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Testosterone Deficiency Associated with Periodontal Disease Increases Alveolar Bone Resorption and Changes the Thickness of the Gingival Epithelium

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Authors' contributions

This work was carried out in collaboration between all authors. Author CGJ did the experimental studies, data and statistical analysis, manuscript preparation, editing and review. Authors RWW, MAM and LGAC did the experimental studies, participated in the acquisition and data analysis and manuscript preparation. Authors JPAA and EMPA were definition of intellectual content, design, experimental studies, data and statistical analysis, manuscript preparation and editing. All authors read and approved the final version of the manuscript.

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ABSTRACT

Aim: The relationship between steroid sex hormones and periodontal disease has been extensively investigated in females; however, studies with males are still scarce. The aim of the present study was to analyze the influence of testosterone deficiency on alveolar bone loss and on the histological structure of the periodontal tissues of castrated rats with experimental periodontitis.
Materials and Methods: To test the hypothesis, we used 28 male Wistar rats obtained from the

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Unioeste's Central Bioterium. When the animals reached 80 days of age, they were separated into four groups (N =7 animals/group): Control without ligature (CON), Control with ligature (CON+LIG), Castrated without ligature (CAST), and Castrated with ligature (CAST+LIG). At 90 days of age, the orchiectomy was performed in the appropriate groups. Sixty days after castration, the periodontal disease was induced by a ligation technique. At the end of the trials (90 days after castration), the animals were weighed and sacrificed using a CO2 chamber. Their jaws were removed, dissected, separated into the right and left counterparts, fixed in 10% (v/v) buffered formalin for 24 h, decalcified and processed for histological and radiological techniques.

Results: The results of this study showed that the ligature model was effective in inducing periodontitis in animals. The animals of the CAST and CAST+LIG groups showed significant reduction in body weight at the end of the trial period when compared to the CON and CON+LIG groups. Castration led to a significant bone loss in the animals, which was aggravated by the induction of periodontal disease. Animals with periodontal disease showed increased gingival epithelium area and connective tissue area when compared to the animals free of periodontitis.

Conclusion: We conclude that testosterone is an important physiological regulator of alveolar bone metabolism. Testosterone deficiency associated with periodontal disease increases alveolar bone resorption and changes the thickness of the gingival epithelium.

Keywords: Periodontal disease; testosterone deficiency; castration; periodontal tissues; bone loss.

1. INTRODUCTION

Testosterone (T) is the main circulating androgen in men, mostly (about 95%) produced and secreted by the Leydig cells in the testicles. The primary function of T is related to the development and maintenance of the reproductive organs and secondary sexual characteristics, essential for controlling the reproductive function and fertility. In addition to its role in reproductive endocrinology and fertility, T is an important hormone in the regulation and functioning of other organs and body tissues such as the kidney, heart, skeletal striated muscle, immune system, salivary glands, bone tissue, and oral and periodontal tissues [1,2].

It is well established in the scientific literature that the concentrations of T in men decrease with advancing age, specifically after 40 years of age, where a reduction of ~ 1-2% of the total concentration is expected [3,4]. At 75 years of age, the plasma concentration of the hormone is approximately two-thirds of the testosterone concentration at 25 years of age [5].

Studies using humans and experimental animals have demonstrated that the decrease of T concentration is a risk factor for the development of cardiovascular diseases [6], inflammation [7], metabolic syndrome [8], osteoporosis [9], and periodontal disease [10]. Periodontitis or periodontal disease (PD) is a chronic inflammatory infectious disease that affects the periodontium and, along with dental cavity, is one of the main causes for the loss of teeth [11]. Furthermore, it is also reported that a decrease in the concentration of T in men is related to changes in alveolar bone, such as increased alveolar porosity, change in trabecular pattern, alveolar bone resorption, alterations in the bone mineral density in the jaw and mandible, and an increase in the secretion of interleukins (IL-1 and IL-6) [12,13]. Following chronic periodontitis, these changes are more pronounced and may lead to tooth loss [10]. In experimental animals, Steffens et al. [14] showed that a decrease in T concentrations, induced by bilateral ligation, is associated with periodontal disease and increased bone loss.

