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SCANNING AND IMMUNOHISTOCHEMICAL STUDY ON THE EFFECT OF TACROLIMUS ON THE LINGUAL MUCOSA OF ALBINO RATS

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ABSTRACT

Aim: The present investigation aimed to evaluate the immunosuppressive drug (Tacrolimus) effect of on the lingual mucosa through histological examination, immunohistochemical detection of proliferating cell nuclear antigen (PCNA) and scanning electron microscopic examination.

Materials & Methods: Twenty rats were utilized in this study. The rats were distributed into two groups (10 animals each); **group 1:** rats served as control, **group 2:** rats were administrated daily oral dose of tacrolimus (0.5 mg/kg body weight). At the end of the experiment, the animals of the different groups were sacrificed, the lingual mucosa specimens were dissected out and prepared to be stained with H&E for histological evaluation and for immunohistochemical detection of PCNA. Statistical analysis of data was carried out using one way ANOVA followed by T-test to compare between the groups under study. The differences were considered significant at p value < 0.05. Tongue samples from each group were prepared to be scanned under scanning electron microscope.

Results: The H&E stained lingual specimens of **group I** revealed normal histological structure of surface epithelium and underlying lamina propria of both dorsal and ventral surfaces of the tongue while g**roup II** treated with tacrolimus revealed histopathological changes of both surfaces of the tongue. The immunohistochemical localization of PCNA showed negative to weak positive staining reaction of PCNA of the epithelial surface of the tongues. The scanning electron micrographs of the dorsal surface of the tongues of tacrolimus treated rats revealed atrophic changes.

Conclusion: Tacrolimus administration showed structural and ultrastructural histological changes in the lingual mucosa and cause alteration in the taste sensation.

KEY WORDS: Tacrolimus, Organ transplant, Tongue, PCNA.

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INTRODUCTION

One of the most difficult and complicated fields of modern medicine is transplantation medicine. Transferring an organ from one body to another or from one area on the recipient's own body to another in order to replace a damaged or absent organ is known as organ transplantation. It is the treatment of choice for end stage organ failure.[1] Autografts, isografts, allografts and xenografts are different forms of transplants.[2]

The potential risks of transplant rejection, in which the immune response of the body reacts to the transplanted organ, which may result in transplant failure and have to remove the organ from the recipient shortly after transplantation, are some of the major areas for medical management.[3] When possible, serotyping to find the best donor-recipient match and using immunosuppressant medications can help to decrease transplant rejection[4]

The improvement in knowledge of the cellular and molecular mechanisms underlying graft rejection is reflected in the development of immunosuppressive drugs. Combinations of medicines that work synergistically to give the potency, safety from adverse effects, ease of administration, and affordability appropriate for each patient make up the best immunosuppressive strategy.[5]

Immunosuppressive drugs include Cortico-steroids (Prednisolone and Hydrcortisone), anti-proliferative (Azathioprine and Mycophenolic acid), mTOR inhibitors (Sirolimus and Everolimus) and Calcineurin inhibitors (Cyclosporin and Tacrolimus). [6]

An immunosuppressive medication (Tacrolimus) is frequently administered following an allogeneic organ transplant to reduce the risk of organ rejection. It accomplishes this by preventing the synthesis of interleukin-2, a chemical that encourages T cells, which are essential for the body's immunological response, to mature and proliferate.[7]

Tacrolimus (FK506), a widely used immunosuppressive drug, possesses neurite-promoting action in peripheral neurons and PC12 cell culture. Tacrolimus influences functional recovery following photothrombotic spinal cord injury as well as the expression of the protein linked with neuronal development, GAP-43 (Growth Associated Protein 43) [8].

