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Evaluation Of Gingival Overgrowth Induced By Anti-Hypertensive, Anti-convulsant And Immunosuppressive Drugs In Albino Rats

(Histological, Histochemical and Immunohistochemical study)

Thesis Submitted To The Faculty Of Oral And Dental Medicine, Cairo University, In Partial Fulfillment Of The Requirements For The Degree of Doctor Of Philosophy In Basic Dental Science

(Oral Biology)

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TO

MY WIFE

and MY PARENTS

Contents

List of Abbreviations

FGF	Fibroblast growth factor
TGF	Transforming growth factor
GO	Gingival overgrowth
DGO	Drug induced gingival overgrowth
NIF	Nifedipine
ATP	Adenosine tri phosphate
PHT	Phenytoin
CsA	Cyclosporin A
PDGF	Platelet derived growth factor
MMP	Matrix metalo proteinase
DMSO	Dimethyl sulfoxide
LSAB	Labelled streptavidin biotin
PBS	Phosphate buffered saline
H&E	Hematoxylin and eosin
CT	Connective tissue
BV	Blood vessel
Th	Helper T - cell
Ts	Supressor T – cell
Tc	Cytotoxic T – cell
ACTH	Adino cortico trophic hormone
KGF	Keratinocyte growth factor
CTGF	Connective tissue growth factor

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تقييم النمو الزائد باللثة الناتج عن عقار مضاد لارتفاع ضغط الدم وعقار مضاد للتشنجات وآخر مثبط للمناعة

(دراسة نسيجية – هستوكيميائية و هستوكيميا - مناعية)

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مقدمه من

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الملخـص العربـــى

أجريت هذه الدراسة على ٧٠ فأر ذكر من فئران الالبينو البيضاء يبلغ وزن الفأر حوالي ٢٠٠ – ٢٥٠ جرام وذلك لتقييم نمو اللثة الزائد الناتج عن تناول بعض العقاقير الطبية مثل أدوية ارتفاع ضغط الدم , أدوية الصرع ومثبطات المناعة .

تم تقسيم حيوانات التجربة إلى ٧ مجموعات متساوية :

* المجموعة الأولى (المجموعة الضابطة): لم تتلقى حيوانات هذه المجموعة أي علاج تم إعطاء حيواناتها ماء مقطر بنفس الطريقة المتبعة مع فئران المجموعات الأخرى وكذلك حقنها بمادة الـ DMSO المستخدمة فى إذابة العقاقير المدروسة.

* المجموعة الثانية: (مجموعة النيفيبن) تم إعطاء حيواناتها ٤٠ مللجم / كجم من عقار النيفيبن يومياً عن طريق الفم بواسطة أنبوبة حلقية .

* المجموعة الثالثة: (مجموعة الفينوتوين) تم حقن حيوانات هذه المجموعة ١٠٠ مللجم / كجم من عقار الفينوتوين يومياً بتركيز ٢,٥ مللجم / ملليتر داخل البطن بواسطة سرنجة أنسولين ١ مللى .

* المجموعة الرابعة: (مجموعة السيكلوسبورين) تم إعطاء حيوانات هذه المجموعة ٢٠ مللجم / كجم من عقار السيكلوسبورين يومياً عن طريق الفم بواسطة أنبوبة حلقية.

* المجموعة الخامسة: (مجموعة النيفيبن والفينوتوين) تم إعطاء حيوانات هذه المجموعة مزيج من المعالجة الخاصة بالمجموعتين٢ & ٣ مع حقنها بنفس الصورة السالفة الذكر .

* المجموعة السادسة: (مجموعة النيفيبن والسيكلوسبورين) تم إعطاء حيواناتها المعالجة الخاصة بالمجموعتين ٢ & ٤ يومياً .

* المجموعة السابعة: (مجموعة الفينوتوين والسيكلوسبورين) تم إعطاء حيواناتها المعالجة الخاصة بالمجموعتين ٣ & ٤ يومياً .

– تم قتل الحيوانات بعد استمرار إعطاء العقاقير للمدة المحددة للتجربة وهى شهران واستئصال نسيج اللثة من الفك السفلى للمنطقة الصدغية عن طريق مشرط معقم.

– وقد تم استعمال صبغة الهيماتوكسلين والأيوسين للدراسة النسيجية بينما تم استخدام القطاعات المجمدة لدراسة النشاط الإنزيمي في الدراسة الهيستوكيميائية وفى

الدراسة الهيستوكيميا – مناعية تم استخدام أجسام مضادة لدراسة كثافة وتوزيع بروتين الكولاجين أنواع ١ & ٤ & ٥ وكذلك احد مكونات النسيج الضام (الفيبرونكتين) .

– وقد أتضح من نتائج الدراسة النسيجية

المجموعة الأولى (المجموعة الضابطة)

باستخدام صبغة الهيماتوكسلين والأيوسين وجد أن نسيج اللثة يتكون من :

١ – **نسيج طلائى** يتكون من أربعة طبقات طبقة الخلايا القاعدية – طبقة الخلايا ذات الأشواك – طبقة الخلايا ذات الحبيبات والطبقة الحرقفية .

٢ – **نسيج ضام .**

المجموعة الثانية (مجموعة النيفيبن)

أوضحت الدراسة أن استخدام عقار النيفيديبين يؤدى إلى زيادة طفيفة فى انقسام خلايا النسيج الطلائى خاصة خلايا الطبقة القاعدية والطبقة ذات الأشواك ، كما يؤدى إلى زيادة سمك الطبقة الحرقفية ، كما وجد زيادة الخلايا الالتهابية بالنسيج الضام .

المجموعة الثالثة (مجموعة الفينوتوين)

أظهر هذا البحث أن استخدام عقار الفينوتوين له تأثير مشابهة لعقار النيفيبن على النسيج الطلائى بينما تأثيره على النسيج الضام يتميز بإحداث تليف واضح به.

المجموعة الرابعة (مجموعة السيكلوسبورين)

أوضحت هذه الدراسة أن استخدام عقار السيكلوسبورين له تأثير شديد على النسيج الطلائى مما يترتب عليه زيادة سمكه نتيجة زيادة واضحة فى معدلات الانقسام وأيضاً زيادة سمك الطبقة الحرقفية بينما تأثيره على النسيج الضام أقل من العقارين السابقين .

أما بالنسبة لباقى المجموعات التى تم استخدام عقارين من العقاقير السابقة فقد وجد أن هناك تأثير تراكمى مما يترتب عليه ظهور تأثير هذه العقاقير بصورة أكبر على كل من النسيج الضام والنسيج الطلائى .

– كما أوضحت نتائج الدراسة الهيستوكيميائية أن النمو الزائد للثة ناتج عن قلة نشاط الإنزيمات المسئولة عن تكسير بروتين الكولاجين مما يؤدى إلى تراكمه في النسيج الضام ولذلك يحدث نمو زائد باللثة.

– أما الدراسة الهيستوكيميا- المناعية أوضحت أن النمو الزائد باللثة ينتج عن زيادة تصنيع بروتين الكولاجين خاصة نوع ١ والفيبرونكتين مما يؤدى إلى زيادة نمو اللثة وتضخمها .

ومن نتائج الدراسة يمكن استخلاص ما يلى:

** النمو الزائد للثة مشكلة متعددة الأسباب فهي تنتج عن :

١. زيادة تكاثر الخلايا ذات الأشواك للنسيج الطلائى .
٢. زيادة سمك الطبقة الحرقفية للنسيج الطلائى .
٣. زيادة الخلايا الالتهابية بالنسيج الضام .
٤. زيادة تصنيع بروتين الكولاجين .
٥. نقص تكسير بروتين الكولاجين .

** كما إتضح أن استخدام أكثر من عقار يسبب نمو زائد باللثة بنسبة اكبر من استخدام عقار واحد وذلك نتيجة للتأثير التراكمي لهذه الأدوية.

– وقد وجد أن تأثير معظم هذه الأدوية على نسيج اللثة يكاد يكون متشابه وذلك ربما يدل على أن طريقة عمل هذه الأدوية فى إحداث هذا النمو أيضاً به نسبة كبيرة من التشابه إلا انه وجد أن :

١. عقار السيكلوسبورين يؤثر بنسبة اكبر على النسيج الطلائى ويسبب تضخم ملحوظ به .
٢. بينما عقار الفينوتوين يؤثر بنسبة اكبر على النسيج الضام ويسبب تليف به
٣. أما عقار النيفيبن فهو اقل تأثير ولكن يحدث زيادة في عدد الخلايا الالتهابية بالنسيج الضام .

– وحيث أن مرض ارتفاع ضغط الدم وعمليات زراعة الأعضاء قد أصبحت في تزايد مستمر لذلك يوصى باستحداث أنواع أخرى من العقاقير ذات تأثير اقل أو منعدمة التأثير على نسيج اللثة وذلك لما يواجهه المرضى من تشوهات في اللثة تؤثر على حالتهم الصحية والنفسية.

Introduction :

Overgrowth :

Increased tissue volume and cell numbers are characteristic features of hyperplastic pathologies which occur in many tissues including : kidney, liver, prostate , breast and gingiva . Normal tissue architecture is maintained by a homeostatic balance between cell proliferation (mitosis) and cell death (apoptosis) . Apoptosis plays an important role in the control of tissue overgrowth [Lovas , 1986 and Marshall and Bartold , 1998].

Appropriate cell number and organ size in a multicellular organism are determined by coordinated cell growth , proliferation , and apoptosis . Disruption of these processes can cause tissue overgrowth or cancer [Lai et al., 2005].As well as, apoptosis plays a critical role in the regulation of inflammation and the host immune response [Alaaddinoglu et al., 2005] .

The reproducible pattern of organismal growth during development is the product of genetically controlled signaling pathways . Patterned activation of these pathways shapes developing organs and dictates overall organismal shape and size [Moberg et al., 2005] .

There are multiple examples for tissue overgrowth in the body as in kidney [Torrezan et al., 2005] . In prostate it occurs in the form of benign prostatic hyperplasia which is characterized by : (i) lymphocytic infiltration with production of proinfla-mmatory cytokines and (ii) increased basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF –beta 1) production leading to stromal proliferation , transdifferentiation and extracellular matrix production [Untergasser et al., 2005] . While the overgrowth in the breast declared a direct relation between the degree of hyperplasia and p53 gene expression [Wang et al., 2005].

In the bone , tissue overgrowth appears in the form of bony exostosis . The dental literature describes buccal bony exostosis development at sites where free gingival grafts have been used to increase the amount of gingiva [Chambrone and Chambrone, 2005] .

Meanwhile in the dental clinic , this tissue overgrowth is widely common to be seen in the gingival tissue .

Review of Literature :

Gingival overgrowth (GO) :

Gingival enlargement is the term now used to describe medication – related gingival overgrowth or gingival hyperplasia condition commonly induced by three main classes of drugs : anticonvulsants, antihypertensive, calcium antagonists and the immunosuppressant cyclosporin [Eggerath et al., 2005].

Gingival overgrowth is the enlargement of the attached gingiva due to an increased number of cells . The most prevalent types of gingival overgrowth in children are drug-induced gingival overgrowth as it occurs in gingival fibromatosis and neurofibromatosis I (von Recklinghausen disease). According to epidemiologic studies, it is more prevalent in male children and adolescents . Genetic heterogeneity seems to play an important role in the development of the disease (Hereditary gingival fibromatosis) . HGF is the most common syndromic gingival enlargement in children . This autosomal dominant disease usually appears at the time of eruption of permanent dentition characterized by highly collagenized connective tissue.

Neurofibomatosis I is an autosomal dominant disease more common in mentally handicapped individuals . Gingival overgrowth is caused by the formation of plexiform neurofibromas in the connective tissue of the gingiva . Plexiform neurofibromas are charteristic of the disease and consist of hypertrophic nerves arranged as lobules in the connective tissue . Complications of the disease are multiple and severe due to neurofibromas and their occasional malignant transformation [Doufexi et al ., 2005] .

Causes of gingival overgrowth :

Gingival tissues are generally in a state of injury and repair that involves repetitive cycles of production of chemotactic factors , inflammatory cell recruitment , as well as tissue resorption , replacement , and remodeling. Collagen turnover is unusually high in periodontal and gingival tissues [Sodek and Ferrier , 1988 and Clark, 1998] .

Gingival overgrowth is a common side-effect of the administration of cyclosporin A (CSA) , phenytoin , and calcium blockers . Signaling mechanisms possibly involved in the overgrowth [Butler et al., 1987 and Bostrom et al., 2005] .

Gingival overgrowth can be inherited , or is of idiopathic origin . The gingival enlargement is one among several common signs of gingival disease . It frequently results from inflammatory changes induced by prolonged deposition of plaque and removal of which often leads to resolution . It has been also associated with neoplastic conditions [Carranza, 1990 ; Hassell , 1990 ; Mendieta, 1995 and Brunet et al., 1996] .

Complications of gingival overgrowth :

The clinical presentation of gingival overgrowth varies from a non inflamed, firm and fibrous condition to an edematous haemmorrhagic appearance with tendency to spontaneous bleeding . It usually begins at the interdental papilla in a lobular form, while at later stages it affects the entire gingiva and extends coronally interfering with occlusion, mastication , speech and dental hygiene resulting in a greater risk of infection and higher incidence of dental caries and periodontitis [Williamson et al., 1994 and Marshall and Bartold, 1998] .

Gingival overgrowth , which normally begins in the region of the interdental papilla, may favor the appearance of clinical symptoms and signs that include pain, bleeding and friability of the tissue, abnormal movement of the teeth , changes of appearance , phonetics, and occlusion as well as the appearance of dental caries and other periodontal disorders [Carranza , 1990] .

Enlarged gingival tissue may allow further accumulation of oral bacteria into the pockets, suggesting that this unwanted side effect may greatly influence the clinical course of marginal periodontitis and subsequent systemic health if complicated . Therefore, it is obvious that a better understanding of the pathogenesis of drug-induced gingival overgrowth is one of the important subjects in clinical periodontology [Marshall and Bartold , 1998] .

Drugs inducing gingival overgrowth :

Drugs inducing gingival overgrowth as a side effect are : immuno-suppressive agents, anti-epileptics and calcium channel blockers but the exact mechanisms underlying the pathogenesis of drug-induced overgrowth are still unclear [Seymour et al., 1996] .

Although the exact mechanisms underlying the pathogenesis of drug-induced overgrowth are still unclear, the use of these drugs, particulaly immuno-suppressive, is estimated to be increased rapidly due to an expected increase in organ transplantation in the 21st century .

The pathogenic mechanisms involve different factors such as : dental plaque , presence of genetically predetermined gingival fibroblasts (named responders) , and effect of the drug itself [Hefti et al., 1994] .

The clinical and pathologic features in drug – induced gingival overgrowth are independent of the drug adminstration, which suggests a common pathway of induction [Akimoto et al ., 1991] .

Etiological factors causing and underlying drug – induced gingival overgrowth (DGO) have been recently reviewed, and it has been reported that local, genetic and systemic factors may also contribute to the development of the overgrowth lesions [Marshall and Bartold , 1999 and Kantarci et al., 1999] .

The incidence of drug induced GO is higher in children [Daley and Wysocki , 1984] and in females and it usually appears within 2-3 months from the first dose [Tyldesley and Rotter ,1984] .

In case of drug induced gingival overgrowth , the esthetic disfiguring (gingival enlargement) is more conspicuous on the buccal than on the lingual gingiva and less severe in the maxilla than in the mandible [Butler et al ., 1987 and Camargo , 1989] .

Drugs implicated in gingival overgrowth :

1 **Nifedipine (NIF)** :

Nifedipine is a calcium channel blocking agent of the dihydropyridine group . Gingival enlargement in patients treated with nifedipine was originally reported in 1984 by Lederman et al., and Ramon et al., 1984 . However , Miranda et al.,2001 found that patients treated with nifedipine are at high risk for gingival enlargement, and gingivitis acts as predisposing factor .

More recently, gingival enlargement also has been described in patients treated with other dihydropyridines, such as : nitrendipine, nicardine, fleodipine, oxidipine and verapamil [Brown et al., 1991 and Jorgensen, 1997] . Moreover this gingival enlargement was time and dose dependant [Brkic , 2005] .

The Pharmacological action of nifedipine :

Nifedipine belongs to the class of agents pharmacologically known as calcium channel blockers [Hillis , 1980] . Nifedipine is a calcium - ion – influx inhibitor which does not change serum calcium levels [Antman et al ., 1980] .

Nifedipine is widely used as a vasodilating agent for the treatment of hypertension and ischemic heart disease [Florez, 1992 and Tamargo and Delpon, 1992] .

Mode of action of Nifedipine :

Nifedipine is a potent vasodilator which causes long lasting coronary artery vasodilatation and reduces coronary artery spasms. The drug is widely used in the treatment of angina and occasionally used to control hypertension . The prescise mechanisms by which it relieves angina is believed to be : prevention of calcium dependant adenosine triphosphatase (ATpase) from breaking down formed ATP, thereby decreasing high energy phosphate consumption , mechanical tension and oxygen requirements of the myocardium [Stone et al ., 1980 a.] .

Nifedipine inhibits both the entry of calcium ions into cells and calcium mobilization from intercellular stores . It inhibits calcium ion influx in cardiac and smooth muscles without changing serum calcium concentration [Stone et al., 1980 b.] .

2 Phenytoin (PHT) :

Anti – epileptics or anti- convulsants are drugs used in the treatment of epilepsy and other convulsive disorders. These drugs include beside phenytoin (Dilantin) , phenobarbitone, primidone , carbamazepine, ethosuximide and valproic acid [Seymour and Heasman , 1988] .

The term epilepsy describes a group of central nervous system disorders, which have in common, the occurance of sudden and transitory episodes (seizures) of abnormal phenomena of motor (convulsions) , sensory, autonomic or psychic origin [Commission on classification and terminology of the international league against epilepsy, 1981] .

Drug – induced gingival overgrowth was first reported in 1939 as a consequence of the chronic adminestration of the anti-epileptic agent phenytoin [Kimball, 1939] .

Phenytoin is an anti-convulsant drug which is commonly used for prevention of seizures [Trackman and Kantarci, 2004].

A common side effect of phenytoin therapy is gingival hyperplasia, occasionally so severe that it requires surgical intervention [Lacopino et al., 1997] .

Individual susceptibility to the development of phenytoin induced gingival overgrowth appears to be genetically based [Hassell and Gilbert, 1983] .

The Pharmacological action of phenytoin :

Pharmacologically phenytoin is a weak acid with poor solubility , that is usually given orally and is slowly absorbed from the gastro-intestinal tract . The drug is extensively bound to plasma protein . It is metabolized in the liver by microsomal enzymes . The metabolites together with 5 % of the unchanged phenytoin are excreted in urine [Richens , 1979] .

Mode of action of Phenytoin :

Phenytoin lowers the exitatory thresheld of the affected neuron in the motor cortex by supressing the sodium- potassium ATpase pump [Pincus and Hsiano , 1970] .

It exerts such anti-convulsant properties by stabilizing neural cell membrane to the action of sodium , potassium and calcium ions thus protecting the nervous tissues from the effects of repetitive activity [Woodbury, 1980] .

Thus such anti – epileptic drug has two general modes of action . Firstly , this drug can act on the pathologically altered neurons when subjected to seizure foci and prevent or reduce the excessive discharges of impluses in them . Secondly , it can reduce the spread of excitation from seizure foci . [Seymour and Heasman , 1988] .

3 **Cyclosporin A (CsA)** :

Cyclosporin is a fungal metabolite produced by the fermentation of the specises trichoderma polysporum and cylindocarpon lucidum first discoved by [Borel et al., 1977] . Cyclosporin is a hydrophobic cyclic polypeptide derived as a metabolite from the fungus Toly pocladium inflatum gams . it is a nonmyelotoxic immunosuppressant .

The experimental studies had shown that CsA suppresses the cell mediated response but had no effect on the humoral response [Bird and Britton 1979] . Beside being one of the keydrugs used to achieve the immuno-suppression necessary for the prevention of interception of transplant rejection, cyclosporin has a theraputic value in a number of disorders where dysfunction of immuno-regulation is considered an etiologic factor, such as : occular Behcet's syndrome, endogenous uveitis, psoriasis , atopic dermatitis, rheumatoid artheritis and pemphigus vulgaris [Gilman, 1995 and Irshied and Bimstein, 2001] .

Dangerous systemic drawbacks of CsA are nephrotoxicity and hepatotoxicity [Rateitschak – Pluss el al ., 1983] .

One of the significant dental side effects of CsA therapy is the esthetically disfiguring overgrowth of the gingiva [Rateitschak - Pluss et al., 1983 ; Chestnykh, 2005 and Ramalho et al., 2003] . The severity of cyclosporin – induced gingival hyperplasia has been shown to be significantly related to the plasma concentration of the drug [Seymour et al ., 1987] .

The severity of the clinical picture of the gingiva in CsA treated patients is directly related to - but not entirely depended on - external irritants such as dental plaque , calculus, appliances, mouth breathing and inadequate dental restorations [Butler et al ., 1987] .

The Pharmacological action of Cyclosporin:

Immuno-suppressants act on various components of the immune system causing selective inhibition and suppression for organ rejection in transplant surgery . Drugs used for this purpose include corticosteroids (mainly prednisone and prednisalone) , azathioprin and more recently cyclosporin . Cyclosporin A is an immuno-suppressant which is widely used to prevent rejection phenomena following organ and bone marrow transplantation and also to treat many systemic diseases with immunologic etiology [Hassell and Hefti, 1991 and Seymour et al., 1996] .

The primary target of cyclosporin is helper inducer T-lymphocytes. Azathioprine is a nonspecific myelosuppressant, it is commonly used together with prednisolone and CsA (triple medication) in organ-transplantation [Seymour et al ., 1996] .

Mode of action of Cyclosporin :

CsA up regulates monocyte and macrophage platelet derived growth factor (PDGF) . PDGF is a dimeric poly peptide consisting of A and B chains in homodimer (AA, BB) or heterodimer (A B) continuations [Ross et al ., 1986] . The A chain is belived to play a minor role in tissue repair, however, the B chain (contained in the PDGF , A B or B B) is a major mitogen and chemoattractant for fibroblasts . It stimulates fibroblast proliferation and synthesis of collagen [Martin et al ., 1992] . The previous investigations indicatd that drugs such as CsA and (PHT) can infleunce macrophage phenotype cytokine and growth factor production . However , it would appear that the pro-inflammatory cytokine is up regulated in inflammatory conditions but not in proliferative conditions associated with drug induced gingival hyperplasia [Dill et al ., 1993] . PDGF on the other hand appears to represent an essential polypeptide growth factor which is specifically regulated in response to drugs which cause proliferation of gingival tissue [Nares et al ., 1996] .

Nifedipine and cyclosporin are widely prescribed drugs in patients who have undergone renal transplantation . The immuno supperssant properties of cyclosporin prevent graft rejection , whilst nifedipine controls hypertension and reduces cyclosporin induced nephrotoxicity [Calne, 1980 and Feehally et al., 1987]

Histological structure of gingiva :

The healthy gingiva is attached to the external part of the alveolar bone and the cervical portion of the tooth to protect and maintain the integrity of the periodontium. [Page, 1972 ; Schluger et al ., 1977] .

The gingiva is belonging to the masticatory mucosa , it extends from the dentogingival junction to the alveolar mucosa it is subjected to the friction and pressure of mastication [Bhaskar , 1990] .

Dale et al., 1990 reported that the two main tissue components of the gingiva are a stratified squamous epithelium, called oral epithelium, and an underlying connective tissue layer called the lamina propria . The interface between epithelium and

connective tissue is usually irregular, and shows upward projections of connective tissue , called the connective tissue papillae, interdigitate with epithelial ridges or pegs, sometimes called the rete ridges or pegs . The papillae of the connective tissue are characteristically long slender, and numerous .

In a typical hematoxylin and eosin stained sections , the interface between the epithelium and connective tissue appears as a structureless layer about 1 to 2 μ m thick, termed the basement membrane .

Ten Cate 1996 reported that the epithelial surface of the gingiva shows the following layers of cells :

1 - **The basal cell layer** :

The basal layer (frequently given the Latin name stratum basale) is a layer of cuboidal or columnar cells adjacent to the basement membrane . Occasionally, the term proliferative or germinative layer (stratum germinativum) is used to describe the cells in the basal region that are capable of division .

2 – **The prickle cell layer** :

Above the basal layer are several rows of larger elliptical or spherical cells known as the prickle cell layer or stratum spinosum. This term arises from the appearance of the cells

prepared for histologic examination, they frequently shrink away from each other, remaining in contact only at points known as intercelluar bridges or desmosomes . This alignment gives the cells a spiny or prickle like profile . The Greek word for prickle, akanthe , is used frequently in pathologic descriptions of an increased thickness (acanthosis) or a separation of cells caused by loss of the intercellular bridges (acantholysis) in this layer .

The basal and prickle cell layers together constitute from half to two thirds of the thickness of the epithelium .

3 - **The granular cell layer** :

The next layer consists of larger flattened cells containing small granules that stain intensely with acid dyes such as hematoxylin. This layer is the granular layer , or stratum granulosum, and the granules are called keratohyalin granules . In the gingiva these granules are difficult to be seen clearly under the light microscope .

4 - **The keratinized cell layer** :

The surface layer is composed of flat (squamous) cells, termed squamae , that stain bright pink with the histologic dye eosin and do not contain any nuclei, this layer is the keratinized

layer or stratum cornium . Other names sometimes used include cornified layer and horny layer . The pattern of maturation of these cells often is termed orthokeratinization .

The gingiva shows variation of keratinization in the form of parakeratinization . In parakeratinized epithelium the surface layer stains for keratin, as described previously, but shrunken (or pyknotic) nuclei are retained in many or all of the squamae. Keratohyalin granules may be present in the underlying granular layer .

In 1990 Bhaskar reported that the lamina propria of the gingiva consists of a dense connective tissue that does not contain large vessels. Small numbers of lymphocytes, plasma cells, and macrophages are present in the connective tissue of normal gingival subjacent to the sulcus and are involved in defense and repair .

The tissue of the lamina propria contains only few elastic fibers, and for the most part they are confined to the walls of the blood vessels . Other elastic fibers known as oxytalan fibers are also present .

The gingival fibers of the periodontal ligament enter into the lamina propria, attaching the gingiva firmly to the teeth . The gingiva is also immovably and firmly attached to the periosteum of the alveolar bone . Because of this arrangement it is often referred to as mucoperiosteum . Here a dense connective tissue, consisting of coarse collagen bundles , extends from the bone to the lamina propria .

The gingiva contains dense fibers of collagen , sometimes referred to as the gingival ligament , which are divided into the following major groups : dentogingival , alveologingival , circular and dentoperiosteal fibers .

The collagenous component of the gingival lamina propria:

In the healthy gingival connective tissue the main structural and fibrillar component is collagen . The healthy gingival lamina propria is composed of collagen type I and III (interstitial collagens) which form thick fibrous bundles or reticular fibers and constitute the main collagenous component of gingival connective tissue [Ballard and Butler , 1974] .

