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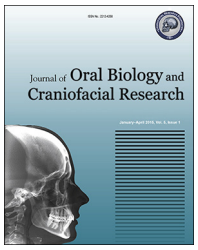

Original Article

Efficacy of azithromycin and metronidazole combined therapy on rats' gingival overgrowth induced by cyclosporine-A: An experimental animal study

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ABSTRACT

Aims: To evaluate the therapeutic efficacy of azithromycin (azm) and/or metronidazole (mtz) on the histopathological features of rats' gingival overgrowth (GO) induced by cyclosporine-A (CsA) in an animal model.

Methods: Ninety male albino rats were divided randomly into six equal groups. The rats of group I received corn oil via gastric feeding for 7 weeks. Group II rats were administered CsA for the same period. Groups III, IV, and V rats received CsA for 6 weeks and simultaneously in the 7th week received a monotherapy of placebo gel, azm suspension, mtz gel, respectively. Group VI rats were handled as groups III, IV, and V and instead received a combined therapy of azm suspension, and mtz gel. Rats were euthanized at the end of the experiment and routine tissue processing was carried out. The obtained specimens were stained with H&E, TGF-β, MMP-1, and IL-6 antibodies.

Results: One-way MANOVA test for TGF-β, MMP-1, and IL-6 revealed an overall significant difference between the different groups ($P = 0.000$). LSD post hoc test for multiple comparisons of TGF-β revealed nonsignificant difference between groups I and VI and between groups IV and V. Nonsignificant difference was found between groups II and III considering the amount of MMP-1 immune expression. In addition, nonsignificant difference was found between groups V and VI regarding the amount of immune expression for IL-6.

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Conclusion: Combined therapy of azm suspension and mtz gel significantly improved the histopathological features of CsA-induced GO better than a monotherapy of azm suspension or mtz gel.

1. Introduction

There are many risk factors involved in gingival overgrowth (GO): hereditary factors as gingival fibromatosis, factors associated with metabolic imbalances, including diabetes and endocrinopathies or may occur among patients as an adverse effect to certain systemic medications, such as antiepileptic – phenytoin, calcium pump inhibitor – nifedipine, or immunosuppressive drugs – cyclosporine-A (CsA).[1]

CsA is a potent immunosuppressive drug used to prevent graft rejection. Prevalence of GO ranges from 20% to 35% for CsA-treated patients. Certain risk factors are directly correlated and affect condition, such as level of plaque control, gender (men three times more sensitive), and drug daily dose, or are inversely correlated, such as age.[2] GO usually affects facial surface of interdental papilla and it may appear on gingival margins and lingual surfaces. It interferes with patient's oral hygiene leading to an increase in susceptibility to infections, caries, and periodontal disease.[3] There is no effective and predictable method of managing this condition. Treatment may involve optional drug therapy, for example, replacing CsA with tacrolimus as an alternative immunosuppressant.[4]

Plaque control and removal of local irritants have been shown to be of some benefit.[5] However, no effective medical treatment is available and gingival surgery is sometimes necessary. Wahlstrom et al.[6] have described an improvement of gingival hyperplasia following treatment with azithromycin (azm), an azalide antimicrobial agent derived from the macrolide antibiotic erythromycin. The azm possesses good activity against common gram-positive and some gram-negative pathogens.[7] It has been approved by the Food and Drug Administration for pediatric use and is available in intravenous or oral preparation. Oral absorption is rapid with 37% bioavailability and it is not thought to affect hepatic enzyme metabolism.[8] Local delivery of metronidazole (mtz) in association with nonsurgical periodontal therapy has been recommended in patients affected by adult chronic periodontitis. The mtz is a synthetic antibacterial and antiprotozoal agent, which is effective against many microbes, including anaerobes, *Trichomonas*, and *Clostridium* species. It is well absorbed orally and metabolized in the liver.[9]

