti-collagenase host modulatory agents such as doxycycline hydrochloride regulates the extent of resultant tissue destruction and provides an ideal therapeutic approach in the treatment of periodontitis. However, data regarding the effect doxycycline hydrochloride on gelatinases has not been extensively documented. Therefore, the aim of this study was to identify the expressions of MMP-2 and 9 by an immunohistochemical assay in experimentally induced-periodontitis animal models and to evaluate the effects of a prepared doxycycline hydrochloride-impregnated collagen scaffold on their expresssion.

**INTRODUCTION:** Periodontitis is an inflammatory disease of the periodontium which is characterized by a progressive destruction of the tissues supporting the tooth. [1] The components of the periodontal tissue extracellular matrix, particularly collagens, appear to be the main targets of degradation in periodontitis primarily as a result of the host's response to matrix metalloproteinases(MMPs). [2]

MMPs are proteolytic enzymes that can collectively degrade all the components of the extracellular matrix and are secreted in latent, inactive pro-enzyme forms; with plasmin, bacteria and their antigenic products, [3] pro-inflammatory cytokines, growth factors, hormones and other MMPs as likely activators. [4] MMP activity is further modulated by the tissue inhibitors of metalloproteinases (TIMP-1, -2, -3 and -4) and alpha-macroglobulin.

In normal steady state tissues, only low levels of MMPs are detected intra and extracellularly as MMPs are tightly regulated at the level of transcription and secretion[5] while in inflamed periodontal tissue, MMPs are significantly expressed as compared with healthy gingival tissue. [6]

Gelatinase A or 72 kDa gelatinase (MMP-2) and gelatinase B or 92 kDa gelatinase (MMP-9) belong to a specific group of MMPs referred to as gelatinases with similar characteristics having a high affinity for gelatin both in the latent and activated forms. Besides gelatin, they also cleave types IV, V, VII collagen and elastin.[7] They have been commonly identified in keratinocytes, monocytes and in a number of other normal and transformed cells.[8] MMP-2, being the most widely distributed of all MMPs, has also been identified in skin fibroblasts, chondrocytes, endothelial cells and osteoblasts;[9] while MMP-9 is also produced by alveolar macrophages and PMN leukocytes.[10]

It has been demonstrated that epithelial migration at wound healing sites to cover the denuded connective tissue surface may be closely linked to the expression of one or more MMPs and is co-regulated by pro-inflammatory cytokines such as IL- 1β/IL-lα and by the growth factors EGF and TGFα[11] as seen during epithelial apical migration in pocket formation. MMP-2 and MMP-9 have been identified in playing an essential part during healing of epithelial wounds, tumour invasion and metastasis.[12-14] MMP-2, which localizes in the connective tissue fibroblasts and endothelial cells, is thought to have an important function during granulation and the early remodeling phases of repair while MMP-9 is stimulated in response to injury and is mostly expressed in the migrating keratinocytes sheet resulting in cleavage of type IV collagen and other basement membrane components.[15]

Golub and coworkers explained the host modulatory effects of the antibiotic tetracycline which was capable of exhibiting anti-MMP properties and their use in arresting periodontal disease with gains in clinical attachment levels and reduction of periodontal pockets.[16] This unique feature was incorporated into various agents to deliver the drug to sites with periodontal disease, a concept referred to as local drug delivery (LDD).[17] The same authors identified the ability of doxycycline to arrest periodontal destruction seen in animal models as well as in humans.[18] Doxycycline non-selectively inhibits MMPs by binding to the active zinc site and secondarily to the inactive calcium ion site causing conformational changes and loss of enzymatic activity.[19] Currently few intrapocket delivery systems incorporating the use of doxycycline have been used viz. a gel and a microparticle form[20] amongst which several studies have reported the efficacy of 10% doxycycline hyclate gel as a local delivery antimicrobial agent for attaining probing depth reduction and gaining clinical attachment.[21-23] Currently, few studies have demonstrated the effects of doxycycline on gelatinases (MMP-2 and 9) and the role of MMP-9 in pocket formation by way of immunolocalization techniques.