On the other hand , little is known about the localization and function of collagen type IV and V in oral mucosa . Type V collagen originally isolated from fetal membranes

[Burgeson et al ., 1976] , later on it was found in virtually all organs such as in liver fibrosis [Rojkind et al., 1979] , scar formation in chronic fibrous diseases [Morton and Barnes , 1982] and it seems to play a major role in the formation of granulation tissue [Kurita et al., 1985] ,

Type IV collagen is considered the principle scaffold for the basement membrane which play an important role in cell differentiation, morphogensis and wound healing. It also regulates nutrition of the epithelium and acts as selective filter between circulatory and stromal components [Vracko, 1982; Martinez and Amenta, 1983 and Sanders 1983] . The normal distribution of collagen type IV is present in the basement membranes of the epithelium , blood vessels and nerves [Abou El Fotoh and Osman , 2004] .

Johnson , 2003 found a synergistic enhancement of collagenous protein synthesis by human gingival fibroblasts exposed to nifedipine .

Collagen metabolism and upregulation (Collagen turnover) :

Collagen is the most abundant protein in mammals and its metabolism is precisely balanced by collagen synthesis and degradation to maintain a steady state (Perz , 1978) . Collagen is degraded via an extracellular pathway involving

secretion of collagenase and via an intracellular pathway involving phagocytosis [Everts et al ., 1985] . The collagenase-mediated route is accompanied by a loss of tissue architecture (e.g., inflammation) , while the collagenase-independent route is important during normal turnover [Sodek and Overall, 1988] .

The primary enzyme responsible for matrix degradation is a family member of matrix metalloproteinase [Woessner, 1991]. Lysosomal cystein proteinase (cathepsin B and L) is also considered to be important for further digestion of matrix components [Burleigh et al , 1974] . In addition, patients with the congenital disease termed mucolipidosis II (I- cell disease) , which is characterized by an abnormal loss of intracellular lysosomal enzymes and elevated level of serum lysosomal enzymes have been reported to manifest severe gingival hyperplasia similar to drug-induced gingival hyperplasia [Patel and Ambani, 1980] . This suggest that lysosomal enzymes play an important role in maintaining the homeostasis of connective tissues.

The non collagenous component of the gingival lamina propria:

Although the main interest has been focused on the collagenous component of gingiva, very little is known about non collagenous proteins especially fibronectin [Narayanan and Page , 1983] .

There is no doubt that fibronectin interacts strongly with other matrix constituents and participates in structural organization of connective tissue . it had been found to promote the substrate attachment of cell grown in cultures [Klebe, 1974]. It was reported that fibronectin molecule contains multiple binding sites for major cell surface components such as collagen, heparan sulfate, proteoglycans, fibrinogen and integrins. These binding sites are involved in the organization of the extra-cellular matrix and the adhesive interactions of cells [Yamada , 1983] .

The function of fibronectin is of great importance because it stimulates the cell locomotion, cell adhesion and spreading as well as mediates and ensures the attachment of cells to the extracellular matrix [Yamada, 1983 ; Robert and Labat, 1991] . In addition, fibronectin also participates in the clearance of injured tissue fragments [Yang et al . , 1990] .

Other studies indicated that a large number of fibronectin isoforms exist due to alternative splicing of the fibronectin primary transcript [Schwarzbauer et al . , 1989] .

Aim of study :

1. The aim of this study is to make quantitative and qualitative evaluation of the histological changes of the gingival tisssue induced by Nifedipine (anti-hypertensive drug) , Phenytoin (anti-convulsant drug) , Cyclosporin (Immuno-supperssive drug) and combinations of these drugs .

2. Searching the causes of gingival overgrowth whether it is due to epithelial or connective tissue overgrowth or both together .

3. Demonstrating the lysosomal activities in the gingiva treated with the previously mentioned three drugs and their combinations to identify if there is decreased degradation of collagen as a result of decreased lysosomal enzyme activity .

4. Investigation of collogen pattern and distribution in the previous groups as well as the extra cellular matrix glycoprotein fibronectin .

Material & Methods

Seventy adult male albino rats weighting about 200-250 gm. were selected for this study. The experimental animals were kept under optimum conditions of proper ventilation and cleaning measurements and allowed to food and water ad-libitum . All animals were fed ground barely, powdered milk, vegetables and water. The experimental animals were purchased from the animal house of the research institute of ophthalmology and kept in wire cages . The animals were divided into 7 main groups , 10 rats for each group .

Control group (Group 1)

The animals of this group received di-methyl sulfoxide DMSO and distilled water orally by intra oesphageal tube as in the same manner of the treated groups .

Nifedipine group (Group 2)

The experimental animals of this group received orally by an intra oesphageal tube a daily dose of 40 mg./kg. rat body weight epilat for 2 months. The contents of the capsules were dissolved in DMSO and distilled water [According to O'Valle et al., 1995].

Phenytoin group (group 3)

The experimental animals of this group were injected intra-peritoneally with a daily dose of 100 mg./ kg. rat body weight phenytoin in a concentration of 2.5 mg./ml. dissolved in DMSO 10% and distilled water 90% for 2 months. [According to Dill et al., 1993] .

Cyclosporin group (group 4)

The experimental animals of this group received orally (by intra-oesophgeal tube) a daily dose of 20 mg./kg. rat body weight cyclosporin dissolved in DMSO 100% and diluted with distilled water to a concentration of 2.5 mg./ml. for 2 months. [According to O'Valle et al., 1995] .

Nifedipine and phenytoin group (group 5)

The experimental animals of this group received a combination of drugs of group 2 and 3 .

Nifedipine and cyclosporin group (group 6)

The experimental animals of this group received a combination of drugs of group 2 and 4 .

Phenytoin and cyclosporin group (group 7)

The experimental animals of this group received a combination of drugs of group 3 and 4 .

The animals were sacrificed after 2 months and the buccal gingiva of the lower jaw of each specimen was dissected out gently and carefully using sterile sharp blade [According to the method of Tyldesley and Rotter 1984] .

In the pilot study we found that the plane of cutting differes greatly where if it passed through the area of the gingiva it showed normal gingival tissue and part of the adjacent sulucular epithelium . While if it passed through the area of the gingival ligament it showed thick collagen fiber bundles which sometimes were miss interpreted as muscles but in fact they were the fibers of the gingival ligament .

Also this plane of section demonstrated a difference in the inflammatory reaction as the degree of inflammation below the oral epithelium was less than that below the sulucular epithelium .

For histopathological investigation :

The specimens were fixed in freshly prepared 10% calcium formole solution for about 24-48 hours at 4^{o}c, then dehydrated by passing them in ascending grades of alcohol , then cleared in xylol .

The specimens were embedded in paraffin wax , sectioned by microtome to about 5 µ thickness and stained with (H&E) [According to the method of Bancroft et al., 1977] .

For histochemical examination :

Fresh frozen tissue sections were prepared for histochemical examination using a tissue Tek microtome cryostat which is found in the National Cancer Institute . The specimens were washed with cold saline to remove blood.

Each specimen was placed on a holder with some of the embedding medium below and around them in order to obtain a solid block of material . The specimen on the holder was then frozen by carbon dioxide and left inside the cryostat at-15° C for 15 min. Sections of 6 – 8 µ thickness were obtained to study the acid phosphatase enzyme activity according to the method of Goldberg and Barka ,1962 .

For acid phosphatase activity examination

Azo dye coupling method was used as follows

Solutions required :

(1) 0.1 M acetate buffer, pH 5.0 .

(2) Sodium α - naphthyl phosphate .

(3) Diazonium salt , Fast Garnet GBC .

Preparation of incubating medium :

- Sodium α - naphthyl phosphate 10 mg.
- 0.1 M acetate buffer, pH 5.0 10 ml.
- Fast Garnet GBC 10 mg.

The sodium α - naphthyl phosphate was dissolved in the buffer and the diazonium salt added . The solution was then filtered and used immediately .

Procedure :

1- Incubate at 37^0 C for 15 – 30 minutes.
2- Wash in distilled water.
3- Counter stain in 2 % methyl green (chloroform extractes)
4- Wash in running water.
5- Mount in glycerin jelly .

The prepared sections were examined according to the intensity of acid phosphatase reaction which was seen as red to brown spots with different intensties ranged from weak to strong. [According to Goldberg and Barka ,1962] .

For immunohistochemical examination:

Specimens were immediately fixed in 10% neutral buffered formalin for 8 hours and then routinely processed to paraffin sections 5 μ m thickness .

Sections were then mounted on positively charged glass slides and immunoperoxidase stained .

Preparation of the solutions used :

I - **Buffered formalin** :

Formalin (40% formaldehyde)	100 ml.
Dibasic sodium phosphate anhydrous	6.5 mg.
Monobasic sodium phosphate monohydrate	4.0 gm.
Distilled water	up to 1 litre.

II- **Immunoperoxidase staining solutions** :

A- **Primary antibodies :**

① **Rabbit anti-mouse collagen I polyclonal antiserum**

Catalog number : AB765

Lot number : 2210542

Immunogen : Puirified mouse collagen type I from mouse skin

Format : Rabbit antiserum .

(**supplied by novocastra laboratories Ltd . Balliol business park west , Benton lane , Newcastle upon type NE12 8EW, United kingdom**) .

② **Collagen Type IV (Ab-2) Polyclonal Antibody**

Host : Mouse Epitope/immunogen : Immunized with human glomeruli .

Clone: CIV 22 Species Reactivity : Bovine , human , rat not canine or porcine .

Iso type : IgG, : Not canine or porcine .

Application : Frozen sections , paraffin sections

(Supplied by Chemicon international single Oak drive , Temecula , CA 92590)

③ **Rabbit anti-mouse collagen type V polyclonal antiserum**

Catalog number : AB763

Lot number : 20020227

Immunogen : Collagen type V extracted and purified from human placenta .

(Supplied by Chemicon international single Oak drive , Temecula , CA 92590)

④ **Fibronectin** :

Lyophilised Monoclonal (NCL – FIB)

Clone 568 : F⌋ P⌋

Fibronectins are glycoproteins composed of two 200KD disulphide-linked subunits. They are found in basement membranes and in the extracellular connective tissue matrix . NCL – FIB is specific for the cell attachment domain of human and rat fibronectin .

Typical working dilution in immunohistochemistry is : 1 : 100 – 1 : 200 .

(Supplied by NeoMarkers , Inc 47790 Westinghouse Dr. Fremont , CA 94539 , USA) .

B – **Universal Kit** :

Labelled streptavidin biotin (LSAB) / horse radish peroxidase (HRP) kit .

Contents of the kit :

Bottle 1 (Blocking reagent) :

15 ml aqueous 3% hydrogen peroxide solution .

Bottle 2 (non specific blocking agent) :

15 ml non immune goat serum prediluted in 0.05 M Tris/HCl buffer, pH 7.6 containing 6% carrier protein and 15 m. M sodium azide as preservative .

Bottle 3 :

A storage container for the diluted primary antibody solution .

Bottle 4 (monoclonal mouse linking reagent) :

15 ml biotinylated antimouse immunoglobulin (Ig) prediluted in 0.05 M Tris/HCl buffer, pH 7.6, containing 25% carrier protein and 15 m. M sodium azide .

Bottle 5 (Streptavidin enzyme label solution) :

Supplied as a concentrated form and then was diluted as follows:

- Add 4 drops of 1.25 M. Tris/HCl buffer in a labeled test tube.
- Add one drop of streptavidin concentrate .
- Mix contents by gently inverting several times .

Bottle 6 (chromagen) :

3 ml. diaminobenzidin (DAB) in distilled water .

Bottle 7 (Colour reagent buffer) :

15 ml. 0.1 M. acetate buffer , pH 4.8 .

Bottle 8 (hyprogen peroxide) :

1 ml. 0.3% hydrogen peroxide in distilled water .

(Supplied by Novocastra Laboratories Lrd., 24 Claremont place , Newcastle upon tyne NE2 4AA. UK)

The substrate solution was prepared from contents of bottles 6 , 7 , 8 as follows :

- add 5 ml. distilled water in a test tube .
- add 2 drops of the buffer (bottle 7) .
- add 2 drops of the 0.3% hydrogen peroxide (bottle 8) .
- add 4 drops of the chromagen (bottle 6) .
- a fresh substrate solution usually used for colour development .

C- **Negative control (non specific serum) :**

Purified mouse immunoglobulin in a carrier protein preservative solution extracted from non immunized mouse .

It was used at the same dilution as its respective primary antibody.

D- **Phosphate buffered saline (PBS)** :

- Sodium chloride .	8.5 gm.
- Monobasic potassium phosphate	0.266 gm.
- Dibasic sodium phosphate .	1.14 gm.
- Distilled water	up to 1 litre .

Adjust the pH to 7.4-7.6 with 0.1 N sodium hydroxide .

Reserve in a cool place (4-8^{o}C) .

Bring the solution to room temperature just before use .

E- **<u>Trypsin solution</u>** :

- Prepare 0.1% trypsin solution in prewarmed distilled water containing 0.1% calcium chloride .
- Adjust the pH to 7.8 with 0.1 N sodium hydroxide .
- Preserve in a cool place (4-8^{o}C) .

Staining Procedures :

Immunoperoxidase staining :

1 - **Blocking endogenous peroxidase** :

- 3 -4 drops of 3% hydrogen peroxide were applied to cover the section .
- Sections were incubated in a humid chamber for 5 minutes
- Sections were gently rinsed in PBS solution .
- Excess liquid was removed by sharply tapping the edge of the slide on a filter paper then carefully dried around the section with an absorbent tissue .

2 - **Trypsin digestion** :

- Sections were incubated in an incubator at 37^{o}C with 0.1% trypsin solution (pH 7.8) for period of 5 minutes .
- Sections were bathed in 2 changes of PBS solution bath each for 5 minutes .
- Sections were washed in 2 changes of distilled water each for 5 minutes .
- Sections were carefully dried as before .

3 - **Blocking non specific staining** :

- 3-4 drops of the blocking serum were added to cover the section .
- Sections were incubated in a humid chamber for 20 minutes .
- Sections were gently rinsed and dried as before .

4 - **Exposure to the primary antibody** :

- 3-4 drops of the primary antibody were added to cover the section .
- Sections were incubated in a humid chamber for 20 minutes .
- Sections were rinsed and dried as before .

5 - **Exposure to the linking antibody** :

- 3-4 drops of the linking antibody were added to cover the section .
- Sections were incubated in a humid chamber for 20 minutes .
- Sections were rinsed and dried as before .

6 - **Exposure to streptavidin enzyme label** :

- 3-4 drops of the diluted enzyme were added to cover the section .
- Sections were incubated in a humid chamber for 20 minutes .
- Sections were rinsed and dried as before .

7 - **Colour development** :

- 3-4 drops of the prepared substrate solution were added by a Pasteur pipette to cover the section .
- Sections were incubated in a humid chamber for 10 minutes .
- Sections were rinsed in PBS bath for 2 minutes by dipping them several times .
- Sections were rinsed in distilled water for 2 minutes .

8 - **Counterstaining** :

- Sections were immersed in a staining jar containing Harris' haematoxylin for 1 minute .
- Sections were dehydrated in ascending grades of alcohol (70%, 90% and absolute alcohol) .
- Sections were cleared in xylene and placed on positive slides.
- For the negative control sections , the primary antibody in step 4 was substituted by the non-specific serum of the same dilution as its respective primary antibody and the procedures were continued as usual .
- All the immunoperoxidase staining procedures were performed in a humid chamber at room temperature (approximately 25ºC) .
- The sections were always placed on a flat surface and were not allowed at any time to dry out .

9 - **Interpretation** :

- The immunostained sections were examined under the L/M and interpreted together with examination and correlation with the negative control .
- The positive staining reaction appeared in the form of an orange to brown staining .
- The intensity of staining was assessed and scored as follows :
- weak
- Mild
- Moderate
- Marked
- Strong
- Intense

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Results

Histopathological Results

Group 1 (Control Group)

Through examination for the gingiva of the present work revealed normal rat gingiva composed of keratinized stratified squeamous epithelium and lamina propria [Fig. 1] .

The epithelium consisted of the four known layers : the basal cell layer , the prickle cell layer , the granular cell layer and the keratinous layer [Fig. 2] .

The basal cell layer in which the cells were variable in shape and size but mostly were columnar with basal nuclei and their long axis were more or less perpendicular to the basement membrane [Figs. 2 – 4] .

The prickle cell layer , the cells were polyhydral with spherical centrally placed muclei , normal mitotic activities were demonstrated in the basal cells and the lower part of the prickle cells (stratum germinativum) [Figs. 3, 4] .

The granular cell layer which consisted of 2 – 3 layers of granular flattened cells with flat nuclei and keratohyaline granules [Figs. 2 , 3] .

The keratinous layer (stratum cornium) , the layer next to the granular layer which composed of compact layers of keratin in most of the specimens without nuclear remnants (orthokeratinized) [Figs. 1 – 3] .

The epithelial component formed of long , slender , irregular and numerous rete pegs lying in the lamina propria which contained the blood supply and other connective tissue elements .

The lamina propria composed of two layers , the papillary and reticular layers . The papillary layer was composed of long , narrow, numerous connective tissue papillae interdigitated with epithelial rete pegs [Figs. 1 – 4] .

The papillary layers consisted of fine , collagen fibers and small sized blood capillaries while the reticular layer was a narrow layer composed of coarse collagen fibers arranged in longitudinal and cross fashion . The connective tissue was highly cellular and vascular with few inflammatory cells [Figs. 1 – 4] .

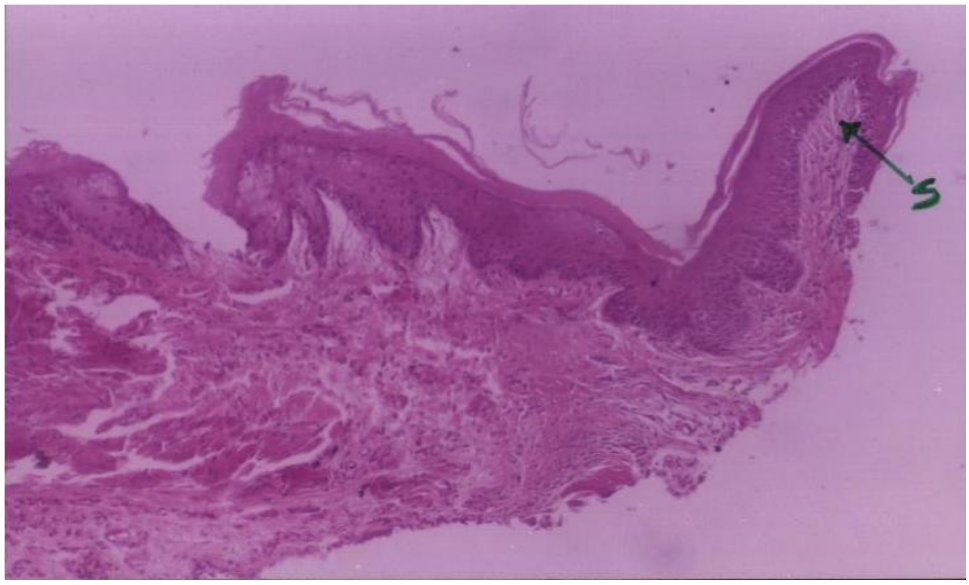

Fig. 1 : A photomicrograph of a specimen of rat gingiva of the control group (Group 1) showing normal structure of the gingival tissue with a part of sulucular epithelium (S) .
[H. & E. stain X 100] .

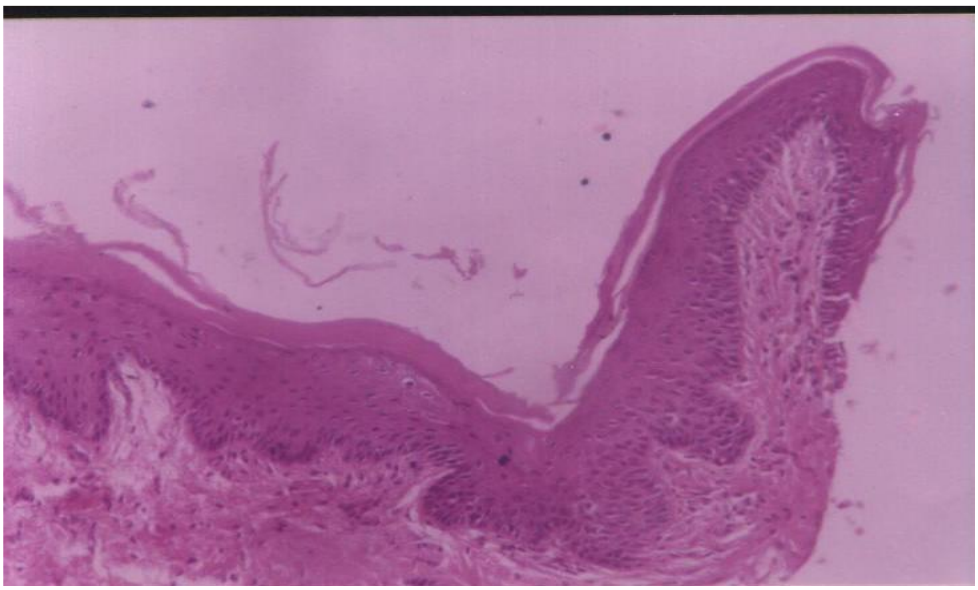

Fig. 2 : Higher magnification of Fig. 1 showing normal epithelial component of gingiva with long , numerous slenderical irregular rete pegs and homogenous keratinous layer
[H. & E. stain X 160] .

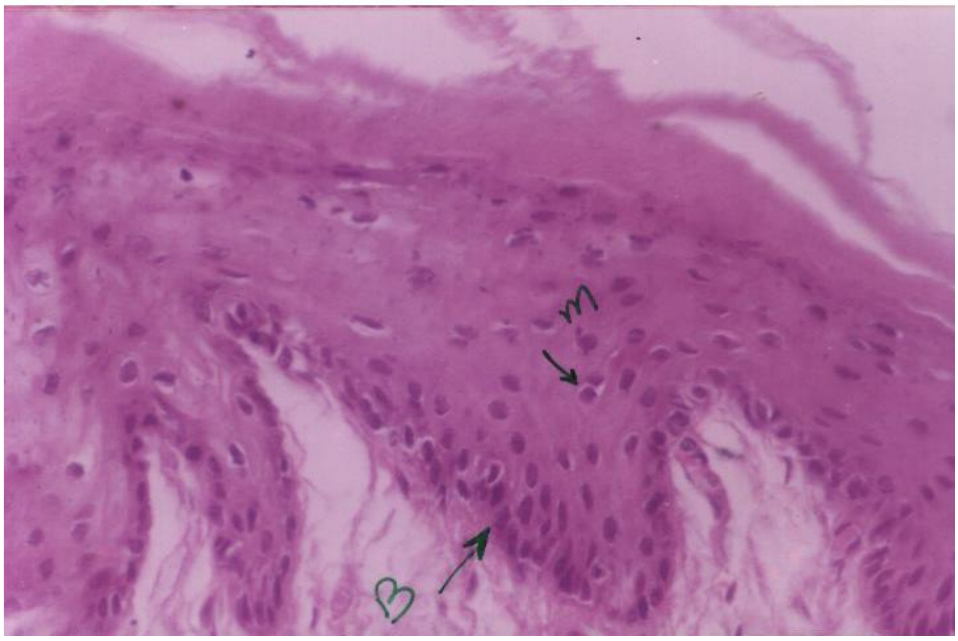

Fig. 3 : The epithelium of control gingiva showing the four layers of the epithelial component of the gingiva . Note that the basal cell layer (B) and the stratum germinativum showed normal mitotic activity (m). [H. & E. stain X 400] .

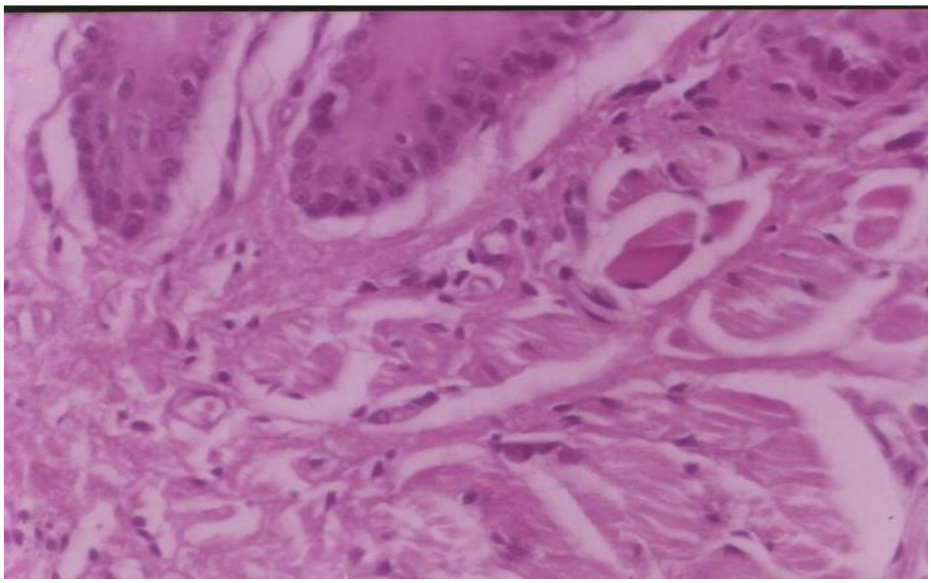

Fig. 4 : A photomicrograph of the control group showing the reticular layer composed of coarse collagen fibers arranged in longitudinal and cross fashion . Note that the C.T of the lamina propria is highly cellular . [H. & E. stain X 400] .

Group 2 (Nifedipine Group)

Examination of the gingival specimens of this group showed hyperplastic parakeratotic epithelium and inflammatory reaction in the subjacent connective tissue. There was also loss of normal tissue architecture compared with the control group [Figs. 5 , 6] .

The basal cell layer shows crowding , disorientation and increase number of cells . The prickle cell layer shows increased mitotic figures . There is also increased thickenss of keratin as compared to the control group . Some of the epithelial rete pegs were relatively long and thin (test - tube) shaped and were inserted in very deep areas of connective tissue , while others are short and broad [Fig. 7] .

The lamina propria revealed areas of fibrous connective tissue determined by the abundance of fibroblasts and collagen fibers . The wall of the blood vessels was relatively thickened and the blood vessel was dilated and congested with red blood cells . The connective tissue also showed dense perivasculer inflammatory cell infiltrate characterized by the presence of lymphocytes [Fig. 8] .

