THESIS SUMMARY
DOCTOR'S DEGREE THESIS IN BIOMEDICAL SCIENCES
Study of the Mandibular Bone Dynamics and of the Alveolar Crest Resorption Processes in Diabetic and Control Rats

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When healthy[1][2], the gingiva is a specialized mucosal tissue adapted to the tooth. The junctional epithelium attaches the gingiva to the tooth and interacts as a seal between the buccal environment and the supporting tissues. The periodontal ligament connects the tooth roots to the dental alveolus.

The tooth supporting tissue is affected by two pathologies: gingivitis and periodontitis. Gingivitis is an inflammatory response of the gingiva to the dental plaque with no destruction of the tissue that surrounds the tooth. Periodontitis is an extension of the inflammatory process with destruction of the connective tissue and the ligament, and bone loss[3]. The bacteria in plaque are the main cause of the periodontal illness.

The structure of the periodontal tissue in rats is similar to that in human beings[4]. Therefore, the former has been used in studies of periodontal illness[5][6][7][8].

Periodontal illness has been termed the sixth complication of diabetes[9]. Most of the clinical and epidemiological evidence reveals that people with diabetes have higher rates of periodontitis, and a more severe or fast-progressing form of periodontitis than those with no diabetes[10][11][12]. The defects in the function of neutriphils, including disturbed chemotaxis, phagocytosis, and death have been informed in people with diabetes[13][14][15]. Published studies associate the delay in cicatrization and periodontitis in diabetes with the alteration of the genetic expression of cytokines produced by macrophages[16-19]. Thus, the deficit of cytokines -produced by hypofunction of the macrophages induced by diabetes-, can lead to a general systemic alteration. This alteration produces a delay or defect in cicatrization, leading to severe periodontitis. Alterations induced by diabetes in the host's defense mechanisms or in tissue oxygenation can favor the growth of certain germs. This creates an environment in which the anaerobic or more virulent periodontal microflora prevails[20-24].

"Bone metabolism illnesses" are those illnesses in which the bone formation and bone resorption coupling mechanism are disturbed. In accordance with this definition, the loss of periodontal bone is a focal osteoporosis. The increase of concurrent osteoclastic activity is produced by the inflammation originated by bacterial activity. Bisphosphonates are synthetic compounds -similar to pyrophosphate- which are firmly attached to calcium phosphate crystals, inhibiting their growth, and eliminating the bone resorption mediated by osteoclasts. As a consequence, they are used in the treatment of disorders of the skeleton.

MATERIALS AND METHODS

Male rats were used, IIM/Fm line, "m" sub line, 2 to 15 weeks old, produced by the School of Medical Sciences' laboratory animals' facility (biotery). The experiments were carried out pursuant to the principles stated in the guide produced by the United States National Health Institutes[25].

At the time the animals were entered into the study protocol, their identification number and body weight were recorded. Animals were overexposed to ethyl ether vapor and killed at the ages indicated below. The mandibles were dissected and placed in boiling water for 5 minutes. Tissues were removed with surgery tools. They were kept in peroxide at 3% for 24 hours and dried at 37º C until they reached constant weight.

Dry weights were reached using a Sauter analytical scale, with precision of one tenth of a milligram.

The volume of hemimandibles was measured by the Archimedes method. The pieces were weighed, hung, and dipped in distilled water. The difference (g) at weighing measures the volume in milliliters.

For the study of evolution of spontaneous periodontal illness in rats, the following techniques were used:

a) X-ray densitometry

Clean hemimandibles and a variable-thickness aluminum scale were x-rayed on an X-ray plate. The plates were scanned and digitized. Digital image analysis software (Image Pro Plus 1.0) permitted absorbance comparison.

b) Measurement of the supporting periodontal bone (HPS)

Measurements were taken on the digital image. On each root, measurements were taken:

- between the amelo-cementum line and the apex (LAC-A).
- between the bottom of the bone defect at its greatest depth and the apex (AB).

Pixel lengths were converted into millimeters using the aluminum scale width (5mm). HPS was calculated with the following formula:

$$HPS = \frac{[(AB\ Distance)\ X\ 100\ ]}{(LAC\text{-}A\ Distance)}$$

c) Measurement of the distance between the amelo-cementum line and the alveolar bone crest (LAC-COA)

The left hemimandibles were colored for a minute with methylene blue (1 g/100 ml) to clearly indicate the amelo-cementum line (LAC). The distance between the amelo-cementum junction and the alveolar bone crest (COA) was measured with a binocular

microscope (30X) with a graded scale on each eyepiece. The shortest appreciable distance was 37 μmm per division. Measurements were taken on the free surfaces of each molar. The measured lengths are expressed in millimeters.

