Influence of diltiazem in combination with a sucrose-rich diet on gingival alterations in rats

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Abstract: The aim of this study was to evaluate the influence of diltiazem in combination with a sucrose-rich diet on gingival alterations in rats. One hundred and twenty male Holtzman rats were randomly assigned to 10 groups (n = 12), being 2 control groups treated with saline and 8 test groups treated with diltiazem in daily doses of 5, 25, 50 and 100 mg/kg during 40 or 60 days. Afterwards, the mandibles were removed for macroscopic, histologic and histometric analyses of the buccal gingiva of the mandibular right first molar. No macroscopic characteristic of gingival overgrowth was observed in any of the groups. The microscopic analysis showed characteristics of normality with inflammatory cells only adjacent to the crevicular epithelium in all groups for both periods. The histometric analysis showed significant differences only for the epithelial tissue area in the 40-day period (Kruskal-Wallis; $P = 0.032$). Comparing the periods, significant differences regarding the connective and epithelial tissue areas were observed only in the group treated with a 25 mg/kg dose (Mann-Whitney; $P = 0.004$ and $P = 0.007$, respectively). Oral administration of diltiazem in combination with a sucrose-rich diet did not induce gingival alterations in rats.

Descriptors: Diltiazem; Sucrose; Gingiva; Rats.

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Introduction

Calcium channel blockers have been widely used in the treatment of cardiovascular diseases and may be classified into four structural subclasses according to their chemical and pharmacological characteristics: phenylalkylamines (e.g., verapamil), dihydropiridines (e.g., nifedipine, nicardipine, isradipine, amlodipine, nitrendipine, felodipine), benzothiazepines (e.g., diltiazem) and T-channel antagonists (e.g., mibefradil).[1]

The side-effects of calcium channel blockers include headache, facial redness, dizziness, peripheral edema and gingival overgrowth.[2] The development of gingival overgrowth in patients treated with nifedipine, associated or not with other drugs such as cyclosporin-A, has been extensively reported.[2,3] On the other hand, there are few reports of diltiazem-induced gingival overgrowth in humans.[4-7] The findings of a recent study[8] showed that in rats with non-inflamed gingival tissue, diltiazem did not induce gingival overgrowth. Nevertheless, the pathogenesis of drug-induced gingival enlargement is uncertain and conflicting and has been related to several factors, namely age, gender, treatment duration, plasmatic and salivary drug concentration, presence of bacterial biofilm, gingival tissue inflammation, genetics and synergism with other medicaments.[9,10]

Considering that these factors are important determinants of gingival overgrowth, the purpose of this study was to evaluate the influence of diltiazem, in different doses and periods, in combination with a sucrose-rich diet on gingival alterations in rats.

Material and Methods

The study protocol was approved by the Ethics in Animal Research Committee, São Paulo State University, Araraquara, SP, Brazil (Process No. 04/2004).

One hundred and twenty male Holtzman rats (*Norvegicus albinus*) weighing approximately 70 g were randomly assigned to 10 groups of 12 animals each. The room temperature was thermostatically regulated to 22°C ± 1°C and the humidity was maintained at 60% ± 5%. The time of exposure to light was automatically controlled (12h30min/day).

Two groups were used as control and received saline (JP Indústria Farmacêutica, Ribeirão Preto, SP, Brazil) orally during the experimental periods. The other 8 groups received diltiazem (Alcon Biosciences Pvt. Ltd., Munbai, India) therapy. Diltiazem was administered orally in daily doses of 5, 25, 50 or 100 mg/kg body weight during 40 or 60 days, using a hypodermic needle and an injector device assembled manually.

The animals were fed daily a sucrose-rich diet composed of 56% refined sugar, 30% integral powdered milk and 14% standard ground chow,[11,12] which were weighed in an electronic balance and mixed manually. The food was prepared daily by adding a small amount of water to obtain a pasty consistence.[2,13] The goal of this diet was to prevent self cleaning during mastication and facilitate bacterial biofilm accumulation in order to induce an inflammatory process.[14] During the experimental period the body weight gain of all animals was evaluated. Afterwards, the animals were sacrificed and the mandibles were carefully removed together with the gingival tissue surrounding the teeth and stored in 10% buffered formalin solution (Synth, Diadema, SP, Brazil).