Hence, the current study was undertaken to determine the immunolocalisation and the concerted action of MMP – 2 and more importantly that of MMP- 9 in a ligature-induced periodontitis model in Wistar rats and the anti-MMP effects using a doxycycline hydrochloride impregnated collagen scaffold.

## MATERIALS AND METHODS:
The collagen scaffolds were prepared according to the patented protocols elaborated by Aishwarya et al 2008.[24] Doxycycline hydrochloride collagen scaffold was prepared by applying the drug on the surface of the membrane at a concentration of $0.3mg/cm^2$.

## Animal Experimental Protocol:
The study design conformed to the guiding principles of The Institutional Animal Ethics Committee (IAEC), Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

A total of 18 two- month old female Wistar rats, weighing 180–200g raised in ambient environmentally controlled conditions were taken up for the study of which 12 were anesthetized with an intraperitoneal injection of ketamine and a floss ligature was tied around the maxillary right and left second molars. The animals were maintained on a soft diet. To promote plaque accumulation and subsequent periodontal disease, the remaining untreated 6 rats were fed regular pellet food.

The animals were divided into three groups of six each as follows:

**Group 1** – Healthy (those with healthy periodontium)

**Group 2** – Diseased (those with ligature induced periodontitis)

**Group 3** – Treated (those subjected to doxycycline coated collagen periodontal dressing treatment)

**Histological Specimens:**
Following 3 weeks into the study, the rats were anaesthetized and gingival tissue was biopsied from the maxillary left second molar region for histological analysis for all rats among the three groups. Simultaneously, the ligatures were removed from the maxillary right second molar region and the site was cleared of all local factors using hand instruments for the rats belonging in Group 3. The site was then irrigated with saline and a doxycycline-coated collagen scaffold was placed into the periodontal pockets formed. At 4 weeks, gingival biopsies were obtained from the maxillary right second molar region. All gingival samples were fixed in 10% formal-saline, dehydrated through graded alcohol series, cleared in xylene and embedded in paraffin wax (melting point – $56^0$C). Serial sections of 5µm thick were cut and stained with hematoxylin and eosin (H & E). The sections were examined using light microscope and photomicrographs were taken.

**Immunohistochemistry:**
5µm thick sections of gingival tissue were mounted on poly –L-lysine-coated slides and fixed in acetone at $4^0$C for 5 min and washed in 0.1 M PBS, pH 7.4, at 22ºC before the inhibition of endogenous peroxidase by immersing the sections in 0.3% hydrogen peroxide in methanol for 20 min. The slides were placed in a humid chamber, and the sections were incubated sequentially with 1) goat polyclonal anti MMP-2 and 9 antibody (Santa Cruz Biotechnology, Inc, U.S.A), diluted 1:100 in PBS and 0.1% w/v BSA for 1 h; 2) Rabbit anti goat IgG-ALP (Genei pvt. Ltd, Bangalore, India), diluted 1:100 in PBS with 0.1% w/v bovine serum albumin for 30 min; and 3) the sections incubated for 5 min in a chromogen solution of 5- Bromo-4-chloro-3-indolyl phosphate/ nito blue tetrazolium (BCIP/NBT). All incubations were performed at 22

$^0$C, and slides were washed twice in PBS between each step. All slides containing consecutive sections were processed further with and without counterstaining with hematoxylin before dehydration in ethanol, clearing in xylene, and mounting. Omission of primary antiserum and use of normal rabbit serum (diluted 1:100) were included as controls.

**Masson's Trichrome Staining**
Masson's trichome staining was performed in order to identify collagen fibers and their organization in the histological specimens. Briefly, slides were deparaffinized and sections rehydrated in xylene and graded series of ethanol. Sections were placed in Bouin's Solution (Sigma) at room temperature overnight in a hood. The slides were then washed in water and stained in working Weigert's Iron Hematoxylin (Sigma) Solution for 5 minutes. They were further washed in running tap water for 5 minutes and subsequently deionized water.

