UNESP – Universidade Estadual Paulista

Faculdade de Odontologia de Araraquara

JOÃO PAULO STEFFENS

EVIDÊNCIAS DA ASSOCIAÇÃO ENTRE TESTOSTERONA E DOENÇA PERIODONTAL NO SEXO MASCULINO

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Tese apresentada ao programa de Pós-Graduação em Odontologia - Área de Periodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista para título de doutor em Odontologia.

Orientador: Prof. Dr. Luis Carlos Spolidorio

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COMISSÃO JULGADORA

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(Martin Luther King)

Steffens JP. Evidências da associação entre testosterona e doença periodontal no sexo masculino [tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

RESUMO

A hipótese deste trabalho é que hormônios sexuais participam da etiopatogenia da doença periodontal (DP). Diferentes níveis de evidência científica testaram esta hipótese, avaliando: i. associação entre hormônios sexuais e DP em homens; ii. a influência de níveis sub- e suprafisiológicos de testosterona (T) sobre a DP em ratos; iii. se este mecanismo de ação envolve respostas de osteoblastos e osteoclastos in vitro. Dados do III NHANES relacionados com diagnóstico de DP e mensuração hormonal em homens com 30+ anos foram analisados para correlacionar estas duas variáveis. Em ratos, níveis subfisiológicos foram alcançados através da orquiectomia e níveis suprafisiológicos pelo tratamento com T. Metade dos animais em cada grupo foi submetida à DP utilizando–se modelo de ligadura. In vitro, células RAW264.7 foram diferenciadas em osteoclastos na presença de T (1nM-1μM) e identificados por TRAP. Cultura primária de osteoblastos murinos foi utilizada para avaliar a expressão de osteocalcina, RANKL e OPG na presença de T. Em homens, altos níveis de T biodisponível e baixa razão estradiol:T se correlacionaram significativamente com DP. Em idosos, baixos níveis de AAG, metabólito da dihidrotestosterona, também apresentaram correlação significativa. Em ratos, níveis sub- e suprafisiológicos de T aumentaram significativamente a perda óssea e modularam a expressão de citocinas inflamatórias. In vitro, doses fisiológicas de testosterona preveniram a osteoclastogênese e diminuíram a expressão de osteocalcina, RANKL e razão RANKL:OPG por osteoblastos. Concluiu-se que a T modula a resposta do hospedeiro à DP no sexo masculino, regulando a diferenciação de osteoclastos direta e indiretamente (via osteoblastos).

Palavra – chaves: Hormônios Esteroides Gonadais, Periodontite, Inflamação

Steffens JP. Evidence of the association between testosterone and periodontal disease in males [tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

ABSTRACT

The hypothesis of this work is that sex hormones participate in the etiopathogenesis of periodontal disease (PD). Different levels of scientific evidence tested that hypothesis, evaluating: i. The association between sex hormones and PD in men; ii. The influence of sub- and supraphysiologic testosterone (T) levels on PD in rats; iii. If that mechanism of action involves osteoblast and osteoclast responses *in vitro*. Data from NHANES III related to diagnosis of PD and hormones measurement in 30+-year-old men were assessed to correlate those two variables. In rats, subphysiologic levels were obtained by orchiectomy and supraphysiolgic levels by T treatment. Half of the animals in each group received PD using a ligature model. *In vitro*, RAW264.7 cells were differentiated into osteoclastos in the presence of T (1nM-1μM) and identified by TRAP-staining. Murine osteoblast primary culture was used to evaluate the expression of osteocalcin, RANKL and OPG in the presence of T. In men, high levels of bioavailable T and low estradiol:T ratio significantly correlated with PD. In older men, low levels of AAG, a metabolite of dihydrotestosterone, also presented a significant correlation. In rats, low and high T levels significantly increased bone loss and modulated the expression of inflammatory cytokines. *In vitro*, physiologic T doses prevented osteoclastogenesis and decreased the expression of osteocalcin, RANKL and RANKL:OPG ratio produced by osteoblasts. We concluded that T modulates host response to PD in males, regulating osteoclast differentiation direct and indirectly (through osteoblasts).

Keywords: Gonadal Steroid Hormones; Periodontitis; Inflammation

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INTRODUÇÃO

1 Introdução

O envelhecimento populacional é observado como uma tendência em muitos países do mundo. No Brasil, o Instituto Brasileiro de Geografia e Estatística (IBGE) estimou que, para a próxima década, a taxa de crescimento da população de 0 a 24 anos seja negativa, enquanto a população com 75 anos ou mais aumentará em 3,81%, chegando a um crescimento de 7,43% entre 2030 e 2050[4]. A transição demográfica, e consequente envelhecimento populacional, levam a uma transição epidemiológica, o que significa que a prevalência e incidência de doenças infecto-contagiosas decresce enquanto doenças crônicas se tornam mais frequentes. Dentre os vários fatores tempo-dependentes que podem desencadear diferentes doenças crônicas, a diminuição/supressão da produção de hormônios sexuais deverá constituir um importante problema, sendo que a mesma está intimamente relacionada com a resposta do hospedeiro às condições do organismo e externas[14].

A testosterona, principal hormônio sexual no homem, é sintetizada pelas células de Leydig e pelo córtex adrenal,[9] apresentando efeitos biológicos morfogênicos irreversíveis e efeitos reversíveis excitatórios/de manutenção[13]. A manutenção de níveis fisiológicos de testosterona é crítica para a saúde masculina. Níveis baixos de testosterona são extensamente correlacionados com uma série de alterações da normalidade clínica, incluindo perda de libido e função sexual, perda de força muscular, fadiga, alterações cognitivas e de humor,[2] bem como aumento de marcadores para doença cardiovascular,[3] mortalidade,[7] diabetes mellitus,[15,16] síndrome metabólica[6] e risco aumentado para fratura óssea[8,11,12,17-19].

Por outro lado, doses suprafisiológicas de testosterona, como aquelas apresentadas por usuários de anabolizantes esteroidais androgênicos que buscam estética e/ou melhora na performance de atividades físicas, também têm sido

associadas com consequências médicas graves, incluindo complicações cardiovasculares, endócrinas e psiquiátricas[1,5].

Recentemente, o termo “Endocrinologia Reprodutiva Periodontal” foi proposto para a representar a área de estudo que avalia a possível participação dos hormônios esteroides sexuais na patogênese e progressão da doença periodontal[10]. Nossa hipótese principal foi que a testosterona exerce importante influência sobre a resposta imunoinflamatória e metabolismo ósseo, não apenas em situações homeostáticas e fisiológicas, mas também em condições associadas à inflamação crônica, como a doença periodontal. O objetivo deste trabalho de tese foi avaliar a relação entre testosterona e doença periodontal em diferentes níveis de evidência científica:

- Associação entre níveis hormonais e presença e severidade de periodontite em homens;
- Relação causal entre modulação dos níveis séricos de testosterona e alterações no periodonto e na resposta do hospedeiro à periodontite experimental em ratos;
- Mecanismo de ação das observações in vivo utilizando cultura de osteoblastos e precursores de osteoclastos.

CAPÍTULOS

2 Capítulos

A relação entre testosterona e a doença periodontal no sexo masculino foi avaliada em diferentes níveis de evidência científica:

- Em humanos, através de um estudo observacional transversal utilizando dados de uma amostra representativa da população norte-americana:

Capítulo 1: Steffens JP, Wang X, Spolidorio LC, Van Dyke TE, Starr J, Kantarci A. *Sex hormones correlate with periodontitis in men: Results from NHANES III*. Artigo a ser submetido ao *Journal of Dental Research*.

- Para se avaliar a relação causal entre andrógenos e severidade da doença periodontal, foi realizado um estudo piloto em ratos para validação de metodologia:

Capítulo 2: Steffens JP, Coimbra LS, Ramalho-Lucas PD, Rossa Jr. C, Spolidorio LC. *The effect of supra- and subphysiologic testosterone levels on ligature-induced bone loss in rats - A radiographic and histologic pilot study*. J Periodontol 2012; 83: 1432-9.

- Para aprofundar a compreensão desta relação, e também para se identificar possíveis mecanismos de ação, foram realizados ensaios in vivo (ratos) e in vitro:

Capítulo 3: Steffens JP, Coimbra LS, Rossa Jr C, Kantarci A, Van Dyke TE, Spolidorio LC. *The impact of testosterone on inflammation-induced periodontal bone loss in rats*. Artigo a ser submetido ao *Journal of Bone and Mineral Research*.

Capítulo 4: Steffens JP, Herrera BS, Stephens D, Spolidorio LC, Kantarci A, Van Dyke TE. *Resolvin D2 ameliorates testosterone-derived downregulation of*

osteocalcin and osteoprotegerin on primary murine osteoblasts. Artigo submetido ao *Hormones and Metabolic Research*.

- Para sugerir novas perspectivas sobre a relação entre envelhecimento e doença periodontal, que não pelo mecanismo hormonal, realizou-se a revisão da literatura:

Capítulo 5: Steffens JP, Masi S, D'Aiuto F, Spolidorio LC. *Telomere length and its relationship with chronic diseases - New perspectives for periodontal research*. Arch Oral Biol 2013; 58: 111-7.

Uma descrição detalhada dos materiais e métodos utilizados pode ser encontrada no Apêndice A. Todos os procedimentos experimentais foram aprovados pela Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Araraquara – Unesp (Anexo A). As permissões das revistas científicas para reprodução dos artigos publicados estão dispostas no Anexo B.

CAPÍTULO 1

Sex Hormones Correlate with Periodontitis in Men: Results from NHANES III*

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ABSTRACT

Sex hormones have the ability to influence inflammation and bone turnover. The goal of this study was to explore the potential association between sex hormones and periodontitis using data from the Third National Health and Nutrition Examination Survey (NHANES).

The dataset from 772 individuals aged 30+ years who had blood tests for assessment of serum testosterone, estradiol, sex hormone binding globulin (SHBG) and androstenediol glucuronide (AAG) levels were included in the analysis. Bioavailable testosterone (CBT) and estradiol over testosterone ratio (ETR) were also calculated. Periodontitis was defined as a combination of clinical attachment loss (CAL)≥3mm and probing pocket depth (PPD)≥4mm. The presence and severity of periodontitis were correlated with categories of the sex hormones.

The prevalence of persons presenting PPD≤3mm or CAL≤2mm was decreased when low or high testosterone levels were present, as well as in men with low estradiol. When adjusted for confounding factors, high CBT and low ETR displayed a significant *odds ratio* for both presence and severity of the disease. The strength of association between sex hormones (or their interactions) and periodontitis was different according to the age intervals studied.

Our findings suggest that sex hormones present an age-dependent association with both presence and severity of periodontitis in men.

Keywords: gonadal steroid hormones; periodontitis; inflammation; androgens.

INTRODUCTION

Sex steroid hormones are known to regulate a variety of functions, such as growth, reproduction and differentiation (Nava-Castro et al., 2012). In men, the main sex steroid hormone is testosterone, which is primarily produced by the Leydig cells in the testicles, but can also be derived from the adrenal precursor dehydroepiandrosterone. The production of testosterone by the testes is regulated through a hypothalamus-pituitary axis feedback mechanism (Mawhinney and Mariotti, 2013). Testosterone can act directly, by binding to the nuclear androgen receptor, or indirectly. The indirect action is mediated by the enzyme 5α-reductase, expressed in tissues like the liver, kidneys, skin and prostate, that converts testosterone to dihydrotestosterone (DHT), which is metabolically more active than testosterone and binds the androgen receptor (AR) with greater affinity. Also, testosterone can be converted to estradiol by the enzyme aromatase, produced by macrophages and fibroblasts in the liver, kidneys, brain and adipose tissue, which exerts its action by binding to estrogen receptors (ER) α, β and G protein-coupled receptor 30 (GPR30) (Ohlsson and Vandenput, 2009; Vandenput and Ohlsson, 2010).

Physiologic testosterone levels vary greatly between individuals and also throughout a man's life. Peaks of testosterone are achieved three times in life: in the middle of embryo development, during the 2nd-5th month of age, and in puberty (Harvey and Berry, 2009). However, after the 40 years of age, total testosterone levels start to decline gradually and continuously at a rate of 1-2% a year (Gray, 2005). Also, the globulin that binds the hormone with great affinity, SHBG, increases, further reducing the amount of bioavailable testosterone (free and albumin-bound testosterone) (Yeap, 2009).

Low testosterone levels have been extensively correlated with medical disorders, such as low libido, sexual dysfunction and muscle strength loss, fatigue, cognitive and mood alterations (Bain, 2010). Low testosterone has also been reported to be associated with increased low grade systemic inflammation biomarkers (tumor necrosis factor-alpha, macrophage inflammatory protein 1-alpha and 1-beta) (Bobjer et al., 2013), markers for cardiovascular disease (Del Fabbro et al., 2010) and mortality (Laughlin et al., 2008), diabetes mellitus (Selvin et al., 2007; Stellato et al., 2000), metabolic syndrome (Laaksonen et al., 2004) and increased risk for bone fracture (Meier et al., 2008; Mellstrom et al., 2006). In rats, we have described that both low and high serum testosterone levels modulate periodontal bone loss (Steffens et al., 2012). However, it is not clear whether the impact of testosterone in men's health, especially in bone metabolism, is derived from the activation of ARs, by testosterone and DHT, or through its aromatization to estradiol (Clarke and Khosla, 2009).