Macroscopic analysis

The presence of characteristics of gingival overgrowth such as volume increase of the gingival margin and interdental papilla and clinical inflammatory alterations, namely alterations in gingival contouring, color and brightness, were examined by a single calibrated blind examiner using a magnifying glass.

Histological analysis

After macroscopic examination, the mandibles were demineralized in Morse solution (Synth, Diadema, SP, Brazil) (50 ml 50% formic acid and 50 ml 20% sodium citrate) during 2 months, the decalcifying solution being changed every 3 days. Five-micrometer-thick serial paraffin-embedded sections (buccolingual) were obtained from the region of the mandibular right first molar and stained with hematoxylin (Mallinckrodt Baker Inc., Paris, KY, USA) and eosin (Nuclear, Diadema, SP, Brazil).

Microscopic analysis

The microscopic analysis was performed on the buccal gingiva of the mandibular right first molar by a single calibrated blind examiner using a light transmission microscope BX51 (Olympus, Melville, NY, USA). Cell adhesion, number of cell layers and presence/absence of deep papillary interdigitations were examined in the epithelial tissue whereas the morphology of collagen fibers and the number of cells and blood vessels were examined in the connective tissue.

The inflammatory response was evaluated according to a score scale:

1. presence of inflammatory cells only adjacent to the epithelium;
2. presence of inflammatory cells throughout the connective tissue;
3. presence of inflammatory cells close to the alveolar bone.

Histometric analysis

For the histometric analysis, four glass microscope slides were examined *per* animal, using a previously described technique.[8] Briefly, the slides were selected in a standardized manner, representing the initial, intermediate and final portions of the gingival margin, thus totalizing 10 readings of histological sections at 60-μm intervals for each animal. The histometric analysis was performed using a DIASTAR optical microscope (Leica Reichert & Jung Products, Wetzlar, Germany) with an objective lens 10/0.25 adapted to a video camera (Sony DXC - 107A, Sony Electronics Inc., Shinagawa-ku, Tokyo, Japan) and connected to a computer. Gingival epithelium and connective tissue areas were measured using image-analysis software (Sigma Scan®; Mocha; Jandel Scientific, San Rafael, CA, USA). The lower limit of the connective and epithelial tissue areas measured with the software was the end of the junctional epithelium. Statistical analysis was based on the average obtained for each animal.

Statistical analysis

Statistical analysis of the histometric data was performed by the Mann-Whitney test using *dose* as a factor and by the Kruskal-Wallis test using *administration period* as a factor. The significance level was set at 5%.

Results

Macroscopic and microscopic data

All rats survived the experimental periods. Compared to the control animals, no animal treated with diltiazem had its body weight gain affected by any of the diltiazem doses for either the 40-day ($P = 0.271$) or the 60-day ($P = 0.186$) administration periods.

No macroscopic characteristic of gingival overgrowth was observed in any of the groups, either control or experimental, for both evaluations periods.

In addition, no microscopic alteration suggestive of gingival overgrowth was observed, regardless of the treatment protocol. In all groups, the epithelium had 3-5 cell layers without hyperplasia or deep papillary interdigitations. The connective tissue had a normal number of collagen fibers and blood vessels and few macrophages (Figure 1).

The application of the score scale to evaluate the inflammatory response showed the presence of inflammatory cells only adjacent to the crevicular epithelium (score 1) in all groups for both evaluation periods.

Histometric analysis

Table 1 shows the results of the Kruskal-Wallis test used to evaluate the effect of the different doses (5, 25, 50 and 100 mg/kg) of diltiazem on the connective and epithelial tissue areas of the marginal gingiva of the buccal face of mandibular right first molar. No statistically significant difference was observed in the connective tissue area for both periods [40 days ($P = 0.128$) and 60 days ($P = 0.278$)]. Regarding the epithelial tissue area, there was significant difference only for the 40-day period ($P = 0.032$). The application of the multiple-comparison test (Dunn's Method) did not show statistically significant difference between the comparisons. However, the results of the groups in which 25 mg/kg and 100 mg/kg were administered were close to significance when compared to the control groups.