When damaged rats were given tacrolimus daily for one week instead of only a vehicle, the proportion of neurons expressing GAP-43 mRNA and protein more than doubled. A considerable improvement in neurological function as measured by open-field and inclined plane tests coincided with this rise in GAP-43-positive cells. Tacrolimus may thereby improve functional outcomes following CNS damage in people.[9]

Tacrolimus has been used successfully to treat refractory eosinophilic granulomatosis with polyangiitis (EGPA) exacerbated by invasive aspergillosis and chronic hepatitis B. It has been demonstrated to suppress cytokine receptor signalling in addition to calcineurin activity.[10]

Tacrolimus treatment for kidney transplant patients has been proven to be safe and effective in preventing graft rejection, renal dysfunction, and cardiovascular risk factors (hypertension and hyperlipidemia). Over the past ten years, clinical trials and registry studies have shown tacrolimus to be a key immunosuppressant in renal transplantation. Tacrolimus has been proven to reduce acute and chronic rejection, enhance kidney function over the long term post-transplant, and reduce the prevalence of hypertension when compared to cyclosporin therapy.[11]

Tacrolimus is a well-recognized substitute for cyclosporin in both primary and rescue therapy due to its 100 times greater immunosuppressive potential.[12]

Tacrolimus has less glaring side effects than cyclosporin, for instance, gingival overgrowth is less common and less severe in adult transplant patients using tacrolimus compared to cyclosporin. .[13]

Proliferating cell nuclear antigen (PCNA) was assessed as a cell proliferation marker in tissue and PCNA labeling technique was effective for estimating cell proliferation rates.[14]

So, the present investigation was conducted to study the effect of oral administration of immunosuppressive drug Tacrolimus on the tongues of male albino rats.

Aim of the study: The present research aimed to assess the effect of Tacrolimus on the lingual mucosa specimens of rats through histological examination, Immunohistochemical localization of PCNA and Scanning electron microscopic analysis of the dorsal surface of the tongues for detection of any possible alterations.

MATERIAL AND METHODS

The present search was commenced after the approval of the Research Ethics Committee of the Faculty of Pharmacy, Suez Canal University with ethical code# 202306RA2.

Twenty male adult albino rats were used in this investigation with body weight ranging from 130 to 150 gm. The rats were allocated and divided into two groups (10 animals each). They were drinking tap water adlibitum and supplied standard natural diet. The rats were divided as follows: **group 1**: served as normal (control) rats and received distilled water using gastric tube**, group 2**: rats received the immunosuppressive drug tacrolimus dissolved in distilled water in a daily oral dose of 0.5 mg/kg body weight using a gastric tube. Tacrolimus is provided in tablet form that were ground to be easily dissolved in distilled water in a concentration of 0.15 mg/ml. The rat with body weight 150 gm took 0.5 ml per day.[15]

Examination of the head and neck of each rat was performed throughout the whole experimental interval which lasted for three months.

The animals of different groups were sacrificed by cervical dislocation at the end of the experiment. The tongue samples of half rats of each group were dissected out, then immediately fixed in 10% neutral buffered formalin, processed, dehydrated in ascending grades of alcohol, embedded in paraffin and sectioned to be stained with hematoxylin and eosin (H&E) for histological evaluation.

Immunohistochemistry

Labelled streptaviden-biotin for immunohistochemical detection of the proliferating cell nuclear antigen (PCNA) and calculating its labeling index to be treated statistically. Images were obtained through digital camera (Olympus Dp25, japan). For localization of percentage area of immunostained cells with PCNA, images of elected parts of lingual mucosa were analyzed using image J software. Analysis of data was carried out employing the statistical set for social sciences, version 21(SPSS software). One -way ANOVA (Analysis of variance) were used to compare between the groups under study followed by T-test for pair wise comparison in each two groups at P value < 0.05. The differences were considered significant at p value <0.05. Results are presented as mean $\pm$ SD.

Scanning electron microscope

Tongue samples of the other half of rats of both groups were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PH 7.4) for 4 hours.

Tongue specimens were washed with phosphate buffer and post fixed on 1% osmium tetroxide for 90 minutes then the samples were washed again with phosphate buffer and dehydrated through series of ascending concentrations of ethanol to 100% amylacetate. After that, samples were coated with gold under vacuum with sputter coater. After gold coating, tongue samples were examined and photographed with JEOL, JSM-53009 scanning electron microscope in the National Research Centre, Cairo.