Recently, the term 'Periodontal Reproductive Endocrinology' was proposed to represent the field of Periodontology dedicated to studying the interactions between sex steroid hormones and the periodontium (Mariotti, 2013). Along those same lines, the objective of this study was to explore the potential impact of abnormal serum levels of sex steroid hormones on the prevalence and severity of periodontitis in men using data from the Third National Health and Nutritional Examination Survey (NHANES III).

MATERIALS AND METHODS

Study Design

Third National Health and Nutrition Examination Survey (NHANES III) was a cross-sectional study conducted from 1988 to 1994 by the National Center for Health Statistics (NCHS), which is part of the Centers for Disease Control and Prevention (CDC). This program of studies was designed to assess the health and nutritional status of adults and children in the United States. It was designed as a multistage, stratified, clustered probability sample of the US civilian non-institutionalized population at least 2 months old. The protocols for the conduct of NHANES III were approved by the institutional review board of the NCHS, CDC. Informed consent was obtained from all participants (Plan and operation of the Third National Health and Nutrition Examination Survey, 1988-94. Series 1: programs and collection procedures, 1994).

A subset of male participants in the first phase of NHANES III (1988-1991) had concentrations of testosterone, SHBG, androstenediol glucuronide (AAG, a metabolite of DHT) and estradiol recorded. These measurements were made on stored serum specimens from 1,637 men aged 12 or more who were examined in the morning sample of the first phase of NHANES III (1988-1991). Blood was drawn after an overnight fast for participants during either an examination at the medical examination center or during an abbreviated examination at home (Selvin et al., 2007). Competitive electrochemiluminescence immunoassays on the 2010 Elecsys autoanalyser (Roche Diagnostics, Indianapolis, IN, USA) were used to quantify testosterone, estradiol and SHBG concentrations, while AAG was detected by an enzyme immunoassay (Diagnostic Systems Laboratories, Webster, TX, USA). The lowest detection limits [and coefficients of variation] of the assays were: testosterone 0.02ng/mL [5.9%(2.5ng/mL), 5.8% (5.5ng/mL)]; estradiol 5pg/mL [6.5%(102.7pg/mL), 6.7%(474.1pg/mL)]; AAG 0.33ng/mL [9.5%(2.9ng/mL), 5%(10.1ng/mL)]; SHBG 3nM

[5.3%(5.3nM), 5.9%(16.6nM)]. The assay of stored serum specimens was conducted at the Children's Hospital, Boston, MA, and the protocol was approved by the Institutional Review Boards at the Johns Hopkins Bloomberg School of Public Health and the NCHS, CDC (Rohrmann et al., 2011).

Among individuals who had their serum specimens assessed, we identified participants who were 30 years old or older, had at least 6 teeth present and had received periodontal examination (n=772). Thirty years of age was chosen because of the rarity of periodontitis in younger individuals. According to NHANES criteria, each individual had 2 randomly selected quadrants evaluated: one upper and one lower quadrant. Only fully erupted teeth were analyzed (excluding third molars), and a maximum of 14 teeth per individual was examined. Periodontal measurements used in this study included clinical attachment loss (CAL) and probing pocket depth (PPD). These measurements were performed in the mesio-buccal and mid-buccal sites of each tooth. PPD was described as the distance between the free gingival margin and the bottom of the pocket/sulcus, while CAL was defined as the distance between the cement-enamel junction and the bottom of the pocket/sulcus. All measurements were performed by trained dentists using NIDR periodontal probes (Albandar et al., 1999).

Classification of Sex Hormone Levels

Total testosterone levels in serum were classified as very low (≤2.3 ng/mL), low (2.3-3.5 ng/mL), and reference (>3.5-7.67 ng/mL) (Wang et al., 2008), whilst the 90th percentile was used as cutoff for 'high' values (>7.67 ng/mL) (Rohrmann et al., 2011). Calculated bioavailable testosterone (CBT) was performed based on previously described equations (Vermeulen et al., 1999) and the albumin value was standardized as 4.5 g/dL. Low CBT (<1 ng/mL) and high CBT (>4.2 ng/mL) cutoffs

were based on generally accepted levels. Estradiol over testosterone ratios (ETR) were calculated by dividing estradiol levels by total testosterone values. The 10th and 90th percentiles were used as lower and higher limit cutoffs, respectively, for all measurements and interactions (CBT, estradiol, ETR, SHBG and AAG) (Rohrmann et al., 2011).

Classification According to Extent and Severity of Periodontitis

We used the classification of extent and severity of periodontitis proposed in the latest NHANES periodontal analysis by Eke et al., 2012:

- Severe periodontitis: presence of 2 or more interproximal sites with CAL≥6 mm (not on the same tooth) and 1 or more interproximal site(s) with PPD≥5 mm.
- Moderate periodontitis: 2 or more interproximal sites with 4 mm ≤CAL<6 mm (not on the same tooth), or 2 or more interproximal sites with PPD≥5 mm, also not on the same tooth.
- Mild periodontitis: 2 or more interproximal sites with 3 mm ≤CAL<4 mm and 2 or more interproximal sites with PPD≥4 mm (not on the same tooth) or 1 site with PPD≥5 mm.

Total periodontitis was the sum of severe, moderate and mild cases of periodontitis (Eke et al., 2012).

Statistical Analysis

All data analyses were performed with STATA version 12 with SVY package, which uses weights to account for the multi-stage stratified, clustered sampling method of NHANES III. Possible confounding factors considered in this analysis were defined *a priori* as follows: age (continuous), current smoking (yes/no), alcohol drinking frequency (drinks per week, continuous), waist-to-hip ratio, race/ethnicity (non-

Hispanic white / non-Hispanic black / others), diabetes mellitus (self-reported, yes/no). We fit logistic regression models to estimate the association between presence or absence of periodontal disease and the various hormones. Ordinal logistic models were used to further evaluate the association of covariates with the severity of periodontal diseases. Unless otherwise stated, all values are expressed as mean ± SEM.

RESULTS

After assessing and screening the NHANES III dataset, 772 men (age 45.5±0.5 years) were included in this study. The baseline characteristics of the study population are shown in table 1.

Among the people presenting with the reference total testosterone levels, 61.1 ± 3.2% had PPD ≤3 mm and 46.8 ± 2.5% had AL ≤2 mm, indicating periodontal health. The prevalence of periodontal health was decreased in men presenting with testosterone levels lower or higher than the reference group. Table 2 contains the raw non-adjusted data for prevalence of periodontitis by category (PPD and CAL) for various sex hormone concentrations.

After classification of presence and severity of periodontitis and statistical adjustments, high CBT levels and low ETR were the only variables to correlate with periodontitis. The adjusted *odds ratios* for presence and severity of periodontitis based on sex steroid hormone concentrations can be found in table 3.

Table 4 demonstrates the prevalence and adjusted *odds ratio* for presence and severity of periodontitis by sex steroid hormone concentrations grouped according to age. Low ETR correlates with the presence and severity of periodontitis in young adult men (30-45 years). In middle-aged adults (46-60 years), we observed

a correlation with the severity, but not presence of periodontitis. We noted a similar trend of association of age with high total testosterone levels. In addition, low estradiol and high ETR were correlated with the presence of periodontitis. In older men (>60 years) only low AAG correlated with presence and severity of periodontitis.

DISCUSSION

Sex hormones are important for a variety of characteristics that individuals develop throughout life. Several factors may contribute to hormone variations in males, which can be physiologic (*e.g.* puberty), pathologic (*e.g.* Klinefelter syndrome and hypergonadotropic hypogonadism), pharmacologic (*e.g.* steroid abuse and testosterone replacement therapy), genetic (*e.g.* race and ethnicity) or behavioral (*e.g.* smoking and alcohol consumption). Physiological testosterone levels are believed to regulate inflammation, since low testosterone levels have been linked to the presence of several inflammatory medical disorders (Maggio and Basaria, 2009; Traish et al., 2009a; Traish et al., 2009b; Traish et al., 2009c). Similarly, anabolic-androgenic steroid abuse that results in high levels of testosterone has adverse actions, such as alterations in the cardiovascular, central nervous and endocrine systems (Basaria, 2010). We have previously demonstrated bimodal actions of testosterone; both low and high testosterone levels increase bone resorption in an inflammatory dental model in rats (Steffens et al., 2012).

Many epidemiological studies have shown men at higher risk for presence and severity of attachment loss and destructive periodontal disease than women (Albandar, 2002; Corbet et al., 2001; Mack et al., 2004; Susin et al., 2005). Although it is plausible that this difference could be hormonally mediated, gender-based differences in behavior could also explain, at least in part, the greater prevalence.

Men tend to have poorer oral hygiene, increased consumption of alcohol and tobacco, and less utilization of oral health care services than women (Haytac et al., 2013). In the elderly population, men were reported to have higher prevalence and greater severity of periodontitis, as shown in cross-sectional (Holtfreter et al., 2010; Mack et al., 2004) and longitudinal studies (Hirotomi et al., 2002; Ogawa et al., 2002).

Several mechanisms have been proposed to explain the regulation of periodontal disease by sex steroid hormones, including: (i) hormones increase growth of pathogenic microflora; (ii) promote an alteration in vascular characteristics; (iii) periodontal tissue responses are exacerbated by immune-endocrine interactions; (iv) specific populations of fibroblasts and epithelial cells are modulated by sex steroid hormones (Mariotti and Mawhinney, 2013). However, the host response in a multifactorial disease, such as periodontal disease, most probably cannot be simplified and represented by one mechanism of action only; a combination of factors is likely involved.

Previous observational studies conducted to evaluate the possible role of sex hormones in the development of destructive periodontal disease reported no significant correlations between hormone levels and the prevalence of periodontitis (Daltaban et al., 2006; Unsal et al., 2008). However, design features of some of these studies limit conclusions and preclude direct comparisons with our data (for instance, small sample size and restriction to hypogonadic men). In a prospective study of a cohort of community-dwelling ambulatory men aged 65 years or older, no correlation was observed between sex hormones and the progression of periodontitis (Orwoll et al., 2009). The criteria for defining periodontitis were different (proximal CAL≥5mm in at least 30% of teeth examined), but the outcome was similar showing

no significant correlation between total testosterone or estradiol and periodontitis in our oldest age group.

We observed a linear relationship between total testosterone levels and presence or severity of periodontitis, though the 95% confidence intervals were very wide. If the clinically generally accepted level of 10 ng/mL is used as a high cutoff value, the correlation is still significant, but the small sample size could bias the result (n=10). To our knowledge, we are the first group to report that high CBT and low ETR are correlated with periodontitis. This observation is consistent with high testosterone and low estradiol levels being harmful to the periodontium. The sex hormone estradiol alone, which is generally accepted as the main regulator of bone loss in men, did not associate with periodontitis in any age group. However, we assessed the levels of the hormone only in serum, whereas estradiol can be generated locally in the tissues (such as bone) using testosterone as a precursor.

As a matter of fact, some studies have shown that testosterone treatment has a suppressive effect on leukocyte count on orchiectomized mice and young male rats (Kamis and Ibrahim, 1989; Yao et al., 2003). Those results also showed that testosterone treatment decreases monocyte count, CD4+/CD8+ ratio, and also inhibits proliferative responses of lymphocytes (Yao et al., 2003). Due to the immunosuppressive properties of testosterone, it can be suggested that high endogenous levels of that hormone increases susceptibility to infectious diseases, such as periodontitis.

Even though a smaller prevalence of healthy subjects (as inferred from CAL≤2 mm and PPD≤3 mm) can be observed in the reference levels of total testosterone when compared to the other groups (Table 2), we could not see this same trend after adjusting for confounding factors. After adjustments, very low and low testosterone

levels were associated with a non-significant decrease in the *odds ratios* for prevalence and severity of periodontitis (Table 3), a direction opposite to what we had proposed. This difference with other medical disorders and our animal findings can be attributed to the fact that periodontitis is a multifactorial disease and highly depends on patient-based biofilm control. That was also the rationale for investigating presence and severity of periodontitis, as to test whether sex hormones could interfere in the initiation or progression of periodontitis.

The sex hormones (and their interactions) exhibit age-dependent associations with periodontitis. ETR correlates with the presence and severity of periodontitis in young and middle-aged adults. In middle-aged adults, low estradiol correlates with the presence of periodontitis and high total testosterone correlates with severity of periodontitis. In older men only low AAG correlates with presence and severity of periodontitis, reinforcing the importance of androgens on this disease.

Among the various limitations of a reliance on NHANES III data is the difficulty in developing accurate measures of disease and disease severity (Albandar, 2011; Eke et al., 2010). The primary disadvantage is that data collection was from only two sites per tooth in two randomly assigned quadrants, an approach that underestimates the prevalence of periodontitis. However, our objective was not to give the absolute prevalence of periodontitis in the studied population, but rather compare the prevalence in groups that were evaluated with the same criteria.

Our findings suggest that high CBT and low ETR correlate with periodontitis prevalence and severity in men, and these correlations are age-dependent. In older men (>60 years) low AAG, a metabolite of DHT, significantly correlates with higher prevalence and severity of periodontitis. Causality remains to be determined.