There is an increase in the expression of specific cytokines, especially transforming growth factor-β (TGF-β) in drug-induced GO.[10] TGF-β induces fibroblast differentiation into myofibroblasts, which are considered predominant cells in matrix synthesis, leading to accumulation of extracellular matrix.[11] In addition, CsA inhibits matrix metalloproteinase-1 (MMP-1) expression at both mRNA and protein level in a dose- and time-dependent manner. MMP-1 mRNA expression was significantly reduced in overgrown compared to normal tissue.[12] One of the pathogenic mechanisms underlying drug-induced GO may be enhanced secretion of interleukin-6 (IL-6) by gingival fibroblast in response to these medications.[13] The levels of IL-6 extracted from overgrown gingival tissue were significantly higher than in inflamed or normal tissue.[14] The present study was designed to evaluate the combined therapeutic efficacy of azm and mtz on the treatment of rats' GO induced by CsA. The null hypothesis of this animal study was that there were no differences between the monotherapy with either azm or mtz and combined therapy of azm and mtz.

2. Materials and methods

2.1. Animals and design

Ninety male pathogen-free white albino rats, aged 2 weeks, were selected. Calculation of sample size was done by power analysis (G* Power, version 3.0.10). The effect size and the standard deviation were calculated from a pilot study. Power analysis was based on type 1 error value of 5% ($\alpha = 0.05$) and on a power of 0.80 sample size; the direction of the effect was two tailed. The rats were kept in a light-controlled room with a 12:12-h light–dark cycle, and 22 °C temperature. Relative humidity of 65–70% was kept constant. They received commercial diet and water. All experimental procedures were performed under a protocol approved by the ethical committee of Faculty of Dentistry, Mansoura University, Egypt. The rats were divided randomly into 6 equal groups of 15 animals each.

Group I: The rats received corn oil via gastric feeding for 7 weeks and were considered as a control.

Group II: A soft gelatinous capsule of CsA (100 mg, Sandimmune, Novartis Pharmaceuticals Corporation, Hanover, Germany) was dissolved in 16.5 cm^3 corn oil. The dissolved drug was administered by gastric syringe for 7 weeks; each rat received 30 mg/kg daily.[15]

Group III: The rats received CsA in corn oil for 6 weeks, and simultaneously in the 7th week, placebo gel was carefully placed topically in the bottom of the gingival sulci of all anterior and posterior teeth using syringe with a blunt cannula.

Group IV: The rats received CsA in corn oil for 6 weeks and simultaneously in the 7th week; azm suspension (Zithromax, Pfizer Labs, New York, USA) was given in a dose of 10 mg/kg via gastric feeding.[15]

Group V: The rats received CsA in corn oil for 6 weeks and simultaneously in the 7th week; mtz gel (Elyzol 25%, Cabon SpA, Milan, Italy) was topically placed in the

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bottom of the gingival sulci of all anterior and posterior teeth.[16]

Group VI: The rats received CsA in corn oil for 6 weeks and simultaneously in the 7th week; azm suspension was given in a dose of 10 mg/kg via gastric feeding and additionally mtz gel was topically placed in the bottom of the gingival sulci of all anterior and posterior teeth.

2.2. Biopsy collection

Rats were anesthetized with a ketamine and valium mix (75 mg/ml ketamine, Parke Davis, Sydney, Australia, and 5 mg/ml valium, Roche Products Ltd, Selwyn Garden City, UK). Then, the rats were euthanized with an overdose of halothane. Scissors were used to cut the angles of the mouth backwards between the upper and lower jaws, and the muscles were completely removed. Then, the lower jaw was dislocated by pulling it down, and biopsies were collected by the following two methods. First, a spoon excavator was used to reflect the marginal gingiva; then, it was continually resected from the right side of the mandible opposite molar region using a scalpel. Second, mandibular specimens from the left side of the mandible, including molar teeth with surrounding bone and gingival tissue, were cut in a buccolingual direction using a scalpel.

2.3. Histological examination

The obtained specimens were prepared at 4 μm and stained with:

(1) Hematoxylin and eosin stain (H&E)
(2) Immunohistochemical staining that was performed using:
   (a) TGF-β (1:50 dilution, cytoplasmic; AbD SeroTec, Killington, Oxford, UK) to determine the amount of collagen metabolism and collagen biosynthesis in the connective tissue of the gingiva.
   (b) MMP-1 (1:50 dilution, cytoplasmic; Bioss Antibodies, Woburn, MA, USA) to detect the amount of extracellular collagen degradation in the connective tissue of the gingiva.
   (c) IL-6 (1:100 dilution, secreted protein; Bioss Antibodies, Woburn, MA, USA.) as a cytokine responsible for activating the proliferation of T and B cells.