They were then stained in Biebrich Scarlet-Acid Fuchsin for 5 minutes and further rinsed in deionized/ distilled water. The slides were then placed in phosphomolybdic/ phosphotungstic acid solution for 5-10 minutes. The sections were stained in aniline blue solution for 5 minutes, rinsed in distilled water and placed in 1% acetic acid solution for 3-5 minutes. The slides were then dehydrated in xylene and graded series of ethanol. A coverslip was placed on the slides using Polymount (xylene based). After drying overnight in the hood, the cells were viewed under light microscope.

**RESULTS:**
**Rat Gingival tissue: Immunohistochemistry:**
**MMP-2:** While the expression of MMP-2 was absent in healthy tissue sections (**Fig.1.A**); in the diseased specimens, the mucosa was infiltrated with neutrophils (**Fig.1.B**) with the expression of MMP-2 in the basal keratinocytes and some neutrophils (**Fig.1.C**) In the treated section, subepithelial tissue showed granulation tissue. The MMP-2 staining was seen spilt over into the connective tissue matrix and was also positive in a few of the inflammatory cells (**Fig.1.D**). The subepithelial tissue showed fibroblasts and fibrous

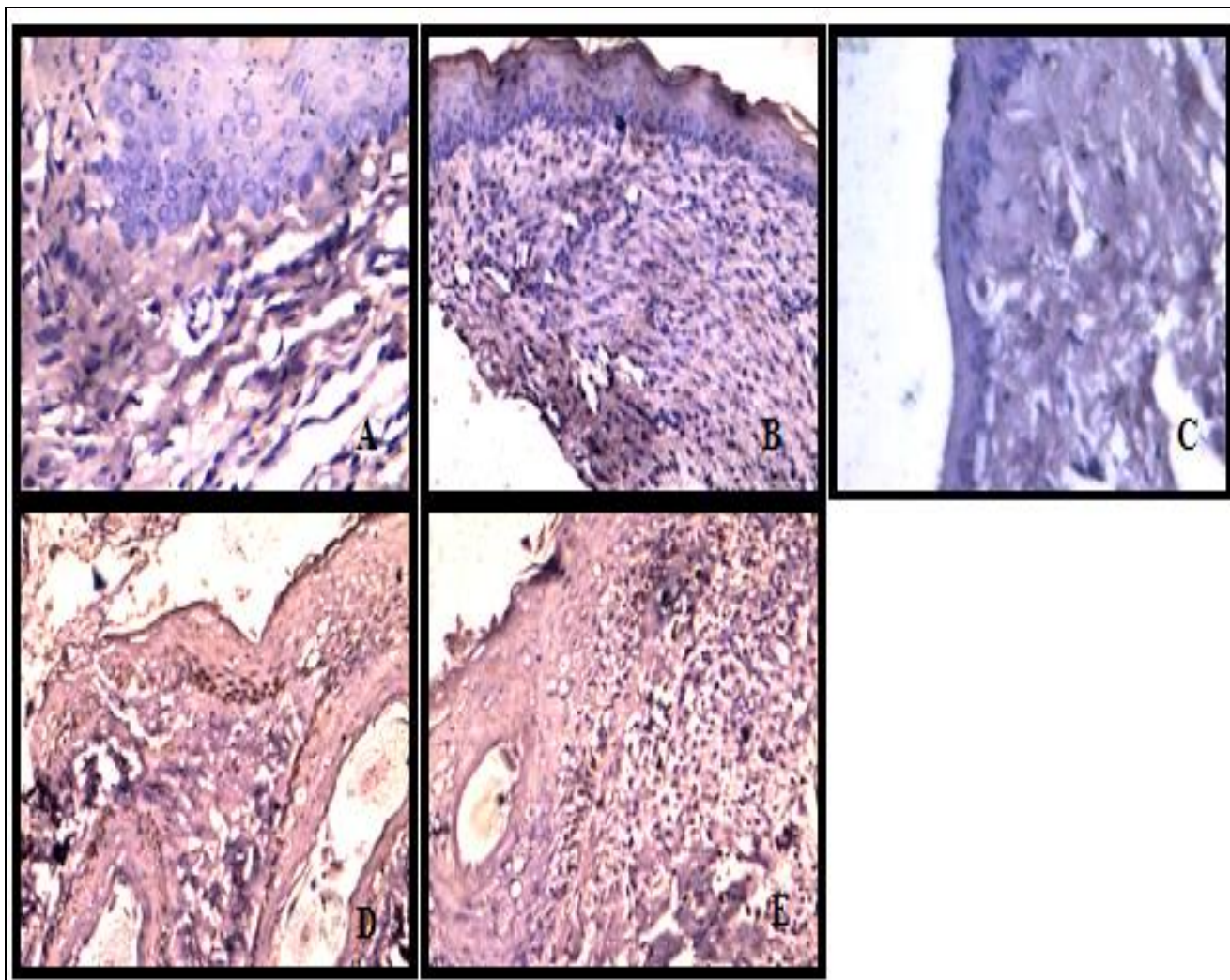tissue showing moderate expression of MMP-2 (**Fig1.E**).