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REFERENCES

Albandar JM, Brunelle JA, Kingman A (1999). Destructive periodontal disease in adults 30 years of age and older in the United States, 1988-1994. *J Periodontol* 70(1):13-29.

Albandar JM (2002). Global risk factors and risk indicators for periodontal diseases. *Periodontol 2000* 29(177-206.

Albandar JM (2011). Underestimation of periodontitis in NHANES surveys. *J Periodontol* 82(3):337-41.

Bain J (2010). Testosterone and the aging male: to treat or not to treat? *Maturitas* 66(1):16-22.

Basaria S (2010). Androgen abuse in athletes: detection and consequences. *J Clin Endocrinol Metab* 95(4):1533-43.

Bobjer J, Katrinaki M, Tsatsanis C, Lundberg Giwercman Y, Giwercman A (2013). Negative association between testosterone concentration and inflammatory markers in young men: a nested cross-sectional study. *PLoS One* 8(4):e61466.

Clarke BL, Khosla S (2009). Androgens and bone. *Steroids* 74(3):296-305.

Corbet EF, Wong MC, Lin HC (2001). Periodontal conditions in adult Southern Chinese. *J Dent Res* 80(5):1480-5.

Daltaban O, Saygun I, Bolu E (2006). Periodontal status in men with hypergonadotropic hypogonadism: effects of testosterone deficiency. *J Periodontol* 77(7):1179-83.

Del Fabbro E, Hui D, Nooruddin ZI, Dalal S, Dev R, Freer G, et al. (2010). Associations among hypogonadism, C-reactive protein, symptom burden, and survival in male cancer patients with cachexia: a preliminary report. *J Pain Symptom Manage* 39(6):1016-24.

Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA (2010). Accuracy of NHANES periodontal examination protocols. *J Dent Res* 89(11):1208-13.

Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ, Cdc Periodontal Disease Surveillance workgroup: James Beck GDRP (2012). Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res* 91(10):914-20.

Gray M (2005). Andropause and the aging man: separating evidence from speculation. *Adv Nurse Pract* 13(4):22-7; quiz 28.

Harvey J, Berry JA (2009). Andropause in the aging male. *J Nurse Pract* 5(3):207-12.

Haytac MC, Ozcelik O, Mariotti A (2013). Periodontal disease in men. *Periodontol 2000* 61(1):252-65.

Hirotomi T, Yoshihara A, Yano M, Ando Y, Miyazaki H (2002). Longitudinal study on periodontal conditions in healthy elderly people in Japan. *Community Dent Oral Epidemiol* 30(6):409-17.

Holtfreter B, Kocher T, Hoffmann T, Desvarieux M, Micheelis W (2010). Prevalence of periodontal disease and treatment demands based on a German dental survey (DMS IV). *J Clin Periodontol* 37(3):211-9.

Kamis AB, Ibrahim JB (1989). Effects of testosterone on blood leukocytes in plasmodium berghei-infected mice. *Parasitol Res* 75(8):611-3.

Laaksonen DE, Niskanen L, Punnonen K, Nyyssonen K, Tuomainen TP, Valkonen VP, et al. (2004). Testosterone and sex hormone-binding globulin predict the metabolic syndrome and diabetes in middle-aged men. *Diabetes Care* 27(5):1036-41.

Laughlin GA, Barrett-Connor E, Bergstrom J (2008). Low serum testosterone and mortality in older men. *J Clin Endocrinol Metab* 93(1):68-75.

Mack F, Mojon P, Budtz-Jorgensen E, Kocher T, Splieth C, Schwahn C, et al. (2004). Caries and periodontal disease of the elderly in Pomerania, Germany: results of the Study of Health in Pomerania. *Gerodontology* 21(1):27-36.

Maggio M, Basaria S (2009). Welcoming low testosterone as a cardiovascular risk factor. *Int J Impot Res* 21(4):261-4.

Mariotti A (2013). The ambit of periodontal reproductive endocrinology. *Periodontol 2000* 61(1):7-15.

Mariotti A, Mawhinney M (2013). Endocrinology of sex steroid hormones and cell dynamics in the periodontium. *Periodontol 2000* 61(1):69-88.

Mawhinney M, Mariotti A (2013). Physiology, pathology and pharmacology of the male reproductive system. *Periodontol 2000* 61(1):232-51.

Meier C, Nguyen TV, Handelsman DJ, Schindler C, Kushnir MM, Rockwood AL, et al. (2008). Endogenous sex hormones and incident fracture risk in older men: the Dubbo Osteoporosis Epidemiology Study. *Arch Intern Med* 168(1):47-54.

Mellstrom D, Johnell O, Ljunggren O, Eriksson AL, Lorentzon M, Mallmin H, et al. (2006). Free testosterone is an independent predictor of BMD and prevalent fractures in elderly men: MrOS Sweden. *J Bone Miner Res* 21(4):529-35.

Nava-Castro K, Hernandez-Bello R, Muniz-Hernandez S, Camacho-Arroyo I, Morales-Montor J (2012). Sex steroids, immune system, and parasitic infections: facts and hypotheses. *Ann N Y Acad Sci* 1262(16-26.

Ogawa H, Yoshihara A, Hirotomi T, Ando Y, Miyazaki H (2002). Risk factors for periodontal disease progression among elderly people. *J Clin Periodontol* 29(7):592-7.

Ohlsson C, Vandenput L (2009). The role of estrogens for male bone health. *Eur J Endocrinol* 160(6):883-9.

Orwoll ES, Chan BK, Lambert LC, Marshall LM, Lewis C, Phipps KR (2009). Sex steroids, periodontal health, and tooth loss in older men. *J Dent Res* 88(8):704-8.

Plan and operation of the Third National Health and Nutrition Examination Survey, 1988-94. Series 1: programs and collection procedures. (1994). *Vital Health Stat 1* 32):1-407.

Rohrmann S, Platz EA, Selvin E, Shiels MS, Joshu CE, Menke A, et al. (2011). The prevalence of low sex steroid hormone concentrations in men in the Third National Health and Nutrition Examination Survey (NHANES III). *Clin Endocrinol (Oxf)* 75(2):232-9.

Selvin E, Feinleib M, Zhang L, Rohrmann S, Rifai N, Nelson WG, et al. (2007). Androgens and diabetes in men: results from the Third National Health and Nutrition Examination Survey (NHANES III). *Diabetes Care* 30(2):234-8.

Steffens JP, Coimbra LS, Ramalho-Lucas PD, Rossa C, Jr., Spolidorio LC (2012). The effect of supra- and subphysiologic testosterone levels on ligature-induced bone loss in rats--a radiographic and histologic pilot study. *J Periodontol* 83(11)*:*1432-9.

Stellato RK, Feldman HA, Hamdy O, Horton ES, McKinlay JB (2000). Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. *Diabetes Care* 23(4)*:*490-4.

Susin C, Valle P, Oppermann RV, Haugejorden O, Albandar JM (2005). Occurrence and risk indicators of increased probing depth in an adult Brazilian population. *J Clin Periodontol* 32(2)*:*123-9.

Traish AM, Guay A, Feeley R, Saad F (2009a). The dark side of testosterone deficiency: I. Metabolic syndrome and erectile dysfunction. *J Androl* 30(1)*:*10-22.

Traish AM, Saad F, Feeley RJ, Guay A (2009b). The dark side of testosterone deficiency: III. Cardiovascular disease. *J Androl* 30(5)*:*477-94.

Traish AM, Saad F, Guay A (2009c). The dark side of testosterone deficiency: II. Type 2 diabetes and insulin resistance. *J Androl* 30(1)*:*23-32.

Unsal B, Saygun I, Daltaban O, Bal B, Bolu E (2008). The relationship between periodontal status and alkaline phosphatase levels in gingival crevicular fluid in men with hypergonadotropic hypogonadism. *Yonsei Med J* 49(1)*:*71-8.

Vandenput L, Ohlsson C (2010). Sex steroid metabolism in the regulation of bone health in men. *J Steroid Biochem Mol Biol* 121(3-5)*:*582-8.

Vermeulen A, Verdonck L, Kaufman JM (1999). A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 84(10)*:*3666-72.

Wang C, Nieschlag E, Swerdloff R, Behre HM, Hellstrom WJ, Gooren LJ, et al. (2008). Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, EAU, EAA and ASA recommendations. *Eur J Endocrinol* 159(5)*:*507-14.

Yao G, Liang J, Han X, Hou Y (2003). In vivo modulation of the circulating lymphocyte subsets and monocytes by androgen. *Int Immunopharmacol* 3(13-14)*:*1853-60.

Yeap BB (2009). Testosterone and ill-health in aging men. *Nat Clin Pract Endocrinol Metab* 5(2)*:*113-21.

Table 1: Characteristics (SEM) of the study population.

	Overall
Unweighted sample number (n)	772
Age (years)	45.5 (0.5)
Race/Ethnicity (prevalence, %)	
Non-Hispanic White	79 (3.0)
Non-Hispanic Black	8 (1.0)
Other	13 (2.0)
Diabetes (prevalence, %)	5 (1.0)
Current smoking (prevalence, %)	30 (2.0)
Education: high school and above (prevalence, %)	54 (4.0)
Body Mass Index (BMI)	26.91 (0.31)
Waist-to-hip ratio (WHR)	0.96 (0.005)
Alcohol drinking frequency (times/month)	14.00 (1.07)
Total Testosterone* (ng/mL)	4.85 (0.10)
Estradiol* (pg/mL)	34.70 (0.77)
SHBG* (nM)	34.96 (0.65)
AAG* (ng/mL)	11.10 (0.36)
PPD, mean (mm)	1.66 (0.04)
CAL, mean (mm)	1.40 (0.06)
Periodontitis (prevalence, %)	30 (2.0)

*geometric mean.
SHBG= sex hormone binding globulin; AAG= androstenediol glucuronide; PPD= probing pocket depth; CAL= clinical attachment loss

Table 2: Prevalence (SEM) of persons presenting categories of probing pocket depth (PPD in mm) and clinical attachment loss (CAL in mm) according to sex hormone concentrations.

		PPD≤3	3<PPD<6	PPD≥6	CAL≤2	2<CAL<5	CAL≥5
TT	Very Low (≤2.3 ng/mL)	51.7(9.6)	29.8(11.2)	18.5(12.6)	38.3(11.9)	45.8(11.1)	15.9(5.2)
	Low (2.3-3.5 ng/mL)	46.9(5.9)	28.3(4.7)	24.9(5.7)	40.5(6.3)	40.1(5.8)	19.4(4.5)
	Reference (>3.5-7.67 ng/mL)	61.1(3.2)	22.5(2)	16.4(2.3)	46.8(2.5)	28.5(2.3)	24.7(2.8)
	90th percentile (>7.67 ng/mL)	46.5(9.7)	38.6(8.9)	14.9(3.9)	38.4(6.6)	35.7(6.2)	25.9(5)
CBT	Low (<1 ng/mL)	49.5(9.6)	32.9(9.2)	17.6(6.3)	23.7(9.7)	43.4(9)	32.9(9.3)
	Reference (1-4.2 ng/mL)	57.9(2.8)	24.4(1.7)	17.7(2.2)	46(1.9)	30.2(2.2)	23.8(2.3)
	High (>4.2 ng/mL)	57.8(11.7)	31.4(7.6)	10.8(8.3)	40(10.5)	41.2(7.5)	18.8(9.3)
Estradiol	10th percentile (<24.88 pg/mL)	70.8(6.1)	15.2(5.2)	14.1(6.9)	52.7(8.8)	25.8(6.7)	21.5(4.6)
	Reference	58.2(2.9)	25(1.9)	16.8(2)	45.6(2)	32.6(2.4)	21.8(2.2)
	90th percentile (>49.2 pg/mL)	38.9(7)	35.1(4.4)	25.9(7.5)	31.1(6)	24.8(6)	44.1(6.1)
ETR (*1,000)	10th percentile (<0.0047)	65.1(7.8)	26.7(7)	8.2(3.4)	62.4(7.4)	19.9(5.3)	17.7(5.5)
	Reference	57.7(2.9)	24.1(1.8)	18.1(2.6)	43.8(2.2)	31.8(2.4)	24.4(2.5)
	90th percentile (>0.0109)	49.3(7.1)	31(5.1)	19.7(6)	36.1(5.5)	38(5.8)	25.9(6.1)
SHBG	10th percentile (<20.7 nM)	56.8(8.3)	25.3(8)	18(5.9)	51.6(8.1)	29.7(6.5)	18.7(5.4)
	Reference	58.3(3.1)	24(2.2)	17.7(2.1)	45.7(2)	31(2.4)	23.2(2.2)
	90th percentile (>59.2 nM)	52.5(6.1)	33.3(5)	14.3(4.3)	30.7(7)	33.4(6.2)	35.9(6)
AAG	10th percentile (<5.26 ng/mL)	51.9(7.9)	35.6(7.4)	12.4(4.4)	21.8(5.3)	35.7(6.6)	42.6(7.3)

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Reference	57(3.4)	23.7(1.8)	19.3(2.7)	48.4(2.4)	28.8(2.5)	22.8(2.4)
90th percentile (>22.28 ng/mL)	68.3(5)	25(5.9)	6.7(3.1)	41.9(6.2)	47.2(6.7)	10.9(3.9)

TT= total testosterone; CBT= calculated bioavailable testosterone; ETR= estradiol over testosterone ratio; SHBG= sex hormone binding globulin; AAG= androstenediol glucuronide

Table 3: Adjusted* OR (95% CI) for presence and severity of periodontitis according to sex steroid hormone concentrations.