2.4. Routine tissue processing

The tissue was fixed immediately using 10% formaldehyde prepared in phosphate buffer saline (PBS) for 24 h. Processing was completed using an automatic tissue processor, which took the tissue through a series of graded alcohol baths to dehydrate the tissues and followed by xylene to clear it. Then, hot paraffin was used to permeate tissues. The processed tissue was embedded in paraffin blocks by warming the block mold on hot plate and filling it with hot paraffin. After the tissue was placed in the mold in desired orientation, the cassette was placed over the mold. Additional melted paraffin was poured onto the cassette. The mold was then cooled and the paraffin block was removed out of the mold.

Samples that contained tooth were decalcified in 4% EDTA solution (Hexis Cientifica, Brazil) for about two weeks. By using a microtome (Leica Biosystems, Nussloch GmbH, Germany), paraffin blocks were cut into sections; the thickness of each is 4 μm. Sections were mounted on opti plus slides. Slides with paraffin sections were then placed in a 65 °C oven for 20 min to bond the tissue to the glass. Slides with paraffin-embedded tissue sections were stored at room temperature and paraffin was dissolved by immersing the sections in xylol.

2.5. Staining techniques

2.5.1. 1-Hematoxylin and eosin stain

Sections were placed in descending grades of absolute alcohol 90%, 70%, 50%, and then distilled water. Staining with the basophilic hematoxylin was carried out first, and then sections were washed in tap water. Counter staining was with the acidic eosin, which stains acidophilic structures red.

2.6. Immunohistochemical staining

Immunostaining was performed using the avidin–biotin complex (ABC) method according to manufacture instructions.

2.7. Procedure

Slides were deparaffinized in xylol and rehydrated in descending grades of alcohol. Blocking endogenous peroxidase using 30% hydrogen peroxide in methanol for 10 min was done followed by washing in PBS. Antigen retrieval was performed in citrate buffer solution at 95 °C to induce refolding of target antigen. Slides were allowed to cool, and then washed in PBS. Serum blocking solution was added for 10 min, and then rinsed without washing. The specific antibody was applied for 1 h at room temperature followed by washing in PBS three times. This was followed by secondary antibody for 10 min, and then washing in PBS.

The slides were then covered by avidin enzyme conjugate for 10 min, and then washed in PBS. Diaminobenzidine (DAB) was used as a chromogen for 5 min at room temperature. The DAB chromogen yielded a reddish-brown reaction end product at the site of target antigen. Slides were counterstained with Meyer's Hematoxylin, dehydrated, and covered.

2.8. Evaluation and scoring of GO using H&E stained slides

Double-blinded calibrated examiners performed the microscopic analysis of the buccal gingiva of decalcified specimens using a light microscope (Olympus, New York, USA). Each examiner had approximately 15 years of experience in investigating biopsy specimens under a light microscope in their practical laboratories. One expert examiner read specimens, and two days later, the same examiner read all the specimens again to evaluate the intraexaminer variability. Then, the same specimens were read by the second examiner to assess the interexaminer variability (Table 1). A modified scoring method was used according to the index described by Banthia et al. in 2014.[17] The crown length was divided into three equal parts (cervical, middle, and occlusal). The hyperplastic

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Table 1 – Statistical results for intraexaminer and interexaminer variability attributed to scored samples.

Features	Intraexaminer variability			Interexaminer variability		
	First reading mean ± SD	Second reading mean ± SD	*P* value	First reading mean ± SD	Second reading mean ± SD	*P* value
Degree of GO	2.9 ± 0.8	2.7 ± 0.7	0.600	0.3 ± 0.7	0.3 ± 0.5	0.726
Shape of gingival crest	0.3 ± 0.1	0.2 ± 0.1	0.153	0.5 ± 0.5	0.6 ± 0.5	0.661

index measured the degree of gingival enlargement in an apico-coronal direction by means of the following 4-point scale:

(0) No GO (gingiva still in the cervical one-third)

(1) Mild (gingiva overgrown to the middle one-third)
(2) Moderate (gingiva overgrown to the lower half of the occlusal one-third)
(3) Severe (gingiva overgrown to the upper half or above the occlusal one-third)

Moreover, the shape of the gingival crest was scored as follows:

(0) Knife edge,

(1) Blunted, or
(2) Bulbous shaped.