RESULTS

Histological Results:

Lingual mucosa sections of control rats stained with hematoxylin and eosin revealed normal histological structures of epithelial surface, underlying lamina propria and submucosa. The filliform and the fungiform papillae were normal protruding from the dorsal surface of the tongue while the superior surface of circumvallate papillae in line with the dorsal surface of the tongue. The filiform

papillae were numerous and distributed evenly over the dorsal surface of the anterior two thirds of the tongue. They appeared as conical projections with pointed tips directed posteriorly and they were covered with keratinized stratified squamous epithelium. The fungiform papillae were seen in between filliform papillae. They were mushroom like with broader upper surface and wide well vascularized connective tissue core. They were seen mostly at the tip and lateral border of the tongue. A single well defined taste bud was seen in the surface epithelium at the top of the papillae. The taste bud is barrel shaped, has a taste pore, outer supporting cells, inner supporting cells and neuroepithelial cells. The circumvallate papillae were located near the v shaped sulcus terminalis. They were surrounded with circular furrow and covered with non-keratinized stratified squamous epithelium with connective tissue core. At the bottom of the furrows opened the ducts of pure serous Von Ebner salivary glands. The underlying lamina propria formed of dense bundles of collagen fibers with connective tissue cells where fibroblasts were clearly the predominant type of cells. The lamina propria was well vascularized. The ventral surface of the tongue was lined with squamous stratified epithelium with numerous broad epithelial ridges extending to the underlying lamina propria. Connective tissue cells, blood vessels and nerves were normally seen **(fig 1 A,B&C).** While the histological slides of lingual mucosa of rats received tacrolimus revealed alteration of their normal structure. The mucous membrane of tongue sections showed marked atrophy and degeneration of the epithelium. The filiform papillae showed marked atrophy, loss of its normal sharp conical shape with apparent decrease in their numbers. The fungiform papillae were also atrophic, losing their characteristic mushroom like shape with degenerated cells of their taste buds. The circumvallate papillae appeared shrunken with deepened furrows around them and aggregation of inflammatory cells below its trough. The basement membrane showed atrophy and lacked its normal appearance.The lamina propria showed marked dissociation and degeneration of collagen fibers with dilatation of blood vessels.**(fig 1 D,E&F).**

Immunohistochemical localization of Proliferating Cell Nuclear Antigen (PCNA):

The sections of mucous membrane of tongue of control rats showed moderate to strong positive immunostaining reaction of the stratum basale cells of the epithelial surface of the tongue to PCNA which indicated normal rate of proliferation denoting normal rate of cell renewal, While the sections of the tongue of animals treated with tacrolimus showed weak immunostaining of PCNA in the basal cell layer of epithelial surface of the tongue with negative to weak positive immunostaining reaction which indicated a reduction in proliferation denoting decline in the turnover and cell renewal rate. **(fig 2 A&B).**

PCNA labeling index: The immunohistochemical analysis of the proliferating cell nuclear antigen (PCNA) revealed that there was significant decline in the PCNA labeling index (P value <0.05) in the epithelial surfaces of both ventral and dorsal surfaces of the tongue of tacrolimus treated rats compared with their controls.

Analysis of data was carried out employing the statistical set for social sciences, version 21(SPSS software). One -way ANOVA (Analysis of variance) were used to compare between the groups under study followed by T-test for pair wise comparison in each two groups at P value < 0.05.

The differences were considered significant at p value <0.05. Results are presented as mean ± SD.

Group	Tongue		P value
	Dorsal surface	Ventral surface	
Group I	0.98±0.03	0.97±0.02	P value < 0.05
Group II	0.35±0.02	0.31±0.05	

Table demonstrates labeling index of PCNA of the epithelial surface of lingual mucosa of the diverse groups. Results are represented as mean + standard deviation (SD).