		Presence OR (95% CI)	Severity OR (95% CI)
TT**	Very Low (≤2.3 ng/mL)	0.31(0.06-1.63)	0.3(0.07-1.34)
	Low (2.3-3.5 ng/mL)	0.45(0.19-1.04)	0.49(0.2-1.24)
	Reference (>3.5-7.67 ng/mL)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th percentile (>7.67 ng/mL)	2.21(0.95-5.11)	2.07(0.96-4.43)
CBT	Low (<1 ng/mL)	0.44(0.12-1.53)	0.36(0.11-1.11)
	Reference (1-4.2 ng/mL)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	High (>4.2 ng/mL)	**4.67(1.04-20.88)**†	**3.62(1.14-11.46)** †
Estradiol	10th percentile (<24.88 pg/mL)	0.88(0.41-1.86)	0.8(0.43-1.49)
	Reference	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th percentile (>49.2 pg/mL)	1.12(0.45-2.77)	0.83(0.4-1.72)
ETR	10th percentile (<0.0047)	**3.28(1.11-9.72)**†	**4.43(1.62-12.11)**†
	Reference	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th percentile (>0.0109)	0.6(0.23-1.59)	0.61(0.23-1.6)
SHBG	10th percentile (<20.7 nM)	0.8(0.27-2.36)	0.76(0.28-2.06)
	Reference	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th percentile (>59.2 nM)	1.91(0.81-4.55)	1.72(0.68-4.35)
AAG	10th percentile (<5.26 ng/mL)	1.81(0.78-4.18)	1.82(0.89-3.72)
	Reference	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th percentile (>22.28 ng/mL)	0.92(0.37-2.27)	1.11(0.42-2.91)

* adjusted for age, race/ethnicity, smoking, education, waist-to-hip ratio, diabetes mellitus, and alcohol drinking.
** further adjusted for estradiol.
† **(bold)** statistically significant.
TT= total testosterone; CBT= calculated bioavailable testosterone; ETR= estradiol over testosterone ratio;
SHBG= sex hormone binding globulin; AAG= androstenediol glucuronide

Table 4: Prevalence [%(SEM)] and adjusted* OR (95% CI) for presence and severity of periodontitis by sex steroid hormone concentrations grouped according to age intervals.

		30-45 years			46-60 years			61+ years		
		%(SEM)	Presence	Severity	%(SEM)	Presence	Severity	%(SEM)	Presence	Severity
TT	Very low	4(2.7)	1.57(0.15-16.19)	1.47(0.16-13.3)	2.3(1.7)	0.08(0-2.93)	0.07(0-5.31)	6.4(2.6)	0.35(0.04-2.72)	0.34(0.04-2.7)
	Low	6(3.2)	0.93(0.12-7.27)	0.91(0.15-5.62)	7.8(2.6)	0.28(0.07-1.09)	0.26(0.07-1.02)	18.9(6.1)	0.29(0.04-2.24)	0.48(0.04-5.13)
	Ref.	70.9(7.5)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	69.6(6.9)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	72(7.7)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th perc.	19.1(7.7)	1.59(0.4-6.29)	1.52(0.41-5.6)	20.3(5.8)	4.31(0.84-22.2)	**3.7(1.02-13.49)**†	2.7(2.3)	2.4(0.15-39.22)	1.97(0.18-21.29)
CBT	Low	-	-	-	3.4(2.1)	2.55(0.53-12.22)	8.58(0.28-266.52)	11.8(3.8)	0.71(0.16-3.08)	0.57(0.2-1.64)
	Ref.	82.2(8.2)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	92.8(3.3)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	88.1(3.8)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	High	11.9(8)	3.28(0.5-21.64)	3(0.62-14.67)	-	-	-	-	-	-
Estradiol	10th perc.	16.3(6.3)	0.7(0.16-3.06)	0.63(0.15-2.63)	18.4(5.7)	8.27(1.69-40.38)	2.15(0.63-7.29)	8.3(3.1)	1.22(0.14-10.89)	0.8(0.18-3.54)
	Ref.	77.4(6.4)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	75.8(5.9)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	75.7(5.4)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th perc.	6.4(4)	0.97(0.17-5.6)	0.95(0.18-5.12)	5.8(2.7)	0.87(0.31-2.47)	0.78(0.3-2.07)	16(5.6)	1.11(0.13-9.41)	0.74(0.13-4.06)
ETR	10th perc.	8.3(5)	**5.24(1.26-21.76)**†	**8.88(1-78.77)**†	13.3(5)	2.43(0.45-13.14)	**5.26(1.31-21.07)**†	3.4(2.4)	1.34(0.22-7.99)	0.95(0.3-3.05)
	Ref.	82.1(6.6)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	76.3(4.9)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	78.6(5.5)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th perc.	9.6(3.1)	1.55(0.19-12.37)	1.41(0.23-8.61)	10.5(2.6)	**0.3(0.14-0.67)**†	**0.26(0.11-0.64)**†	18(4.3)	0.46(0.09-2.45)	0.56(0.08-4.19)
SHBG	10th perc.	8.4(3.3)	0.54(0.19-1.57)	0.53(0.19-1.52)	6.7(1.8)	0.79(0.19-3.34)	0.75(0.19-2.99)	4.8(4.1)	0.3(0.01-10.18)	0.19(0.01-5.83)
	Ref.	84(3.9)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	76.8(4.3)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	73.8(5.7)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th perc.	7.6(3.2)	0.96(0.16-5.82)	0.89(0.16-4.99)	16.5(3.8)	2.51(0.61-10.25)	2.97(0.79-11.19)	21.3(4.9)	0.8(0.21-3.04)	0.56(0.16-1.91)
AAG	10th perc.	11.3(6)	1.62(0.32-8.33)	1.35(0.28-6.55)	18.4(6.6)	0.8(0.2-3.21)	1.09(0.3-3.96)	23.9(6.6)	**7.41(1.51-36.34)**†	**5.15(2.01-13.21)**†
	Ref.	79.5(5.4)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	76.5(7.2)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	63.5(8.7)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	90th perc.	9.2(4.3)	1.19(0.24-5.86)	1.17(0.26-5.22)	5.1(3.1)	0.32(0.08-1.23)	0.41(0.05-3.33)	12.6(4.7)	2(0.52-7.67)	3.13(0.7-14.01)

*adjusted for age, race/ethnicity, smoking, education, waist-to-hip ratio, diabetes mellitus and alcohol drinking.
† **(bold)** statistically significant.
TT= total testosterone; CBT= calculated bioavailable testosterone; ETR= estradiol over testosterone ratio; SHBG= sex hormone binding globulin; AAG= androstenediol glucuronide; Ref.= reference; Perc.= percentile.

CAPÍTULO 2

The Effect of Supra- and Subphysiologic Testosterone Levels on Ligature-Induced Bone Loss in Rats — A Radiographic and Histologic Pilot Study

Joao P. Steffens,* Leila S. Coimbra,* Pablo D. Ramalho-Lucas,* Carlos Rossa Jr.,† and Luis C. Spolidorio*

Background: Testosterone is the primary male sexual hormone, and varying concentrations of the hormone mediated by physiologic, pathologic, or pharmacologic mechanisms may induce large variations in the body. Data regarding the general role of testosterone in mediating inflammation are still inconclusive. Therefore, the purpose of this study is to assess the consequences of supra- and subphysiologic levels of testosterone on ligature-induced bone loss in rats.

Methods: Three male adult Holtzman rats were used to observe the course of serum testosterone concentration following orchiectomy (Ocx) and testosterone injections. Another 60 rats were randomly assigned to the following groups: 1) sham-operation controls (n = 10); 2) sham-operation and ligature-induced bone loss (n = 10); 3) orchiectomy without ligature (Ocx; n = 10); 4) Ocx and ligature (n = 10); 5) Ocx plus 250 mg/kg body weight intramuscular testosterone esters injection without ligature (Ocx+T; n = 10); and 6) Ocx, T, and ligature (n = 10). The ligatures were placed 30 days post-orchiectomy (or sham-operation) and maintained for 15 days. Thereafter, the rats were sacrificed, and their hemimandibles were used for radiographic evaluation of bone loss along with histologic and histometric analyses of gingival tissue.

Results: The results indicated a significant increase in bone loss in the Ocx and Ocx+T groups in the presence and absence of inflammation, respectively. In addition, the Ocx and Ocx+T groups presented increased gingival area accompanying ligature-induced bone loss.

Conclusions: Both sub- and supraphysiologic testosterone levels may influence bone metabolism, but only subphysiologic levels significantly increase ligature-induced bone loss. Moreover, testosterone has a regulatory effect on the gingival area. *J Periodontol 2012;83:1432-1439.*

KEY WORDS

Bone remodeling; gingival overgrowth; inflammation; testosterone.

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Androgens are hormones responsible for regulating primary and secondary sexual characteristics in men.[1] Testosterone, the primary male sex hormone, is related to the development and maintenance of muscle mass, erythropoiesis stimulus, increased brain perfusion, influence of mood and cognition, and bone health.[2] Testosterone rises to comparable adult male levels during three life phases: 1) the midpoint of embryonic development, 2) the first 2 to 5 months of life, and 3) throughout puberty.[1,3]

Approximately 95% of the testosterone in a healthy man is produced by the testes, whereas the other 5% is converted into testosterone from adrenal-produced precursors.[4,5] Testosterone may act directly by binding to the intracytoplasmic androgen receptor or can be converted to dihydrotestosterone (DHT) or estrogen. DHT is responsible for an even greater activation of the androgen receptor, whereas estrogen, the primary regulator of bone homeostasis in men, acts by binding to estrogen receptors.[4,5]

After 40 years of age, testosterone levels in men may decrease gradually and continuously at a rate of 1% to 2% per year. It is estimated that 50% of men aged 60 years or older may have considerably decreased testosterone levels.[3,6] Clinically, low testosterone

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levels may influence sexual function and libido, muscle strength, bone density, fatigue, cognition, mood, adipose tissue, or even affect periodontal disease.[6-8]

In contrast, supraphysiologic levels of testosterone may be induced by synthetic drugs used by body builders and other athletes to increase performance. The effects of these drugs include an increase in body weight, fat-free mass production, and enlargement of muscle size and mass. The reported side effects are sexual dysfunction; liver toxicity; and alterations of the psyche, behavior, and cardiovascular system.[9] Additionally, a case-control study reported that the prolonged use of anabolic androgenic steroids is associated with significant levels of gingival enlargement.[10]

However, the effect of testosterone on inflammation remains unclear. Androgens have been shown to be protective in certain conditions, and harmful in others.[11] They contribute to inflammation by influencing the activity of leukocytes and inflammatory cells such as neutrophils,[12] monocytes,[13] macrophages,[14] mast cells,[15] and platelets.[16,17] Although androgens protect males from inflammatory disorders such as atherosclerosis and rheumatoid arthritis,[18-20] they may also exacerbate wound inflammation.[21,22]

Polymerase chain reaction mRNA analysis suggests that androgen receptors are also expressed in human periodontal and gingival tissue. In addition, testosterone may modulate its own receptors, which indicates that supra- and subphysiologic levels of the hormone could modify tissue response.[23] Many in vitro experiments using periodontal cells and tissues have been performed to identify the role of testosterone on inflammatory markers such as prostaglandins and interleukins, as well as on the proliferative capacity of bone cells.[24-27] However, in vivo experiments are still needed to understand the influence and consequences of the variations of testosterone levels in the periodontium. Therefore, the primary purpose of this pilot study is to evaluate the consequences of supra- and subphysiologic testosterone levels on ligature-induced bone loss in rats.

MATERIALS AND METHODS

Animals

A total of 63 male adult Holtzman rats (*Rattus norvegicus albinus*) weighing 300 to 400 g were housed under similar conditions in cages with access to food and water *ad libitum*. During the entire experimental protocol, the rats were kept in a quiet room with controlled temperature (23 ± 2°C), humidity (65% to 75%), and a 12-hour light–dark cycle. All experimental protocols were approved by the local Ethics Committee for Animal Experimentation and conducted in accordance with the guidelines of the Brazilian College of Animal Experimentation.