- Induction of diabetes by aloxane

Three-week-old animals were employed with an average weight of 39 ± 7.5 g. (average ± standard error). The animals were fed ad libitum with a balanced diet (Cargill). Diabetes was induced according to the Prahl and Steenrod[26] method. The treated animals' glycaemia was measured once a week within the 20-30 days after the aloxane administration. Determination was carried out thru the enzymatic method (Trinder), using the enzymatic glycaemia equipment belonging to Laboratorio Wiener. Plasma was obtained from the blood of non-fasted animal tails in heparinized capillary tubes. The samples were centrifuged to separate the plasma. The tubes were coded: white, standard and samples, which were incubated in double boilers at 37º C and read in a PERKIN ELMER photocolorimeter at 530 nm wavelength. The results were calculated as follows:

Cc. glucose in plasma = Cc witness . sample reading / witness reading

Rats with lower than 300 mg/dl glycaemia were rejected[27]. The animals received no treatment until killed.

- **Treatment with bisphosphonates**

A bisphosphonate solution (20 mg) was prepared in 100 ml. distilled water. The animals received an intraperitoneal dose of 30 micrograms (0.15 ml solution) every 100 g weight, once a week, from the fifth week of age, for seven running weeks. The animals were killed at the age of 12 weeks. The control animals received similar quantities of physiologic saline.

- **Statistics techniques and line adjustment according to time**

For the comparison of experimental groups, Student's t-test and variance analysis were used[28]. The time-related modifications of dry weight, volume, and density were analyzed thru data adjustment with Boltzmann's equation. Adjustments were made with a statistics program which contains the non-lineal adjustment of the function to the data. Given two or more apparently satisfactory functions, the function with the highest R^2 was chosen.

RESULTS

1) CONTROL RATS

A) GROWTH

The body weight and hemimandible dry weight followed a sigmoid function typical of growth. Coincidence of the inflection point of both lines indicated that both processes took place in harmony.
The hemimandible maximum growth rate took place at the 6th week. The head weight / body weight ratio was higher at birth than at maturity.

B) PERIODONTAL ILLNESS

Bone resorption focuses appeared at the 4th week of age. The evolution of the total number of focuses reached a plateau in 5, at the 6th – 7th week of age. The total resorption area, measured in square millimeters (mm^2) in vestibule-lingual projection, increased from the 3rd to the 6th week of age, then reaching the plateau.

Normal periodontal bone mineral density reached its highest point at the 6th week and, from then on, it remained stable. The periodontal bone in resorption did not show a defined trend between the 6th and 15th week of age. The resorption areas lost, on average, per unit of projection area, 56% of the normal periodontal bone mineral.

The distance between the amelo-cementum line and the alveolar bone crest was higher on the lingual surface than on the vestibular surface in the three molars.

Supporting periodontal bone (HPS) kinetics in the three roots (mesial, middle and distal) of the 1st molar followed a lineal function with a gradient that is no different from zero. Average insertion is 60%.

C) HISTOLOGIC STUDIES

At the 4th week of age, mononucleated cell clusters are observed near the gingival sulcus and in the periodontal ligament close to alveolar bone resorption areas. These indicated inflammation or the start of bacterial inflammation. The images analysis indicates that the alveolar bone shows a high rate of bone remodellation, as revealed by the root's normal osteoclastic resorption tunnels, consistent with the rat age, with a high growth rate.

When the 6, 12 and 19-week-old rat hemimandibles were histologically analyzed, no mononucleated cell clusters were observed. Moreover, the histological image of the alveolar bone still showed intense resorptive metabolic activity in the absence of nearby mononucleated cells and without bone formation activity. This is discussed below –as a hypothesis- as an indirect proof of immunological tolerance to the plaque germs.

2) DIABETIC RATS

A) GROWTH

Diabetic and control animals' body weight evolved with a similar kinetics. Diabetic animals weighed, on average, 172.6 g less than the control animals. Loss of body weight produced by diabetes led to a higher hemimandible weight / body weight ratio than the ratio for the control animals.

No significant differences were observed in the dry weight and hemimandible volume, which can be attributed to the low proportion of soft tissue, which are the most damaged when suffering from diabetes. The hemimandible density revealed no significant differences between control and diabetic animals.

B) PERIODONTAL ILLNESS

As diabetes settled, the number of focuses grew more slowly than in the control animals, tending to reach similar or higher values towards the 12^{th} – 15^{th} week. The total area in resorption was smaller in diabetic rats than in control rats.

The mineral density of the periodontal bone without pathology also revealed the impact of diabetes. The evolution of this variable did not correlate with age. The mineral density of the periodontal bone in resorption in diabetic animals was significantly lower than in control animals.

The LAC-COA distance was bigger in the lingual side than in the vestibular side in the three molars.