These observations suggest that periodontal tissues are targets of action by androgens as these hormones are key factors in the pathogenesis of periodontal diseases. In addition, periodontal diseases can be a consequence of the actions and interactions of sex steroid hormones on specific cells found in the periodontium. However, this relationship has been poorly explored in men and their mechanisms are mostly unknown. Therefore, the present study aims to analyze the influence of testosterone deficiency on alveolar bone loss and on the histological structure of the periodontal tissues of castrated rats with experimental periodontitis.

2. MATERIALS AND METHODS

2.1 Animals

A total of twenty-eight adult male Wistar rats (80-days-old) were provided by the Bioterium of Unioeste University, Cascavel-PR. The animals

were adapted and maintained in the Sectorial Bioterium of the CCBS/Unioeste - Campus Cascavel. All animals were housed in polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding and maintained under controlled temperature settings (23 ± 1℃) and lighting conditions (12 h L, 12 h D photoperiod, lights switched off at 0700 h), and had free access to water and standard rodent chow Nuvital® (Nuvilab CR-1, Colombo, PR, Brazil). The rats were handled in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (BCAE) and approved by the UNIOESTE´s Ethics Committee for Animal Experimentation and Practical Classes.

2.2 Experimental Groups

The animals (N = 07/ group) were randomly divided into four experimental groups: Control without ligature (CON), Control with ligature (CON+LIG), Castrated without ligature (CAST), and Castrated with ligature (CAST+LIG).

To perform the castration technique, animals at 90 days of age underwent surgery with intraperitoneal anesthesia of 75 mg of ketamine and 15 mg of xylazine per kilogram of body weight (bw). We opened the scrotum in the midline with 2-cm incision and dissected the wall until the exposure of the testicles. The testicles were removed prior to ligation of the spermatic cord with a 3-0 cotton yarn. For the control group, the testicles were exposed, handled and reinserted in the scrotum, under the same experimental conditions of the castrated animals. All of the animals had their testicular bag closed with sutures with 4-0 nylon threads.

Sixty days after castration, experimental periodontitis was induced in specific groups through the ligature technique. For this procedure, the animals were anesthetized via intraperitoneal doses of ketamine (75 mg/Kg/bw) and xylazine (15 mg/kg/bw), being positioned on the operating table to allow the access to the posterior teeth of the jaw. With the aid of a modified clamp and a probe, a number 40 cotton yarn was placed around the first lower left and right molars. This ligature served as a gingival irritant for 30 days and favored the accumulation of bacterial plaque [15].

2.3 Sample Collection and Processing

At the end of the trial period (90 days after castration), all animals were weighed and sacrificed in a CO^2 chamber. Their jaws were removed, sagitously sectioned, separated into right and left, and fixed in 10% (v/v) buffered formalin for 24 h. After this period, all of the samples were washed in running water and kept in 70% alcohol solution.

The left hemimandibles were X-rayed, and later left decalcified in a descaling acid solution (Allkimia®) for 19 h and stored in 70% alcohol. Then, both samples were dehydrated in growing series of alcohols, put through transillumination in xylene and finally embedded in paraplast. For histomorphometric analysis, coronal plane cuts were made in the mesial to distal direction (anterior-posterior), with 5-µm thickness, using a manual rotary microtome (Olympus 4060), equipped with a steel knife. The sections were dewaxed with xylene, hydrated with distilled water and subjected to the staining technique using Hematoxylin-Eosin (HE) for analysis.

2.4 Radiographic Evaluation

Before descaling, the left hemimandibles were X-rayed by a single trained examiner, on two different days, using dental x-ray apparatus of the X-Dent D70 brand, kva 70 amp/60 Hz, with radiographic sensor of occlusal size by the DURR DENTAL VISTA manufacturer, with time of about 0.10 sec, 50cm focal length, so that the beam is directed perpendicular to the piece and the sensor. The pieces were placed so that the lingual surface of the hemimandible stayed in close contact with the film. The sensor reading was held by the reader DURR DENTAL VISTA SCAN MINI, SNB 224702049 registry. The images obtained were analyzed with the software Image Tool 3.0 and an average was taken from them by linear measure, which covered the distance from the cementoenamel junction to the alveolar bone crest of the mesial side of the first lower left molar of the rat, with the measurements in pixels [15].