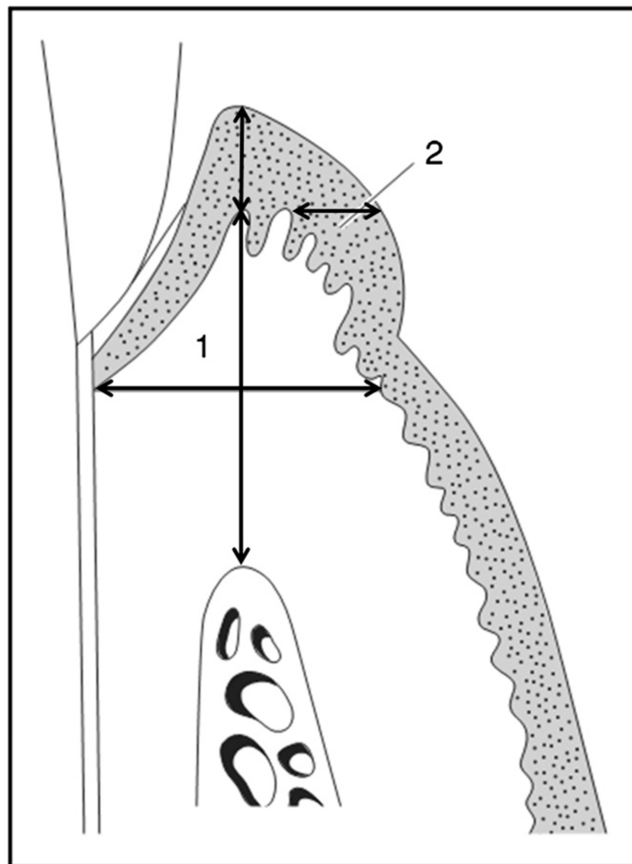

Figure 1.
Diagram illustrating the points used to evaluate the connective tissue and epithelial height and width measurements. Connective tissue (1) and buccal epithelium (2) areas were obtained by multiplying each tissue's height by its width. Modified from Corrêa et al. (2005).[32]

Orchiectomy and the Dynamics of Testosterone Levels After Exogenous Testosterone Administration

Three rats were used to evaluate the dynamics of testosterone levels after orchiectomy and testosterone injections. Surgical orchiectomy was performed under anesthesia using ketamine (1 mL/kg/body weight [bw]) and xylazine (0.4 mL/kg/bw). In brief, after a scrotal incision, both testicles were removed and the incision sutured under sterile conditions. The rats were given acetaminophen (300 mg/kg/bw; orally) for postoperative pain relief and an intramuscular dose of penicillin and streptomycin (1 mL/kg/bw). After the procedure, the animals were kept in separate cages for 7 recovery days. Three days postorchiectomy, the animals were given a single intramuscular injection of a long-lasting mixture of testosterone esters (30 mg testosterone propionate,

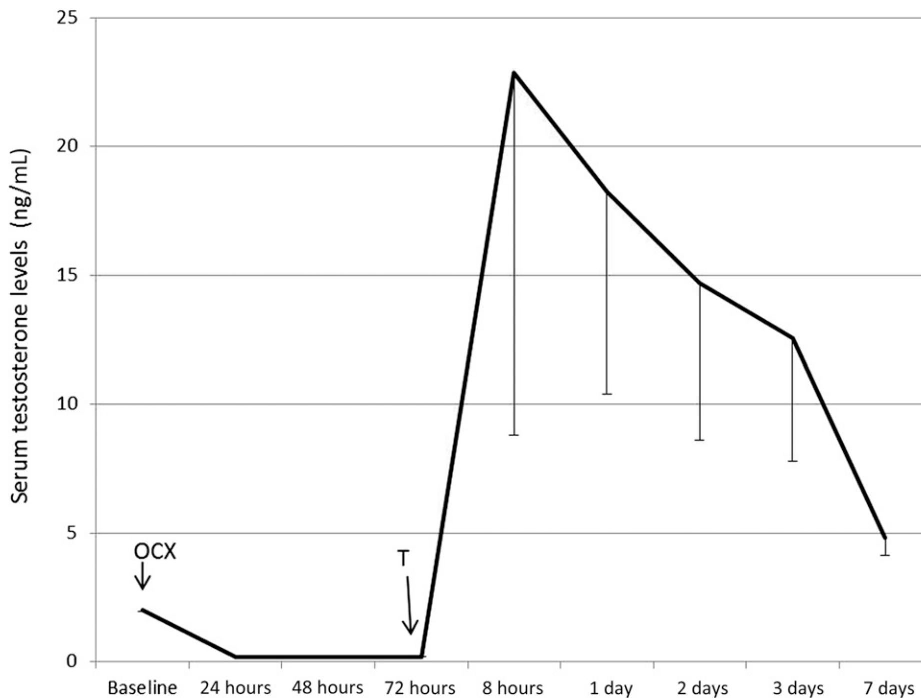

Figure 2.
Mean (± SEM) testosterone serum levels (ng/mL) at baseline, postorchiectomy, and post-testosterone injection (n = 3). OCX = orchiectomy; T= testosterone injection.

60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate, and 100 mg testosterone decanoate),‡ 250 mg/kg/bw, diluted to 0.1 mL in corn oil.[28] Venous blood (400 μL) was collected from the tail at baseline; 1, 2, and 3 days postorchiectomy; and 8 hours, 1, 2, 3, and 7 days post-testosterone injection. A blood sample was centrifuged for 10 minutes at 3,000 rpm to obtain approximately 150 μL of serum. Each serum sample was analyzed for total testosterone levels using a chemiluminescence-based immunoassay.§

Experimental Protocol

Sixty rats were randomly assigned to the following groups: 1) sham-operation controls (Sham; n = 10); 2) sham-operation and ligature-induced bone loss (Sham+L; n = 10); 3) orchiectomy without ligature (Ocx; n = 10); 4) Ocx and ligature (Ocx+L; n = 10); 5) Ocx plus testosterone injection without ligature (Ocx+T; n = 10); and 6) Ocx, T, and ligature (Ocx+T+L; n = 10). Thirty days postorchiectomy (or sham operation), the ligatures were placed bilaterally on the mandibular first molars as previously described and maintained for 15 days.[29] The Ocx+T group received injections of testosterone esters (250 mg/kg/bw, intramuscularly; diluted to 0.1 mL in corn oil)[28] every 7 days until the rats were sacrificed, starting 3 days postorchiectomy. At the end of the experimental period, the rats were sacrificed via an overdose of anesthesia.

Radiographic Evaluation

After the rats were sacrificed, the mandibles were removed, and the right hemimandible was evaluated radiographically as previously described.[30] In brief, digital x-ray images were used to estimate alveolar bone loss by examining the distance between the cemento-enamel junction (CEJ) and the height of alveolar bone in the mesial root surfaces of the mandibular right first molars. The analysis was conducted with the aid of software.‖

Histologic and Histometric Evaluation

The left hemimandibles were soaked in formalin (10%) for 48 hours. A quick decalcifying agent containing EDTA, sodium and potassium tartrate, chloric acid, and deionized water¶ was used for a 10-hour decalcification. Serial paraffin 5-μm-thick sections were made on the mesial and distal aspects of the whole mandibular right first molars and stained with hematoxylin and eosin. The gingival epithelium and connective tissue areas were measured in a modification of a previously described method.[31,32] In brief, histologic sections of each rat were photographed at 100× magnification, and the connective tissue and epithelial height and width were measured with the aid of software.# The connective tissue and buccal epithelium areas were obtained by multiplying each tissue's height by width (Fig. 1).

Statistical Analyses

One-way ANOVA, Tukey post hoc tests, and pairwise comparisons (Student *t* test) were used to assess the differences among the quantitative data. Whenever the groups did not reach homogeneity of variances or did not present a normal distribution, the Kruskal-Wallis (Dunn post hoc) or Mann-Whitney *U* tests were used. A paired *t* test was used to assess baseline and final weight differences. The data were expressed as mean ± SEM. All tests were

‡ Durateston, MSD Animal Health, Milton Keynes, UK.
§ Immulite 2000, Diagnostic Products Corporation, Gwynedd, UK.
‖ ImageTool for Windows 3.0, The University of Texas Health Science Center at San Antonio, San Antonio, TX.
¶ Allkimia, Campinas, São Paulo, Brazil.
\# BioEstat 5.0, Manuel Ayres, Belém, Porto Alegre, Brazil.

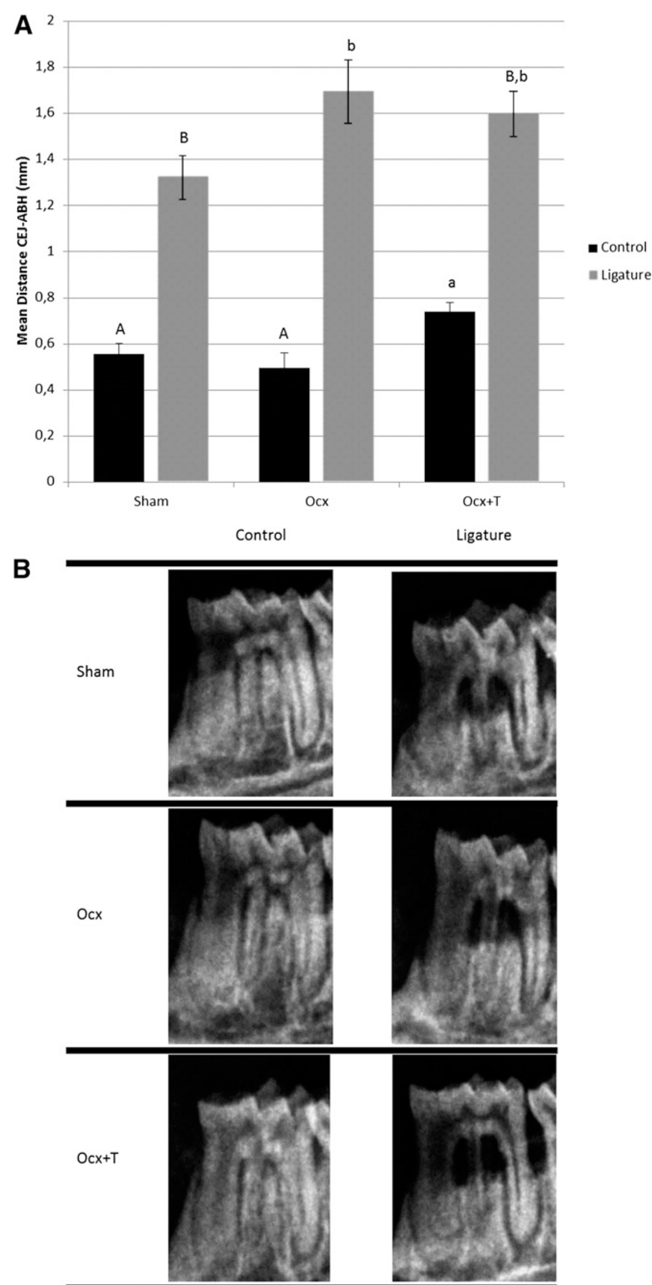

Figure 3.
A) *Mean (± SEM) distance from CEJ to alveolar bone height (ABH) (mm) measured in mesial root surfaces for each group (n = 10 animals/group). Same letters indicate no statistically significant difference (t test; P >0.05).* ***B)*** *Representative radiographs of each experimental group.*

performed using software,** and the significance level was set at $P = 0.05$.

RESULTS

All animals were alive at the end of the experimental period. The animals in the group that received a testosterone injection demonstrated aggressive behavior and were placed in isolated cages. The three animals that were used to evaluate the dynamics of testosterone levels after orchiectomy and testosterone injection presented mean baseline testosterone levels of 2.01 ± 0.07 ng/mL, which decreased to non-detectable levels 24 hours postorchiectomy. The testosterone injections resulted in a mean 11-fold maximum increase compared with the baseline serum levels 8 hours after drug administration. Seven days postinjection, testosterone levels decreased to values comparable to the baseline. The effect of orchiectomy and testosterone injection on the serum levels of testosterone is shown in Figure 2.

The mean baseline weight for each group was: 1) Sham = 388 ± 4 g; 2) Sham+L = 381 ± 2 g; 3) Ocx = 386 ± 5 g; 4) Ocx+L = 384 ± 7 g; 5) Ocx+T = 398 ± 4 g; and 6) Ocx+T+L = 379 ± 6 g ($P = 0.1$). The mean final weight for each group was: 1) Sham = 445 ± 8 g; 2) Sham+L = 445 ± 5 g; 3) Ocx = 442 ± 9 g; 4) Ocx+L = 418 ± 8 g; 5) Ocx+T = 404 ± 4 g; and 6) Ocx+T+L = 379 ± 8 g. Paired statistical testing indicated that the baseline and final weight measurements significantly differed for all groups ($P < 0.0001$), except for the Ocx+T and Ocx+T+L groups ($P > 0.05$).

Bone loss was successfully induced by ligature; the non-ligature groups significantly differed from the ligature-induced bone loss groups ($P < 0.05$). When inflammation was absent (non-ligature groups), a testosterone injection after orchiectomy significantly increased bone loss compared with either the orchiectomy alone or sham operation groups. In contrast, in the presence of inflammation (ligature groups), orchiectomized, but not testosterone-treated rats, presented an increased bone loss compared with sham operation controls ($P < 0.05$). The mean bone loss (± SEM) for each group is shown in Figure 3.

Histometric analyses indicated that, in the presence of inflammation, both supra- and subphysiologic levels of testosterone significantly increased the gingival area, but there was no increase in gingival area when inflammation was absent. Ligature-induced inflammation resulted in a significantly increased buccal epithelium in Ocx and Ocx+T groups compared with sham operation controls ($P < 0.05$), whereas connective tissue was significantly increased in only the Ocx+T group. The mean (± SEM) gingival area (μm^2) for each group at each experimental period is listed in Table 1. Representative histologic images of each experimental group are shown in Figure 4.