Scanning electron results

The scanning electron micrographs of the dorsal surface of the tongues of rats of control group showed the filliform papillae compactly distributed over the entire dorsal surface with uniform shape and direction, fungiform papillae with their taste pores distributed among the filiform and circumvallate papillae that lie in front of the terminal sulcus. **(fig 3 A,B&C).** While, scanning of the dorsal surface of lingual sections of tacrolimus treated rats showed numerous atrophic filiform papillae with distracted alignment and inclination. It became thinner with more slender shape. Others were covered by constricted keratin. Severely destructed filliform papillae with desquamation of its epithelial covering were depicted. Numerous filliform papillae were completely covered by colonies of candida albicans indicating presence of fungal infection and lower immunity of treated rats. Fungiform papillae showed wrinkled heavily keratinized epithelial covering were represented. They were atrophic, shrunken and degenerated. The pore of taste buds was sometimes unclear in central region of taste bud. Sometimes the gustatory pore appeared depressed, irregular& obliterated .The circumvallate papillae were shrunken and atrophic with irregular outline and deepening of the furrow surrounding them. **(fig 3 D,E&F).**

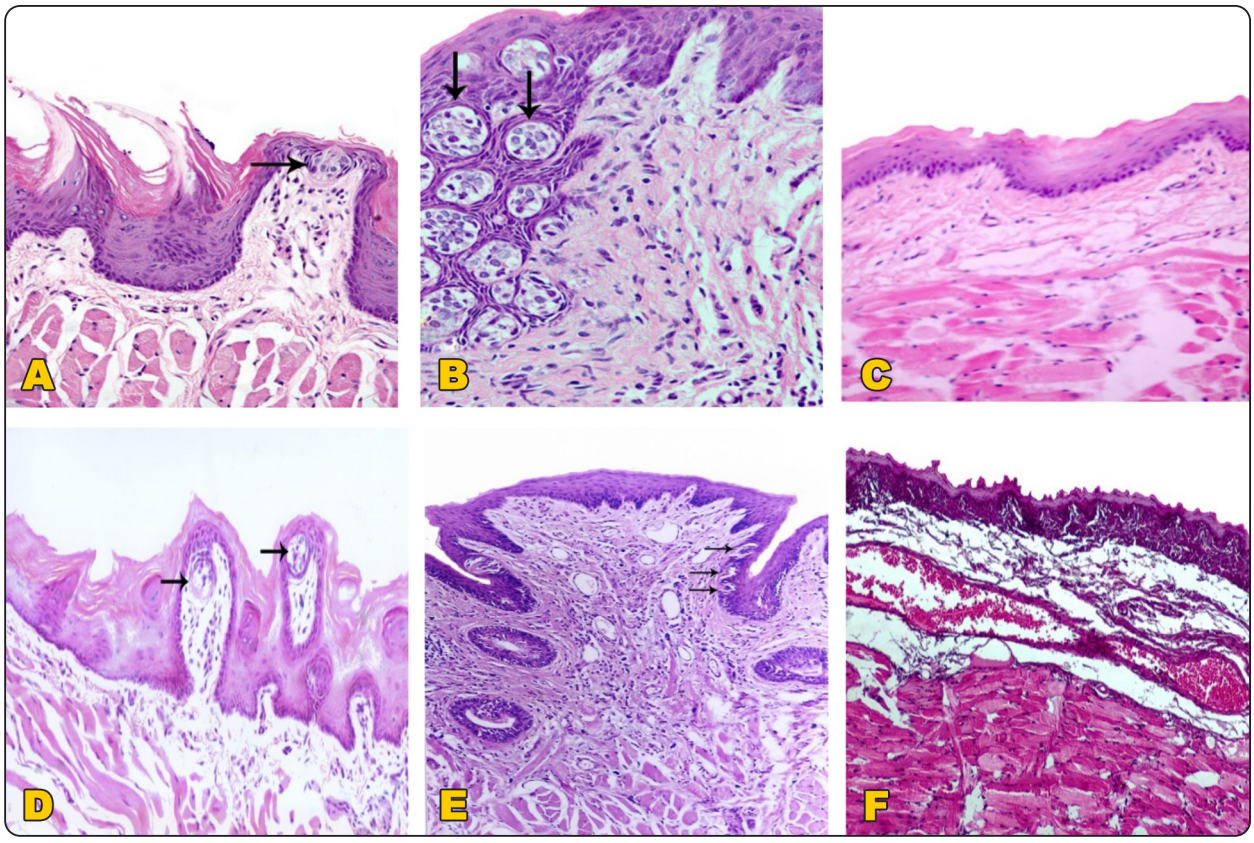

Fig. (1): A, photomicrograph showing filiform and fungiform papillae with normal taste bud (arrow) B, showing circumvallate papilla with normal stratified squamous epithelium, lamina propria and several taste buds in its side walls (arrows) (orig. mag. 400) C, showing the ventral surface of the tongue and underlying lamina propria (orig. mag.200).D, showing atrophic fungiform papillae, degenerating cells of taste bud and dissociation of collagen fibers in the lamina propria (orig.mag.200). E, circumvallate papilla showing degeneration of basal cells, hallowing out of taste buds (arrows) (orig.mag.400). F,ventral surface of the tongue revealing atrophy of epithelial surface, degeneration of collagen fibers and dilated blood vessels of lamina propria (orig. mag. 200).

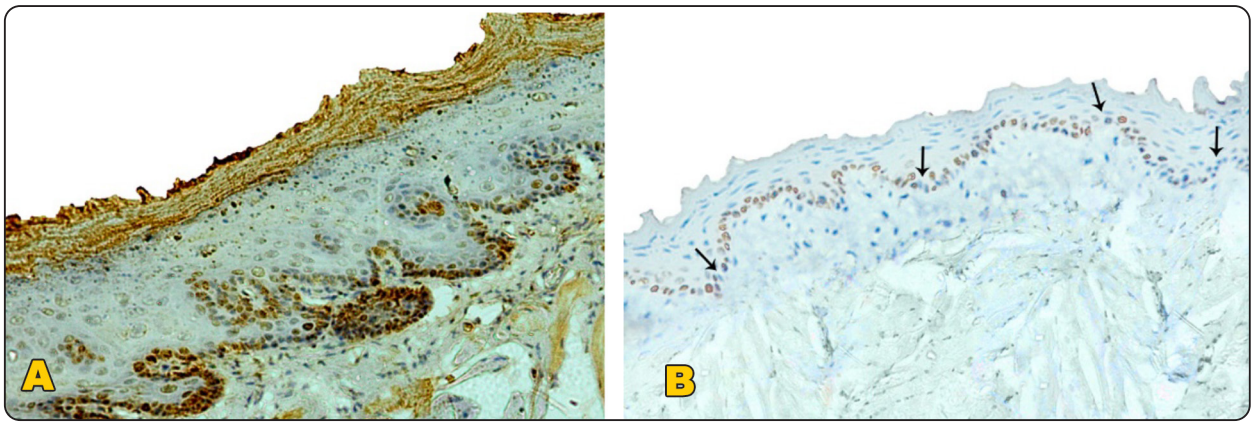

Fig. (2) A, photomicrograph revealing strong positive immunostaining reaction of stratum basal layer of the ventral surface of lingual mucosa of group I animals to PCNA,B, showing the ventral surface of lingual mucosa of tacrolimus treated animals showing weak positive immunostaining reaction of stratum basal layer to PCNA. Notice there is a lot of unstained basal cells (arrows) (orig. mag. 200).