**FIG. 1: A) HEALTH – NORMAL MUCOSA WITH NO EXPRESSION OF MMP. B) MUCOSA INFILTRATED WITH NEUTROPHILS IN DISEASED SITES C) EXPRESSION OF MMP-2 IN BASAL KERATINOCYTES AND FEW NEUTROPHILS. D) SURFACE STAINING OF KERATINOCYTES IN THE MUCOSA AND GRANULATION TISSUE IN THE SUBEPITHELIAL TISSUE IN DISEASED SITES. MMP-2 STAINING SPREAD OVER CONNECTIVE TISSUE MATRIX AND IN A FEW OF THE INFLAMMATORY CELLS. E) FIBROBLASTS AND FIBROUS TISSUE IN -THE SUBEPITHELIAL TISSUE WITH MODERATE EXPRESSION OF MMP-2.**

**MMP -9:**
Healthy gingival tissue was seen to be positive for MMP– 9 (**Fig. 2.A**) while the subepithelial tissue showed high levels of PMNs and the basal layer showed intense positivity for MMP - 9 in the diseased tissue section (**Fig. 2.C**). The stratum corneum was detached from the rest of the mucosa and showed mild diffused positivity (**Fig.2.D**) whereas moderate positivity was seen along the keratin layer (**Fig. 2.E**), keratinocytes and a few fibroblasts (**Fig.2.F**). Granular positivity was seen in **Fig.2. F), G**). The tissue shows diffused positivity while macrophages and fibroblasts showed intense positivity (**Fig. 2.H**). The leading edge was seen to be positive (**Fig.2.G**). Periodontal