2.9. *Quantification of immunohistochemical stained slides*

Five different fields of each slide of the outer surface of the buccal gingiva were imaged at 40× magnification using an Olympus digital camera installed on an Olympus microscope with a 1/2× photo adaptor using 40× objective. The resulting images were analyzed on an Intel Core I3-based computer using Video Test Morphology software (Saint Petersburg) with a specific built-in automated object. The analysis provided an estimated quantification of immunohistochemical results. Immune-reactivity of the cells was scored as the follow:

(0) =100% of cells were negative,

(1) Mild = (>0–<10%) of cells were positive,
(2) Moderate = (>10–<50%) of cells were positive, and

(3) Intense = (>50%) of the cells were positive.

2.10. *Statistical analysis*

The data were tabulated, coded, and then analyzed using statistical package for social science (SPSS version 17.0). For the analytical statistics and due to several dependent variables that include TGF-β, MMP-1 and IL-6, and one independent factor that includes randomization into several groups, the significant differences were tested using one-way multivariate analyses of variance (MANOVA) to compare between more than two groups for the numerical parametric data. MANOVA measures the mean scores between multiple groups and assumes a cause–effect relationship whereby the group factors cause the significant difference of TGF-β, MMP-1, and IL-6 characteristics. MANOVA test was followed by post hoc least significant difference (LSD) for multiple comparisons. LSD test computes the pooled standard deviation from all groups and thus increasing the statistical power. Moreover, the Kruskal–Wallis test was used to compare between more than two groups for categorical data, followed by the Mann–Whitney *U* test to compare two groups. The statistical tests were based on a type 1 error value of 5% ($\alpha = 0.05$) and on a power of 0.85 sample size. *P* value <0.05 represents level of significance.

3. Results

3.1. *Hematoxylin and eosin stain findings*

3.1.1. *Degree of GO*

Groups II, III, IV, V, and VI rats showed GO with variable extents over the crown of teeth, while group I showed score 0 GO with knife edge gingival crest (80%) and 20% with blunt edge. Groups II and III yielded scores 3 or 2 GO with blunt gingival crest (60%) and 40% with bulbous gingival crest. Groups IV and V showed score 2 or 1 GO with knife edge (80%) and 20% with blunt edge. Group VI revealed less pronounced GO (100% score 1) with knife edge.

The Kruskal–Wallis statistical test revealed an overall significance between the different groups ($P = 0.000$). The highest mean ranks were for group II (73.47) followed by group III (71.83), while the lowest one was with group I (8.00). The mean ranks for groups IV, V, and VI were 48.30, 42.90, and 28.50, respectively. The Mann–Whitney *U* revealed significant differences between groups I & II, I & III, I & IV, I & V, I & VI and between II & IV, II & V, II & VI and between III & IV, III & V, III & VI and between IV & VI and between V & VI. Nonsignificant differences were found between groups II & III and IV & V (Table 2).

3.1.2. *Histological findings*

Fig. 1A illustrates normal stratification of the epithelial layers that were separated by flat basement membrane from a fully collagenized connective tissue for group I. Collagenized connective tissue was covered by hyperplastic epithelium with elongated thin and slender rete processes for group II (Fig. 1B). Collagenized connective tissue was covered by hyperplastic epithelium with elongated broad rete processes for group III (Fig. 1C). Collagenized stroma was covered with slightly thickened epithelial layers and flat basement membrane for group IV (Fig. 1D). Collagenized stroma was covered with hyperplastic epithelial layers with broad rete processes. Some spinous cells are enlarged and have pyknotic nuclei for group V (Fig. 1E). Less collagenized connective tissue was

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Table 2 – Nonparametric statistical tests used to determine the degree of GO for all groups.