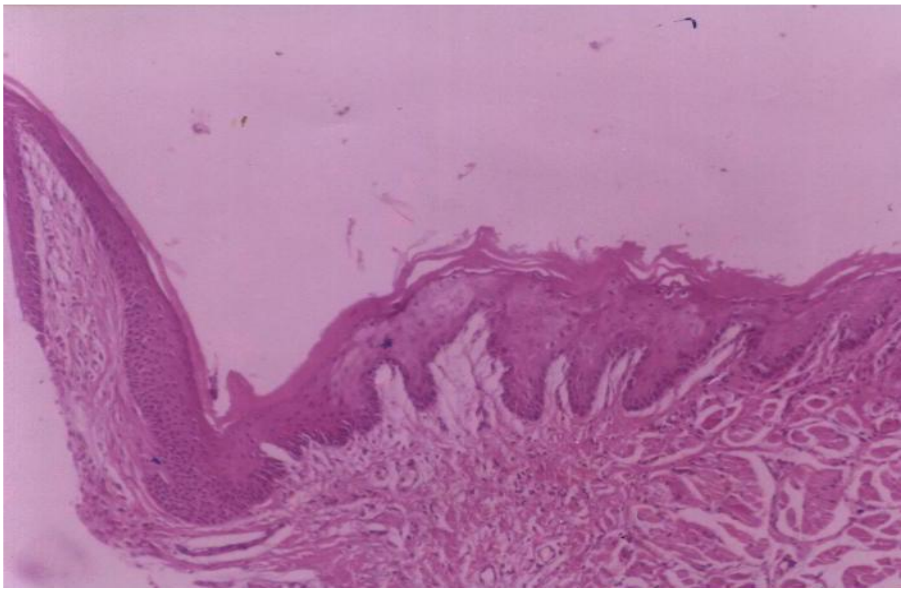

Fig. 5 : A photomicrograph of a rat gingiva of the nifedipine group (group 2) showing part of sulucular epithelium and part of nifedipine induced gingival overgrowth .
[H. & E. stain X 100] .

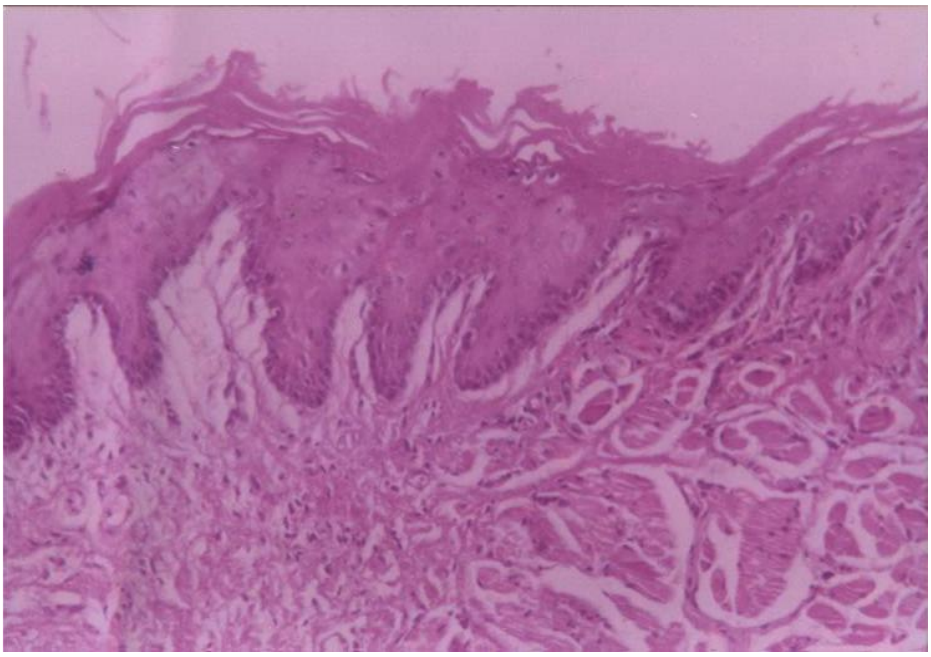

Fig. 6 : Higher magnification of Fig. 5 showing (test - tube) shaped epithelial rete pegs and increased thickenss of keratin .
[H. & E. stain X 160] .

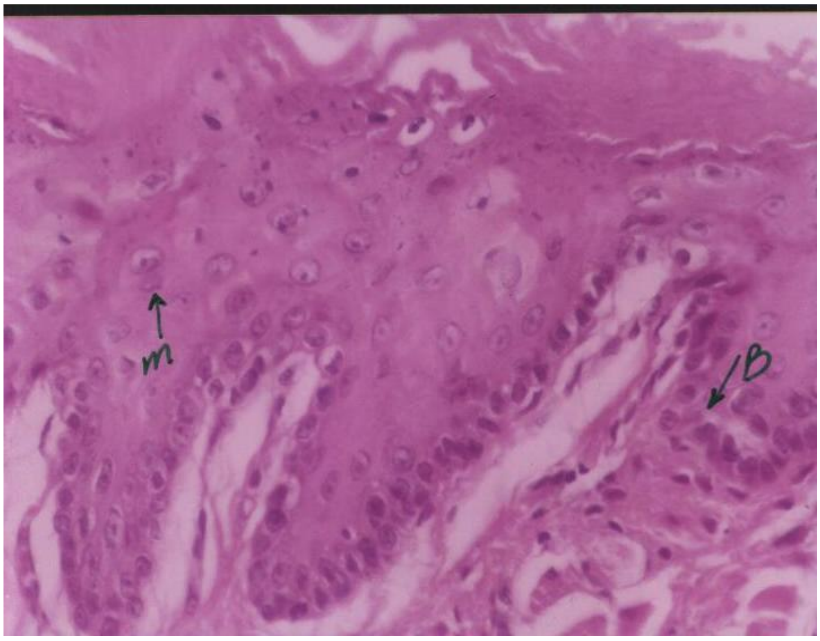

Fig. 7 : Higher magnification of Fig. 5 showing : crowding, disorientation and increase in number of the basal cells (B) . The prickle cell layer showed increased mitotic figures (m) . [H. & E. stain X 400] .

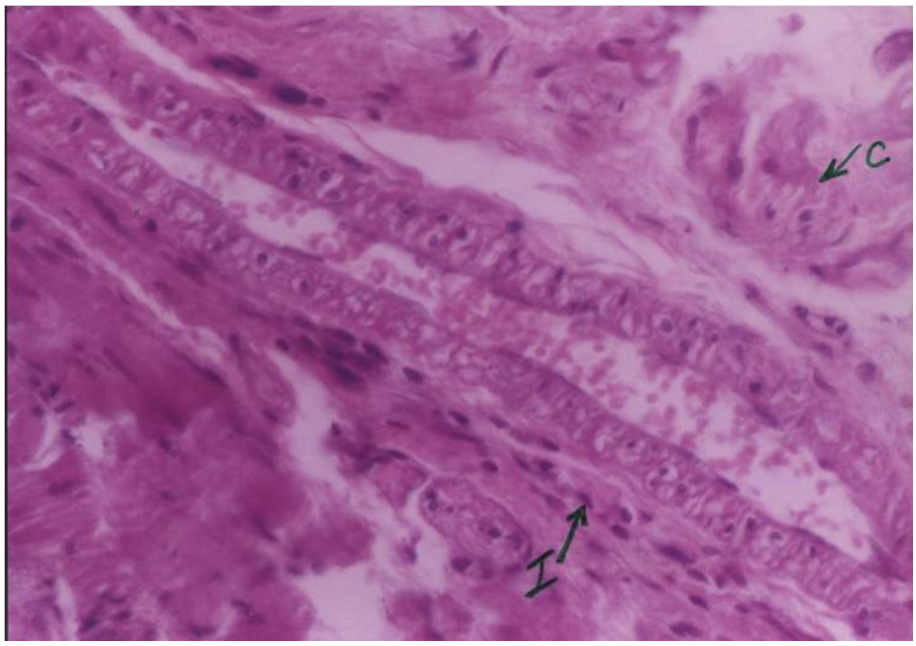

Fig. 8 : a photomicrograph of the same group showing : areas of fibrous connective tissue (C) and dense perivasculer inflammatory cell infiltrate characterized by the presence of lymphocytes (I) . [H. & E. stain X 400] .

Group 3 (Phenytoin Group)

The gingival sections of this group showed gingival hyperplesia in the form of : acanthosis and hyperkeratosis in the form of slightly increased thickness of keratin as compared to that of nifidipine group . The rete pegs were relatively broad [Figs. 9 , 10] .

The lamina propria revealed coarse irregularly distributed dense collagen fiber bundles with abundant fibroblasts and chronic Inflammatory cells , much more fibrosis than the previous two groups (control and nifedipine groups) . Areas of oedema were demonstrated . Inflammatory cells , entirely composed of lymphocytes distributed specially in the subepithelial layer of connective tissue, were seen . [Figs. 10 , 11] .

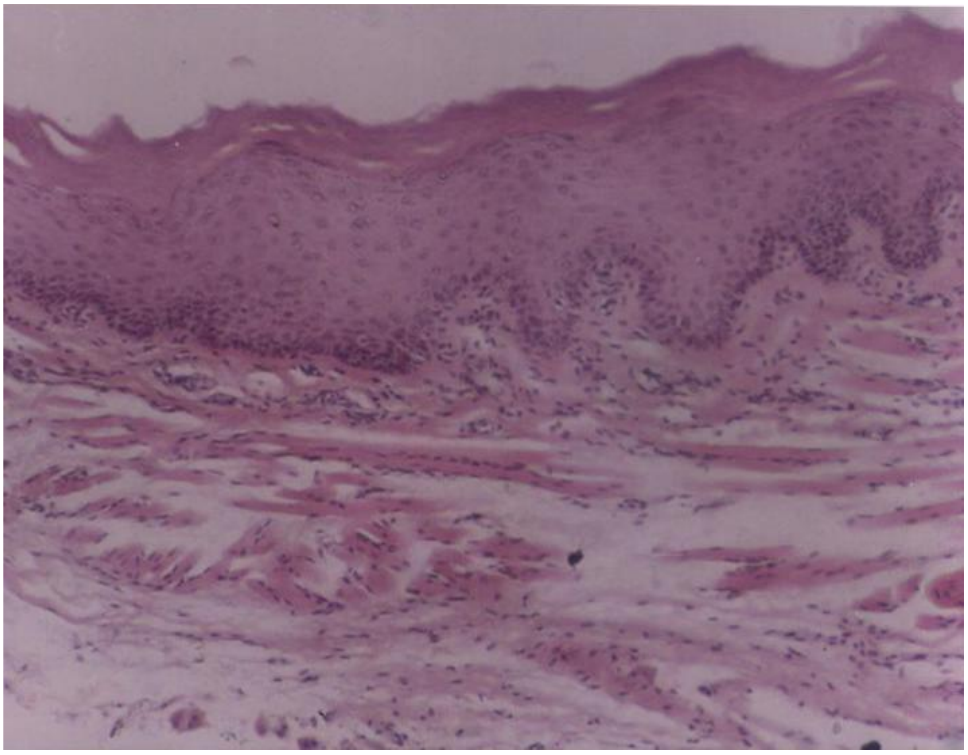

Fig. 9 : A photomicrograph of a rat gingiva of the phenytoin group (group 3) showing hyperplastic hyperkeratotic epithelium and fibrous C.T. as compared to control and nifedipine group .
[H. & E. stain X 100] .

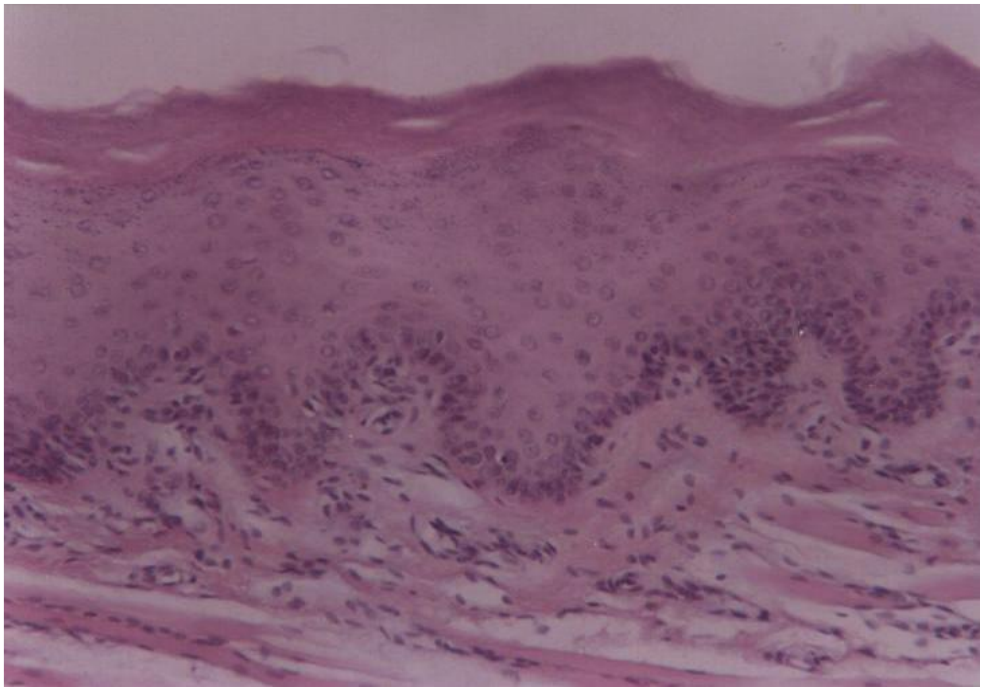

Fig. 10 : Higher magnification of Fig. 9 showing relatively broad rete pegs , increased thickness of the prickle cell layer and slightly increased thickness of keratin as compared to that of nifedipine group .
[H. & E. stain X 160] .

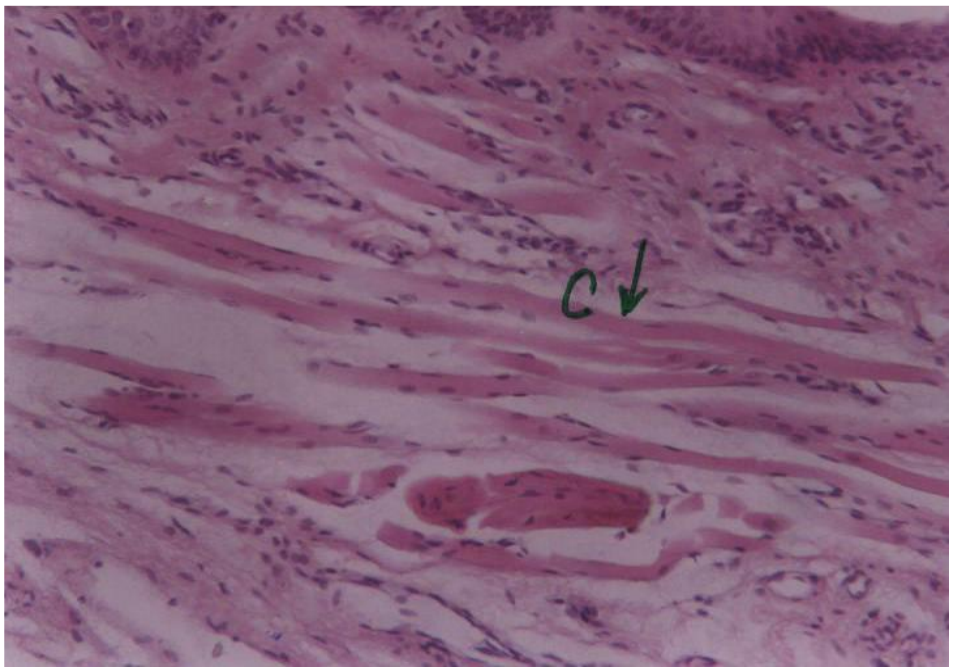

Fig. 11: A photomicrograph of the same group showing :marked C.T. fibrosis, dense collagen fiber bundles (C) and few inflammatory cells as compared to those of nifedipine group .
[H. & E. stain X 400] .

Group 4 (Cyclosporin Group)

The light microscopic examination of the H. & E. stained gingival sections of cyclosporin group showed conspesious enlargement of the epithelium which was obviousely much more greater than the previous groups . It also revealed hyaline like changes in the connective tissue stroma with few inflammatory cells as compared to the phenytoin group [Fig. 12] .

The epithelium of this specimen revealed crowding of the basal cells and marked acanthosis . The prickle cell layer showed increased thickness . There was also increase in the thickness of keratin more than the previous groups . The epithelial ridges were broad and deeply inserted into the subepithelial C.T. [Fig. 13] .

The lamina propria demonstrated changes in the connective tissue stroma in the form of irruglarly arranged collagen fiber bundles (not so dense as in phenytoin group) and few inflammatory cell infilterate most probably lymphocytes . [Fig. 13] .

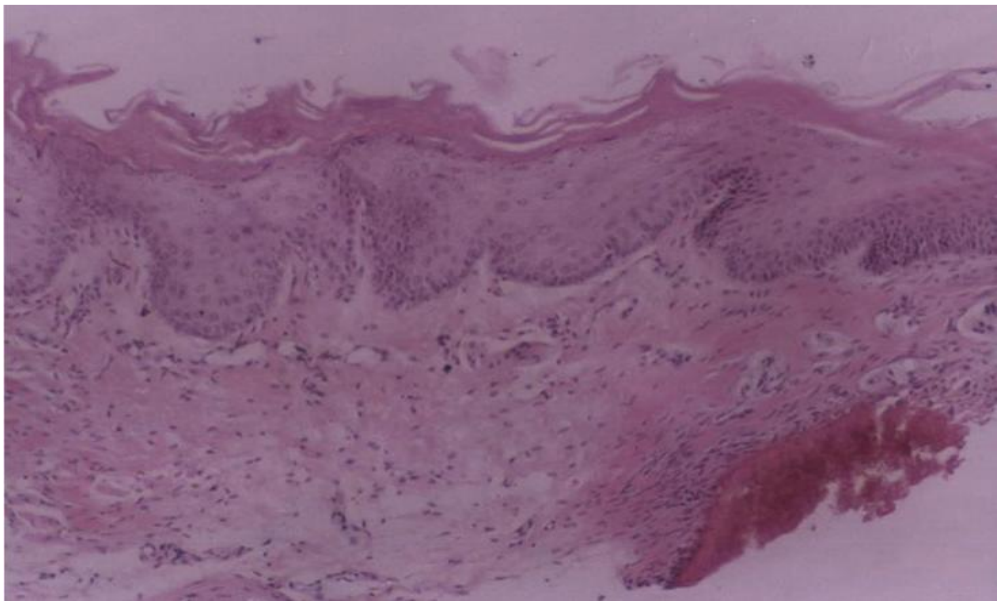

Fig. 12 : A photomicrograph of a rat gingiva of the cyclosporin group (group 4) showing conspesious enlargement of the epithelium and fibrous connective tissue .
[H. & E. stain X 100] .

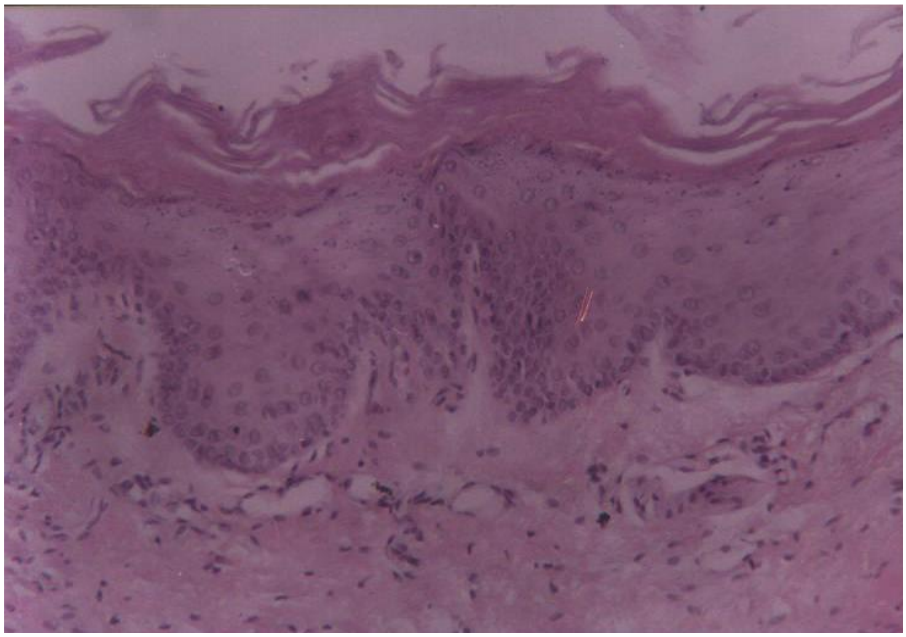

Fig. 13 : Higher magnification of Fig. 12 showing : crowding of the basal cells , (B) , increased thickness of the prickle cell layer (m) , irregularly arranged collagen bundles .
[H. & E. stain X 160] .

Group 5 (Nifedipine and Phenytoin)

The examination of gingival sections of this group demonstrated hyperplastic epithelium and fibrous connective tissue . The lamina propria showed densely arranged thick collagen fibers (similar to that of phenytoin group or slightly greater). Few inflammatory cells (mainly lymphocytes) could be seen. However the lamina propria showed decreased cellularity as in phenytoin group [Fig. 14] .

There was no crowding in the basal cell layer but the prickle cell layer revealed increased mitotic figures and some binucleated cells could be detected. There was also marked increase in the thickness of keratin. The epithelial rete pegs appeared broader than those seen when each drug was used alone (group 2 and 3) [Fig. 15] .

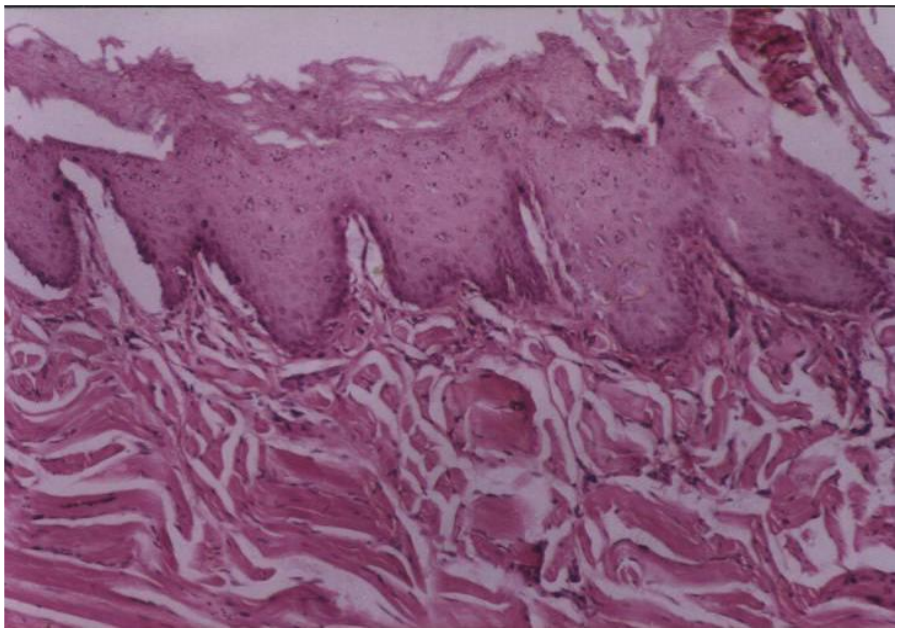

Fig. 14 : A photomicrograph of a rat gingiva of the nifedipine & phenytoin group (group 5) showing hyperplastic epithelium and fibrous connective tissue accompained with few inflammatory cells .
[H. & E. stain X 100] .

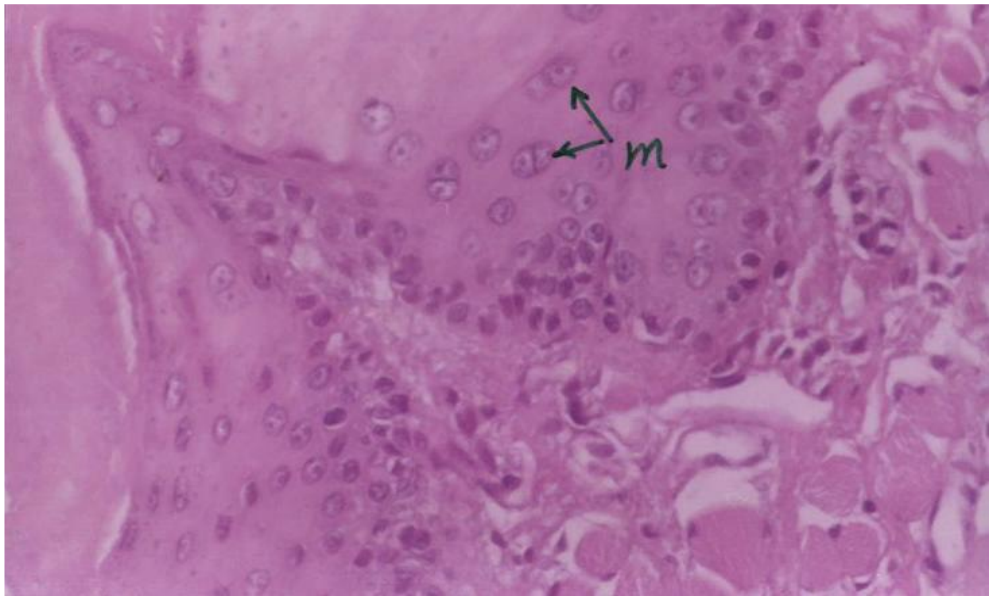

Fig. 15 : Higher magnification of another specimen of this group showing : increased mitotic figures (m) and some binucleated cells (arrow) .
[H. & E. stain X 400] .

Group 6 (Nifedipine and Cyclosporin)

The light microscopic examination of the H. & E. stained gingival sections of nifedipine and cyclosporin group revealed hyperplastic hyperkeratotic epithelium and inflammatory reaction in the underlying connective tissue in a degree lesser than the next group but greater than the control group and the groups of a single drug [Fig. 16] .

The epithelium of this group expressed acanthosis in the form of increase in size and number of cells of the prickle cell layer. The prickle cell layer also showed binucleated cells and mitotic figures. There was also marked increase in the thickness of keratin . The epithelial rete pegs were lesser in length and width as compared with those of cyclosporin and cyclosporin combined with phenytoin groups [Fig. 17] .

The lamina propria was heavily infiletrated with inflammatory cells most probably lymphocyts . The collagen fiber showed dense network. The lamina propria was more cellular than in nifedipine and Cyclosporin alone groups but not more than phenytoin or phenytoin and nifedipine groups [Fig. 17] .