treatment reduced the amount of gelatinases. (**Fig.2.H**) Stroma of the connective tissue interspersed with fibroblasts and a few inflammatory cells suggested ongoing healing. Granulation tissue was seen with fibrosis and positivity along the stratum corneum and scattered positivity in the macrophages (**Fig. 2.I**). The re-epithelialised area along with keratin layer showed diffused positivity **(Fig. 2.J**).
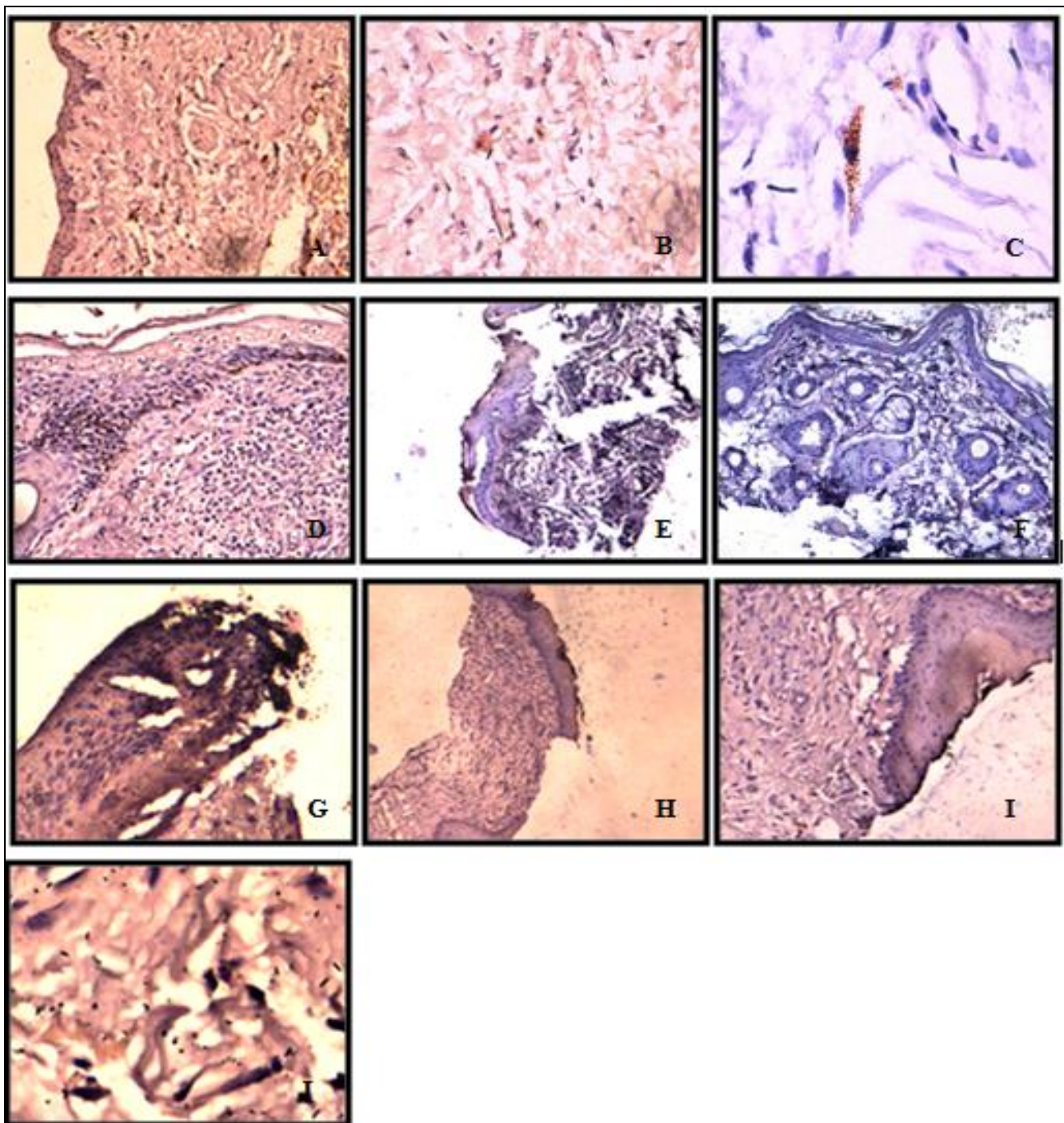
**FIG. 2. A) IN HEALTHY SITES, THE STRATUM CORNEUM AND MONOCYTES STAINED POSITIVE FOR MMP-9 B) GRANULAR POSITIVITY SEEN IN DISEASED SITES C) INTENSE POSITIVITY FOR MMP-9 DISPLAYED IN THE BASAL LAYER. D) STRATUM CORNEUM DETACHED FROM THE REST OF THE MUCOSA WITH MILD DIFFUSED POSITIVITY. E) POSITIVITY ALONG THE KERATIN LAYER. F) FEW KERATINOCYTES AND FEW FIBROBLASTS SHOW POSITIVITY. G) LEADING EDGE DISPLAYING POSITIVITY. H) GRANULATION TISSUE WITH FIBROSIS POSITIVITY ALONG THE STRATUM CORNEUM AND SCATTERED POSITIVITY IN THE MACROPHAGES IN TREATED SITES I) REPITHELIALISATION AREA SHOWS POSITIVITY ALONG WITH KERATIN LAYER. J) HEALED STROMA OF CONNECTIVE TISSUE. GRANULATION TISSUE SHOWS POSITIVITY AND GRANULAR POSITIVITY SEEN IN FIBROBLASTS.**