Kruskal–Wallis		Mann–Whitney *U*				
Groups	Mean rank	Groups	Mann–Whitney *U*	Wilcoxon W	Z	P2 value
I	8.00	I*II	0.000	120.00	−5.14	0.000**
II	73.47	I*III	0.000	120.00	−5.10	0.000**
III	71.83	I*IV	0.000	120.00	−5.14	0.000**
IV	48.30	I*V	0.000	120.00	−5.07	0.000**
V	42.90	I*VI	0.000	120.00	−5.38	0.000**
VI	28.50	II*III	105.0	225.00	−0.39	0.695**,†
Chi-Square	75.39	II*IV	22.00	142.00	−4.13	0.000**
df	5	II*V	16.00	136.00	−4.27	0.000**
P1 value	0.000*	II*VI	0.000	120.00	−5.14	0.000**
		III*IV	27.50	147.50	−3.91	0.000**
		III*V	20.00	140.00	−4.11	0.000**
		III*VI	0.000	120.00	−5.10	0.000**
		IV*V	90.00	210.00	−1.11	0.264**,†
		IV*VI	30.00	150.00	−4.09	0.000**
		V*VI	52.50	172.50	−3.24	0.001**

* Kruskal–Wallis test.
** Mann–Whitney.
P1 < 0.05.
P2 < 0.05.
† P > 0.05 (Mann–Whitney *U*).

covered with thin epithelial layers and flat basement membrane for group VI (Fig. 1F).

3.2. *Immunohistochemical findings*

3.2.1. *TGF-β*

Group I showed moderate expression of TGF-β within epithelium and connective tissue. Group II showed intense immune expression of the protein at epithelial and connective tissue. The epithelial reaction was obvious at basal and suprabasal layer. Group III had an intense immune reaction at both epithelial and mesenchymal cells. Group IV had a moderate reaction within the connective tissue, while the epithelium was negative. Group V had a moderate immune reaction within epithelial cells specifically at the basal cells, while the connective tissue had an intense reaction. Group VI had moderate reaction within epithelial and connective tissue and mostly the reaction was at connective tissue.

3.2.2. *MMP-1*

Group I showed moderate reaction to MMP-1 mainly within the connective tissue, while the epithelial had few and scattered positive cells. Group II showed mild reaction to MMP-1 within epithelium. Group III had mild reaction to MMP-1 within epithelial cells, while moderate reaction to MMP-1 was seen at connective tissue. Group IV had a moderate reaction to MMP-1 within epithelial and connective tissue. Group V had a patchy reaction to MMP-1 within epithelial cells and the connective tissue had mild reaction. Group VI had mild reaction to MMP-1 mainly within basal cells of epithelium.

3.2.3. *IL-6*

Group I showed patchy reaction to IL-6 within epithelium. Group II showed intense reaction to IL-6 mainly within epithelium. The reaction was mild within connective tissue. Group III had an intense reaction to IL-6 at both epithelial and connective tissue. Group IV had a mild reaction to IL-6 within epithelial and connective tissue cells. Group V had a mild reaction to IL-6 within the epithelial cells. Group VI had a mild reaction to IL-6 within the epithelial cells, while connective tissue was negative; the reaction appears as patches.

3.2.4. *Statistical results*

The means and standard deviation for TGF-β, MMP-1, and IL-6 are illustrated in Table 3 and Fig. 2. Regarding TGF-β, the highest mean values were in groups II (69.27 ± 3.24) and III (71.20 ± 2.85), while the lowest mean values were in group I (15.33 ± 1.71). The mean values for groups IV, V, and VI were 19.67 ± 2.02, 19.87 ± 1.84, and 16.20 ± 1.01, respectively. Regarding MMP-1, the highest mean values were in group I (46.47 ± 1.99), while the lowest mean values were in groups II (11.67 ± 1.39) and III (11.20 ± 1.26). The mean values for groups IV, V, and IV were 17.33 ± 1.75, 19.27 ± 1.48, and 33.67 ± 0.90, respectively. Considering IL-6, the highest mean values were for groups II (61.00 ± 2.77) and III (71.00 ± 2.77), while the lowest values were for groups IV (4.13 ± 1.35), V (3.53 ± 0.64), and VI (2.13 ± 0.64). The mean value for group I was 38.47 ± 3.06.