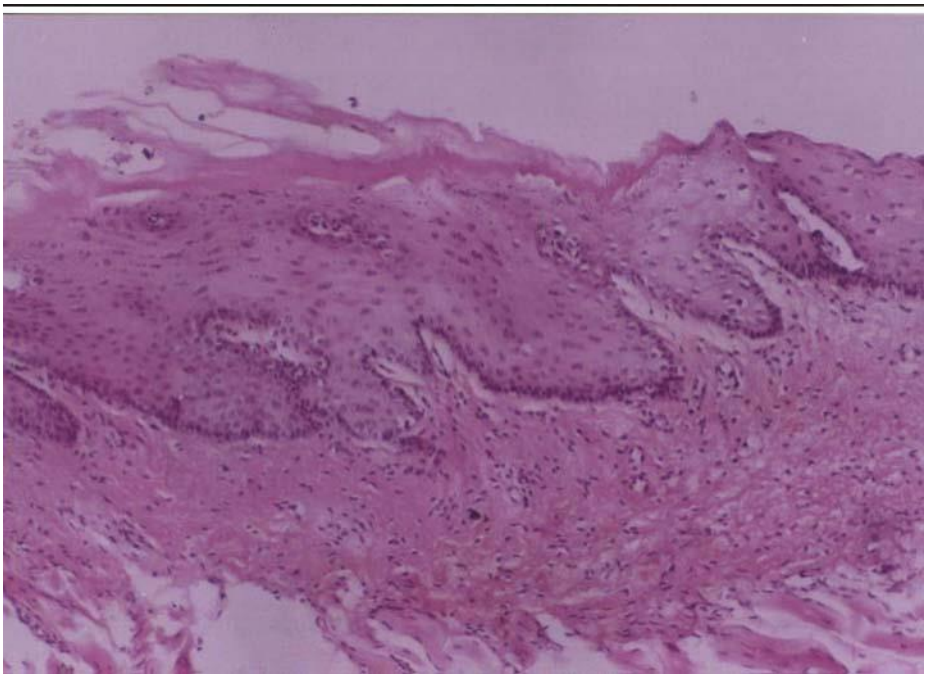

Fig. 16 : A photomicrograph of a rat gingiva of the nifedipine & cyclosporin group (group 6) showing hyperplastic, hyperkeratotic epithelium lesser than (group 7) and more than (group 3 and 4).
[H. & E. stain X 100] .

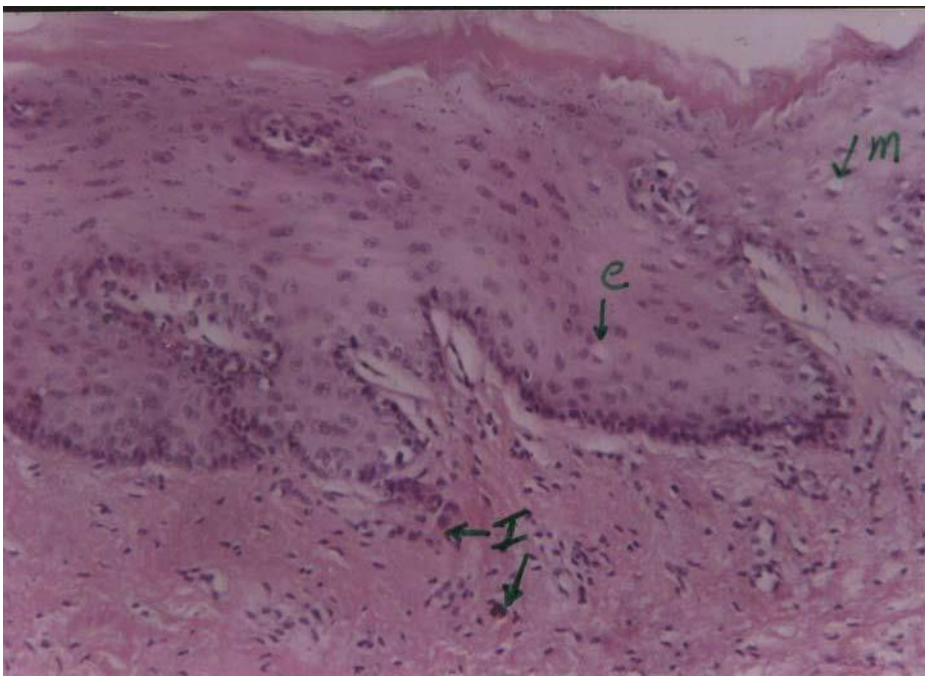

Fig. 17: Higher magnification of Fig. 16 showing heavy infiltration of inflammatory cells most probably lymphocytes (I) in between the interlacing collagen fibers of the lamina propria.
[H. & E. stain X 160] .

Group 7 (Phenytoin and Cyclosporin)

The light microscopic examination of the H. & E. stained gingival sections of this group exhibited hyperplastic epithelium and inflammatory reaction in the underlying connective tissue [Fig. 18] .

The prickle cell layer showed binuclated cells (mitotic figures) more than any of previous mentioned groups . There was also maximum increase in the thickness of keratin than any other group . The epithelial ridges were markedly elongated and broad . It was noted that there was also conspesious enlargement of the epithelial component as in cyclosporin group (group 4) [Fig. 19] .

The basel cell layer showed increase in the number of cells and crowding (basilar hyperplasia) . The lamina propria revealed dense arrangement of collagen fiber bundles. The blood vessels were congested with red blood cells and the inflammatory cells (mainly lymphocytes) were aggregated in different areas of lamina propria in the subepithelial layer [Fig. 20 ,21] .

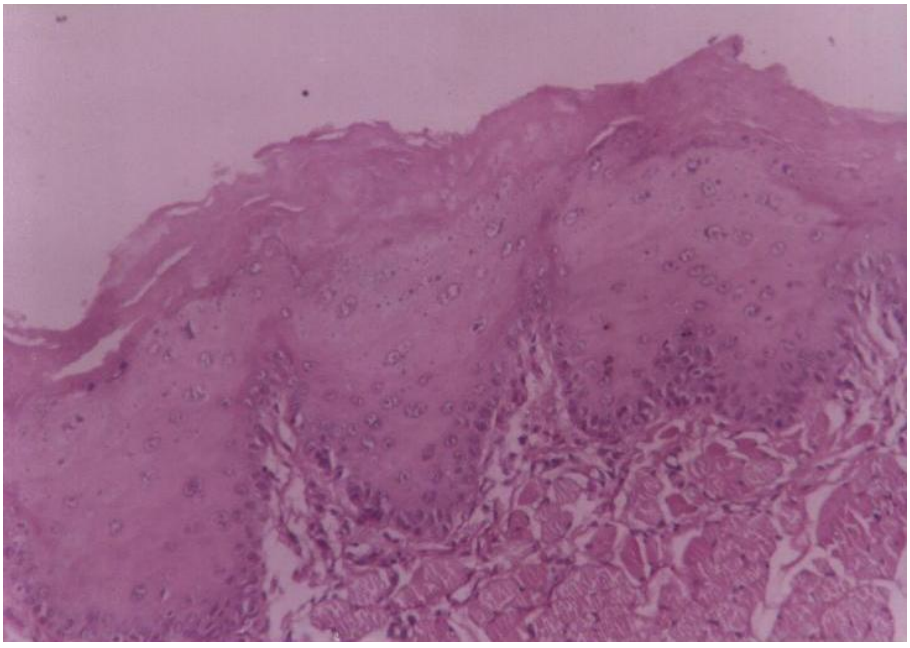

Fig. 18 : A photomicrograph of a rat gingiva of the phenytoin & cyclosporin group (group 7) showing hyperplastic epithelium and inflammatory reaction in the underlying connective tissue .
[H. & E. stain X 100] .

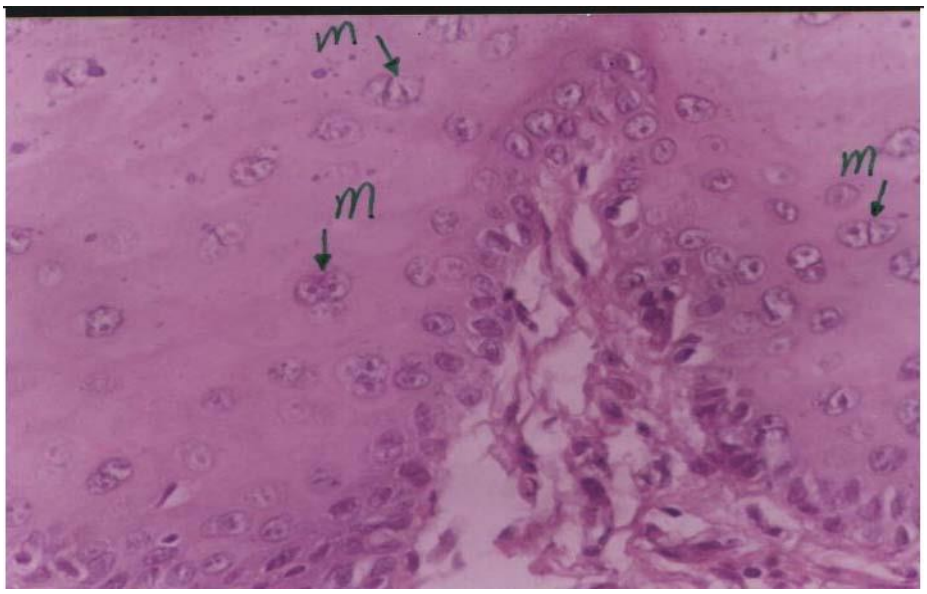

Fig. 19 : Higher magnification of Fig. 18 showing : the prickle cell layer with more than one nucleolus in several cells (mitotic figures) (m) .
[H. & E. stain X 400] .

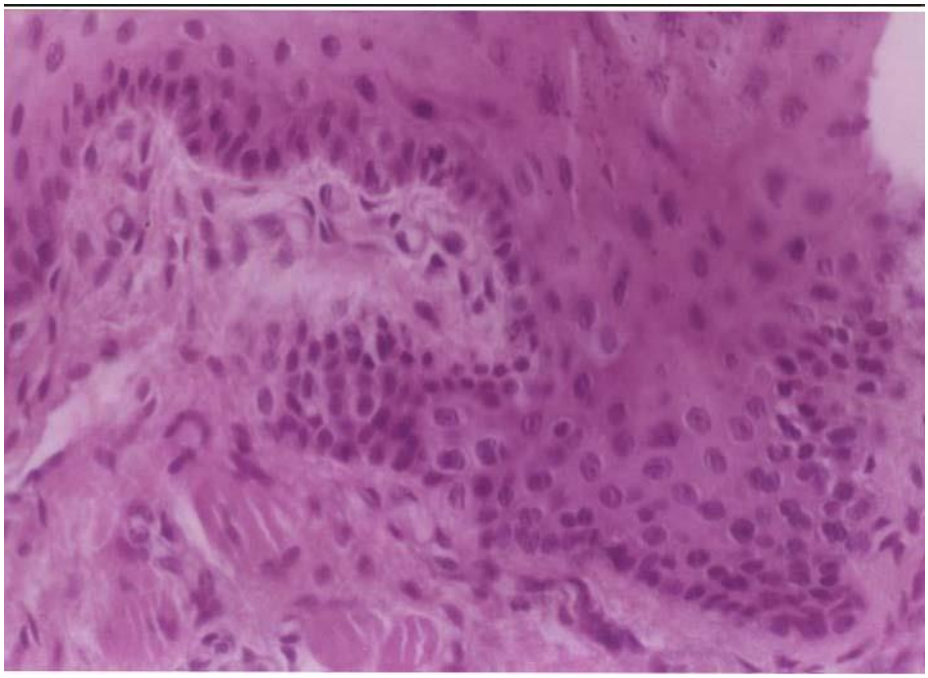

Fig. 20 : Another specimen of (group 7) showing : crowding and increase in the number of basal cells (basilar hyperplasia) .
[H. & E. stain X 400] .

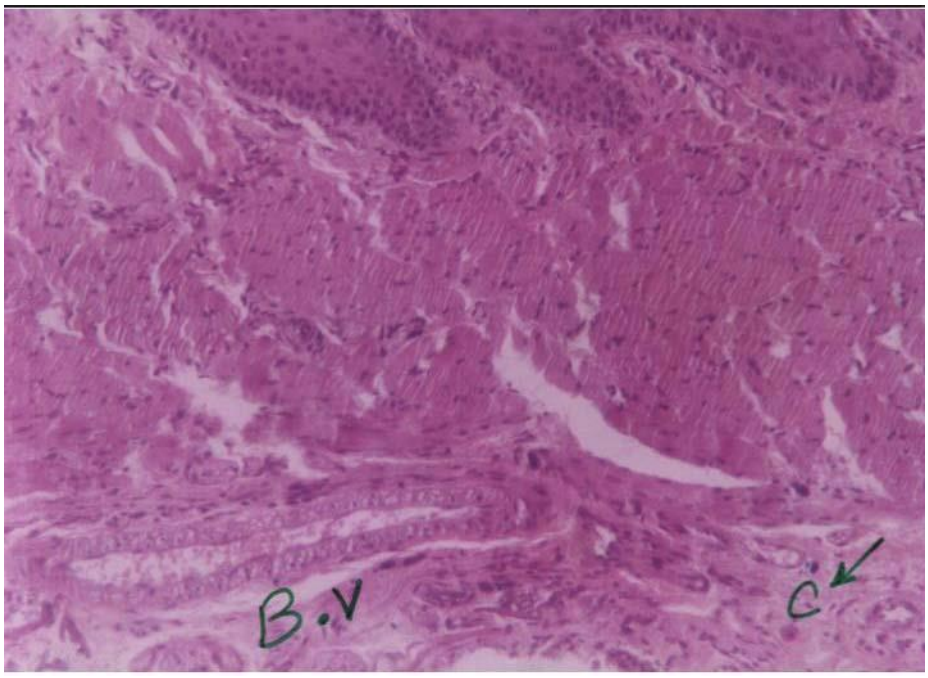

Fig. 21: The gingival lamina propria of the same group showing : dense arrangement of collagen fiber bundles (c) , B.V congested with RBCs (B.V) and inflammatory cells aggregated in different areas of the lamina propria .
[H. & E. stain X 160] .

Summary of the histopathological results (Table1) :

1 - The examination of the gingival specimens of the experimental groups revealed increase in the thickness of keratin in all groups than the control group . This increase was greater in NIF and PHT group as well as NIF and CsA group followed by PHT , PHT and CsA groups and the less degree was observed in NIF group .

2 - There was also increase in the length and width of the epithelial rete pegs which was more characteristic for CsA , CsA and NIF as well as NIF and PHT groups .

3 - Increased rate of cell proliferation in the form of increased mitotic figures was detected in the basal and prickle cell layers was detected more in CsA , CsA and NIF as well as CsA and PHT groups .

4 - These changes were detected in the epithelium while the C.T demonstrated fibrosis in the form of accumulation of collagen fibers in NIF and CsA as well as NIF and PHT groups .

5 - There was also inflammatory cell infilterate mainly of lymphocytes in all experimental groups .

Table (1) : demonstrating
Summary of the histopathological results

Group	Group 1 (Control)	Group 2 (NIF)	Group 3 (PHT)	Group 4 (CsA)	Group 5 (NIF+PHT)	Group 6 (NIF+CsA)	Group 7 (PHT+CsA)
Changes in karatin thickness	±	+	+ +	+ +	+ + +	+ + +	+ +
Changes in epithelial rete pegs	±	+	+ +	+ + +	+ + +	+ + +	+ +
Changes in basal cell layer	±	+	+	+ +	+ -	+ +	+ +
Changes in prickle cell layer	±	+	+	+ +	+	+ + +	+ +
Changes in lamina propria	±	+	+ +	+	+ + +	+ + +	+ +

± Normal + Weak ++ Moderate +++ Strong

Histochemical results (Table 2):

Group 1 (Control group):

The results of reaction to acid phosphatase showed normal lysosomal activity distributed allover the C.T. This activity appeared in the form of purple to red granules [Fig. 22].

Group 2 (Nifedipine group):

The results of the acid phosphatase showed the strongest acid phosphatase reactivity as compared to the next groups at the same time it is slightly less than the lysosomal activity of the control group [Fig.23] .

Group 3 (Phenytoin group):

The acid phosphatase examination revealed mild enzymatic activity with phenytoin compared with the control group. [Fig.24]

Group 4 (Cyclosporin group):

The acid phosphatase immunoreaction exhibited marked lysosomal activity [Fig. 25] .

Group 5 (Nifedipine and Phenytoin group) :

This group showed weak reaction for the acid phosphatase reactivity . This reaction is distributed throughout the papillary layer of the C.T [Fig. 26] .

Group 6 (Nifedipine and Cyclosporin group) :

The results of the acid phosphatase technique of this group showed moderate enzymatic activity which was limited to the subepithelial zone [Fig. 27] .

Group 7 (Phenytoin and Cyclosporin) :

This group showed the weakest reaction to acid phosphatase. This reaction was more confined subepithelialy. [Fig. 28].

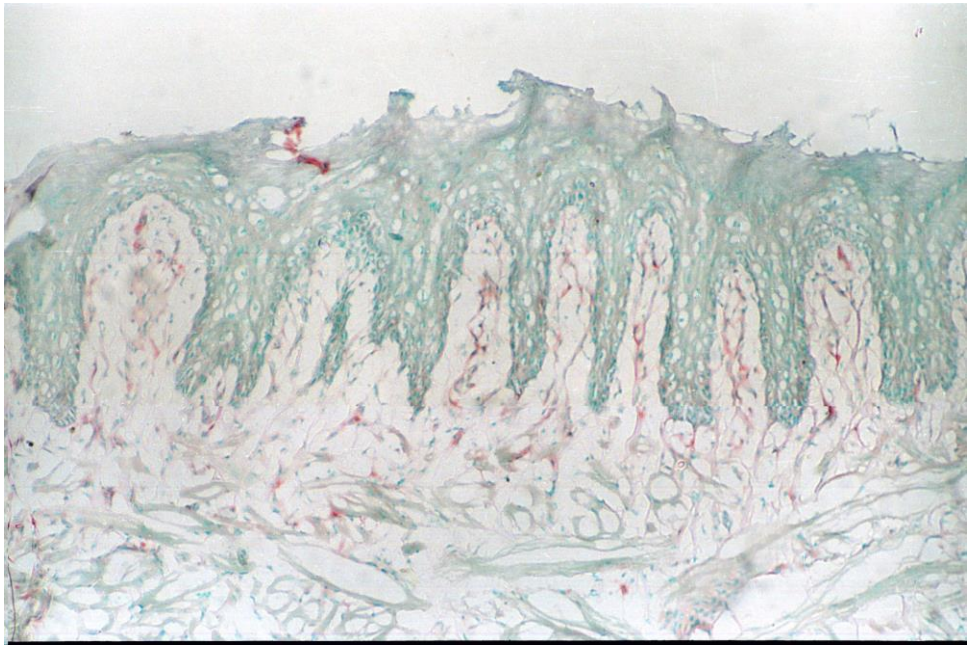

Fig. 22 : A photomicrograph of a rat gingiva of control group (group 1) showing normal lysosomal activity distributed all over the C.T .
[Acid phase. stain X 100] .

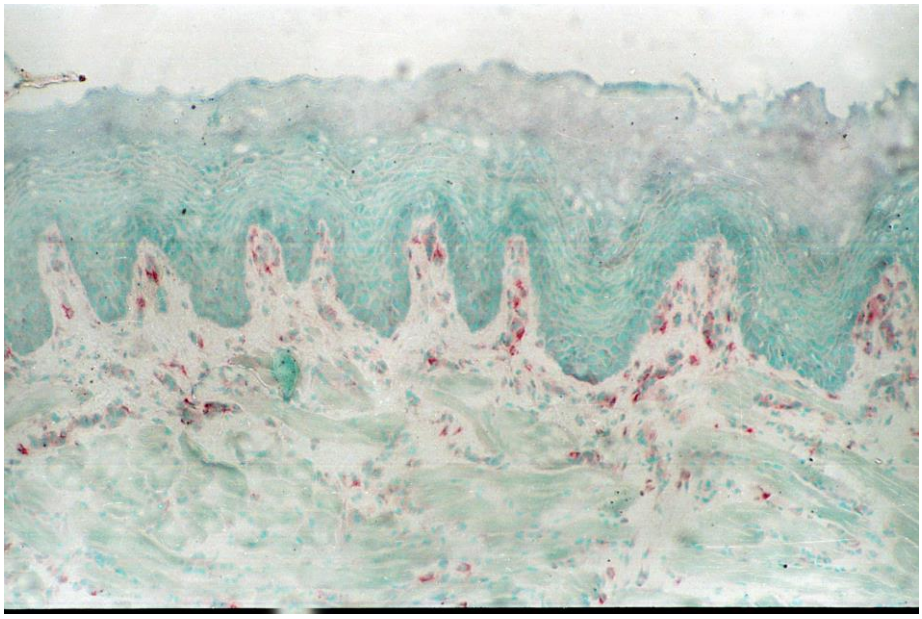

Fig. 23 : A photomicrograph of a rat gingiva of nifedipine group (group 2) showing a strong acid phosphatase reactivity . [Acid phase. stain X 100] .

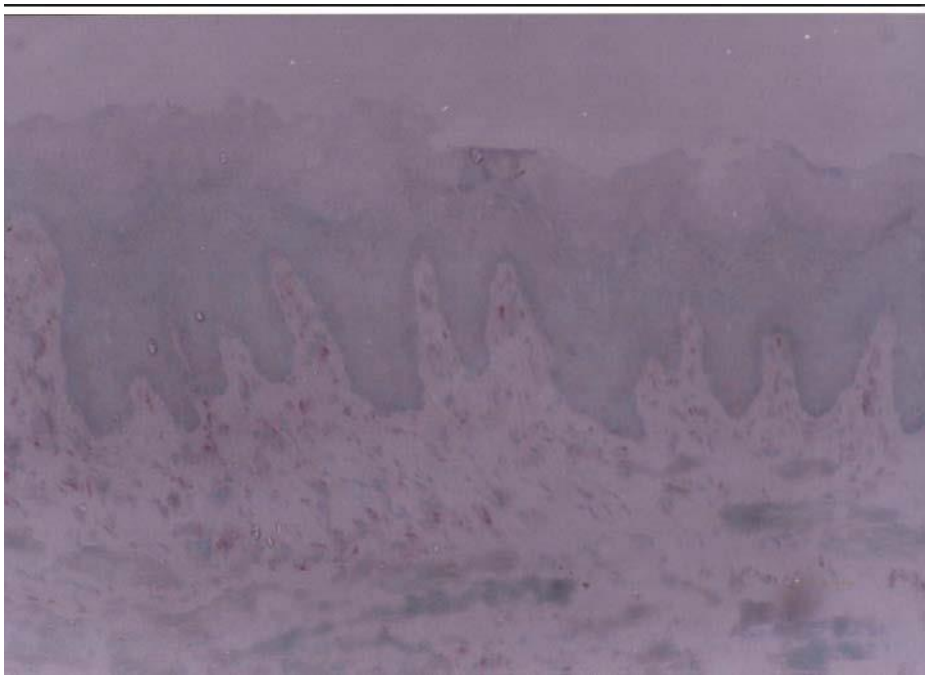

Fig. 24 : A photomicrograph of a rat gingiva of phenytoin group (group 3) showing mild lysosomal activity .
[Acid phase. stain X 100] .

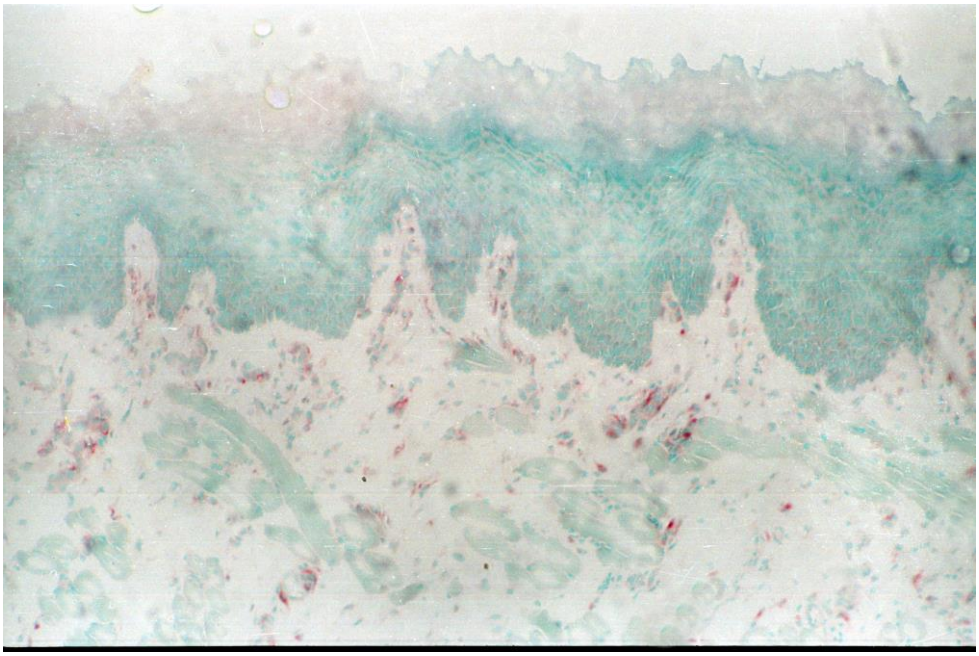

Fig. 25 : A photomicrograph of a rat gingiva of cyclosporin group (group 4) showing marked lysosomal activity .
[Acid phase. stain X 100] .

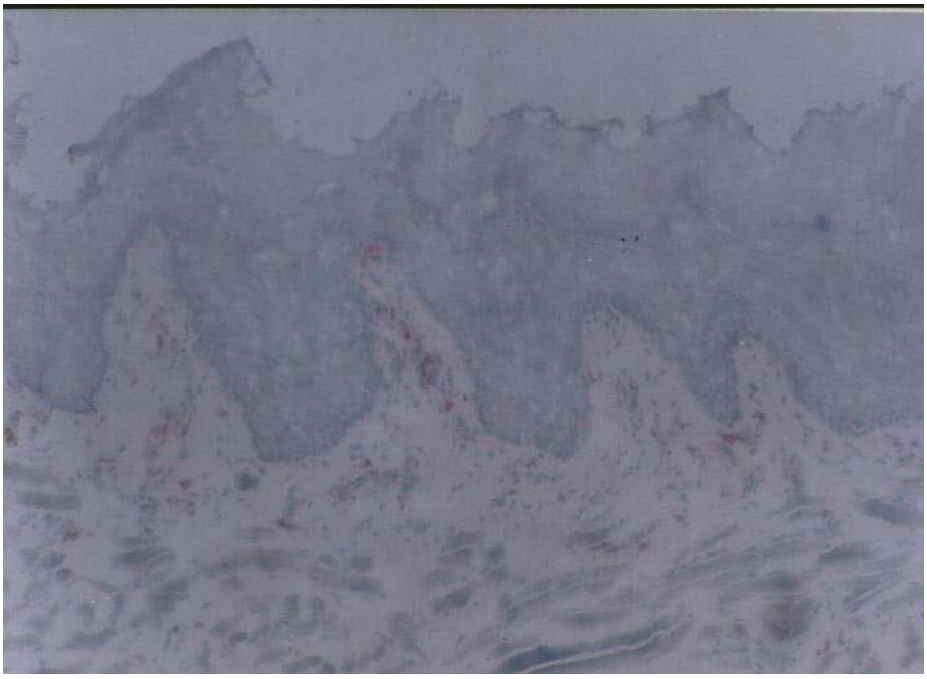

Fig. 26 : A photomicrograph of a rat gingiva of nifedipine and phenytoin group (group 5) showing weak enzymatic activity .
[Acid phase. Stain X 100] .