2.5 Histomorphometric Analysis

The analysis of the gingival epithelium and the underlying connective tissue were performed using histological sections photographed with 100x magnification in an optical microscope (Leica Microsystems, Switzerland), with the use of the counting and capturing system LAS V 4.2 (Leica Microsystem) and 4 measurements were taken: height and width of the gingival epithelium, height, and width of the connective tissue. The calculation of the area was obtained by

multiplying the height and width of the respective regions [14].

2.6 Statistics

For data analysis, we used Analysis of Variance (ANOVA), complemented with the Tukey-Kramer test or the Kruskal-Wallis test complemented with Dunn test for non-parametric distribution, according to the characteristic of each variable. Statistical significance was set at $p<0.05$. The statistical software used was *Graph Pad Instat version 4.0* and *Sigma Plot version 11.0* for graphic design.

3. RESULTS

3.1 Food Consumption Pattern

Animals of the different experimental groups showed no significant differences in the pattern of food consumption following 12 weeks of measurements (Fig. 1).

3.2 Animals Body Weight

At the end of experiment period, all of the castrated animals, with or without periodontitis, showed a significant decrease in body weight when compared to the other groups (Table 1).

3.3 Radiographic Evaluation

Animals with experimental periodontitis (CON+LIG and CAST+LIG) presented greater distance fromthe cement-enamel junction in relation to the alveolar bone crest ($p<0.01$) after comparison to the groups without ligature (CON and CAST). Alveolar bone loss was severely increased in animals from the CAST+LIG group ($p<0.01$), when compared to the CON+LIG group (Fig. 2); These results reinforce the importance of the T in maintaining proper alveolar bone remodeling.

3.4 Histomorphometric Analysis

Animals with experimental periodontitis (CON+LIG and CAST+LIG) showed an increase in the area of the gingival epithelium ($p<0.001$) when compared to the control group (CON). In the absence of T, the area of gingival epithelium was increased in animals with a ligature (CAST+LIG), when compared to the castrated animals without ligature ($p<0.01$) (Table 2).

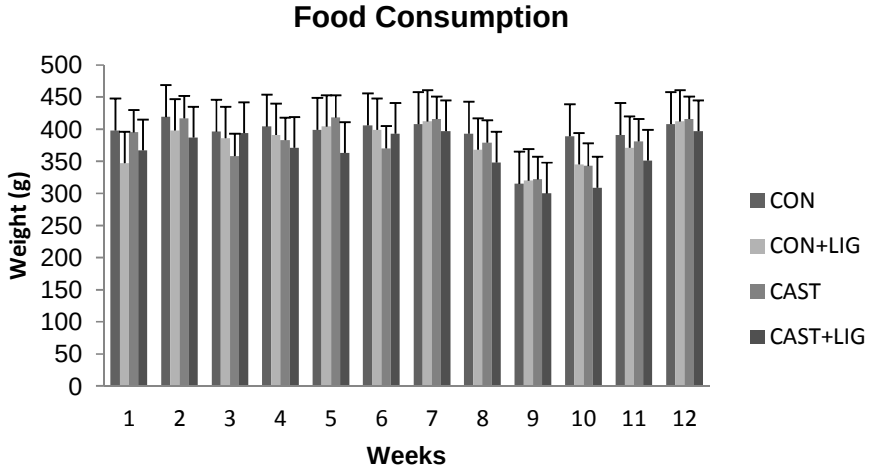

Fig. 1. Weekly food intake for the experimental groups. Values are expressed as the mean ± SD. Tukey-Kramer test; p<0.05, (n=7/group)

Table 1. Initial body weight (g) and final body weight (g) of animals from different experimental groups.