One-way MANOVA test for TGF-β, MMP-1, and IL-6 revealed an overall significant difference between the different groups (P = 0.000) (Table 3). LSD post hoc test for multiple comparisons of TGF-β revealed nonsignificant difference between groups I and VI and between groups IV and V. Nonsignificant difference was found between groups II and III considering the amount of MMP-1 immune expression. In addition, nonsignificant difference was found between groups V and VI regarding the amount of immune expression for IL-6 (Table 3).

4. Discussion

In the present study, groups II and III yielded scores 3 or 2 GO, collagenized connective tissue covered by hyperplastic

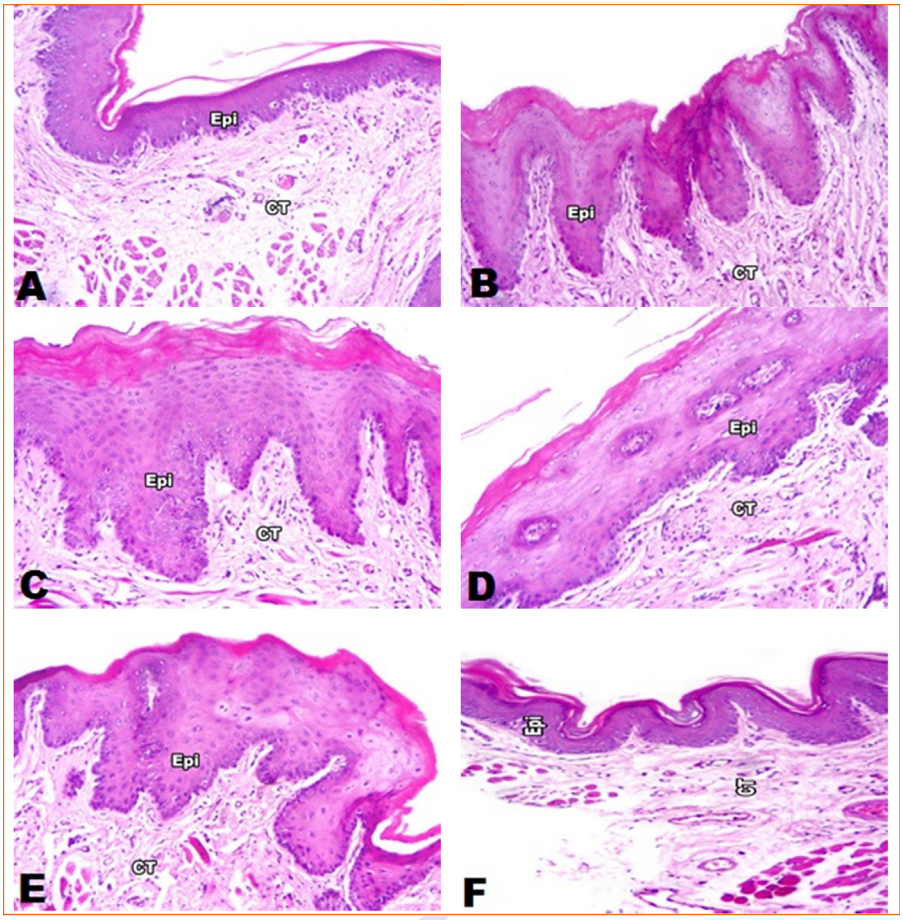

Fig. 1 – Photomicrograph shows normal stratification of the epithelial layers that separated by flat basement membrane from a fully collagenized connective tissue for group I (A). Collagenized connective tissue covered by hyperplastic epithelium with elongated thin and slender rete processes for group II (B). Collagenized connective tissue covered by hyperplastic epithelium with elongated broad rete processes for group III (C). Collagenized stroma covered with slightly thickened epithelial layers and flat basement membrane for group IV (D). Collagenized stroma covered with hyperplastic epithelial layers with broad rete processes. Some spinous cells are enlarged and have pyknotic nuclei for group V (E). Less collagenized connective tissue that covered with thin epithelial layers and flat basement membrane for group VI (F) (H&E 100×).