** BioEstat 5.0, Manuel Ayres.

Table 1.

Mean (± SEM) Gingival Area (μm^2) for Each Group (n = 10 animals/group)

Tissue Type	Treatment	Sham	Ocx	Ocx+T
Buccal epithelium	Control	6,662 ± 947	3,438 ± 383	4,875 ± 1,260
	Ligature	737 ± 271*	7,245 ± 1,826	14,995 ± 8,209
Connective tissue	Control	118,815 ± 15,693	121,159 ± 10,734	99,436 ± 7,806
	Ligature	62,264 ± 24,305†	120,319 ± 16,287	147,186 ± 8,295

* Statistically significant difference compared with Ocx and Ocx+T (Mann-Whitney *U*; *P* =0.01).
† Statistically significant difference compared with Ocx+T (Mann-Whitney *U*; *P* =0.02).

DISCUSSION

The effects of testosterone hormone on the periodontium and periodontal cells have been assessed both in humans[8,10] and in vitro,[33,34] but there is insufficient evidence to fully understand the exact effect of testosterone on periodontal tissues and inflammation in general. This issue is important, given the increasing age of most populations and the associated hormonal imbalances.[1] Using an animal model enables evaluation of the effects of hormonal imbalances in vivo with the additional advantage of enabling ex vivo analyses.

Sex steroid hormones, such as testosterone, carry out their function in adipose tissues by both genomic and non-genomic mechanisms, which leads to lipolysis.[35] As testosterone regulates the amount and distribution of adipose tissues, it would be expected that supraphysiologic levels would lead to fat burning compatible with the lack of statistically significant differences between final and baseline weights observed in the testosterone-treated rats. Additionally, the stress provided by weekly injections of a medication could have contributed to this finding.

Bone loss was significantly induced by ligatures in every ligature group, thus validating the method used. The authors observed that orchiectomized rats presented increased bone loss when compared with sham operation controls, but this difference was not observed when the ligature was not placed around the teeth, even though measuring bone loss only in the mesial surface of the tooth could result in an underestimation of bone loss. Daltaban et al.[8] demonstrated that in humans, clinical attachment levels were not statistically different in patients with hypergonadotropic hypogonadism compared with controls, although these authors observed a negative correlation between gingival index and free testosterone levels. In rats, bone marrow plasma and the bone marrow cell extract receptor activator of nuclear factor-kappa B ligand (RANKL), an essential cytokine for bone resorption, are significantly increased 1 and 2 weeks postorchiectomy.[36] It would, therefore, be expected that a decrease of testosterone levels would also decrease peripheral estrogen levels (also a product from testosterone conversion), which are closely related to bone metabolism regulation. However, in rats, 30-day orchiectomy significantly decreased serum testosterone and DHT levels, but not estrogen, whereas supraphysiologic testosterone treatment was related to significantly increased serum testosterone, DHT, and estrogen levels.[37]

Testosterone injections significantly increased both the connective tissue and epithelial areas in the presence of inflammation when compared with sham operation controls ($P < 0.05$). These results are in accordance with findings in humans that revealed the association between prolonged use of steroids and gingival enlargement.[10] The fact that sub- or supraphysiologic levels of testosterone did not influence the gingival area of non-ligature animals could be at least partially attributed to the inflammation-induced higher conversion of testosterone to DHT.[33,34] Gingival fibroblasts present androgen receptors (but not estrogen receptors), and DHT upregulates their proliferation.[25] However, our results clearly demonstrate that orchiectomized animals also present increased epithelial area when compared with sham operation controls. In fact, one study suggests that bone may contain an intraskeletal reservoir of sex steroids that are capable of producing biologic effects, and 30-day orchiectomy did not alter the testosterone or estrogen reservoir but instead increased DHT levels by 39%.[37] This suggests a role for the increased intraskeletal DHT reservoir following orchiectomy or other testosterone-related mechanisms that may be involved in gingival tissue regulation.

Supraphysiologic levels of testosterone were induced by the injection of testosterone esters, which resulted in a mean 11-fold maximum increase when compared with baseline serum levels 8 hours after drug administration. This represents a light hormone overdose (drug abusers usually take 10 to 100 times higher doses than those used for medical

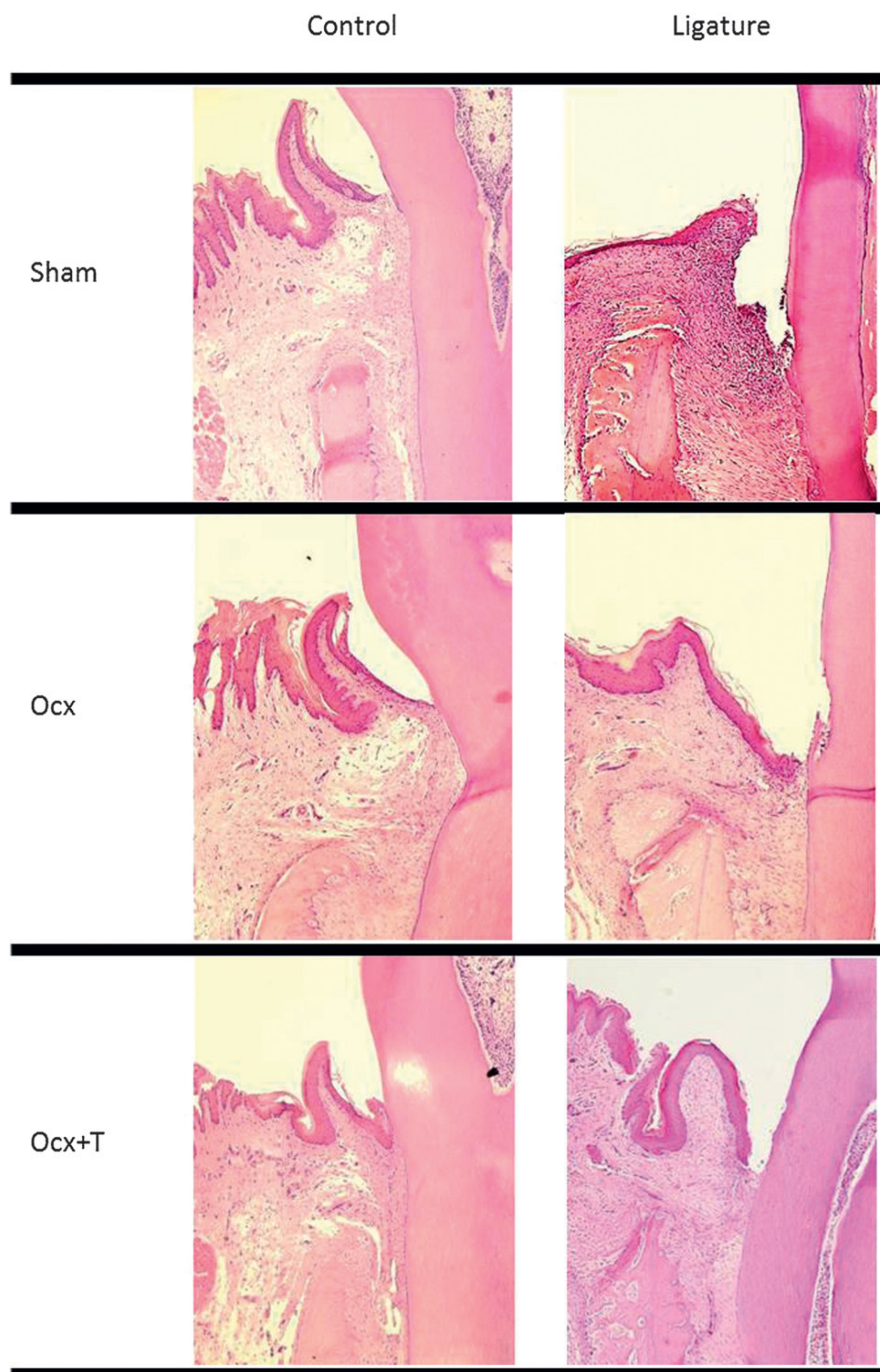

Figure 4.
Representative histologic images of each experimental group (H&E staining).

were also subjected to orchiectomy to verify the effect of synthetic hormone on the tissues and to serve as controls for the orchiectomy group. We did not use a hormone reposition group (physiologic levels) because the non-operation animals served as normal testosterone–level controls.

As this was an initial study, only the consequences of abnormal testosterone levels on rats' periodontal tissues in the presence or absence of inflammation were assessed. However, the mechanisms by which testosterone deficiency excessively influenced bone remodeling or epithelial and connective tissue areas of periodontal tissues in health and disease should be further investigated.

CONCLUSIONS

Both sub- and supraphysiologic levels of testosterone may influence bone metabolism, but only subphysiologic levels significantly increase ligature-induced bone loss, thus suggesting a protective effect of normal testosterone levels on inflammation-induced alveolar bone resorption. Last, testosterone has a regulatory effect on the gingival area.

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conditions).[10] Our results clearly demonstrate that the medication dose used (250 mg/kg) provides 7-day supraphysiologic testosterone levels, thus indicating that the method used provided high levels of testosterone throughout the experiment. Supraphysiologic levels of testosterone groups

REFERENCES

1. Harvey J, Berry JA. Andropause in the aging male. *J Nurse Pract* 2009;5:207-212.
2. Bain J. Testosterone and the aging male: To treat or not to treat? *Maturitas* 2010;66:16-22.
3. Gray M. Andropause and the aging man: Separating evidence from speculation. *Adv Nurse Pract* 2005;13: 22-27, quiz 28.
4. Vandenput L, Ohlsson C. Sex steroid metabolism in the regulation of bone health in men. *J Steroid Biochem Mol Biol* 2010;121:582-588.
5. Ohlsson C, Vandenput L. The role of estrogens for male bone health. *Eur J Endocrinol* 2009;160:883-889.
6. Gooren LJ. Androgens and male aging: Current evidence of safety and efficacy. *Asian J Androl* 2010; 12:136-151.
7. Bhasin S. Regulation of body composition by androgens. *J Endocrinol Invest* 2003;26:814-822.
8. Daltaban O, Saygun I, Bolu E. Periodontal status in men with hypergonadotropic hypogonadism: Effects of testosterone deficiency. *J Periodontol* 2006;77: 1179-1183.
9. van Amsterdam J, Opperhuizen A, Hartgens F. Adverse health effects of anabolic-androgenic steroids. *Regul Toxicol Pharmacol* 2010;57:117-123.
10. Ozcelik O, Haytac MC, Seydaoglu G. The effects of anabolic androgenic steroid abuse on gingival tissues. *J Periodontol* 2006;77:1104-1109.
11. Gilliver SC. Sex steroids as inflammatory regulators. *J Steroid Biochem Mol Biol* 2010;120:105-115.
12. Békési G, Kakucs R, Várbíró S, et al. In vitro effects of different steroid hormones on superoxide anion production of human neutrophil granulocytes. *Steroids* 2000;65:889-894.
13. Miyagi M, Morishita M, Iwamoto Y. Effects of sex hormones on production of prostaglandin E2 by human peripheral monocytes. *J Periodontol* 1993;64: 1075-1078.
14. Chao TC, Van Alten PJ, Walter RJ. Steroid sex hormones and macrophage function: Modulation of reactive oxygen intermediates and nitrite release. *Am J Reprod Immunol* 1994;32:43-52.
15. Mayo JC, Sáinz RM, Antolín I, Uría H, Menéndez-Peláez A, Rodríguez C. Androgen-dependent mast cell degranulation in the Harderian gland of female Syrian hamsters: In vivo and organ culture evidence. *Anat Embryol (Berl)* 1997;196:133-140.
16. Rosenblum WI, el-Sabban F, Nelson GH, Allison TB. Effects in mice of testosterone and dihydrotestosterone on platelet aggregation in injured arterioles and ex vivo. *Thromb Res* 1987;45:719-728.
17. Li S, Li X, Li J, Deng X, Li Y. Inhibition of oxidative-stress-induced platelet aggregation by androgen at physiological levels via its receptor is associated with the reduction of thromboxane A2 release from platelets. *Steroids* 2007;72:875-880.
18. Malkin CJ, Pugh PJ, Jones RD, Jones TH, Channer KS. Testosterone as a protective factor against atherosclerosis — Immunomodulation and influence upon plaque development and stability. *J Endocrinol* 2003; 178:373-380.
19. Mikkola TS, Clarkson TB. Estrogen replacement therapy, atherosclerosis, and vascular function. *Cardiovasc Res* 2002;53:605-619.
20. Spector TD, Ollier W, Perry LA, Silman AJ, Thompson PW, Edwards A. Free and serum testosterone levels in 276 males: A comparative study of rheumatoid arthritis, ankylosing spondylitis and healthy controls. *Clin Rheumatol* 1989;8:37-41.
21. Ashcroft GS, Mills SJ. Androgen receptor-mediated inhibition of cutaneous wound healing. *J Clin Invest* 2002;110:615-624.
22. Gilliver SC, Ashworth JJ, Mills SJ, Hardman MJ, Ashcroft GS. Androgens modulate the inflammatory response during acute wound healing. *J Cell Sci* 2006; 119:722-732.
23. Parkar MH, Newman HN, Olsen I. Polymerase chain reaction analysis of oestrogen and androgen receptor expression in human gingival and periodontal tissue. *Arch Oral Biol* 1996;41:979-983.
24. ElAttar TM, Lin HS, Tira DE. Testosterone inhibits prostaglandin formation by human gingival connective tissue: Relationship to 14C-arachidonic acid metabolism. *Prostaglandins Leukot Med* 1982;9:25-34.
25. Parkar M, Tabona P, Newman H, Olsen I. IL-6 expression by oral fibroblasts is regulated by androgen. *Cytokine* 1998;10:613-619.
26. Gornstein RA, Lapp CA, Bustos-Valdes SM, Zamorano P. Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *J Periodontol* 1999;70: 604-609.
27. Kasperk CH, Wergedal JE, Farley JR, Linkhart TA, Turner RT, Baylink DJ. Androgens directly stimulate proliferation of bone cells in vitro. *Endocrinology* 1989;124:1576-1578.
28. Nolan LA, Levy A. The effects of testosterone and oestrogen on gonadectomised and intact male rat anterior pituitary mitotic and apoptotic activity. *J Endocrinol* 2006;188:387-396.
29. Spolidorio LC, Herrera BS, Coimbra LS, Figueiredo MN, Spolidorio DM, Muscará MN. Short-term induction of thrombocytopenia delays periodontal healing in rats with periodontal disease: Participation of endostatin and vascular endothelial growth factor. *J Periodontal Res* 2010;45:184-192.
30. Herrera BS, Martins-Porto R, Maia-Dantas A, et al. iNOS-derived nitric oxide stimulates osteoclast activity and alveolar bone loss in ligature-induced periodontitis in rats. *J Periodontol* 2011;82:1608-1615.
31. Spolidorio LC, Spolidorio DM, Holzhausen M, Nassar PO, Nassar CA. Effects of long-term cyclosporin therapy on gingiva of rats — Analysis by stereological and biochemical estimation. *Braz Oral Res* 2005;19: 112-118.
32. Corrêa FdeO, Giro G, Gonçalves D, Spolidorio LC, Orrico SRP. Diltiazem did not induce gingival overgrowth in rats. A clinical, histological and histometric analysis. *Braz Oral Res* 2005;19:163-168.
33. Soory M. Targets for steroid hormone mediated actions of periodontal pathogens, cytokines and therapeutic agents: Some implications on tissue turnover in the periodontium. *Curr Drug Targets* 2000;1:309-325.
34. Soory M, Tilakaratne A. Anabolic potential of fibroblasts from chronically inflamed gingivae grown in a hyperglycemic culture medium in the presence or absence of insulin and nicotine. *J Periodontol* 2003; 74:1771-1777.