**Masson's Trichrome Staining**:
Normal mucosa was seen in healthy sites along with normal amount of collagen and RBCs were few in number. The collagen fibers stained green and RBCs stained red (**Fig.3.A, B**). Diseased sections revealed mucosa with intense inflammation in the superficial and deep subepithelial region (**Fig. 3.C,** D) and the collagen content appeared considerably reduced. Treatment with doxycycline showed areas of resolution of the inflammation.
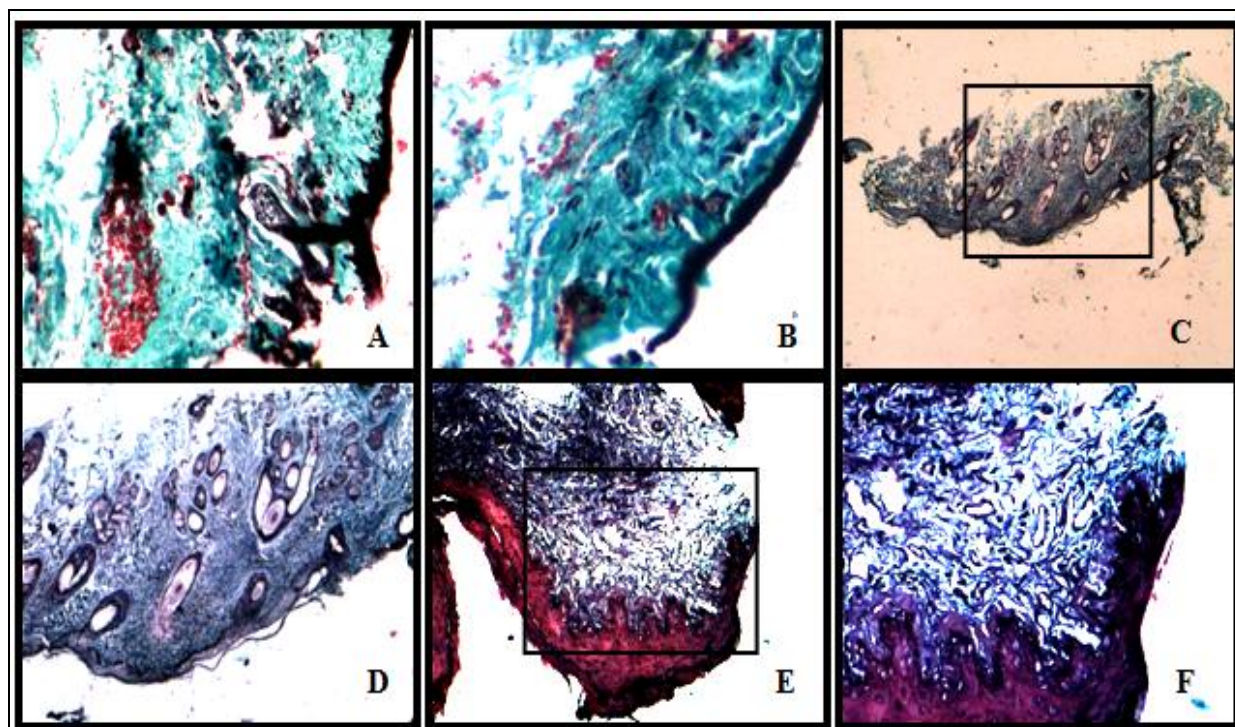
**FIG.3: MASSON'S TRICHROME STAINING: COLLAGEN FIBERS – GREEN, RBC - RED A) NORMAL MUCOSA AND NORMAL AMOUNT OF COLLAGEN PRESENT IN HEALTHY SITES. B) THE PRESENCE OF FEW RBCS C) MUCOSA WITH INTENSE INFLAMMATION IN THE SUPERFICIAL AND DEEP SUBEPITHELIAL REGION IN DISEASED SITES D) REDUCED COLLAGEN CONTENT. E) AREAS OF RESOLVING INFLAMMATION IN THE TREATED SITE. F) PRESENCE OF RESIDUAL INFLAMMATION.**