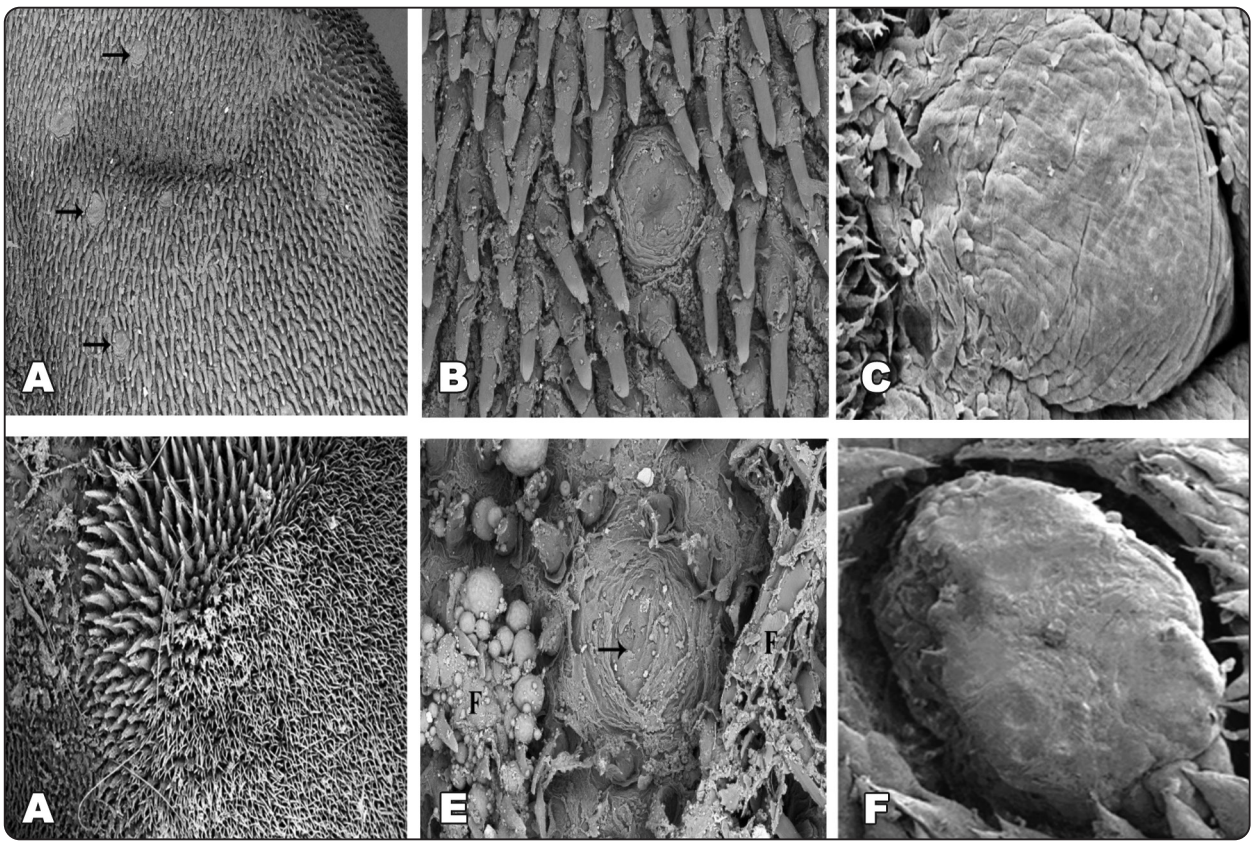

Fig. (3) A, Scanning electron micrographs of the dorsal surface of the tongue of a rat from the group I showing normal uniform antero-posterior inclination of long conical filliform papillae arranged in parallel rows and fungiform papillae among them (arrows)(Mag. X100).B, revealing long conical filliform papillae and large fungiform papilla between them with central taste pore at its top (Mag. X 600). C, showing large circumvallate papilla surrounded by a furrow (Mag. X1200). D, revealing abnormal distribution and direction of the filliform papillae of tongue dorsal surface of group II animals (Mag. X150). E, fungiform papilla with disappearance of its taste pore (arrow) and a lot of growing fungi (F) (Mag. X2000). F, showing circumvallate papilla with marked deepening of the furrow surrounding it (Mag. X 1000).

DISCUSSION

The purpose of immunosuppression is mainly to limit graft rejection. The primary role of the immune system is to protect the vertebrates' bodies against infection. It is composed of billions of lymphocytes comprising different clones and each clone has a unique cell surface receptor to enable them to bind to a particular antigen. B lymphocytes, which produce antibodies, and T lymphocytes, which are in response to cell-mediated immunity, are the two kinds of lymphocytes.[16]

Success in solid organ transplantation with minimal complications is now achieved for most patients in the presence of new immunosuppressive medications such as Tacrolimus[17].

The present study revealed histological results of atrophic changes in the epithelium as well as the lingual papillae of lingual mucosa of tacrolimus treated rats. Loss of normal shape of lingual papillae and degeneration of taste buds cells were observed. Degeneration of collagen fibers in the subepithelial lamina propria of the tongues in addition to a lot of inflammatory cell infiltrate were distinguished in tacrolimus treated rats.