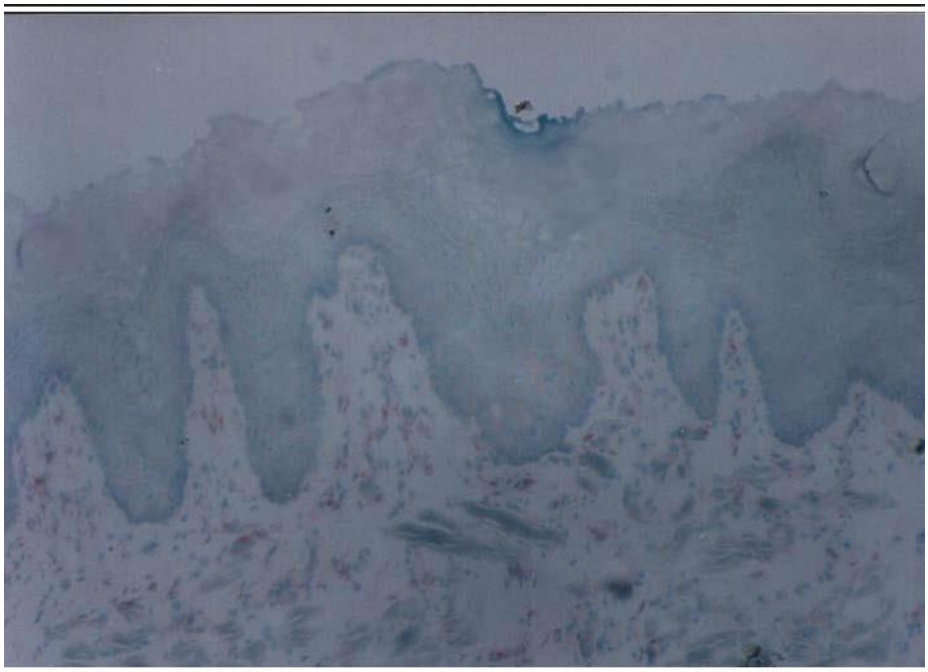

Fig. 27 : A photomicrograph of a rat gingiva of nifedipine and cyclosporin group (group 6) showing moderate enzymatic activity . [Acid phase. Stain X 100] .

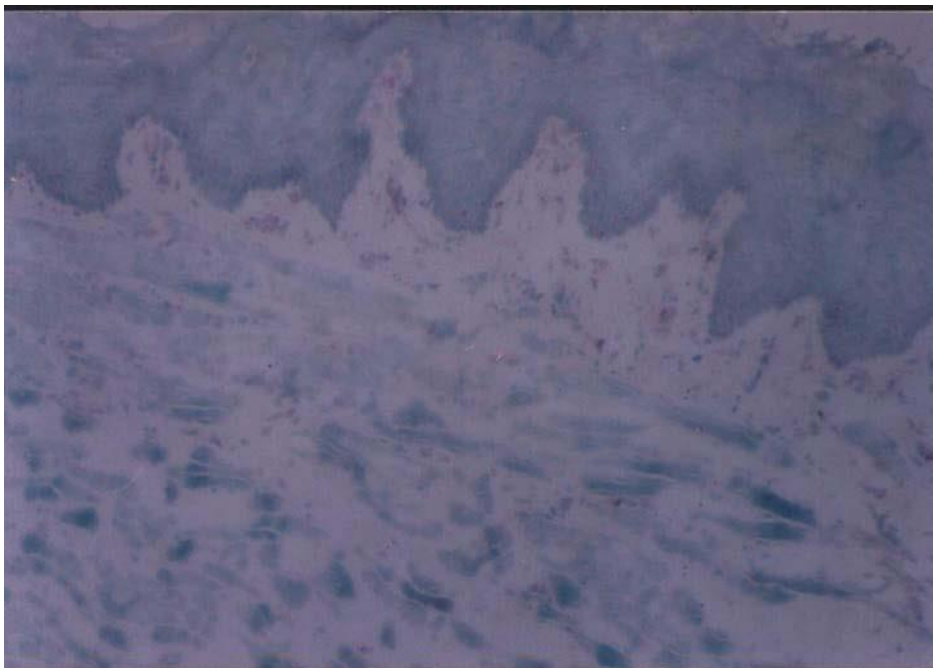

Fig. 28 : A photomicrograph of a rat gingiva of phenytoin and cyclosporin group (group 7) showing the weakest enzymatic activity as compared to the all groups .
[Acid phase. Stain X 100] .

Table (2) : demonstrating
Summary of the histochemical results

Group	Group 1 (Control)	Group 2 (NIF)	Group 3 (PHT)	Group 4 (CsA)	Group 5 (NIF+PHT)	Group 6 (NIF+CsA)	Group 7 (PHT+CsA)
Acid phase. reaction	±	+	– – –	–	– – – –	– –	– – – – –

Normal ± strong + marked – moderate – – mild – – – weak – – – – weakest – – – – –

Immunohistochemical Results (Table 3):

Collagen Type I

Group 1 (Control group) :

The immunohistochemical study using polycolonal antibodies against type I collogen showed normal reaction in the lamina propria . The reaction was uniform and consistent allover the lamina propria [Fig. 29] .

Group 2 (Nifedipine group) :

The immunohistochemical study of this group revealed a weak reaction in the collagen fibers at the subepithelial layer but more than the control group [Fig. 30] .

Group 3 (Phenytoin group) :

The immunohistochemical study using polycolonal antibodies for type I collagen demonstrated a marked reaction. Morever the collagen fiber bundles appeared more thickened and arranged in dense bundles [Fig. 31].

Group 4 (Cyclosporin group) :

The bundles of collagen fibers were mildely reacted as compared to those of the control group. [Fig. 32] .

Group 5 (Nifedipine and Phenytoin group) :

The collagen bundles appeared strongly reacted to collagen type I antibodies [Fig. 33] .

Group 6 (Nifedipine and Cyclosporin group) :

The immunohistochemical examination of the specimens using polycolonal antibodis for type I collagen showed a marked reaction [Fig. 34] .

Group 7 (Phenytoin and Cyclosporin group) :

The immunohistochemical examination using polycolonal antibodies for type I collagen showed the more intense reaction as compared to the all groups [Fig. 35] .

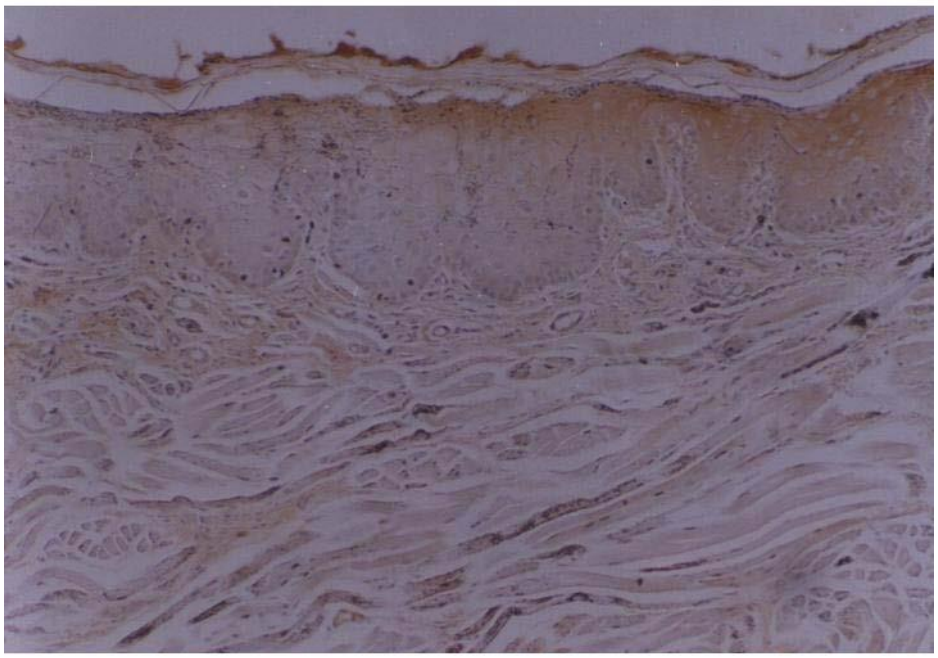

Fig. 29 : A photomicrograph of a rat gingiva of control group (group 1) showing immunostanining of type I collagen demonstrating normal reaction .
[Anti - collagen type I X 100] .

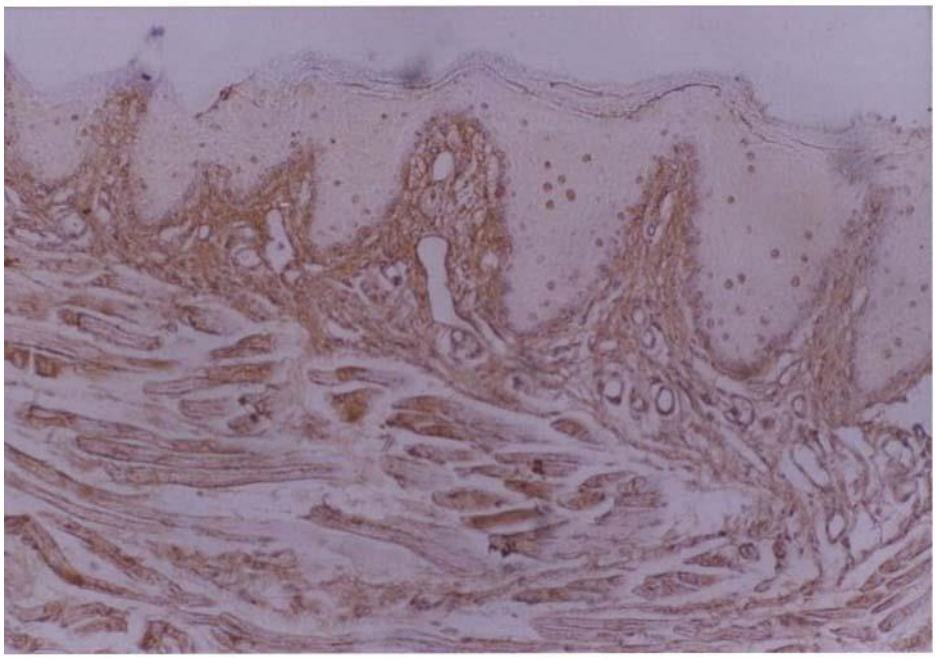

Fig. 30 : A photomicrograph of a rat gingiva of nifedipine group (group 2) showing a weak reaction for anti type I collagen . [Anti - collagen type I X 100] .

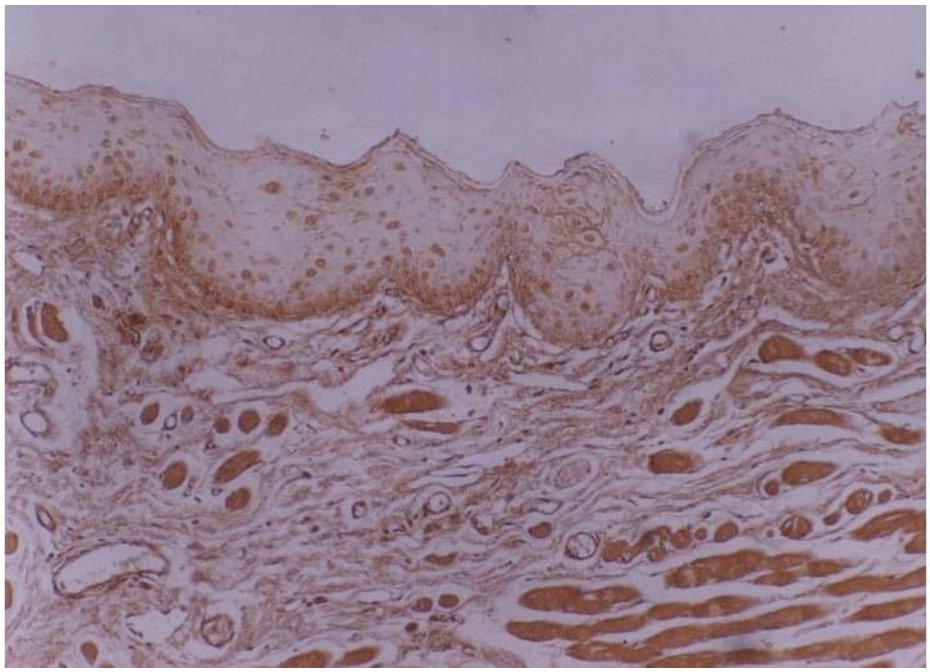

Fig. 31 : A photomicrograph of a rat gingiva of phenytoin group (group 3) showing immunostanining of type I collagen demonstrating marked reaction .
[Anti - collagen type I X 100] .

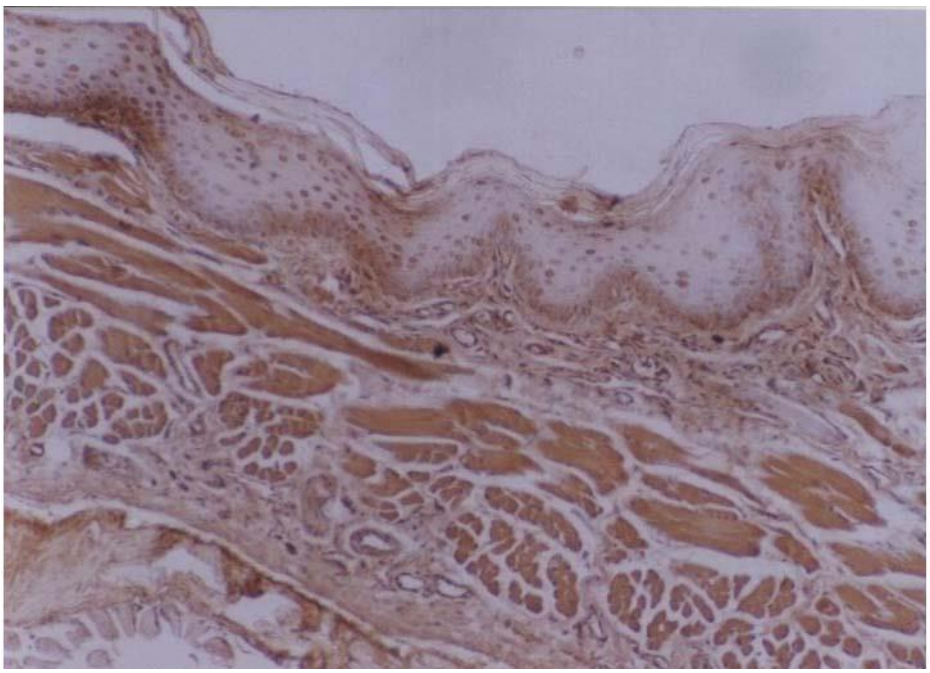

Fig. 32 : A photomicrograph of a rat gingiva of cyclosporin group (group 4) showing mild immunostanining of type I collagen. [Anti - collagen type I X 100] .

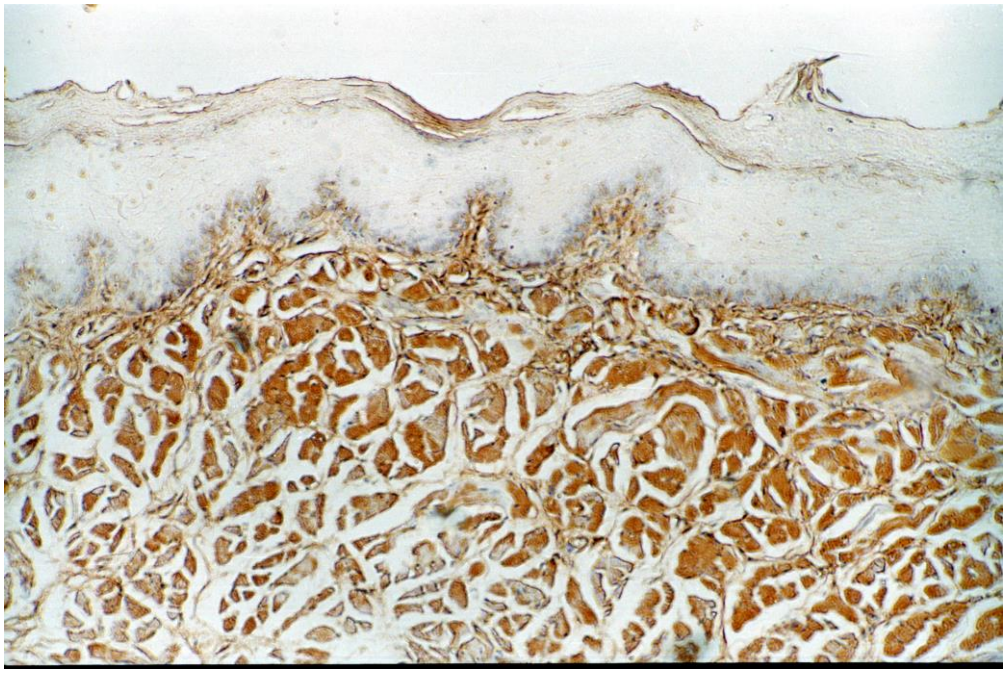

Fig. 33 : A photomicrograph of a rat gingiva of nifedipine and phenytoin group (group 5) showing strong immunostanining of type I collagen.
[Anti - collagen type I X 100] .

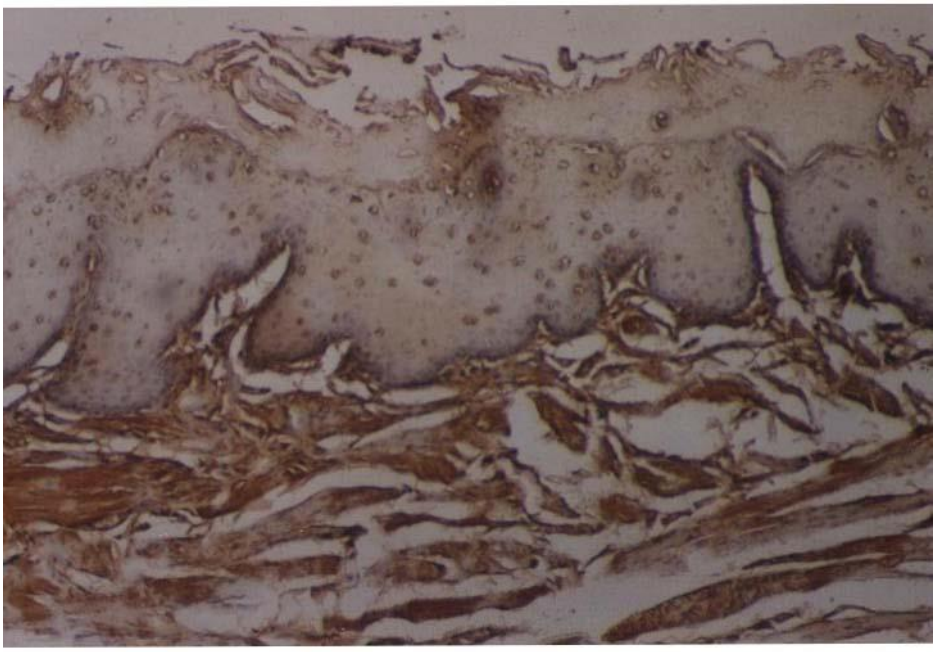

Fig. 34 : A photomicrograph of a rat gingiva of nifedipine and cyclosporin group (group 6) showing marked immunostanining of type I collagen.
[Anti - collagen type I X 100] .

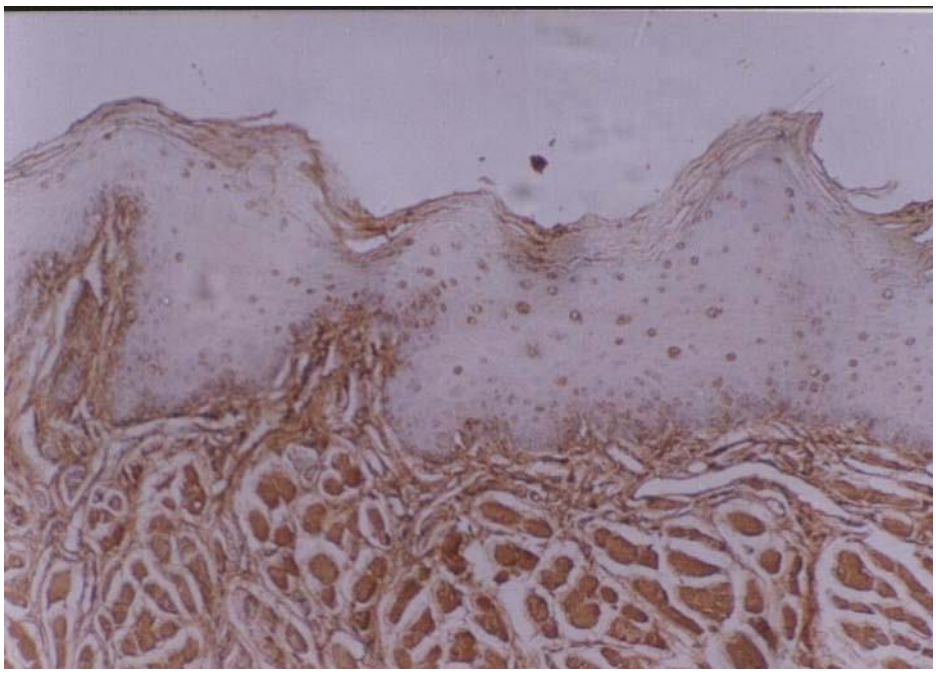

Fig. 35 : A photomicrograph of a rat gingiva of phenytoin and cyclosporin group (group 7) showing the intense reaction of type I collagen.
[Anti - collagen type I X 100] .

Collagen Type IV

Group 1 (Control Group) :

The immunohistochemical study using polycolonal anti bodies for type IV collagen showed that it was normaly present in the basement membrane and wall of blood vessels mainly capillaries in contrast to negative reaction of the epithelium and C.T. [Fig. 36].

Group 2 (Nifedipine group) :

This group showed weak reaction of type IV collagen which is mainly present in the basement membrane as in the control group . In the underlying C.T it was present in the wall of the blood vessels [Fig. 37] .

Group 3 (Phenytoin group) :

Examination of the immuno stained sections using polycolonal antibodies for type IV collagen showed weak reaction denoting a distributation of type IV collagen fibers at the basement membrane and wall of the blood vessels more than that in the control group [Fig. 38] .

Group 4 (Cyclosporin group) :

This group showed mild reaction denoting higher amounts of type IV collagen than the control group [Fig. 39] .

Group 5 (Nifedipine and phenytoin group) :

The immunohistochemical study using polycolonal antibodies for type IV collagen showed moderate reaction to type IV collagen [Fig. 40] .

Group 6 (Nifedipine and Cyclosporin group) :

The immunohistochemical examination of the specimens using polycolonal antibodis for type IV collagen showed marked reaction at the basement membrane and wall of blood vessels [Fig. 41] .

Group 7 (Phenytoin and cyclosproin group) :

The immunohistochemical examination of this group showed a marked reaction to type IV collagen which is mainly present in the basement membrane and in the wall of blood vessels [Fig. 42].

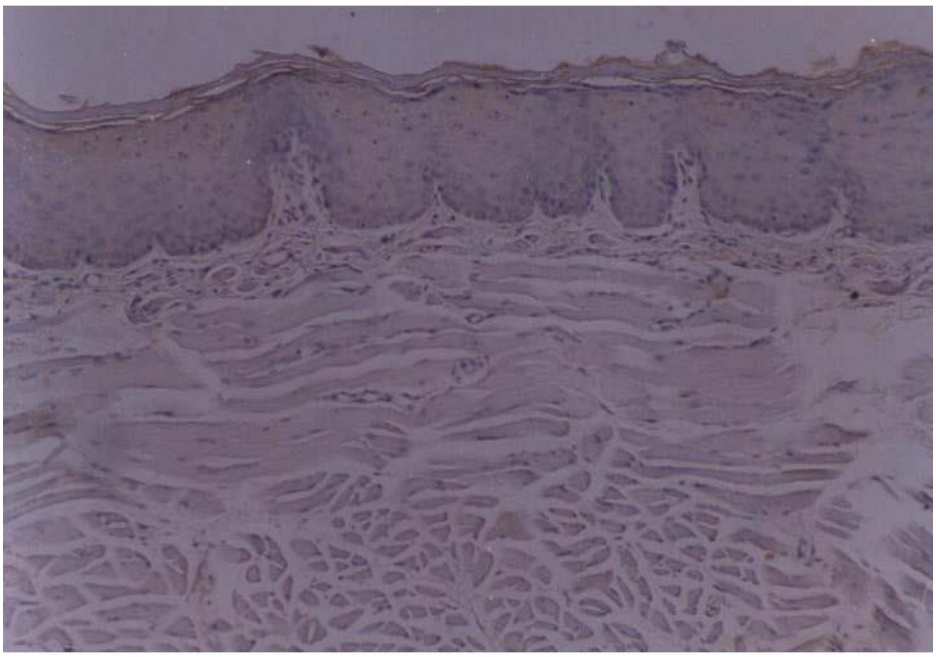

Fig. 36 : A photomicrograph of a rat gingiva of control group (group 1) showing the normal distribution of type IV collagen in the basement membrane and wall of blood vessels mainly capillaries and its absence in the epithelium .
[Anti - collagen type IV X 100] .

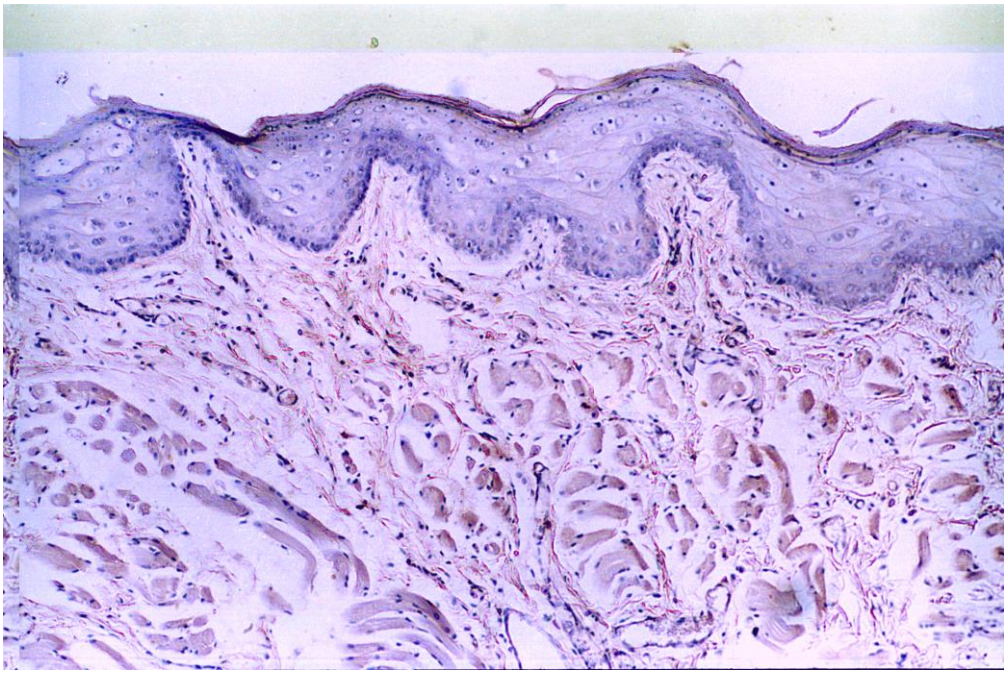

Fig. 37: A photomicrograph of a rat gingiva of nifedipine group (group 2) showing weak distribution of type IV collagen in the basement membrane and in wall of blood vessels .
[Anti - collagen type IV X 100] .