epithelium with elongated rete processes, an intense immune expression of TGF-β and IL-6, and mild reaction to MMP-1. This finding is consistent with the results of Dreyfuss et al.[18] who found an overexpression of TGF-β1 and TGF-β type II receptor in the normal gingiva treated with CsA. They concluded that extracellular matrix accumulation in these diseases was modulated by TGF-β and TGF-β receptor. In addition, Lauer et al.[19] concluded that CsA in low concentrations, as applied in

Table 3 – One-way MANOVA and LSD post hoc test for TGF-β, MMP-1, and IL-6, for all groups.

Pillai's trace (F ratio and P value)				LSD post hoc (Mean ± SD)			
Groups (121.451, 0.000)				Groups	TGF-β	MMP-1	IL-6
One-way MANOVA (F ratio and P value)	Groups	TGF-β	(2201.975, 0.000*)	I	$15.33 \pm 1.71^{**\|\|}$	$46.47 \pm 1.99^{**}$	$38.47 \pm 3.06^{**}$
		MMP-1	(1559.748, 0.000*)	II	$69.27 \pm 3.24^{**}$	$11.67 \pm 1.39^{**\neq}$	$61.00 \pm 2.77^{**}$
		IL-6	(3253.111, 0.000*)	III	$71.20 \pm 2.85^{**}$	$11.20 \pm 1.26^{**\neq}$	$71.00 \pm 2.77^{**}$
				IV	$19.67 \pm 2.02^{**\ddagger}$	$17.33 \pm 1.75^{**}$	$4.13 \pm 1.35^{**}$
				V	$19.87 \pm 1.84^{**\ddagger}$	$19.27 \pm 1.48^{**}$	$3.53 \pm 0.64^{**\dagger}$
				VI	$16.20 \pm 1.01^{**\|\|}$	$33.67 \pm 0.90^{**}$	$2.13 \pm 0.64^{**\dagger}$

* One-way MANOVA.
** LSD.
$P < 0.05$.
Superscripted same symbols in the same column means nonsignificant at level $P > 0.05$.

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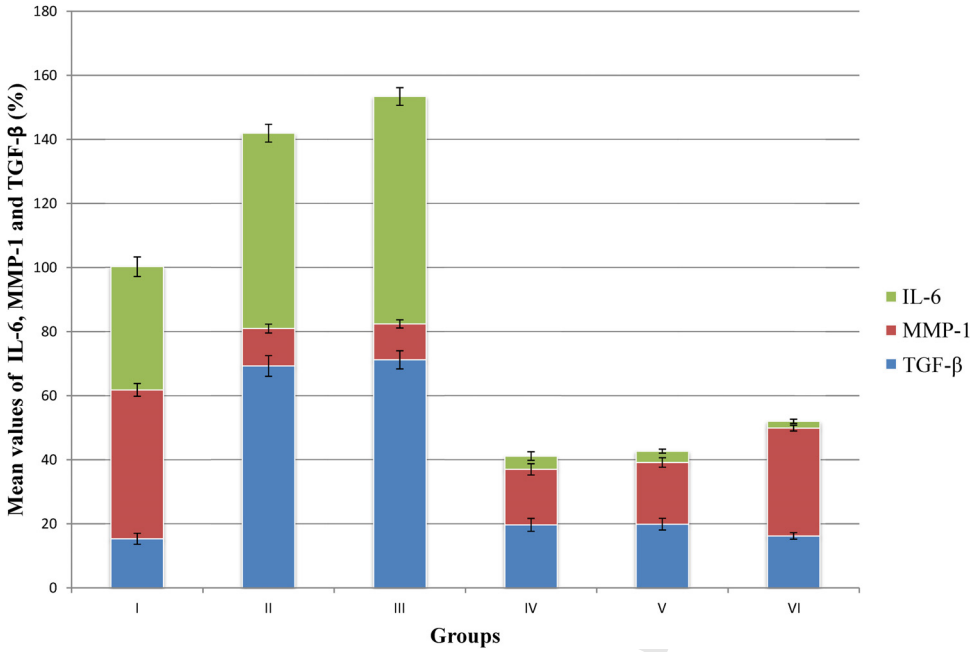