Parameters	CON	CON+LIG	CAST	CAST+LIG
Initial body weight (g)	329.57 ± 18.95	332.57 ± 15.75	331.85 ± 16.19	329.85 ± 15.38
Final body weight (g)	447.83± 22.06[a]	444.87±23.75 [a]	414.71±19.32 [b]	416.61±20.30 [b]

Values are expressed as the mean ± SD. Different letters indicate statistically significant differences between the experimental groups (p<0.01). Tukey-Kramer test; (n = 7/group)

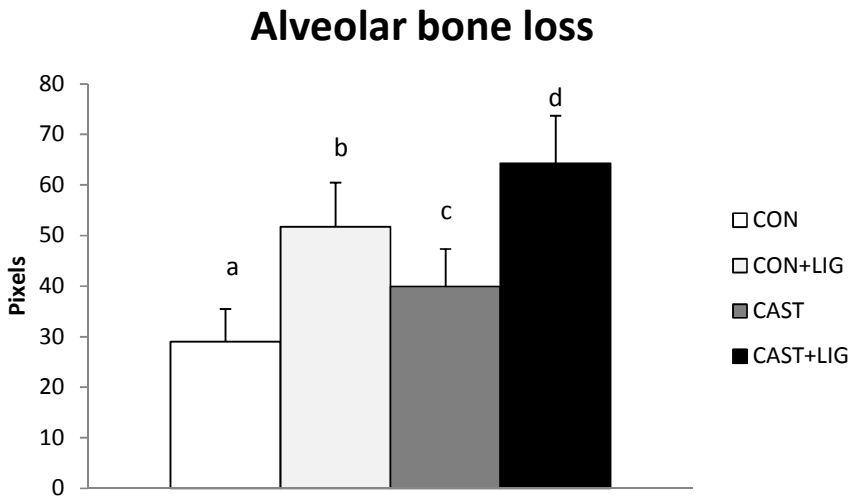

Fig. 2. Distance from the cement-enamel junction in relation to the alveolar bone crest of the animals of different experimental groups. Values are expressed as the mean ± SD

[a,b,c,d]Different letters indicate statistically significant differences between the experimental groups (p<0.01). Tukey-Kramer test; (n = 7/group)

Table 2. Gingival epithelium and connective tissue area (μm^2) of the animals of different experimental groups

Parameters	CON	CON+LIG	CAST	CAST+LIG
Gingival epithelium area (μm^2)	6.86 ± 1.64[a]	8.14 ± 1.47 [b]	7.54 ± 1.61[a,b]	8.85 ± 1.53 [c]
Connective tissue area (μm^2)	149.13 ± 33.78[a]	485.07 ± 38.45[b]	153.12 ± 36.52 [a]	537.33 ± 34.45[b]

Values are expressed as the mean ± SD. Different letters indicate statistically significant differences between the experimental groups ([a,b]p<0.01; [c] p<0.05 from CAST+LIG vs. CON+LIG). Tukey-Kramer test; (n = 7/group).

The area of the connective tissue was larger in animals with experimental periodontitis (CON+LIG and CAST+LIG) when compared to the groups without ligature (CON and CAST; p<0.01) (Table 2).

4. DISCUSSION

T deficiency or hypogonadism is a clinical condition in which the concentration of the hormone in men decreases, leading to various pathophysiological changes in the body. It affects about 30% of men aged 40-79 years [16]. In addition to aging, diseases such as diabetes, hypertension, and obesity are listed among the main causes of the increase in the prevalence of hypogonadism in men [17].

The castration of rodents, performed in this study, has been used experimentally in different studies to better understand the consequences of T deficiency in men with hypogonadism [18].

In addition to its role in the reproductive endocrinology, T is strongly related to body composition, influencing the metabolism of lipids and proteins. In men, the decrease of T is related to a decrease in muscle mass and an increase in the amount of body fat, which can lead to obesity and the associated metabolic syndrome [17,19]. Typical features of the metabolic syndrome, as well as an increase in body weight, have also been observed in neutered cats and dogs [20,21].

Although no change in the pattern of food consumption was observed, the castrated animals showed a decrease in the body weight when compared to uncastrated animals. Similar to our study, other experimental studies with rodents have shown a decrease in body weight and muscle mass in rats and mice after

castration [22,23]. The reasons for the interspecies differences are unclear but may be reflect a number of effects of T deficiency on physical activity and energy expenditure as well as those related to the appetite and food intake [24]. T deficiency is related to a reduction in the production and secretion of growth hormone (GH), an important regulator of the growth, somatic development, and body composition, leading subsequently to a decrease in the concentrations of IGF-1, one of the main mediators of the anabolic actions of GH [25]; this can be related to increased protein catabolism and, consequently, the decrease in muscle mass observed in rodents [23], hence the weight loss observed in our animals.