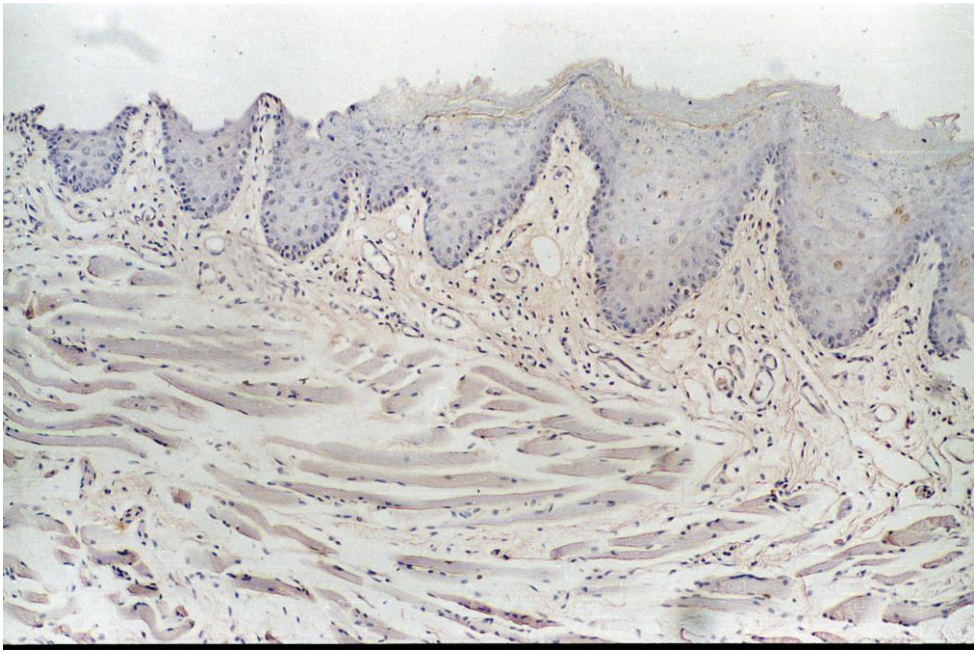

Fig. 38 : A photomicrograph of a rat gingiva of phenytoin group (group 3) showing immunostanining of type IV collagen demonstrating a weak distribution .
[Anti - collagen type IV X 100] .

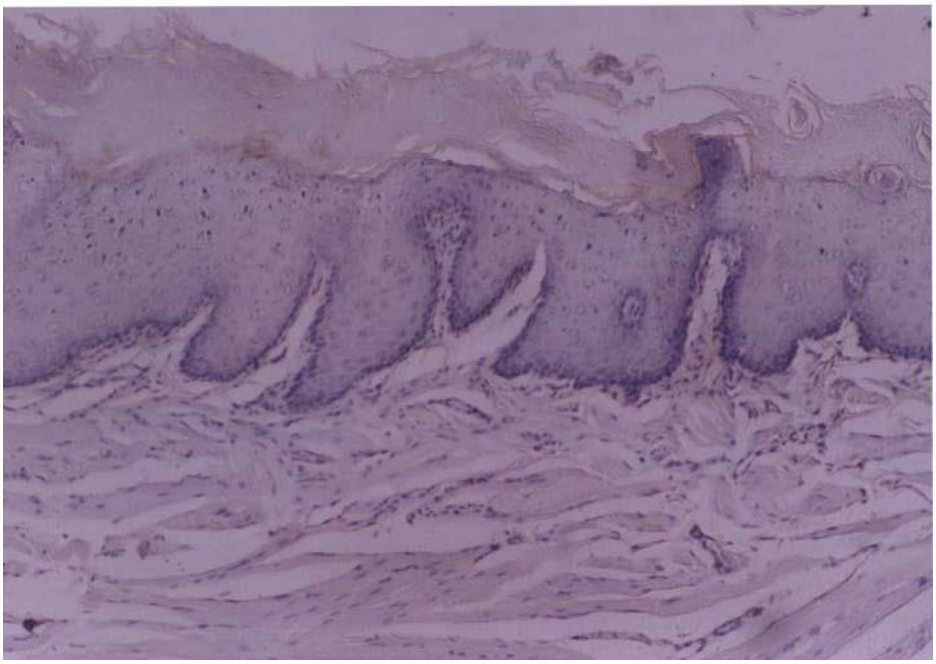

Fig. 39 : A photomicrograph of a rat gingiva of cyclosporin group (group 4) showing mild immunostanining of type IV collagen demonstrating higher amounts of type IV collagen in basement membrane and wall of the blood vessels than the control group . [Anti – collagen type IV X 100] .

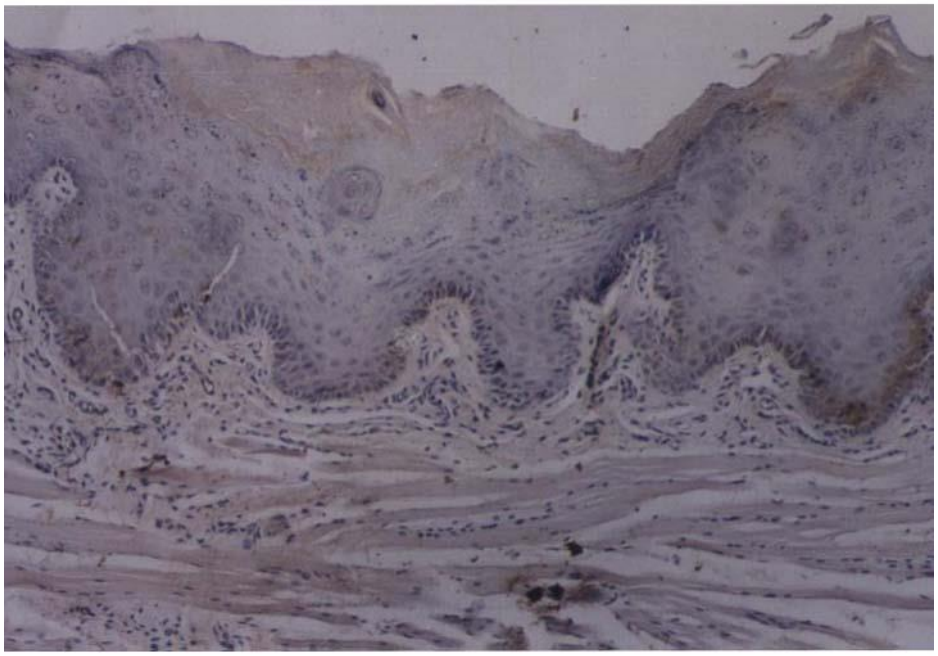

Fig. 40 : A photomicrograph of a rat gingiva of nifedipine and phenytoin group (group 5) showing moderate immunostanining of type IV collagen demonstrating higher amounts of type IV collagen than the control group.
[Anti – collagen type IV X 100] .

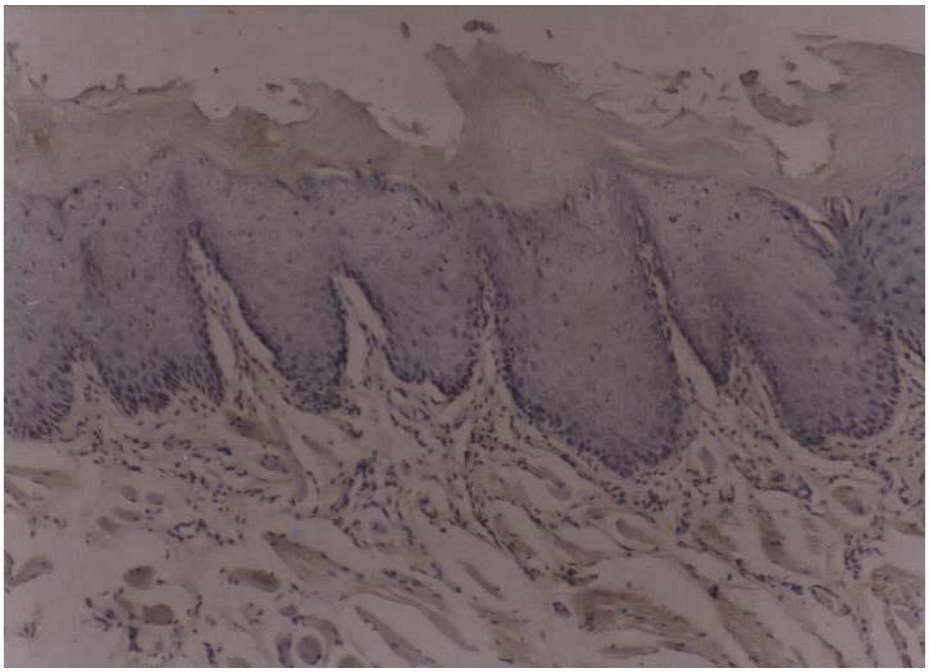

Fig. 41 : A photomicrograph of a rat gingiva of nifedipine and cyclosporin group (group 6) showing marked immunostanining of type IV collagen .
[Anti – collagen type IV X 100] .

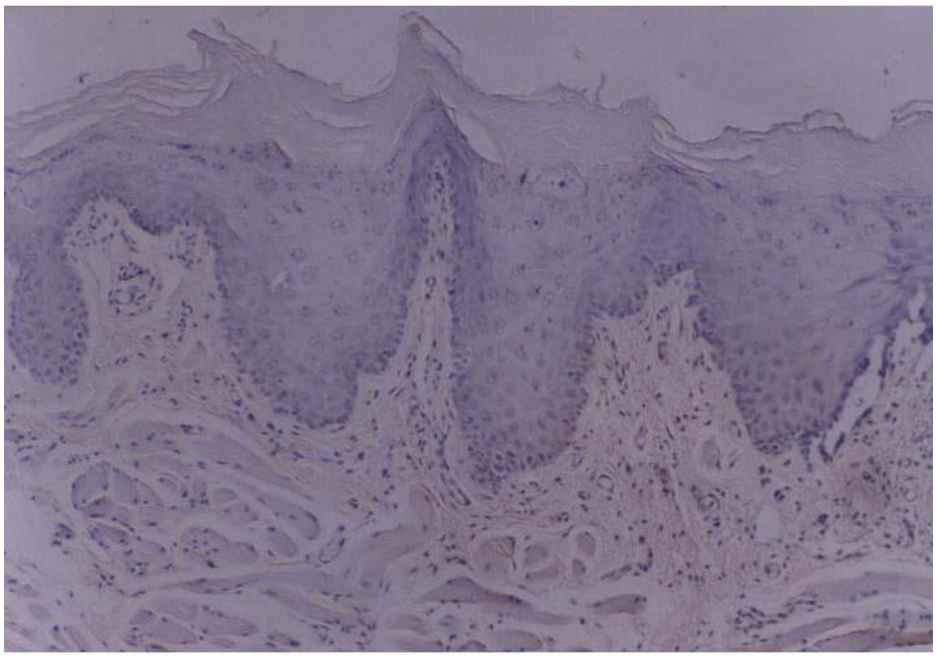

Fig. 42 : A photomicrograph of a rat gingiva of phenytoin and cyclosporin group (group 7) showing marked immunostanining of type IV collagen.
[Anti – collagen type IV X 100] .

Collagen type V

Group 1 (Control group) :

This group showed the normal distribution of collagen type V. which was mainly near the basement membrane in the papillary layer of C.T. and with its characteristic network pattern . [Fig. 43].

Group 2 (Nifedipine group) :

This group revealed mild reaction which is much more than the control group . Type V collagen fibers showed the same arrangement as in group 1 [Fig. 44] .

Group 3 (Phenytoin group) :

The immuno-histochemical study using polycolonal antibodies for type V collagen showed a marked reaction in the C.T. [Fig. 45] .

Group 4 (Cyclosporin group) :

This group showed moderate reaction compared with the control group. [Fig. 46] .

Group 5 (Nifedipine and phenytoin group) :

The immunohistochemical study using polycolonal antibodies for type V collagen exhibited a strong reaction in the lamina properia while the epithelium showed negative reaction. [Fig.47] .

Group 6 (Nifedipine and Cyclosporin group) :

The immunohistochemical examination of the specimens treated with polycolonal antibodis for type V collagen demonstrated a moderate reaction [Fig. 48] .

Group 7 (Phenytoin and cyclosporin group) :

The immunohistochenical examination using polycolonal antibodies for type IV collagen showed the more intense reaction compared to the all groups. [Fig. 49] .

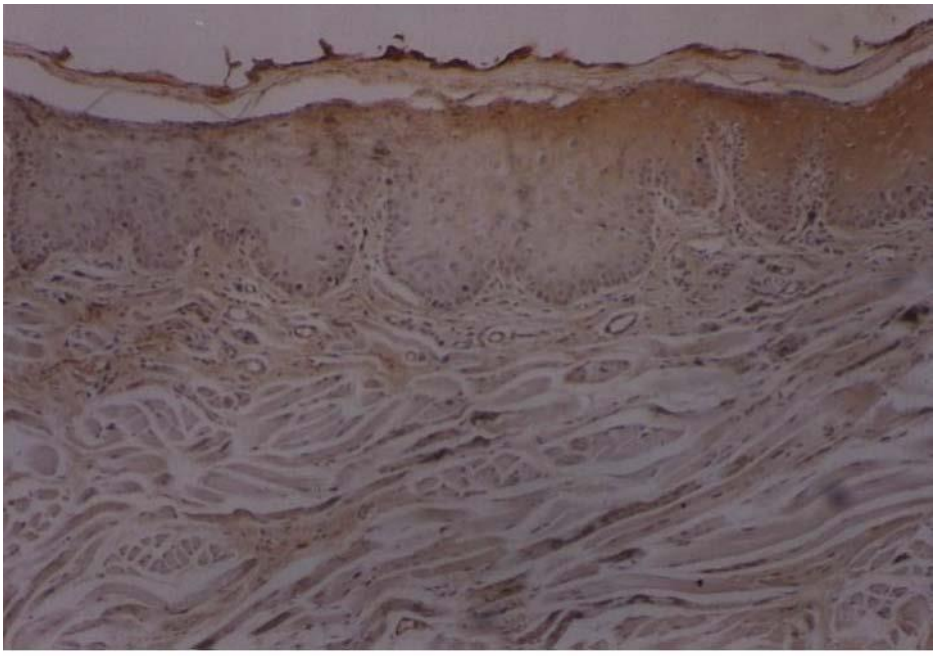

Fig. 43 : A photomicrograph of a rat gingiva of control group (Group 1) showing immunostanining of type V collagen demonstrating normal distribution in the C.T.
[Anti - collagen type V X 100] .

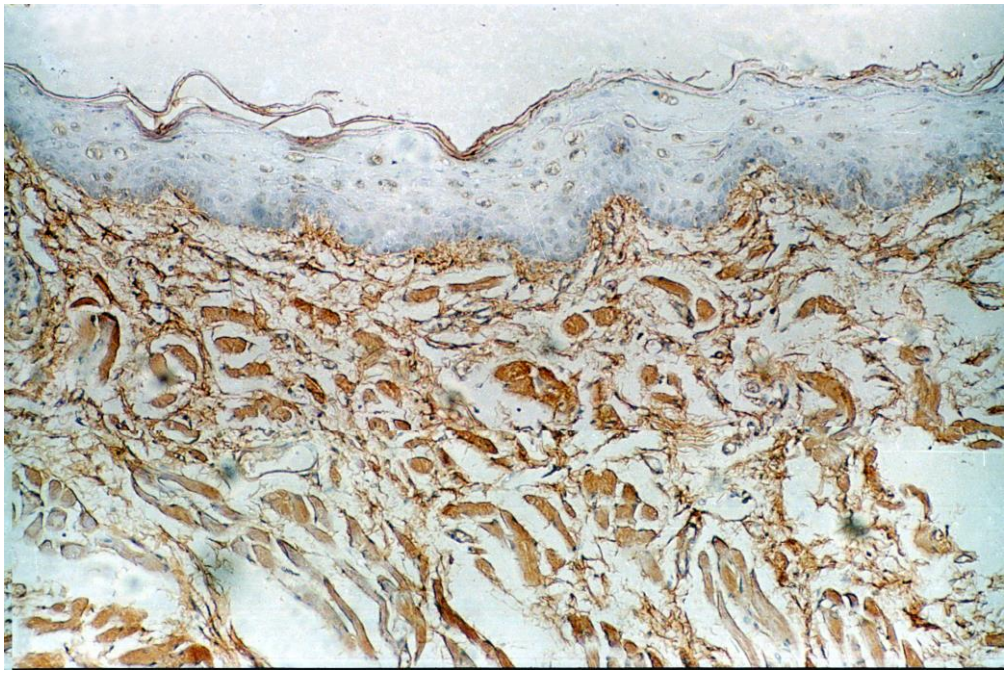

Fig. 44 : A photomicrograph of a rat gingiva of nifedipine group (group 2) showing mild immunostanining of type V collagen which is more than the reaction of the control group.
[Anti – collagen type V X 100] .

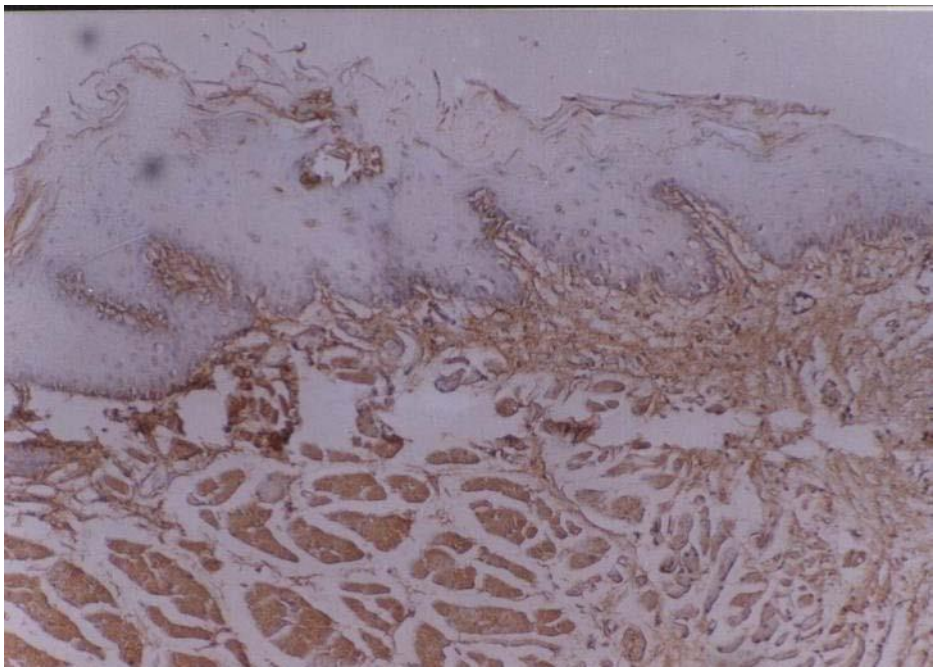

Fig. 45 : A photomicrograph of a rat gingiva of phenytoin group (group 3) showing immunostanining of type V collagen demonstrating a marked reaction.
[Anti – collagen type V X 100] .

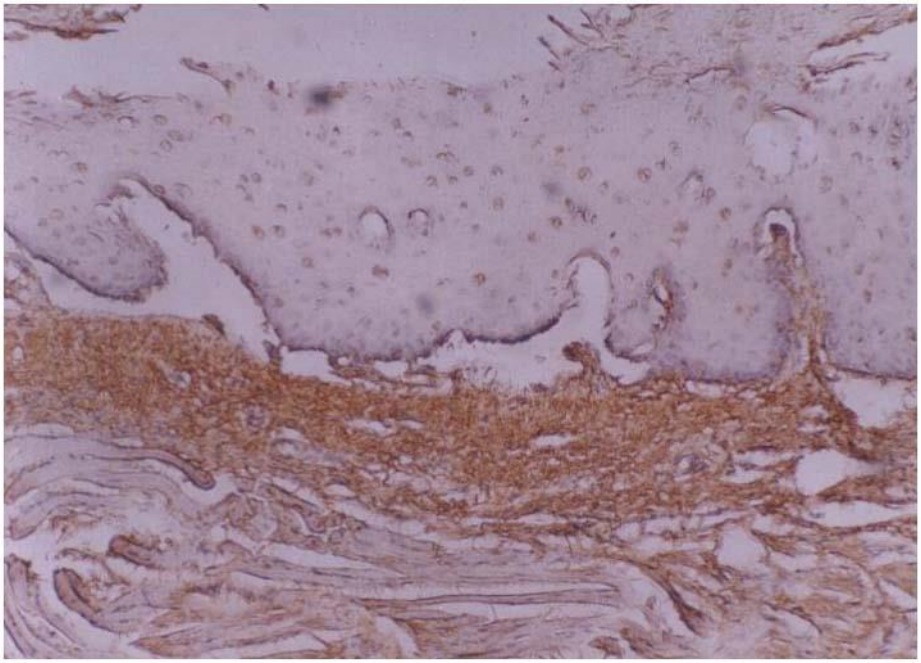

Fig. 46 : A photomicrograph of a rat gingiva of cyclosporin group (group 4) showing immunostanining of type V collagen demonstrating a moderate reaction.
[Anti – collagen type V X 160] .

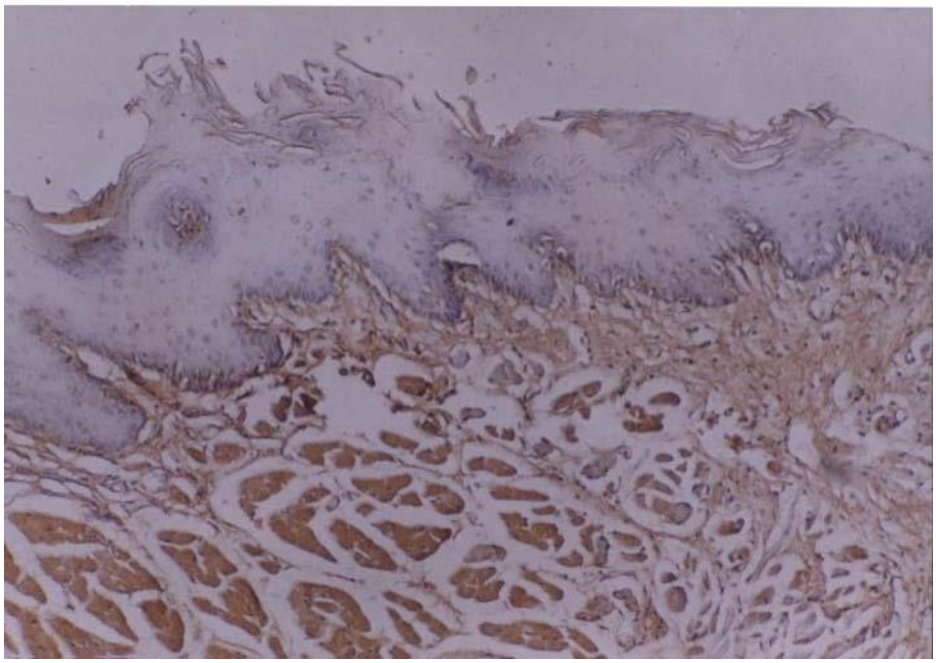

Fig. 47 : A photomicrograph of a rat gingiva of nifedipine and phenytoin group (group 5) showing immunostanining of type V collagen demonstrating a strong reaction .
[Anti – collagen type V X 100] .

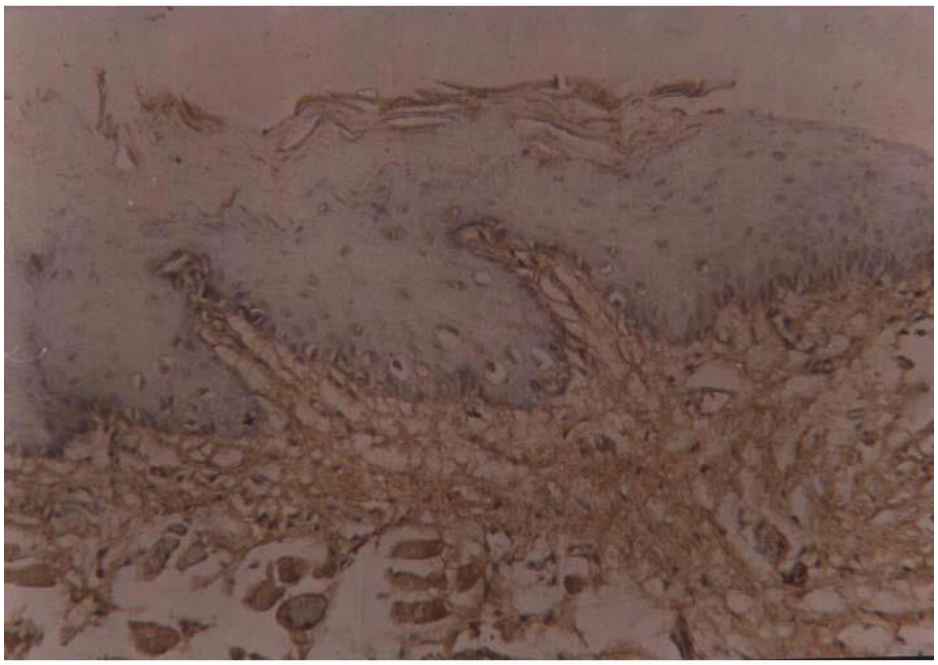

Fig. 48 : A photomicrograph of a rat gingiva of nifedipine and cyclosporin group (group 6) showing immunostanining of type V collagen demonstrating moderate reaction .
[Anti – collagen type V X 160] .