3) THERAPEUTIC ASSAY WITH BISPHOSPHONATES

Results were compared with the variables values from 12-week-old control animals.

A) GROWTH

No significant differences regarding body weight were observed between control animals and treated animals. There were significant differences in the hemimandible dry weight between the control group and the group treated with lidadronate. However, no difference was observed between the control animals and the animals treated with olpadronate. Upon analysis of the mandibular volume, significant differences were observed between the control group and the group treated with lidadronate. However, no difference was observed between the control group and the group treated with olpadronate. The mandibular volume / alive weight (μl/100 gr) ratio was not significant among the groups.

B) PERIODONTAL ILLNESS

The number of resorption focuses revealed no significant differences between control animals and treated animals. The total value of resorption areas in lidadronate-treated rats was lower than the control group values and the olpadronate-treated group values. However, no significant differences were observed between the last two groups.

The mineral density of the periodontal bone with no pathology increased significantly in the olpadronate-treated group in comparison with the control group. No differences were observed between the control group and the lidadronate-treated group. The mineral density of the periodontal bone in resorption revealed no significant differences between the groups.

In the LAC-COA distance measurement, no significant differences were observed for the first and second molars between the control animals and the treated animals. However, in the lidadronate-treated group, the third molar showed a significant decrease in this distance.

DISCUSSION AND CONCLUSIONS

1) Growth

a) Control rats

The overall mandible growth of a normal rat is harmonious with body growth. From birth until the inflection point, growth can be represented by an exponential function which implies that the anabolic processes magnitude exceeds the catabolic processes magnitude. From the inflection point onwards, the catabolic and anabolic processes enter a complex regulation phase, probably genetically determined -undefined yet-, which helps to reach weight asymptote. Application of Parks' function[29] to the experimental results helps to define the inflection point, which might coincide with the start of puberty. As hypothesis support, it should be mentioned that the female rats are placed in the laboratory animals' facility breeding stock at the age of 7 weeks.

The hemimandible growth in dry weight and volume is harmonious with body weight growth. As a consequence, modifications to density (weight/volume) follow the same pattern. Density measured in the indicated terms is a function of matrix mineralization. Healthy periodontal bone mineralization coincides with the previous results.

b) Diabetic rats

Including diabetes in the development of "spontaneous" periodontal illness in rats had two objectives:

- To study the impact of diabetes mellitus on the hemimandible and the periodontium.
- To prove the techniques developed in the study of control animals in this experimental model.

Analysis of the effects of insulin on the bone cells has proved that insulin has an obvious anabolic effect on the matrix and the bone mineral[30-33]. Diabetes has an adverse effect on body growth, since it means severe alteration to the energy distribution efficiency[34]. Some results suggest that diabetes implies aging acceleration: Brody's constant associated with hunger kinetics is reduced regarding the control animals, and diabetic animals reach the body weight plateau before the control animals with lower absolute values[34]. This coincides with the results obtained in this Thesis, since diabetic rats showed a decrease in body weight, despite hyperphagia, when compared to control animals.

In diabetic rats by aloxane, growth stop has an obvious impact on all of the bone tissue variables[35-38]. Bone formation and resorption rates are reduced. The effect is clear even when their bone mass weight unit values are normalized[35]. Wettenhall et al[39] proved that, in comparison with the control group, diabetic rats by aloxane show a progressive decrease in femur dry weight, in diaphysiary cortex thickness, in metaphysiary trabecular bone volume, and in the growth cartilage thickness. The diabetic animals employed in this Thesis showed no significant difference in dry

weight, volume or mandibular density in comparison with the control animals. This may be due to the low proportion of soft tissue in the hemimandible, since the soft tissues are the most damaged in *diabetic status.*

c) Bisphosphonate-treated rats

Given the treatment short period and despite using the right dose of bisphosphonates, growth was not significantly affected. There was a significant increase in dry weight and mandibular volume in animals treated with lidadronate in comparison with control animals. These results are consistent with the well-known antiresorptive effect of such drugs[40].

2) Non-experimental periodontal illness

Periodontal illness was termed "spontaneous" because the periodontal illness that evolved was not experimentally induced by microorganism introduction or foreign elements in the sulcus.

In control rats, the periodontal bone resorption focuses appear on the 4th week, after weaning, and reach their highest point on the 6th or 7th week. Resorption total area follows the same evolution.

Periodontal bone mineral density reaches its maximum value on the 6th week and, then it remains constant. Resorption areas lose, on average, 56% of the normal periodontal bone mineral.

The LAC-COA distance increased in time and proved longer on the lingual surface than on the vestibular surface. This finding concurs with Jossi and Schoeder's study[41]. They discovered a bigger increase of this distance on the molars' lingual side than on the vestibular side. This difference on both sides might be related to the mandibular alveolar bone topography, which is thicker on the vestibular surface than on the lingual surface.