Table 2 shows the results of the Mann-Whitney test used to assess the effect of the duration of diltiazem therapy (40 and 60 days) on the connective

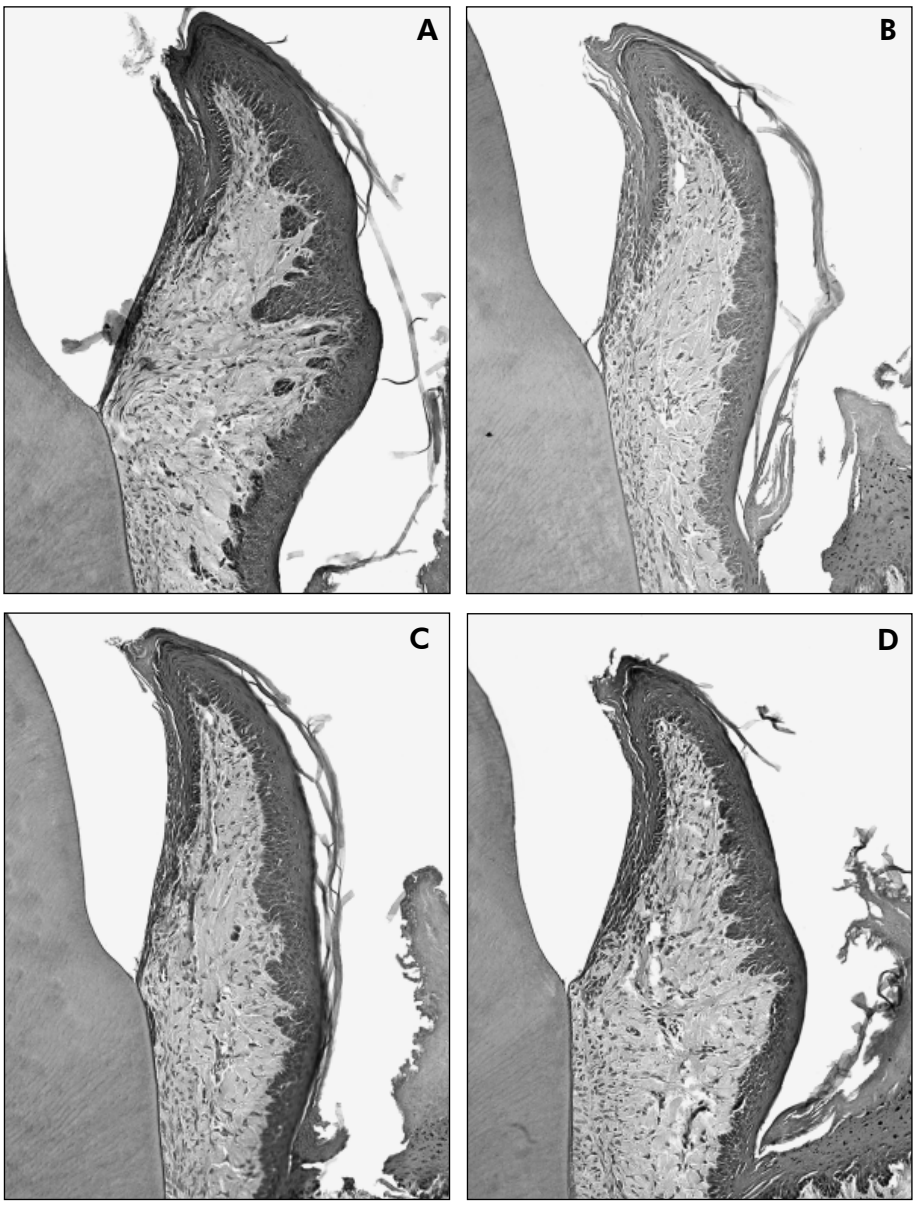

Figure 1 - Buccolingual sections of the buccal region of the first mandibular molar from the control groups after the 40- and 60-day periods (1A and 1C, respectively) and test groups, treated with 100 mg/kg of diltiazem, after the same periods (1B and 1D, respectively). No alteration in the gingival tissues was observed (Hematoxylin and Eosin stain, magnification x40).

and epithelial tissue areas of the marginal gingiva of the buccal surface of mandibular right first molar. Statistically significant differences for the connective and epithelial tissue areas between the 40- and 60-day periods were observed only in the group that received a 25 mg/kg dose ($P = 0.004$ and $P = 0.007$, respectively). For the other doses, the experimental period did not influence the induction of gingival alteration in the animals treated with diltiazem.