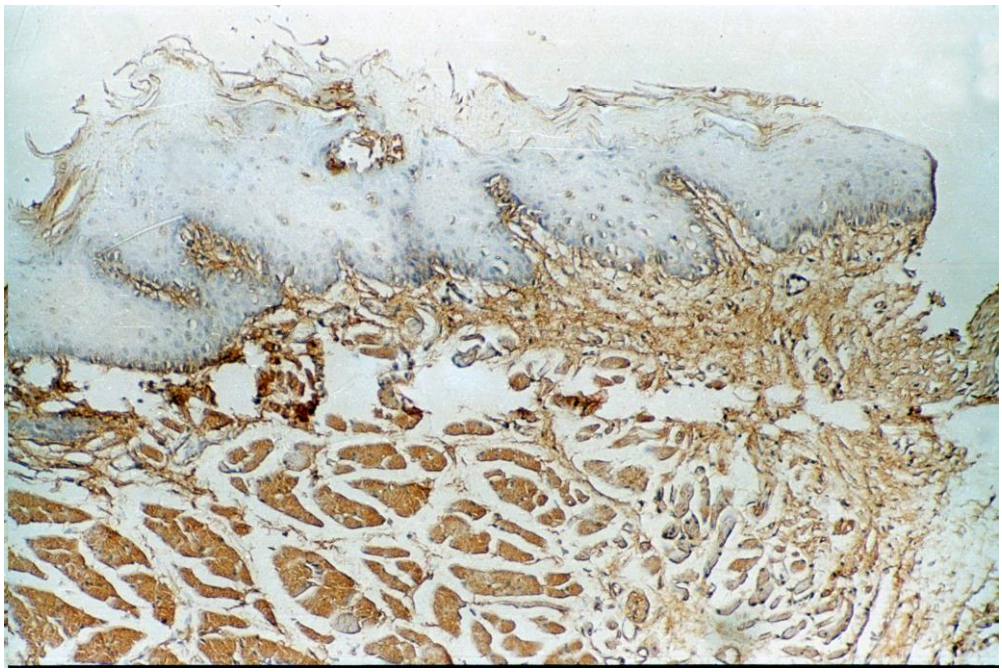

Fig. 49 : A photomicrograph of a rat gingiva of phenytoin and cyclosporin group (group 7) showing immunostanining of type V collagen demonstrating an intense reaction .
[Anti – collagen type V X 100] .

Fibronectin

Group 1 (Control Group) :

The results of the fibronectin labeling showed normal distribution . Both the subepithelial and deep C.T of the gingiva exhibited positive immuno staining for fibronection which appeared in a fibrillar pattern . This pattern was uniform and consistent in its distributation in the lamina properia [Fig.50].

Group 2 (Nifedipine group) :

In this group characteristic microfibrillar labeling for fibronectin was seen in the lamina properia . These stained fibers have different lengths and thicknesses. The reaction was slightly more than in the control group but the weakest reaction compared with the next groups [Fig. 51] .

Group 3 (Phenytoin group) :

The staining reaction for fibronetin was moderate reaction. This reaction was not uniform all over the lamina properia as it appeared amorphous in some areas and absent in others. This group showed strong staining in the subepithelial region in the form of parallel bundles while in deep region it showed a network distributation [Fig. 52] .

Group 4 (Cyclosporin group) :

Fibronectin is mildely expressed in this group [Fig. 53] .

Group 5 (Nifedipine and phenytoin group):

This group showed a strong reaction for fibronectin which could be detected all over the lamina properia [Fig. 54].

Group 6 (Nifedipine and Cyclosporin group) :

The immunohistochemical reaction revealed a strong reaction [Fig. 55].

Group 7 (phenytoin and Cyclosporin group):

The immunohistochemical reaction revealed a more intense reaction as compared to the previous groups [Fig. 56] .

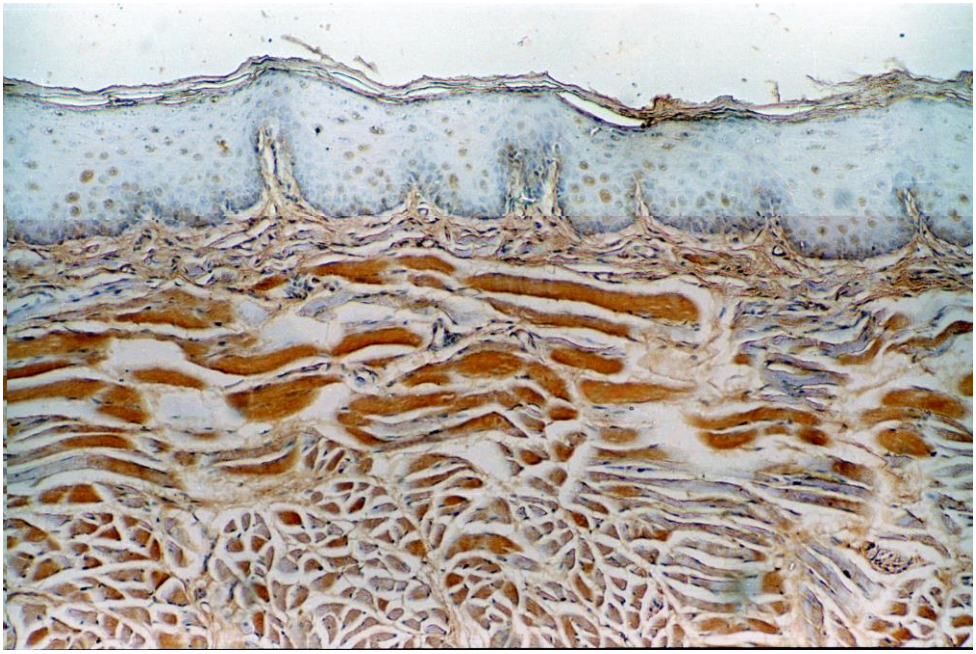

Fig. 50 : A photomicrograph of a rat gingiva of control group (group 1) specimen incubated with anti fibronectin antibody showing normal reaction in the C.T. .
[Anti – fibronectin antibody X 100] .

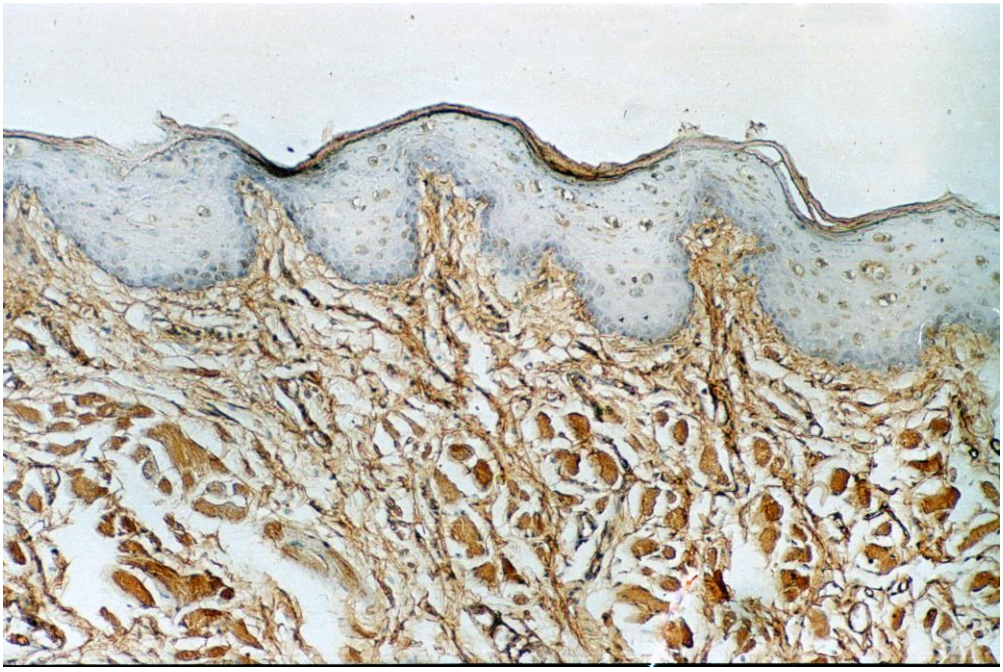

Fig. 51 : A photomicrograph of a rat gingiva of infedipine group (group 2) showing a weak stanining reaction in the subepithelial region of the gingival lamina propria which is slightly more than the control group .
[Anti – fibronectin antibody X 100] .

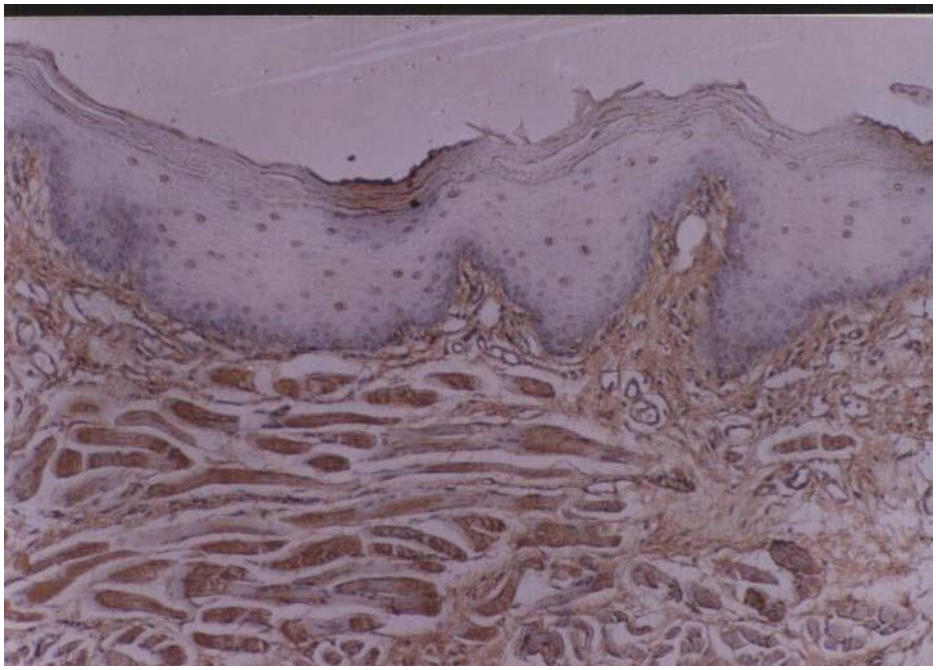

Fig. 52 : A photomicrograph of a rat gingiva of phenytoin group (group 3) showing moderate reaction for fibronectin . [Anti – fibronectin antibody X 100] .

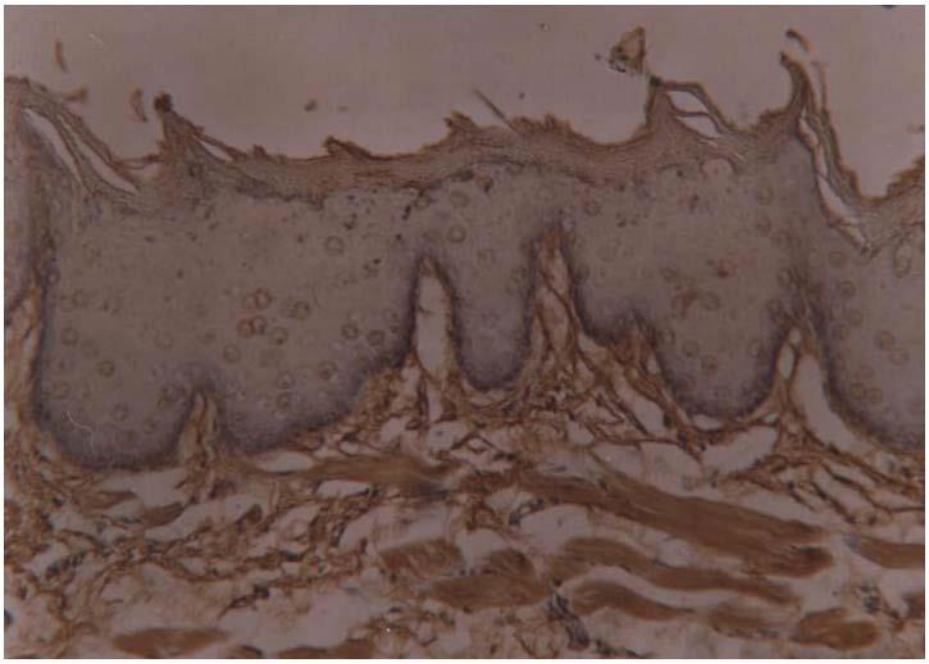

Fig. 53 : A photomicrograph of a rat gingiva of cyclosporin group (group 4) showing a mild reaction for fibronectin .
[Anti – fibronectin antibody X 100] .

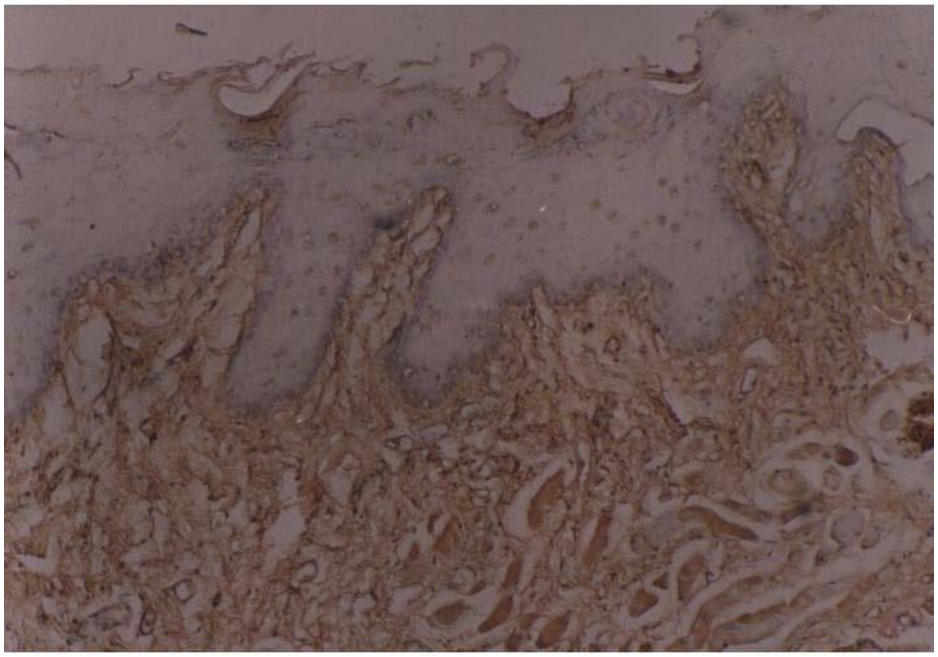

Fig. 54 : A photomicrograph of a rat gingiva of nifedipine and phenytoin group (group 5) showing strong reaction for fibronectin. [Anti – fibronectin antibody X 160] .

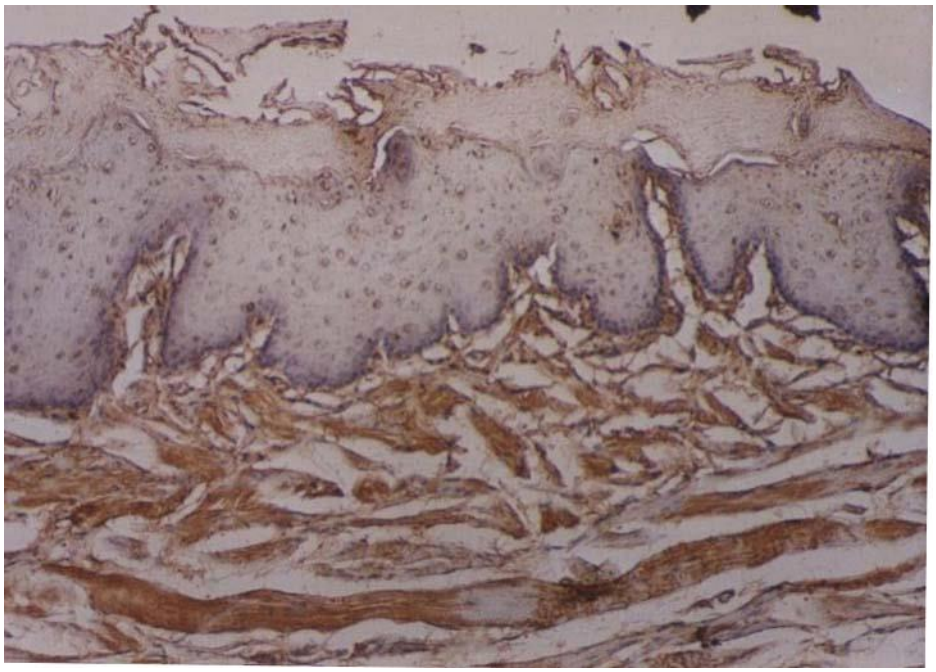

Fig. 55 : A photomicrograph of a rat gingiva of nifedipine and cyclosporin group (group 6) showing a strong reaction for fibronectin .
[Anti – fibronectin antibody X 100] .

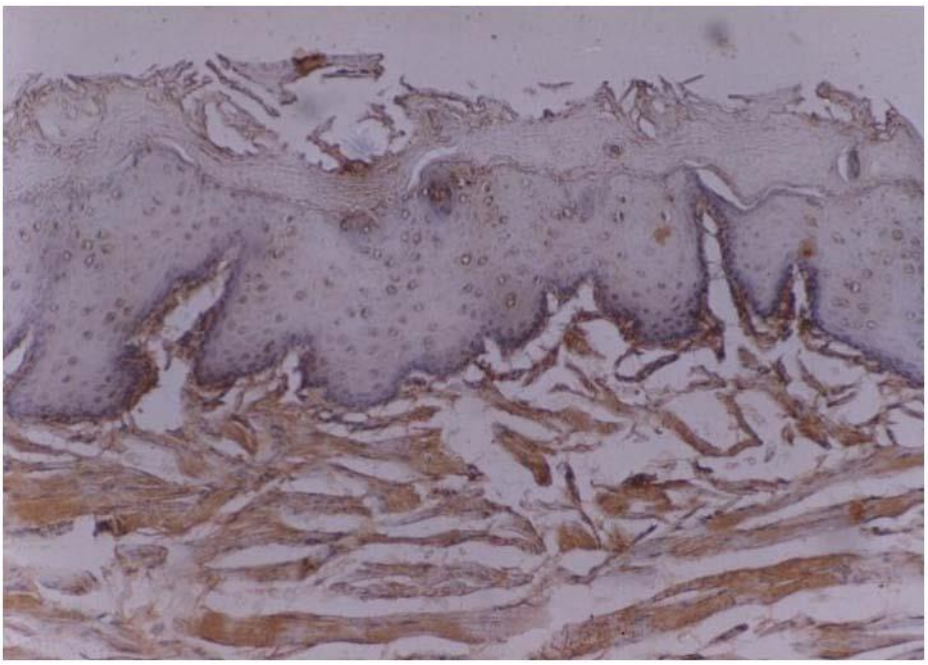

Fig. 56 : A photomicrograph of a rat gingiva of phenytoin and cyclosporin group (group 7) showing an intense reaction for fibronectin .
[Anti – fibronectin antibody X 100] .

Table (3) : demonstrating
Summary of the immuno histochemical results

Group	Group 1 (Control)	Group 2 (NIF)	Group 3 (PHT)	Group 4 (CsA)	Group 5 (NIF+PHT)	Group 6 (NIF+CsA)	Group 7 (PHT+CsA)
Collagen I	±	+	+ + + +	+ +	+ + + + +	+ + + +	+ + + + + +
Collagen IV	±	+	+	+ +	+ + +	+ + + +	+ + + +
Collagen V	±	++	+ + + +	+ + +	+ + + + +	+ + +	+ + + + + +
Fibronectin	±	+	+ + +	+ +	+ + + + +	+ + + + +	+ + + + + +

Normal	weak	mild	moderate	marked	strong	intense
±	+	+ +	+ + +	+ + + +	+ + + + +	+ + + + + +

Discussion :

- Drug – induced gingival overgrowth is a frequent adverse side effect associated principally with the administration of immuno suppressive drug cyclosporin A, and also certain anti epileptic (phenytoin) and anti hypertensive drugs (Nifedipine) [Bulut et al., 2006] .
- As elevated Blood pressure and organ transplantion cases are increased nowadays in particular renal transplant , so the use of these drugs have increased and produced a great problem because of their adverse side effects [Bulut et al., 2004] .
- These drugs produce other serious systemic complications such as nephrotoxcity and hepatotoxicity . The side effects of phenytoin can occasionally lead to morbidity . Phenytoin can affect clotting function and alter vitamin and mineral levels . Prenatal exposure to phenytoin may result in a spectrum of structural, developmental and behavioral changes , known as the fetal hydantion syndrome [Scheinfeld , 2004] .
- So scientists have to find out other substitutional drugs otherwise patients will be subjected for many dangerous side effects .
- Histologically the changes in the oral tissues as a result of these drugs administration were obvious in the gingiva . The

reason for localization of the effect of these drugs in the gingiva is unknown . It is possible that the gingival tissue may be exposed to higher concentrations of these drugs than other tissues , both directly from the blood stream as well as from the oral cavity through the crevicular epithelium [Williamson et al., 1994] .

- These changes were demonstrated as increased thickness of the epithelium and excessive amount of connective tissue . These changes were frequently detected on the buccal side of the gingiva of the lower jaw so the specimens in this experiment were taken from the rat gingiva from this region. The specimens were stained with H. & E. to study the histopathological changes and the acid phosphatase reaction to study the activity of the lysosomal enzymes . Anti collagen and anti fibronectin antibodies were used to reveal changes in the lamina properia.
- However the specimen were taken showing part of the gingival tissue and part of the adjacent sulucular epithelium
- In the previously made pilot study the plane of section revealed part of fibers of the gingival ligament which sometimes were miss interpreted as muscles [Bhaskar , 1990] .
- Also this plane of section helped in explaining the role of the inflammatory reaction as a cofactor in the process of drug – induced gingival overgrowth as the degree of

inflammation below the oral epithelium is less than that below the sulucular epithelium [Nurmenniemi et al., 2001] .

- 70 adult male albino rats weighting about 200 – 250 gm classified to 7 equal groups (10 for each) were utilized to compare the effect of each drug alone and in combinations as in pratice NIF is commonly used with CsA in renal transplanted patients to control hypertension [Thomason et al., 1993].
- Although it was suggested that rat is the highest resistant animal for the environmental conditions [Abou El Fotoh , 1994] it was found that mortality rate was higher in PHT group than CsA and NIF groups so 70 rats were used to compensate this high mortality rate throughout the experiment and to give more accurate results .
- The appearance of the G.O started at the 8^{th} week for PHT , at the 5^{th} week for CsA and at the 4^{th} week for NIF so we selected the time of the experiment to be 2 months to study the variable changes occurring in the gingiva in all groups .
- In the control group the histopathological examination of the specimens of the present work showed normal gingival tissue with the characteristic four layers : basal cell layer formed of one layer of high cuboidal cells, prickle cell layer formed of 4 – 5 layers of polyhydral cells , granular cell layer formed of 2 – 3 layers of flattened cells and normal

thickness keratin layer (keratinized stratified squamous epithelium) . The lamina properia is divided into 2 layers: papillary layer showing fine loosely arranged collagen fibers and reticular layer .

- Histochemically this gingiva showed normal enzymatic activity and so normal degradation of collagen , revealing the importance of the process of homeostasis which cause balance between collagen formation and degradation. This process maintains normal structure of the gingiva and any imbalance of it leads to accumulation of collagen fibers resulting in overgrowth of the gingival tissue [Perez, 1978].
- As well as the immuno histochemical examination of control specimens showed normal pattern of distribution of collagen type I, IV, V and fibronectin which was used as a guide to control and compare the changes caused by different drugs in the other groups .
- The histopathological examination of group 2 (nifedipine group) showed hyperplastic , hyperkeratotic epithelium and moderate vascular fibrous connective tissue with inflammatory reaction .
- This group showed that the mitotic activity of the epithelial cells was increased and higher than in normal gingiva of the control group, the difference was most clearly seen in

the oral epithelium. This finding was supported by the work of [Nurmenniemi et al., 2001] .

- The epithelium consists of keratinocytes, which emerge from the proliferation compartment in the basal cell layer and move upward through various layers of the stratified squamous epithelium, progressively undergoing differentiation, then finally cell death. The normal mechanism of epithelial turn over " renewal " is normally preserved by the homeostatic balance between cell proliferation and cell death (apoptosis) . This balance seemed to be disturbed by nifedipine leading to cell accumulation , resulting in epithelial hyperplasia and gingival overgrowth.
- These results coincide with the study of Bhathal and Gall in 1985 which demonstrated that apoptosis of keratinocytes was severely suppressed before gingival epithelial hyperplasia . In addition , the work of Shimizu et al., 2002 reported that nifedipine induces epithelial hyperplasia in gingival overgrowth not by an increase in keratinocyte proliferation , but by prolongation of cell life through reduction of apoptosis before epithelial hyperplasia is detectable .
- Some studies also showed that gingival keratinocytes, when cultured under low Ca^{2+} culture conditions, express Bcl-xL,

a factor that suppresses apoptosis ; in contrast , keratinocytes induced to differentiate by high Ca^{2+} express [Maruoka et al., 1997].

- Nifedipine is well known as a calcium channel blocker and inhibits calcium influx from extracellular fluid . Threrfore , gingival epithelial hyperplasia may be induced by nifedipine through an increase of keratinocyte bcl-2 expression [Fukuda et al., 1994] . More recently [Abou El Fotoh & El Hemma , 2004] found altered expression of bcl-2 oncogene (apoptosis suppressing protein) in palatal mucosa of nifedipine treated albino rats .
- Furthermore, apoptosis involves a cascade of specific biochemical steps, which require a rise in the level of intracellular Ca^{2+} . Blockage of one or more steps in the cascade by nifedipine may result in a decrease in the apoptosis rate . So , obviously nifedipine affects cell proliferation , DNA and collagen synthesis [Matsumoto et al., 2001] .
- The results in the current study showed that the lamina properia of the nifedipine group revealed increased number of fibroblasts and moderate increase in the collagen fiber content , also there was inflammatory reaction mainly lymphocytes . This finding was supported by the work of [Bullon et al., 2001] who found that nefidipine affects the

inflammatory infiltrate with a great number of lymphocytes (especially B).

- In the previous studies the exact mechanisms of nifedipine induced gingival overgrowth are still unclear , however several possible mechanisms have been proposed by Brown et al., 1991 and Seymour , 1991 . It has been suggested that nifedipine increase fibroblast proliferation as it increases DNA synthesis [Zebrowski et al ., 1986 and Sato et al., 2005]. On the other hand, it was reported that nifedipine has a contrasting effect as it reduces fibroblast protein synthesis [Salo et al., 1990] .
- The histochemical findings revealed decreased lysosomal enzyme activity in nifedipine group as compared with the control group.
- However degradation of collagen is an important pathway for physiological remodeling of connective tissue . Deficiencies in collagen phagocytosis by fibroblasts have been observed in nifedipine – induced gingival overgrowth [Kataoka et al., 2001] .
- In addition these findings agreed with the results of [Thomason et al., in 1993 and McCulloch , 2004] who found that nifedipine caused an impairment of collagenase activity through reducing its synthesis and release from gingival fibroblast, as the release and synthesis of collagen are calcium dependent .