The prevalence of infection in patients with organ transplantation is excessive. 40% of deaths among patients with organ transplantation are due to complications of infections. Between one and six months after transplantation, patients are susceptible to life threatening viral infections such as cytomegalovirus due to heavy immunosuppression.[18]

Scanning electron microscopic examinations revealed marked degeneration of the tongue papillae and a white film coating of candida albicans covering the tongues under investigations.

After six months, the risk of chronic viral infections such as hepatitis B, hepatitis C and fungal infections as candida increase. Millsop J.W. et al, 2016 reported that one of major systemic causes of oral candidiasis is immunosuppression therapy. These findings can explain the presence of signs of inflammation and the presence of chronic inflammatory cells in the lamina propria of tacrolimus treated group of rats. Also explains the huge amount of candida colonies on the mucous membrane of the dorsal surface of the tongues in this investigation seen clinically and confirmed under scanning electron microscope.[19]

It was reported that matrix metalloproteinases (MMPs) are the primary group of enzymes responsible for the breakdown of collagen and other proteins in the extracellular matrix (ECM). Collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and additional unclassified MMPs are the six groups that make up the MMP family. These findings explain the degradation of collagen fibers in lamina propria of mucous membrane of tongues of rats treated with tacrolimus. [20]

The results of the present investigation revealed that the filiform, fungiform and circumvallate papillae were atrophic with marked degeneration of their cells. The taste buds in fungiform and circumvallate papilla were atrophic. Degeneration of collagen fibers in lamina propria of connective tissues and inflammatory cells infiltration mainly as macrophages, neutrophils and lymphocytes were observed.

These inflammatory cells are able to produce inflammatory cytokines such as interleukin-1B and tissue necrosis factor (TNF-α) which are known to cause degenerative changes in the epithelium and connective tissue and IL-1B which stimulates the production of proteolytic enzymes including matrix metalloproteinases and plasminogen activators.[21]

Thus the changes that occurred in the tissues under investigation as a result of immunosuppression may be due to direct toxic effect of the drug used causing cell damage with release of lysosomal enzymes of damaged cells leading to degeneration and necrosis.[22]

According to these results, it is suggested that the administration of tacrolimus in systemic long-term way induces deterioration of antioxidant enzymatic defense of salivary glands of rats, which may lead to altered composition of saliva and alteration in the morphology explaining the results of the present investigation[23].

Proliferating cell nuclear antigen (PCNA) was evaluated as a marker of cell proliferation in formalin-fixed rat liver tissue and PCNA labeling technique was effective for evaluating cell proliferation rates.[24]

PCNA immunohistochemical stained sections showed marked decrease in the staining reactivity and significant decline in labeling index of PCNA; denoting decrease in the proliferation and turnover rate of the stratum basal cells of the surface epithelium. During analysis of the influence of immunosuppressive drugs on apoptosis and PCNA in the ventral prostate of rats.

These results are in accordance with the present results of immunostaining for PCNA which revealed decrease in mitotic activity resulting in atrophy of the surface epithelium of the tongues of group II animals. Moreover it has been reported that tacrolimus is one of the drugs causing hearing loss as a side effect due to cellular degeneration. .[25]

It has been reported that tacrolimus triggered a significant increase of apoptosis in epithelial cells and decrease in proliferation in both epithelial and stromal cells.[26] These apoptotic changes can explain the same results found when the tongues of tacrolimus treated rats were stained for PCNA and seen under light microscope showed decrease in proliferation in basal cells of epithelium.[27]

According to prior opinions and investigations, the use of topical and systemic tacrolimus, after balancing the potential risks and benefits, may be beneficial in immunosuppression as it successfully suppresses graft rejection while lowering death rates. However, due to the high toxicity and side effects of tacrolimus, its use should be limited and under supervision.

CONCLUSION

Administration of tacrolimus revealed alteration in the structure and ultrastucture of the lingual papillae will definitely affect their functions among them mainly the taste sensation.

RECOMMENDATIONS

The utilization of high doses of tacrolimus for long periods should be confined to the demonstrated cases and follow up of these cases is important. Patient treated with oral tacrolimus for long period should preserve great oral hygiene to keep away from the development of fungal infections and lingual salivary glands diseases.

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