Another important target of androgenic actions in the body is in the bone tissue. Hypogonadism in men is associated with decreased mineral bone density, increasing the risk of fractures and osteoporosis [26]. In the context of dentistry, changes in the production, secretion, and concentration of sex hormones can drastically affect bone metabolism and may contribute to the development of periodontal disease and tooth loss. Periodontal disease, or periodontitis, is an infectious and inflammatory disease that results in the destruction of the support tissues of the teeth and can lead to tooth loss [27]. The role of T in the progression of periodontal disease has not yet been fully addressed [10].

In this study, all animals with experimentally-induced periodontitis had agreater alveolar bone loss when compared to the animals in the control and castrated without ligature, thus confirming the effectiveness of the experimental model [28]. Castration alone resulted in an alveolar bone loss greater than that observed in animals in the control group. The mechanism(s) by which T affects bone metabolism remains a matter of debate [29]. The action of T on thebone tissue can be mediated by direct binding to the androgen receptors, or indirectly through the regulation of growth factors.

Studies have shown that the osteoblasts, both in humans and in experimental animals, express androgen receptors [30], and researchers have demonstrated that androgens are able to stimulate the proliferation and differentiation of osteoblastic cells [31]. Thus, bone loss resulting from castration can be the result of damages in the formation of bone tissue. Chin et al. [32] showed that the supraphysiological replacement of T in castrated rats can prevent the degenerative bone tissue changes observed in animals. Gill et al. [33] further reported that T deficiency decreases the production of transforming growth factor (TGF-b) by osteoblasts in rodents. Past studies showed that the TGF-b produced by osteoblasts increases bone formation and inhibits bone reabsorption in humans and mice [34,35].

The most alveolar bone losses were observed in castrated animals with periodontitis. T deficiency is the main cause of osteoporosis in men [9]. Osteoporosis is related to a decrease in bone mineral density in the skeleton, including the jaw and mandible, resulting in increased alveolar bone porosity, altered trabecular pattern and increased the speed of resorption of the alveolar bone, after the invasion by periodontal pathogens. In addition, systemic factors that affect bone remodeling can also modify the local tissue response to periodontal infection, such as increased systemic release of interleukin-1 and interleukin-6, which stimulate osteoclastic activity as a result of bone loss [13,36].

In castrated animals with induced periodontitis, the gingival area was larger than that of the other experimental groups. Yarrow et al. [37] showed that the bone tissue acts as a reservoir of sex hormones, with potential biological effects in different tissues. These authors demonstrated that, although the castration of rodents decreases the concentration of sex hormones in the blood significantly, it did not alter the concentration of hormones stored in bone tissue, but it also did not prevent the decrease of bone mineral density in the animals; this suggest that the intraskeletal sex steroids are sequestered in a bone compartment incapable of protecting against bone loss. Steffens et al. [14] suggest that the conversion of T to dihydrotestosterone (DHT) occur in the bone reservoir after castration, and the increase in intraosseous DHT could be involved in the regulation mechanisms of increased gingival epithelium area.

We did not find significant changes in the connective tissue area that could be attributed to the androgen regulation. Regardless of castration, the connective area was greater in animals with induced periodontitis. This eventis probably related to the inflammatory process characteristic of periodontal disease. The release of the inflammatory mediators and interleukins, particularly interleukin-1, are associated to an increased collagen production by gingival fibroblasts. In addition, the inflammatory process

triggered by periodontal disease leads to an increase in edema and vasculogenesis, with increased formation of blood vessels in the gingival plexus [38].

5. CONCLUSIONS

We conclude that testosterone deficiency associated with the periodontal disease increases alveolar bone resorption and changes the thickness of the gingival epithelium, thus demonstrating that testosterone is an important physiological regulator of the alveolar bone metabolism.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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