35. Mayes JS, Watson GH. Direct effects of sex steroid hormones on adipose tissues and obesity. *Obes Rev* 2004;5:197-216.
36. Li X, Ominsky MS, Stolina M, et al. Increased RANK ligand in bone marrow of orchiectomized rats and prevention of their bone loss by the RANK ligand inhibitor osteoprotegerin. *Bone* 2009;45:669-676.
37. Yarrow JF, Conover CF, Lipinska JA, Santillana CA, Wronski TJ, Borst SE. Methods to quantify sex steroid hormones in bone: Applications to the study of androgen ablation and administration. *Am J Physiol Endocrinol Metab* 2010;299:E841-E847.

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CAPÍTULO 3

The Impact of Testosterone on Inflammation-Induced Periodontal Bone Loss in Rats*

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Abstract

Testosterone, the main androgen, can be present at different levels within an individual's lifespan, which could alter host susceptibility to diseases. Periodontitis is an infectious disease that leads to inflammation and consequent bone loss around the teeth. The objective of this study was to evaluate the impact of different testosterone levels on ligature-induced periodontitis *in vivo*, assessing associated bone markers and cytokines expression, as well as to investigate the impact of testosterone on osteoclastogenesis *in vitro*.

A total of 80 male adult rats were used in the study and subjected to treatments that resulted in subphysiologic (L), normal, or supraphysiologic (H) serum concentrations of testosterone. Forty rats were subjected to bilateral orchiectomy and 40 rats received testicular sham-operation. Twenty of the sham-operated animals received flutamide (F), an androgen receptor antagonist. Three days after orchiectomy, 20 of the rats started receiving testosterone injections. Four weeks after surgery, half of the rats received a subgingival cotton ligature around the lower first molars, which is an experimental model for periodontitis, and were killed two weeks later. *In vitro*, osteoclasts were generated from RAW264.7 precursors for 4 days. Test groups were treated with testosterone at doses of 1, 10, 100 nM or 1µM. F (100nM) was added to the 100nM testosterone group.

In ligated animals, gingival levels of IL-1β were increased in L group, while F significantly reduced gingival IL-6 when compared to sham-operated animals. Linear analysis of bone loss using micro-computed tomography was increased for L, F and H groups when compared to ligated controls. Similarly, the number of osteoclasts was significantly reduced only when 10 and 100nM testosterone was added, which was successfully reversed by F. Testosterone also dose-dependently reduced TNF and RANTES.

Testosterone modulates host response to periodontitis in rats, interfering at a molecular level in the progression of the disease.

Keywords: testosterone; periodontitis; inflammation; androgens; gonadal steroid hormones.

Introduction

Aging can be defined as a gradual and time-dependent decline of various physiological functions, leading to the end of lifespan [1]. Nine hallmarks of aging have been described: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [2]. In 2000, the term "Inflammaging" was proposed to represent the low-grade pro-inflammatory status developed during the aging process, that predisposes the individual to age-related diseases [3]. Indeed, several experiments support this concept; aging mammals express an increase in genes related to the immune-inflammatory response; activation of nuclear factor kappa B (NF-κB) signaling (a key regulator of inflammation); and increased serum pro-inflammatory cytokines [4-8].

It was recently demonstrated that the aging process induces activation of inhibitor of nuclear factor kappa B kinase subunit beta (IKK-β) and NF-κB in the hypothalamus of mice, and mechanistic studies revealed that those proteins inhibited gonadotropin-releasing hormone (GnRH) [1]. In men, hypothalamic GnRH is responsible for regulating the release of interstitial cell-stimulating hormone by the pituitary gland, which in turn stimulates the Leydig cells to produce testosterone [9]. The decline of GnRH, and consequently testosterone, indicates the closing of the reproductive period of life – which is necessary for the quality of the species – but also contributes to systemic aging [1].

Declines in testosterone have broader consequences beyond reproduction. The maintenance of physiologic levels of testosterone is critical for overall men's health. Cross-sectional and prospective studies show that low testosterone levels are related to increased serum soluble interleukin (IL)-6 receptor (sIL-6r) in older men, although results for IL-6 were controversial [10, 11]. In addition, testosterone replacement therapy in hypogonadal men significantly decreased serum pro-inflammatory cytokines IL-1β and tumor necrosis factor (TNF) [12]. A recent cross-sectional study demonstrated that macrophage inflammatory

protein 1-alpha (MIP-1α), 1-beta (MIP-1β) and TNF are negatively associated with total testosterone in young men, before any concurrent manifestation of age-related systemic diseases, suggesting that low testosterone levels also promotes an aging-independent pro-inflammatory status [13].

Other studies have shown that testosterone treatment of orchiectomized mice and young male rats suppresses leukocyte counts [14, 15]. The results also showed that testosterone treatment decreased monocyte counts, $CD4^{+}/CD8^{+}$ ratio, and inhibited proliferative responses of lymphocytes [15]. Due to the immunosuppressive properties of the doses of testosterone used, animals that were treated with testosterone were more susceptible to infection [14].

Periodontitis is a chronic inflammatory disease affecting the supporting structures of teeth that is characterized by an overproduction of innate immune cytokines, such as IL-1β, IL-6, and TNF, leading to tissue breakdown, and consequent loss of clinical attachment and alveolar bone [16]. The loss of bone in periodontitis is thought to be a consequence of altered coupling of bone resorption and formation. The disease etiology is infectious; it is initiated and maintained by specific microorganisms organized on the tooth surfaces as an oral biofilm. Since the pathogens associated with periodontitis are commensal, susceptibility and host response are key regulators of disease initiation and progression, and have been investigated at genetic, cellular and molecular levels [17].

Since periodontitis is an infection-derived inflammatory disease, we used a model of experimental periodontal disease in rats to test the hypothesis that sub- and supraphysiologic testosterone levels impact host susceptibility to periodontal bone loss. The objective of the present study is to evaluate the actions of testosterone in an experimental model of periodontitis *in vivo*, assessing associated bone markers and cytokine expression, as well as to investigate the impact of testosterone on osteoclastogenesis *in vitro*.

Materials and Methods

Animals

Eighty male adult Holtzman rats weighing 300-400g were kept in cages under similar conditions (controlled temperature 23±2ºC, humidity 65-75% and 12-hour light-dark cycles). Food and water were provided *ad libitum*. Randomization of animals was performed using a riffle method. All experimental protocols were approved by the Institutional Ethics Committee for Animal Experimentation (protocol #25/2010) and performed in accordance with the guidelines of the Brazilian Society of Science on Laboratory Animals (SBCAL). This study conforms to the ARRIVE guidelines.

Induction of Sub- and Supraphysiologic Serum Testosterone Levels

After 1 week of acclimatization, forty rats received orchiectomy to suppress testosterone production. Briefly, a scrotal incision was performed for bilateral testicular removal and the incision was sutured under anesthesia using ketamine [1 mL/kg/body weight (bw)] and xylazine (0.4 mL/kg/bw) under sterile conditions. The rats were given acetaminophen (300 mg/kg/bw; orally) for postoperative pain and a single intramuscular dose of penicillin and streptomycin (1 mL/kg/bw). After the procedure, the animals were kept in individual cages for recovery for 7 days. Starting three days after orchiectomy, twenty of the rats received 250 mg/kg of a long-acting mixture of testosterone esters – 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate, and 100 mg testosterone decanoate (Durateston, MSD, Campinas, SP, Brazil). The medication was diluted to 0.1mL in corn oil and injected intramuscularly every 7 days until sacrifice. Twenty other rats received the same surgical procedure except for the testicular removal and were considered sham-surgery controls.

Assessment of the Role of Androgen Receptors on Testosterone-Related Responses

Flutamide (Sigma-Aldrich, Saint Louis, MO, USA; 50mg/kg), an androgen receptor antagonist, was administered intragastrically, every other day, to twenty sham-surgery

controls until sacrifice. Flutamide was diluted to 25mg/mL in distilled water and Tween-20. The treatment was started three days after sham-surgery [18].

Induction of Experimental Periodontal Disease

Four weeks after orchiectomy (or sham-surgery), half of the rats in each group (n=10/group) were anesthetized as above. A 3.0 cotton ligature was placed in a subgingival position around the lower first molar tooth. Ligatures enable bacterial accumulation leading to inflammation and bone loss. The other 10 animals in each group served as controls. The ligatures were maintained for 2 weeks at which time all rats were sacrificed. A schematic representation of the *in vivo* study design is shown in figure 1.

Blood Assessment

A blood sample was collected from every animal at the end of the experiment. After clotting for 45 minutes at room temperature, the sample was centrifuged for 10 minutes at 3,000 rpm to obtain serum. Each serum sample was analyzed for total testosterone levels using a chemiluminescence-based immunoassay (Immulite 2000, Diagnostic Products Corporation, Gwynedd, UK); biochemical colorimetric tests for calcium (Ca^{2+}), alkaline phosphatase (ALP) and phosphorus (P) (Bioclin, Quibasa, Belo Horizonte, MG, Brazil); and enzyme-linked immunosorbent assays (ELISA) for the detection of interleukin (IL)-1β and IL-6, using commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Micro Computed Tomography (μCT) Analyses

For quantitative and qualitative three-dimensional (3D) analysis of the alveolar bone, mandibles of 5 animals per group were scanned using a μCT system (Skyscan, Aartselaar, Belgium). The specimens were scanned at a resolution of 18 μm in all three spatial dimensions. CTan/CTvol software (Skyscan) was used for imaging and analysis. The region of interest (ROI) was interpolated and drawn including all bone medial to the mesial roots of

the second molar to the mesial surface of the first molar, using a slice-based method. Bone volume fraction (BV/TV) was analyzed by the CT-scan software.

Additionally, the linear distance between the cemento-enamel junction and alveolar bone was measured on the mesial surface of the first molars using Dataviewer 1.4.3, (Skyscan) software. The measurement was performed three times by a calibrated individual, who was blinded to the treatment groups, and under the same background conditions. The mean of all three measurements was considered one sample and used for statistical analysis.

Local Expression of Cytokines in the Tissue

The mucogingival tissues around the first molars of the remaining 5 animals per group were removed and processed for concentrations of IL-1β and IL-6 using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions. Total protein content in each sample was determined using the Bradford method and the results were used for normalization.