Discussion

The hypothesis investigated in the present study was raised from our recent findings, which showed that the treatment with increasing doses of diltiazem in rats did not induce gingival alterations.[8] At the same time, it has been suggested that drug-induced gingival overgrowth is related to factors such as treatment duration, presence of bacterial biofilm and gingival inflammation.[9,10]

Therefore, our hypothesis was to explore in rats treated with diltiazem whether gingival inflammation, secondary to a sucrose-rich diet, would induce gingival alterations.

The animal model used in this study (rat) has

Table 1 - Histometric analysis of the influence of different administration doses of diltiazem on the connective tissue (A1) and epithelial tissue (A2) areas (mm^2) of the buccal gingiva of the mandibular right first molar of rats.

Area	Period (days)	Dose (mg/kg)	Means ± SD	Kruskal-Wallis
A1	40	Control	16.73 ± 5.64	$P = 0.128$
		5	17.15 ± 8.06	
		25	12.58 ± 3.19	
		50	17.37 ± 9.51	
		100	12.75 ± 3.54	
A2	40	Control	29.53 ± 5.21	$P = 0.032$*
		5	28.39 ± 6.61	
		25	23.34 ± 5.00	
		50	27.96 ± 8.18	
		100	23.69 ± 3.32	
A1	60	Control	19.81 ± 11.58	$P = 0.278$
		5	15.36 ± 5.73	
		25	21.50 ± 8.29	
		50	20.14 ± 9.33	
		100	16.44 ± 11.15	
A2	60	Control	32.66 ± 13.34	$P = 0.163$
		5	26.25 ± 7.93	
		25	32.42 ± 8.08	
		50	32.83 ± 9.87	
		100	26.75 ± 9.35	

*Statistically significant difference.

Table 2 - Histometric analysis of the influence of different administration periods of diltiazem on the connective tissue (A1) and epithelial tissue (A2) areas (mm^2) of the buccal gingiva of the mandibular right first molar of rats.

Area	Period (days)	Dose (mg/kg)	Means ± SD	Mann-Whitney
A1	Control	40	16.73 ± 5.64	$P = 0.908$
		60	19.81 ± 11.58	
	5	40	17.15 ± 8.06	$P = 0.525$
		60	15.36 ± 5.73	
	25	40	12.58 ± 3.19	$P = 0.004$*
		60	21.50 ± 8.29	
	50	40	17.37 ± 9.51	$P = 0.324$
		60	20.14 ± 9.33	
	100	40	12.75 ± 3.54	$P = 0.908$
		60	16.44 ± 11.15	
A2	Control	40	29.53 ± 5.21	$P = 0.686$
		60	32.66 ± 13.34	
	5	40	28.39 ± 6.61	$P = 0.326$
		60	26.25 ± 7.93	
	25	40	23.34 ± 5.00	$P = 0.007$*
		60	32.42 ± 8.08	
	50	40	27.96 ± 8.18	$P = 0.291$
		60	32.83 ± 9.87	
	100	40	23.69 ± 3.32	$P = 0.564$
		60	26.75 ± 9.35	

*Statistically significant difference.

been widely employed to investigate the effect of drugs on the oral environment because it provides more uniform responses compared to humans and allows better control of several important variables including genetic predisposition, gender, age, dose and duration of drug administration.[15]

The diltiazem-treated rats did not present clinical evidence of toxicity that compromised their weight gain or survival. In fact, the findings of previous investigations have shown that the administration of diltiazem in the doses used in the present study did not induce toxicity and that the treatment was well supported by the animals.[8,13,16,17]