- The drug altered calcium flux in gingival fibroblast may produce a disturbance in the balance between production and removal of collagen [Seymour, 1991].Moreover it was reported that the drug suppress Cathepsin B & L leading to thickening of the skin and gingival overgrowth [Nishimura et al.,2002].
- The immunochemical investigations demonstrated increased extracellular matrix proteins, e.g., collagens (Type I, IV, V) and fibronectin . There was increase in collagen expression than the control group and this result was also declared by Timar et al., 1992 and Kozlowska et al., 1996 . These authors found that systemic scleroderma (SSc) is a disease characterized by an excessive deposition of extracellular matrix proteins, collagens and fibronectin in the skin and various organs, and decreased expression of α 2 β1 integrin of fibroblasts derived from SSc patients has been reported . Both α 1 β1 and α 2 β1integrins are cell surface receptors for collagen [Dickeson et al., 1999]. Cells expressing the α1 β1 integrin preferentially adhere to Type IV collagen, whereas cells expressing the α 2 β1 preferentially adhere to type I collagen [Dickeson et al., 1999] . It has been shown that the initial binding step of collagen phagocytosis relies in adhesive interaction between cells and collagen and that

α 2 β1 integrin plays a critical role in the phagocytic regulation of collagen internalization [Timar et al., 1992] . Timar et al., 1992 reported the inhibition of integrin expression on cell surface by nifedipine in vitro . From these reports , there is a possibility that nifedipine may inhibit the phagocytosis of fibroblasts through reduced integrin expression . Furthermore Kataoka et al., 2005 supported this finding where they reported the implication of intracellular calcium in the regulation of integrin-mediated binding activity .

- Fibrosis and granulation tissue formation confirm that nifedipine – induced hyperplastic gingiva contain extreme amounts of collagen in general [Brkic , 2004] and type V in particular , as was previously reported by Narayanan and Page, 1983 . This might be accounted for the fact that type V collagen is more resistant to the bacterial collagenase than other collagens .
- In addition transforming growth factor TGF - β and connective tissue growth factor CTGF were found to stimulate the production of type 1 collagen integrin α 5 , and fibronectin in fibroblasts [Igarashi et al., 1995].
- CTGF stimulates fibroblastic cell proliferation and extracellular matrix synthesis [Uzel et al., 2001] . While

FBGF affects cell cycle in human gingival fibroblasts from nifedipine responder [Takeuchi , 2004] .

- Previous studies on the levels of TGF - β exprsssion in gingival overgrowth demonstrated that this cytokine is elevated in nifedipine gingival overgrowth tissues as shown by elevated immunostaining for TGF- β compared to controls [Lacopino et al., 1997] . This staining varied in different tissue areas . While connective tissue showed higher TGF - β staining , epithelia of nifedipine tissues did not contain elevated levels of TGF - β .
- Calcium channel blockers have dose-dependent action . Brkic 2005 found that nifedipine induced gingival hyperplasia in the experimental animal which recieved high doses of the drug during extended period of time . Calcium channel blockers have a reversible, immunomodulating effects on lymphocytes in vitro . They inhibit lymphocyte activation following mitogenic stimulation , cytokine-induced lymphocyte migration, the cytotoxic activity of natural killer cells and intracellular protein synthesis . Nifedipine inhibits mitogen-dependent production of interleukin-2 in human [Morgano et al., 1990] .
- Nifedipine suppresses T helper cells (Th_1) and cytotoxic T cells (T_c cells) without affecting T suppressor cells (T_s cells).

Activation of Th_1 and T_c cells is interleukin-2 dependent. Nifedipine is known to suppress interleukin -2 production in T- cell cultures . Changes in Th_1/Th_2 and T_c/T_s ratios would most likely alter the cytokine profile and consequently, affect the inflammatory response in the gingiva [Dupuis et al., 1993].

- As well as [Huang et al., 2003] found that taking nifedipine may alter cytokine profile of T-cells in susceptible oral tissue e.g. gingiva .
- At the same time nifedipine upregulates keratinocyte growth factor (KGF) where Das and Olsen 2000 suggested that KGF may have an important role in the molecular pathology of gingival hyperplasia by increasing proliferation of the epithelial cells .
- In phenytoin group the histopathological examination showed hyperplastic ,hyperkeratotic epithelium , however the connective tissue was more fibrotic than nifedipine and control group with inflammatory reaction . The results revealed that the histological appearance of the G.O is a function of its stage of development. Thus, the incipient developing lesion manifested histologically as a true cellular hyperplasia . While the mature fibrous end stage overgrowth lesion was characterized by a normal degree of cellularity and these findings were similar to Hall results in 1979 .

- The research work of Mallek and Nakamoto in 1981 suggested that the mechanism of phenytoin-induced folic acid depletion is uncertain . The drug may reduce absorption of folic acid from the gastro-intestinal tract or block its transport across intestinal epithelium . Alternatively, phenytoin may inhibit the enzyme folate reductase . The latter is an enzyme found in the upper small intestine which hydrolyses dietary folate in the polyglutamate form to the monoglutamate form, thus facilitating absorption . It has been suggested that phenytoin-induced gingival overgrowth is related to folic acid deficiency . Folic acid is essential for DNA synthesis , thus a deficiency will affect those cells with a high rate of turnover (i.e., bone marrow and also oral epithelium) . A deficiency of folic acid may result in an impaired maturation of the gingival sulcular epithelium, thus rendering the underlying connective tissue more susceptible to inflammation . It has also been shown in animal studies that folic acid supplements reduce the incidence and severity of phenytoin-induced gingival overgrowth . [Mallek & Nakamoto 1981] .
- Phenytoin therapy is reported to cause an alteration in the metabolism of the adrenal glands causing a suppression of adenocorticotrophic hormone (ACTH) production . Consequently an alteration of adrenal-cortical function

resulting in a reduction of glucocorticoid synthesis . This has been suggested as an explanation for the gingival overgrowth . When ACTH production is suppressed by phenytoin , there is a compensatory increase in the production of scmatotrophic hormone . The latter hormone may cause fibroblast proliferation [Hassell 1981] .

- Previous reports concerning the histologic appearance of proliferating connective tissue in the rat model indicated that there were large numbers of macrophages present . In vitro and in vivo studies demonstrated that PHT induces macrophages to synthesize and release essential polypeptide growth factors such as PDGF . The accumulation of macrophages and subsequent production of growth factors have also been associated with the processes of tissue repair and fibrosis . Thus, PHT is not only a mitogen for fibroblasts but also appears to modulate the activity of macrophages, possibly generating the molecular signals required for the appearance of myofibroblasts [Lacopino et al ., 1997] . The same finding was discussed by [Sano et al., 2004] .
- Cohen et al., 1992 suggested that macrophages play an important role in regulating fibroblast proliferalion, growth as well as production of the basic components of connective tissue such as extracellulr matrix, collagen and blood vessels . It is belived that macrophage perform its keyrole in

connective tissue turnover through the release of specialized cytokines termed polypeptide growth factors [Martin et al., 1992] .

- One of these polypeptide growth factors is platelet derived growth factor (PDGF) which is thought to be a major mitogen and chaemoattractant for fibroblast . In addition it stimulates fibroblast proliferation and synthesis of glycoseamino- glycans, proteoglycans, fibronectin and collagen [Seppa et al., 1982] . Nares et al., 1996 reported that phenytoin upregulates monocyte and macrophage platelet derived growth factor gene expression in vivo and in vitro in hyperplastic gingival tissue .
- They found that the gingiva of the phenytoin-treated animals differed from the control group in that the collagen appeared more dispersed and irregular with large areas of interstitial material between the fibers. It has been demonstrated that the amount of collagen and protein produced per cell was markedly elevated in fibroblast cultures from phenytoin induced gingival overgrowth compared to normal gingival fibroblasts . The collagens synthesized by these cells (Fibroblasts in phenytoin induced gingival overgrowth) were found to be of normal types and molecular ratio . Futhermore , there was a significant decrease in the collagenolytic enzymes secreted by these

cells . These enzymes proved to be incapable of degrading collagen in vitro [Hassell, 1982] . Howerver in 1982 Hall and Squier explained that increased amount of collagen in phenytoin induced gingival overgrowth might be due to increased production of collagen, increased number of fibroblasts , decreased collagen degradation or combination of these factors . This also was discussed by Kato et al., 2005 who found that phenytoin caused an impaired degradation of collagen by suppression of enzymatic degradation with matrix metalo proteins (MMPs).

- Both connective tissue growth factor (CTGF) synthesis and extracellular accumulation are elevated in phenytion-induced gingival overgrowth . PHT samples contained an increased abundance of fibroblastic cells and connective tissue fibers in histologic samples compared to the nifedipine and control groups . This indicates that CTGF expression may be a marker for a pathway characterized by increased fibroblastic activity and fibrosis [Uzel et al., 2001]. Indeed , studies indicate that CTGF stimulates fibroblast proliferation and insoluble collagen accumulation by some tissue and cells including human gingival fibroblast [Duncan et al., 1999] .

- The overall pathogenesis of drug-induced gingival hyperplasia might be multifactorial . It has been suggested that the pathogenesis of the disease is strongly associated with the increased expression of essential growth factors, such as platelet-derived growth factor, by accumulated macrophages but not by residual fibroblasts . If this was the case, gingival fibroblasts may produce more matrix components stimulated by growth factors, which are produced from macrophages triggered by the drugs . Gingival fibroblasts are unable to degrade these overproduced matrix components due to the impaired ability of protein breakdown . This phenomenon may further accelerate the increase of tissue mass, leading to enlarged gingival tissue [Dill et al., 1993] . In conclusion, the decreased ability of protein degradation by lysosomal enzymes is, at least, one of the factors in the pathogenesis of this unwanted side effect [Lacopino et al., 1997] .
- Furthermore there is another concept of phenytoin effect on the production of interleukin -6 and interlukin-8 in human gingival fibroblasts . Modeer et al., 2000 demonstrated that PHT may give a prerequisite for the establishment of an interaction between cytokines and connective tissue cells in the periodontal tissue , which is suggested to lead to gingival overgrowth .

- In cyclosporin group the histopathological examination showed conspicuous enlargement of epithelium forming several layers of basal cells. The gingiva consists of highly vascularized connective tissue with an overlying irregular multi-layered parakeratinized epithelium of variable thickness . Some epithelial ridges were seen penetrating deeply into the subepithelial connective tissue , with irregularly arranged collagen fiber bundles associated with them . In the connective tissue there were accumulations of inflammatory cells, in which the most predominant cell type were lymphocytes .
- The results of the present work showed that mitotic activity was observed to be higher in cyclosporin -induced gingival overgrowth specimens than in control and NIF specimens . The difference was most clearly seen in the oral epithelium (the basal and prickle cell layers) .
- This was explained by Williamson et al., 1994 who reported that gingival overgrowth , wound healing and collagen turn over are regulated by cytokines and growth factors . The expression of these mediators and their corresponding receptors is thus likely to be of fundamental importance in the pathophysiology of GO .

- Keratinocyte growth factor KGF was isolated initially as a product of fibroblast cells and shown to stimulate epithelial cell growth where it stimulates the growth and activity of the epithelial cells [Das et al., 2002] . Both the naturally occurring and recombinant forms of KGF have been implicated in tissue repair and regeneration, with a primary impact on the proliferation and differentiation of epithelial cells [Werner et al., 1992] .
- Although KGF has originally been shown to be synthesized and secreted by stromal cells , other studies suggested that this growth factor might also be produced by certain T lymphocytes [Finch et al., 1996] . Recently Chin et al., 2006 found another relation between gingival overgrowth and the expression of epidermal growth factor which increased upon cycloporin treatment and this may lead to epidermiod carcinoma .
- An association between CsA- induced gingival overgrowth and clinical inflammation has been recorded in several researches . Gingival inflammation induces a proliferative response in epithelial cells [Odile et al., 1997] .
- Some studies which suggested that CsA induce gingival overgrowth concluded that : 1- Gingival epithelial hyperplasia is not due to an increase in keratinocyte proliferation . 2 - It is rather to a prolongation of cell life in

CsA-induced gingival overgrowth as tissue hyperplasia involves proliferative and / or survival components [Niimi et al., 1996] .

- One of the possible mechanisms responsible for cyclosporine induced gingival overgrowth is the increased fibroblastic activity through alterations in levels of various growth factors and cytokines [Lacopino et al., 1997] . It is assumed that the gingival overgrowth observed in CsA treated patients was not due to an increase in tissue collagen. It is rather to an increase in epithelium with an accumulations of non collagenous extra cellular matrix material [Pisanty et al., 1988] .
- CsA inhibits the proliferation of T-lymphocytes stimulated by either antigen or mitogen . The drug acts at 3 distinct but related stages on T-cell . These are as follows :

(1) It inhibits T-cell helper function to accessory cells (i. e marcrophage) for the synthesis of interlukin I (Perviously known as lymphocyte activation factor) .

(2) It prevents the formation of receptors to interleukin I on the membrane of the T – cell . Activation of interleukin I receptor is an essential stage in the production of Interleukin II (T-cell growth factor) . Production of the latter is therefore suppressed .

(3) It renders T – cells unresponsive to interlukin II . As a result of these three mechanisms , there is a suppression of T-helper cells which are more affected by cyclosporin

than are suppressor cells resulting in a net imbalance of T-suppressor cells and immunosuppression. Cyclosporin does not directly inhibit natural killer cells (N.K) thus maintaining the role of these cells in tumour surveillance [Luapacis et al., 1982]. In addition Bulut et al., 2002 found that low numbers of natural killer cells are important in the expression of plaque-induced inflammatory changes in CsA-associated GO.

- So it has been suggested that CsA has a unique modulating effect on the cells of the immune response .
- It seems to inhibit the production and release of interlukin II. Inhibition of (I.LII) release is most sensitive to CsA administration more than that of interlukin I [Bunjes et al., 1981] . This finding was also supported by the work done by Grassi et al., 2006 who demonstrated that CsA interfers with T cell activity and cell-mediated activity .
- It has been found also that CsA increases the level of interlutkin 6 (I. L6) [Willimson et al., 1994].
- However, it would appear that the pro-inflammatory cytokine is upregulated in inflammatory conditions but in proliferative conditions associated with drug induced gingival hyperplasia [Dill et al., 1993]. PDGF on the other hand appears to represent an essential polyptide growth factor which specifically regulated in response to drugs which cause proliferation of gingival tissue [Nares et al., 1996] .

- The histochemical findings of the present work revealed decreased lysosomal enzyme activity in cyclosporin group than the control group and this finding agreed with Thomason et al., 1998 . They suggested that Cyclosporin A suppressed the expression of matrix-degrading enzymes as seen in cathepsin L and matrix metalo protein MMP-1 . Recently, it has been reported that MMP-1 expression was reduced in cyclosporine A induced gingivally overgrown tissues compared to healthy gingival tissue [Yamada et al., 2000] .
- The interacellular ability of protein degradation was suppressed, as judged by the low expression of cathepsin L and its impaired activity . Additionally, depressed cathepsin activity may increase the amount of new collagen deposition in the extracellular matrix by decreasing intracellular degradation of newly synthesized procollagen prior to its secretion into the matrix. This phenomenon may account for the increase in cell mass often seen in cells obtained from long-term gingivally overgrown tissues . McCulloch, 2004 suggested that a common pathway targeted by cyclosporin is the regulation of the intracellular pathway of collagen degradation .
- Taylor and Shuff 1994 reported that patients with mucolipidosis-II (I-cell disease), a congenital disease characterized by a defect in intracellular lysosomal function such as cathepsins, exhibited sever gingival enlargement . Yamaguchi et al., 2004 emphasized the same fact that the lysosomal enzymes play an important role in the pathogenesis of gingival hyperplasia .

- In addition, KGF is 100-fold more effective than bFGF at inhibiting the expression of epithelial cell specific collagenase . Thereby KGF is possibly contributing further to the excessive accumulation of extracellular matrix, which occurs in GO and other hyperplastic pathologies, more than bFGF [Koos et al., 1993].
- The immunohistochemical investigations of the current study showed increased extracellular matrix proteins collagens type I, IV, V and fibronectin compared with control group . This was similar to somewhat extent to the work done by Dannewitz et al., 2006 who found that cyclosorin A induced gingival overgrowth included the accumulation of extracellular matrix constituents, collagen type-I and type-III and proteoglycans, in subgingival connective tissue .
- The pathogenesis of drug-induced gingival hyperplasia has been frequently described in relation to the growth of gingival fibroblasts, and to the ability of these cells to produce extracellular matrix components such as type I collagen and heparin sulfate proteoglycan [Seymour et al., 1996] .
- The immunosuppressive agent cyclosporin A is known to induce gingival overgrowth . It was demonstrated the possibility that CsA-induced gingival overgrowth is induced by a decrease in Type I collagen degradation. Predominantly through a reduction in collagen phagocytosis, by using a similar animal experimental model involving 20 day old rats [Kataoka et al., 2000] .

- In nifedipine and phenytoin group the histopathological examination revealed more thickness of the keratinous cell layer compared with the group of nifedipine alone or the group of phenytoin alone . Also the epithelial rete pegs demonstrated more width and length than in case of using single drug therapy . These results coincide with the work of Sano et al., 2004 who declared that the use of phenytoin and nifedipine stimulate the response of gingival fibroblasts from humans with gingival fibromatosis . These findings suggest that PHT-and NIF-induced gingival proliferation may be mediated by endogenously generated immuno-reactive endothelin .
- The histochemical findings of the present work revealed decreased lysosomal enzyme activity . The enzymatic activity was markedly suppressed when both drugs were used in combination resulting in decreased collagen degradation .
- The immunohistochemical investigations of the current study showed increased extracellular matrix proteins collagens type I, IV, V and fibronectin more obviously than in case of using nifedipine or phenytoin alone due to the cumulative effect of both drugs together .

- In nifedipine and cyclosporin group the histopathological examination showed hyperplastic ,hyperkeratotic epithelium and vascular fibrous connective tissue with inflammatory reaction .
- The results of this work showed that the effect of the combined drug therapy is more severe than single drug therapy . This was in contrary to the work done by Chiu et al., 2001 where they observed that the gingival dimensions increased after CsA or NIF therapy, although they were more prevalent with CsA. But the augmenting pattern in gingival morphology observed with CsA therapy decreased when the animals received additional NIF.
- Several hypotheses have been advanced to explain drug-induced gingival overgrowth . Nifedipine , cyclosporin and phenytoin may directly influence gingival fibroblasts increasing cell proliferation and matrix formation .
- Essentially, drug-induced gingival overgrowth is a result of an interaction between drug and / or metabolite with the gingival tissue . The target cell appears to be the gingival fibroblast .
- Drug may directly influence human gingival fibroblasts causing increased cell proliferation, DNA synthesis and matrix formation . Interactions may also exist between drugs . Significantly increased cell proliferation , DNA, and collagen synthesis have been observed in gingival fibroblast cultures from NIF-induced overgrowth in presence of phenytoin . The combination of drugs such as cyclosporin and NIF, was shown to increase the prevalence of gingival overgrowth [Bartold , 1989] .

- Defects in collagen degradation had been observed with drug-induced gingival overgrowth . Deficiencies in collagen phagocytosis by fibroblasts and interference with collagenase activity by drugs aggravate the imbalance of collagen degradation . Also , intracellular calcium imbalance was reported to play a role [Gelfand et al., 1987].
- Dental bacterial plaque may enhance drug-induced overgrowth through an inflammatory pathway as the inflammation started and enhanced by the plaque lead to an hyperemic and edematous gingival tissue [Vescovi et al., 2003] . Inflammatory changes within the tissue may enhance this interaction where variable responses in fibroblast behavior have been reported when they have been grown in tissue culture with cyclosporin or Nifedipine [Seymour and Smith,1991].
- Some studies showed that the effect of these drugs is time-dependent while other studies had shown a dose dependent increased rate of cell proliferation with cyclosporin . A similar finding had been reported for nifedipine [Hassell et al., 1991] .
- There were conflicting findings regarding drug-induced gingival overgrowth among patients taking CsA alone or in combination with nifiedipine . A combined administration of CsA and nifedipine induced more severe gingival overgrowth in patients than CsA alone in some studies, while CsA-induced gingival overgrowth was found not to be influenced by a combined treatment with nifedipine in another study . Slavin and Taylor 1987 reported a

significant increase in the incidence of gingival overgrowth among patients taking both CsA and nifedipine compared with those taking only CsA . Furthermore Khoori et al., 2003 declared that the severity of gingival overgrowth in renal transplant patients who had received both drugs. There was a clear relationship between gingival overgrowth and duration of cyclosporine and nifedipine use. On the other hand, Thomason et al., 1993 found that the combined use of CsA and nifedipine increased the severity, but not the incidence, of gingival overgrowth in renal and cardiac transplant patients . In addition the fibroblast and collagen density increased in parallel with the severity of the overgrowth when both CsA and nifedipine were used in combination [Spoildorio et al., 2002] .

- It is also considered that gingival overgrowth in rats treated with CsA and nifedipine or diltiazem as all considered as calcium channel blockers [Thomason et al., 2003 and Prisant and Herman , 2002] . Concurrently gingival overgrowth is induced in an additive mode rather than a synergistic mode . Although the blood level of nifedipine was elevated by the simultaneous treatment with CsA, that of diltiazem was reduced by CsA . [Bullon, 1985].
- The combination between NIF and CsA cause gingival overgrowth by altering the immune response [Pernu and Knuuttila , 2001] .

Concerning phenytoin and cyclosporin group the histopathological results expressed : The greatest thickness of keratin and the epithelial rete pegs that showed the maximum length and width . These results might be due to the synergetic effect of both drugs when they are used in a combination .

The histochemical findings of the present work revealed decreased lysosomal enzyme activity . The enzymatic activity was markedly suppressed when both drugs were used together . This finding agreed with the work done by Yamada et al., 2000 who found that besides the suggested effects of these drugs on gingival fibroblasts and/or on accumulated cells in the gingival tissues , extracellular matrix-degrading ability, particulary that by cathepsin L, is also suppressed by cyclosporin A and phenytoin in gingival fibroblasts . The lysosomal enzyme plays an important role in the pathogenesis of drug – induced gingival hyperplasia.

The immunochemical investigations of the current study showed the maximum amount of the extracellular matrix proteins, e.g., collagens (Type I, V) and fibronectin more obviously than in case of all the previous groups . This is most probably due to the effect of both drugs together on the gingival fibroblasts stimulating its proliferation and collagen synthesis.

Summary & Conclusions

- Patients coming to the dental clinic with different problems. One of the most important patient chief complain is gingival enlargement . Gingival enlargement may be so sever that it requires surgical intervention . It may interfere with occlusion , speech and lead to bleeding , periodontitis or caries .

- Gingival enlargement may be idiopathic , hereditary , inflammatory but the most common is drug induced gingival overgrowth .

- The use of systemic medications was found to cause gingival overgrowth such as : antihypertensive , antiepileptic and immuno suppressive drugs . The use of these drugs have been increased nowadays due to the increased cardiovascular diseases and organ transplantation cases .

- Drug induced gingival overgrowth is neither typical hyperplasia nor typical hypertrophy and the exact mechanism by which these drugs induced gingival overgrowth is still unclear .

- So our research work was interested in this phenomenon to identify the mechanism of each drug in producing gingival overgrowth .

- Normally the gingiva consists of epithelial component which is stratified squamous keratinized epithelium and connective tissue component which is the lamina propria .

- The normal gingiva is in a state of homeostatic balance as the rate of keratinocyte proliferation is in balance with apoptosis of the epithelial cells . Also the rate of collagen fiber synthesis by fibroblasts is in balance with the rate of degradation and phagocytosis .

- Disturbance of this balance lead to increased thickness of epithelium and / or accumulation of collagen and other matrix glycoproteins in the lamina propria .

- In this study 70 adult male albino rats were selected and classified into 7 groups :

Group 1 control group

Group 2 nifedipine group

Group 3 phenytoin group

Group 4 cyclosporin group

Group 5 nifedipine & phenytoin group

Group 6 nifedipine & cyclosporin group

Group 7 phenytoin & cyclosporin group

The experimental animals were sacrificed after 2 months .

Specimens were taken from the buccal gingiva to be examined .

Histopathological investigation using H &E to identify the causes of gingival overgrowth whether it is due to epithelial or connective tissue overgrowth or both together .

Histochemical examination using acid phosphatase to demonstrate the lysosomal activities in previously mentioned three drugs and their combinations to identify if there is decreased degradation of collagen as a result of decreased lysosomal enzyme activity .

Immunohistochemical examination : using polycolonal antibodies for type I , IV , V collagen and fibronectin to detect collagen pattern and distribution in the previous groups as well as the extra cellular matrix glycoprotein fibronectin .

From examination of these specimens the **histopathological results** demonstrated that the drugs cause increase in the mitotic activity of the epithelial cells - with variable degrees – specially in the basal and prickle cell layer . This increase in the rate of proliferation of keratinocytes was more obvious in cyclosporin group .

Also the histopathological examination of the specimens showed increased collagen fiber content in the lamina propria . This increase in the collagen fibers is specially of type I and more detected in phenytoin followed by cyclosporin group . The H.&E. study also revealed inflammatory reaction in the lamina propria - mainly lymphocytes - which was more obvious in nifedipine group .

While the **histochemical examination** of the specimens revealed decreased enzymatic activity in the experimental groups than the control group explaining the accumulation of collagen fibers in the lamina propria of the gingival specimens of these groups due to decreased degradation rather than increased synthesis .

The immunohistochemical examination demonstrated increased amount of collagen specially type I and the extracellular matrix glycoprotein fibronectin in the lamina propria of the gingival specimens of the experimental groups than the control group.

The overall pathogenesis of drug-induced gingival hyperplasia might be multifactorial . It has been suggested that the pathogenesis of the disease was strongly associated with the increased expression of essential growth factors, such as : keratinocyte growth factor , platelet-derived growth factor , connective tissue growth factor , transforming growth factor and epidermal growth factor .

The clinical and pathologic features in drug – induced gingival overgrowth are independent of the drug adminstration, which suggests a common pathway of induction .

* From the present research work it can be concluded that

1 - Gingival overgrowth is a multi-fuctorial problem which might be due to :

a) Increased mitotitic activity of the epithelial cells , thickness of keratin and the inflammatory cells in the lamina properia.

b) Increased collagen synthesis .

c) Decreased collagen degradation.

2 - The effect of the used drugs on type IV collagen was lesser than their effect on the other types of collagen (I & V) and fibronectin.

3 - The use of more than one causative drug lead to more gingival overgrowth than the use of single drug due to the cammulative effect rather than synergistic effect.

4 - The use of more than one causative drug lead to increased severity of G.O but not increase in its incidence

5 - The drugs causing gingival overgrowth share a common mechanism of action where:

a- Cyclosporin cause conspicuous enlargement of the epithelium.

b- Phenytoin cause fibrosis of the lamina properia.

c- Nifedipine has least effect in comparison to the other two drugs with obvious inflammatory reaction in the lamina propria.

Thus we recommend patients undergo to nifedipine , phenytoin and cyclosporin treatment :

1 – Not to use them for a long time to avoid their tissue hyper plastic effect which may be by time become irreversible reaction and continue up to cause tumorigenesis .

2 – Not to use a mix between two drugs of them to avoid the cammulative drastic hyperplastic effect .

Discussion

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&

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