Fig. 2 – Bar chart for TGF-β, MMP-1, and IL-6 mean values for all different groups.

long-term therapy stimulates gingival keratinocyte growth and therefore might be related to hyperplasia of the gingiva. Maita et al.[20] reported that MMP-1 secretion was inhibited significantly by CsA and the amount of its secretion from normal and periodontitis gingival fibroblast specimens did not differ, but that from the overgrown gingiva was significantly less than the other types. Mohamed et al.[21] found that rats administered CsA revealed the highest immunoreactivities for TGF-β and for IL-6 and the lowest level to MMP-1 expression.

Group IV and V showed score 2 or 1 GO, collagenized stroma covered with slightly thickened epithelial layers and flat basement membrane, moderate to an intense immune reaction for TGF-β, mild to moderate reaction to MMP-1, and mild reaction to IL-6. These findings are consistent with the results of Condé et al.[22] who found that that roxithromycin treatment was effective in reducing CsA-induced GO in rats. Both connective tissue and epithelium showed a decrease in thickness and a significant reduction in TGF-β2 expression, with a lower number of fibroblasts, reduction in fibrotic areas, and decrease in inflammatory infiltrate. They suggested that the downregulation of TGF-β2 expression may be an important mechanism of action by which roxithromycin inhibits GO. In addition, Kim et al.[23] reported that azm elevated the reduced MMP-1 and MMP-2 activities in CsA-treated renal transplant fibroblasts and normal fibroblasts. In CsA-treated renal transplant fibroblasts, azm blocked the accumulation of total collagen in culture media and the increase in type I collagen mRNA level, but recovered the reduced MMP-2 mRNA level to the control. Moreover, Namazi et al.[24] suggested some immunomodulatory effects for azm, such as a decrease of IL-1α and tumor necrotizing factor-α (TNF-α) as a mechanism for the treatment of phenytoin-induced GO in epileptic patients.

Elyzol 25% dental gel contains metronidazole in the form of benzoate as active principle and it is a proprietary medicine conceived to be applied in the periodontal pocket. Rizzo et al.[25] found that mtz inhibits the production of IL-1β, IL-6, IL-8, IL-12, and TNF-α cytokines induced by *Porphyromonas gingivalis*-lipopolysaccharide treatment on human periodontal ligament cells. Montebugnoli et al.[16] concluded that mtz and placebo are equally effective in reducing plaque index, bleeding on probing, probing depth, and GO when associated with scaling and root planing. Long-term results, however, showed greater efficacy of mtz with respect to placebo in controlling CsA-induced GO. In contrary, Aufricht et al.[26] concluded that oral metronidazole does not improve CsA-induced GO.

Group VI revealed less pronounced GO (100% score 1) with knife edge, less collagenized connective tissue that covered with thin epithelial layers and flat basement membrane, moderate reaction to TGF-β, mild reaction to MMP-1, and a negative connective tissue reaction to IL-6. This can be attributed to the good results obtained by the combined therapy of azm and mtz for treating CsA-induced GO. A number of treatment options are utilized in the treatment of GO, including CO_2 laser surgery, improved oral hygiene, the use of antibiotics, such as metronidazole and azithromycin, and surgical intervention.[27]

The *P* value was less than 0.01 and thus the null hypothesis of the present study was rejected. Thus, the combined therapy was better for the treatment of CsA-induced GO compared to monotherapy of azm or mtz. To the best of our knowledge, this is the first animal study aimed to evaluate the combined therapy of azm and mtz for the treatment of CsA-induced GO using TGF-β, MMP-1, and IL-6 as diagnostic immunohistochemical markers. In previous studies, they evaluated a monotherapy of azm,[22–24] mtz,[16,26] or metronidazole vs. azithromycin for treatment of CsA-induced GO.[28,29]

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5. Conclusion

Combined therapy of azm suspension and mtz gel significantly improved the histopathological features of CsA-induced GO better than a monotherapy of azm suspension or mtz gel.

Conflicts of interest

The authors have none to declare.

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