Macroscopically, the sucrose-rich diet induced dental biofilm accumulations in all animals though none of them exhibited clinical characteristics of gingival overgrowth, which confirms our previous findings.[8] However, Morisaki *et al.*[16] (2000) observed gingival overgrowth in rats after administration of diltiazem in doses greater than those used in the present study during a similar experimental period. Microscopically, there was only a mild inflammation in the animals (Figure 1), which is particularly relevant because the diet followed by the animals was expected to cause gingivitis. Based on previous studies[11,12,18] that described the sucrose-rich diet as a model to induce periodontal disease (gingivitis and periodontitis), we aimed to promote natural accumulation of supragingival plaque on the dentogingival area and consequently experimental

gingivitis. However, our result was similar to that of Galvão *et al.*[19] (2003), who reported that a sucrose-rich diet was not capable of promoting more inflammation than that seen in rats treated with standard rat lab chow.

Another possible explanation for our results would be the influence of diltiazem on the biochemical composition of saliva. Dehpour *et al.*[20] (1995) evaluated the effects of calcium channel blockers on the functions of the parotid and submandibular glands of rats. They reported that the administration of 10 mg/kg of diltiazem decreased significantly the flow and the concentration of calcium (both glands) and amylase (parotid gland) in the saliva of these animals. Moreover, Rudiger *et al.*[21] (2002) have demonstrated that there is a greater concentration of salivary amylase in the presence of gingival inflammation caused by a greater biofilm accumulation. They have also shown that the concentration of proteins present in saliva may modify the bacterial adhesion and the initial composition of dental biofilm. Given the alterations relative to modification of the amount of salivary amylase that might be triggered by diltiazem, it may be speculated that there was less formation of dental biofilm or an alteration of its composition in the present study.

In humans, the presence of inflammation in the pathogenesis of gingival growth is debatable. Miranda *et al.*[7] (2005) observed that individuals in therapy with diltiazem or nifedipine presented greater gingival enlargement when the use of these drugs was associated with the presence of gingivitis. On the other hand, Morisaki *et al.*[17] (1993) reported that nifedipine induced gingival overgrowth in rats with or without gingival inflammation and/or dental biofilm, these factors being able to potentialize the effect of the drug.

In the present study, the histometric analysis showed no statistically significant difference among the groups for most part of the sample. Only the group that received a dose of 25 mg/kg of diltiazem presented a significant increase of the connective and epithelial tissue areas when comparing the 40- and 60-day periods (Table 2). Since this finding was not observed when the higher doses were administered, there is reason to believe that it might be a casual finding.

The results of this study showed that different doses of diltiazem did not influence the development of gingival overgrowth (Table 1). Correa *et al.*[8] (2005) had similar results, although using lower doses of diltiazem. Another study[16] reported gingival overgrowth in rats after administration of diltiazem in doses > 1,000 mg/kg body weight for 40 days, which is much greater than the maximum dose used in our study (100 mg/kg). The rationale for choosing these doses of diltiazem was to simulate as close as possible the doses used in humans based on the fact that, in rats, 5 mg/kg correspond to the average daily intake of a hypertensive adult patient.

Another factor taken into account was the administration route. In our previous study,[8] the drug was administered subcutaneously while in the present investigation the drug was given orally. The rationale was to simulate the administration route of diltiazem in humans. Nevertheless, this factor was proved not to have a significant influence on the outcomes, given that the results of the present study were similar to those reported by Correa *et al.*[8] (2005).

Most studies that evaluated diltiazem-induced gingival enlargement in humans[4-7] had to deal with a heterogenous sample regarding age, duration of drug use, dose and other variables. Therefore, further research using animal models should be undertaken to evaluate the behavior of gingival tissue after long-term use of diltiazem, in order to evaluate other mechanisms that might be involved in gingival overgrowth, not only gingival inflammation.

Conclusion

According to the methodology proposed and based on the results of this study, the following conclusions may be drawn: the oral administration of diltiazem associated with a sucrose-rich diet did not induce gingival alterations in rats regardless of the dose (5, 25, 50 and 100 mg/kg) and the duration of the treatment (40 or 60 days).

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