This Thesis studied "spontaneous" periodontal illness on 3 to 15-week-old rats, a fast-growing stage in a rat's life. During this period, as the molars erupt, there is a high rate of bone remodellation. As a consequence, three phenomena were combined to measure the supporting periodontal bone:

a) active eruption. This phenomenon, which is clinically or radiographically assessed, occurs between the third and the fifth weeks of age.
b) passive eruption. This physiological phenomenon occurs when the molars are in occlusion. By depositing cement by apposition on its apices, it compensates the attrition caused by chewing.
c) periodontal illness.

Therefore, this method is not applicable in the animal's active growth phase. Measurement of the supporting periodontal bone is a useful resource *after the animal has stopped growing*.

Effects of diabetes on the development of periodontal illness

The periodontal illness developed by the diabetic animals used in this Thesis was characterized by:

a) Higher number of resorption focuses than in control animals towards week 11, but slower growth. In spite of this, there was less resorption total area involved. The decrease in the bone turnover, and the likely immunological tolerance to the usual germs both in food and in the mouth, would account for this observation.
b) The mineral density of the periodontal bone with no pathology was, on average, 34% lower than in the control animals.
c) The mineral density of the periodontal bone in resorption was significantly lower than in the control animals.
d) Considering animals between the 6^{th} and 11^{th} weeks of age, and comparing the bone mineral density –with and without the pathology-, diabetic animals lose approximately 49% of mineral density in resorption areas.
e) Evolution of the LAC-COA distance regarding time for both surfaces in the three molars did not differ between diabetic and control animals.

Effect of the bisphosphonates treatment on the development of periodontal illness

Equal doses of two bisphosphonates did not produce the same results. Lidadronate reduced the resorption total area to values lower than the control group's values. Olpadronate did not produce any significant modification in this variable.

The mineral density of the periodontal bone with no pathology increased significantly in the olpadronate-treated group. This variable was not modified by the effects produced by the administration of lidadronate.

In the first and second molars, the distance between the amelo-cementum line and the alveolar bone crest did not differ between the control animals and the treated animals. However, in the third molar, this distance was significantly shorter in the lidadronate-treated group.

At first, these results confirm that the techniques employed permit observation of the modifications which are consistent with the bisphosphonates' antiosteoclastic effect.

The results from this research suggest two hypotheses:

A. Is "spontaneous" periodontal illness an example of immunological tolerance?

B. Are the modifications observed in very young rats' periodontium a result of the environmental antigens in a phase of immaturity of the immune system?

A. Is "spontaneous" periodontal illness an example of immunological tolerance?

The evidence accumulated in literature regarding periodontal bone loss has produced the following consensus: plaque bacteria produce inflammation. The cells typical of inflammation secrete cytokine interleukine-1 (IL-1), which has a well-known osteoclast recruiting ability. Osteoclasts begin the resorption of alveolar bone, thus originating the periodontal illness[42-46]. Tolerance to the dental plaque germs toxins may be explained as an immunological tolerance phenomenon.

Literature[47] reveals that foreign antigens can be administered so as to induce tolerance rather than immune response. In general, proteic antigens administered with adjuvants favor immunity, whereas high doses of antigens systemically administered without coadjuvants tend to produce tolerance. The most probable reason for this phenomenon is that adjuvants stimulate the expression of co-stimulators in antigen-presenting cells (CPA) and, in the absence of co-stimulation, T-lymphocytes, which recognize antigen, may become anergic. Tolerogenic antigens may also activate T-lymphocytes, which regulate or favor the differentiation of T lymphocytes in cytokine-producing cells, such as interleuquine-4 (IL-4), which do not induce cellular immunity.

B. Are the modifications observed in very young rats' periodontium a result of the environmental antigens in a phase of immaturity of the immune system?

The immune response, produced by immunization at an early age, is known to differ from the immune responses produced by adults. Certain studies indicate that the quantitative responses in young animals are feeble and slowly developed. Qualitatively, the response has a strong TH2 bias, with predominance of antibody production and poor cellular reaction[48-50].

In line "I" rats from this School of Medicine's laboratory animals' facility, Pascutti et al[51] have observed that inoculation with trypanosoma cruzi at weaning (3-4 weeks old) is spontaneously solved, although there is concurrence of high parasitemia. In adult animals (24-28 weeks old) the same treatment produced light illness with very low parasitemia. During the first week of infection, young rats showed significantly lower levels of immunoglobulin M (IgM), and immunoglobulin G (IgG) anti-T.-cruzi antibodies. In adult animals, the resistance increase seems to be the result of proper antibody production.

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