**DISCUSSION:** The gelatinases MMP-2 and 9 have been demonstrated to play a pivotal role during destruction and remodelling of the extracellular matrix.[8] The influential role of MMP-2 in periodontal tissue destruction is well established [25, 26] while MMP-9 is stimulated in response to injury, keratinocyte migration and the cleavage of type IV collagen and other basement membrane components. [27] Wound fluid from chronic ulcers persistently contain high levels of MMP-2 and 9 that probably contribute to abnormal healing of the lesion [28] [29] as they degrade growth factors and ECM components required for the restoration of ECM bed that support migration of the cells colonizing the wound. Oral fluid samples from both healthy and periodontitis patients contain gelatinolytic activity, and is further increased with periodontal disease severity. [30, 31] Various investigators have proved beyond doubt that doxycycline is capable of anti-MMP activity in periodontitis with beneficial effects by means of arresting the disease progression.[32, 33]

In the present study, immunolocalization techniques were used to detect the expression of gelatinases MMP-2 and 9 in healthy, diseased and treated rat gingival tissues. The healthy tissue samples showed negligible expression of MMP-2 and positive expression of MMP-9 in the stratum corneum and monocytes. The Masson's trichrome staining showed normal mucosa and normal amount of collagen present in healthy sites with scattered RBCs.

In diseased tissues, MMP-2 was seen to be expressed in basal keratinocytes and a few neutrophils in the mucosa and granulation tissue in the subepithelial layer. During inflammatory conditions, the expression of MMP-2 is bound to be high owing to the intense infiltration of inflammatory cells in the diseased connective tissue The expression of MMP-9 showed intense positivity displayed in the basal layer, keratin layer and in the stratum corneum which was detached from the rest of the mucosa showing mild diffused positivity. Masson's trichrome staining of the diseased tissue samples showed mucosa with intense inflammation in the superficial and deep subepithelial region with reduced collagen content and deranged collagen fibers - a typical finding in

sites of active tissue destruction. An *in vitro* study demonstrating the molecular events during the healing process involving keratinocytes showed that MMP-9 is further capable of being stimulated by injury in normal human keratinocytes. [34, 35] This cycle of extracellular matrix degradation by MMP-9 and the subsequent detachment of the epithelium, causes further expression of MMP-9.

The treated samples showed a comparatively moderate positivity for MMP-2 in fibroblasts and fibrous tissue of the subepithelial layer. MMP-9 was expressed along the stratum corneum, in the macrophages, in areas of re-epithelialisation along the keratin layer with granular positivity in fibroblasts of the connective tissue. Masson trichrome staining showed areas of resolving inflammation as evident by more organized collagen fibers in the treated sites with presence of residual inflammation. In agreement with previous studies, the use of doxycycline against gelatinases appears to prevent further epithelial invasion and destruction of the underlying mesenchyme.

**CONCLUSION:** Within the limits of the present study, MMP-2 and 9 play a significant role in periodontal tissue destruction with MMP-9 contributing to apical migration and proliferation of the junctional epithelium during pocket formation. Furthermore, the use of doxycycline hydrochloride as a local drug delivery system significantly decreases periodontal tissue destruction by suppressing MMP-2 and 9 activity with characteristic reduction of MMP-9 contributing to reduced periodontal pocket progression.

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