Increasing Testosterone Concentration and Osteoclast-like Cells

The RAW264.7 murine monocyte/macrophage cell line was obtained from ATCC (Manassas, VA, USA) and cultured in T-75 flasks containing MEM-alpha supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin until approximately 90% cell confluence, with media replacement every three days. On day 4, cells were seeded at $2.5x10^4$ cells/mL in 96-well plates (200μL/well) in the same media with the addition of 50ng/mL recombinant murine sRANKL (PeproTech, Rocky Hill, NJ, USA).

Testosterone (Sigma-Aldrich) was diluted in DMSO to a stock concentration of 100 mM and further dilutions were performed using medium. The tested doses included 1nM, 10nM, 100nM and 1μM. 1% DMSO was added to each control well. Flutamide (Sigma-Aldrich), 1μM, was used alone or in addition to testosterone to evaluate the role of androgen receptors on cell responses. Each treatment was performed in quintuplicate, and each plate was repeated three times.

After 5 days, the supernatants were collected under denaturizing conditions for the analysis of IL-6, IL-10, Tumor Necrosis Factor (TNF) and Regulated on Activation Normal T cells Expressed and Secreted (RANTES), using a multiplex ELISA approach (Millipore, Billerica, MA, USA). The attached cells were then washed with PBS, fixed with 10% formalin for 5 minutes, and stained using tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Sigma-Aldrich), according to the manufacturer's instructions. After staining, each well was divided into 4 quadrants, photographed at 40x magnification, and osteoclasts were counted using Image J software (National Institute of Health, Bethesda, MD, USA); and ImageTool, (UTHSCSA, San Antonio, TX, USA). Osteoclasts were defined as TRAP-positive cells containing 3 or more nuclei.

Caspase-3 Analysis

The apoptotic marker caspase-3 was analyzed in separate experiments as above ending on day 3. The supernatant was discarded and the cell lysate was obtained using the buffer supplied in the Caspase-3 Colorimetric Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). All procedures were conducted according to the manufacturer's instructions. The samples were read at 405nm.

Androgen Receptor Expression Analysis

The expression of androgen receptors was assessed by Western Blot. After 5 days, the cell lysate was obtained using RIPA buffer containing 1% protease inhibitor cocktail (P8340, Sigma-Aldrich). Incubation with the primary antibody (sc-816, Santa Cruz Biotechnology, Dallas, TX, USA), diluted 1:200 in 5% milk/TBS-T, was performed overnight at $4^{o}C$. β-actin reactive bands served for densitometry normalization, which was performed with the aid of software (ChemImager 5500 system, Alpha Innotech Corp., USA).

Statistical Analysis

Tests for homogeneity of variance and normal distribution were performed. If data satisfied both criteria, one-way analysis of variance (ANOVA) and post-hoc Tukey tests were

performed. Pairwise analyses were also performed using t tests where appropriate. If normality criteria were not met, the non-parametric Kruskal-Wallis and Dunn's post-test were performed. Alternatively, Mann-Whitney was performed for pairwise comparisons. All tests were performed using GraphPad Prism 5.0 for Mac OS X software (GraphPad Software, San Diego, CA, USA). The significance level was set at $p<0.05$. Fold-change calculations were performed by dividing all data by the average of the control group. Data are expressed as mean ± SEM, unless otherwise stated.

Results

Orchiectomy significantly decreased the levels of testosterone to below the detection point of the assay; administration of testosterone provided a 20-fold increase in the serum hormone concentration. Calcium serum concentration was not modulated by any treatment in the control group; was increased in orchiectomized and testosterone-treated animals with ligature induced periodontitis. Phosphorus and alkaline phosphatase levels were significantly decreased in the control and ligated testosterone-treated animals, and ligature also reduced ALP concentration in sham and orchiectomized animals when compared to their controls (Figure 2).

Three-dimensional analysis of bone volume fraction (BV/TV) demonstrated that ligature successfully induced periodontal bone loss, but only testosterone-treated animals had higher bone loss than the ligated sham-operated animals. Linear analysis of the bone on the mesial surface of the first molars showed that the distance between cemento-enamel junction and alveolar bone was increased for both orchiectomized and testosterone-treated ligated animals. The same pattern was observed *in vitro*, where the number of osteoclasts was significantly diminished in the presence of 10 and 100nM testosterone, but the lower and higher tested doses were not different from control. Caspase-3 activity was dose-dependently

increased by testosterone treatment, with a significant increase at the 100nM dose. At the highest dose, caspase-3 activity decreased again (Figure 3).

Orchietomy significantly increased gingival levels of IL-1β in ligated animals, which was reversed by testosterone treatment, but had no impact in controls. In vitro, IL-6 and IL-10 were not modulated by testosterone treatments, but TNF and RANTES were dose-dependently decreased (Figure 4).

Flutamide-treated animals had serum testosterone levels 5 times as high as sham-operated controls, and that difference was also significant when compared to orchiectomized animals. In the ligature animals, the linear bone loss evaluation demonstrated similar loss to that observed in orchiectomized animals, and was also significantly higher than sham-operated animals. Local expression of IL-1β with ligature, as opposed to orchiectomy alone, was not increased in flutamide-treated animals. IL-6 levels, however, were significantly decreased in the local tissue in flutamide-treated ligated animals (Figure 5).

In vitro, testosterone treatment dose-dependently increased the expression of androgen receptor on osteoclasts, with a significant increase at the dose of 1μM. Flutamide treatment antagonized the actions of the 100nM dose of testosterone, but failed to reverse the impact of the 1μM dose on TNF and RANTES production (Figure 6).

Discussion

Sex hormones have many functions including modulating the host response. With respect to ‘Inflammaging’, it is still unclear whether the decline in testosterone levels leads to a deregulation of systemic inflammation or it is a consequence of the aging process and its associated inflammation. Periodontitis is a chronic inflammatory disease triggered and maintained by specific pathogenic microorganisms in the oral biofilm that involves soft and hard tissue loss. Many *in vitro* studies have assessed the role of androgens on different periodontal soft tissue cells: in gingival connective tissue, testosterone inhibits prostaglandin

formation [19], and in inflamed gingiva there is an increase in 5-alpha-reductase activity [20]. In fibroblasts, increasing concentrations of androgens inhibits IL-6 production [21, 22] and increases cell proliferation [23]. Our group previously demonstrated that low and high testosterone levels in rats increase both gingival and connective tissue areas [24]. The impact of sex hormones on periodontal health has recently been revisited in publications, with the proposal of the term 'Periodontal Reproductive Endocrinology' [25].

In this study using adult rats, it was demonstrated that orchiectomy decreases serum testosterone levels that can be reversed with exogenous administration of testosterone to achieve supraphysiologic levels providing a model for the impact of testosterone levels in periodontitis and oral health. The testosterone concentration used therapeutically was effective in maintaining supraphysiologic levels for up to 7 days, when a second injection was required [24]. Ligature-induced periodontal disease in sham-operated animals did not change serum calcium or phosphorus levels when compared to their respective non-periodontitis controls, which is in accordance with Nassar et al., 2004 [26]. In contrast, we observed a significant decrease of alkaline phosphatase in sham-operated animals due to the insertion of the ligature, which is in accordance with the findings of Goes et al., 2012 [27]. A previous study had also reported that orchiectomy does not change serum calcium levels [28], suggesting that the body may exert a strict regulation on ionic absorption/secretion, most probably to protect the neuromuscular system and other calcium-dependent processes rather than maintaining the skeleton integrity. Some studies had also reported no statistically significant difference in alkaline phosphatase levels, which is in accordance with our results [28, 29]. However, low levels of free androgen have been correlated with low levels of bone markers in men [30]. Interestingly, the pattern of the impact of testosterone modulation on bone markers is similar in health and disease. One should notice that the most profound effects on the studied bone markers are those presented by testosterone-treated groups.

Our results showed that testosterone modulates periodontal bone loss in an inflammatory environment, but it was not enough to increase bone loss in control rats. Again, high testosterone levels presented the highest impact on bone loss, as it was the only group that differed from sham-operated rats with ligature when three-dimensional analysis was performed. On the other hand, both orchiectomy and orchiectomy plus testosterone rats presented with higher bone loss during inflammation than sham-operated controls. As a matter of fact, some of our previous studies also demonstrated that measuring the mesial surface of the ligated teeth was a more sensitive analysis than the three-dimensional one. This may be due to the great impact that 14 days of ligature have on bone loss at all periodontal areas (furcation, mesial and distal sites), and also that the knot is placed on the mesial surface of the tooth.

Similar results have been found using an osteoclast differentiation assay. Apart from the 1µM dose, we showed here that testosterone dose-dependently decreases osteoclast formation from RANKL-stimulated RAW264.7 cells. A similar study showed that this happens through a selective regulation of *c-Jun*, a key transcription factor that is essential for osteoclast formation [31]. Other studies have shown that androgens decrease osteoclastogenesis from stimulated mouse unfractionated bone cell culture [32] and human peripheral blood CD14+ monocytes [33], as well as decrease bone resorption by primary avian cell culture, primary murine cell culture, and human osteoclasts from giant cell tumors [34]. Our findings also suggest that this observation is due to increased apoptosis, as can be derived from the increase in caspase-3 apoptotic marker. However, just like in the *in vivo* experiment, we also demonstrate that there is a saturation limit where the impact is reversed, as can be seen in the dose of 1µM.

Indeed, testosterone levels in men may vary greatly among individuals and in different race/ethnical populations [35]. A nationally representative sample of Americans, for example, present with a total testosterone concentration mean of 20.68nM (IC 95%: 19.54-21.93) when

they are 20-29 years old, declining to 15.89nM (IC 95%: 14.85-17.00) at late adulthood (50-59 years) [36]. In rats, we have shown that mean serum concentration of the hormone was approximately 7nM and a 10-fold reduction in these levels was obtained following orchiectomy as a model of hypogonadism [24]. On the other hand, abusive anabolic androgenic steroid users, such as bodybuilders and those who use it for athletic purposes, generally use these drugs in a concentration 10-100 times as high as those used for medical conditions [37]. However, it is possible that the concentration of the hormone in bone is higher than in serum [38]. Based on that, even though many studies used 10nM testosterone to represent 'physiologic' levels, we tested 4 different testosterone concentrations in an attempt to cover some possible clinical presentations of testosterone in bone tissue.

We demonstrated that orchiectomy upregulates IL-1β production only at the site of inflammation (and not systemically), but it had no effect on IL-6 either locally or systemically. Both IL-1β and IL-6 are important cytokines that regulate osteoclast number and activity and are therefore related with bone loss [16]. Our findings are not in accordance with experiments in male mice and murine bone marrow-derived cells, where it was shown that testosterone decreases the expression of IL-6 gene and that IL-6 mediates the upregulation of osteoclastogenesis and bone loss [39]. At the same time, IL-10 production and release in osteoclast culture was not affected by testosterone treatment. IL-10 is an anti-inflammatory cytokine produced by T cells, B cells, eosinophils, keratinocytes, mast cells and monocytes/macrophages [40] and it downregulated osteoclastogenesis when RAW264.7 cells were used as precursors [41]. Previous studies have shown that testosterone increases IL-10 production by CD4+ T lymphocytes in mouse splenocyte culture [42]. Also, in humans, IL-10 was significantly increased in hypogonadal men who received testosterone replacement therapy when compared to the control group [12].

RANTES, also known as CCL-5, is a chemokine that is induced when human bone resorbing cells differentiate from monocyte precursors *in vitro* and can stimulate

differentiation of cells with visual appearance of osteoclasts [43]. Additionally, 6-month-old RANTES-deficient mice display osteopenia associated with decreased bone formation and increased osteoclastogenesis [44]. Taken together, these data may imply that RANTES expression can limit or modulate osteoclastogenesis. We demonstrated here that testosterone treatment dose-dependently inhibits RANTES expression, which is in accordance with the osteoclast number finding, although we expected the highest dose to increase again. That suggests that low and high testosterone doses have different pathways and mechanisms to maintain the osteoclast number similar to the control group. As for TNF, a cause and effect relationship between this cytokine and bone loss in several chronic inflammatory diseases have been demonstrated [45]. Similarly to RANTES, our results show that testosterone dose-dependently decreased TNF, even though the difference was significant only for the two higher hormone doses. In humans, some conflicting results have been demonstrated regarding the relationship between cytokines and testosterone. A cross-sectional study demonstrated an inverse correlation between testosterone and soluble IL-6 receptor in older men (sIL-6r), but no correlation was found for other markers, such as IL-6, IL-1β or TNF [10]. Differently, induction of 4-week hypogonadism in older men increased both IL-6 and sIL-6r in older men [11], but those results seem not to be maintained in longer periods of hypogonadism [46]. Testosterone replacement therapy in hypogonadal men significantly decreased IL-1β and TNF, but had no effect on IL-6 levels [12]. When testosterone replacement therapy was withdrawn, a significant increase in IL-6 and decrease in TNF levels was observed two weeks later [47].

Flutamide was used to isolate the impact of low testosterone levels, which could also imply in lower estrogen levels, to those effects related to androgen receptor activation. Estrogen is a key regulator of bone turnover, and its impact in men has been documented [48]. We showed that periodontal bone loss is increased in flutamide-treated animals in a fashion similar to that observed in orchiectomized animals, even though serum testosterone

concentration was increased. This finding suggests that the androgen receptor activation is necessary for the beneficial effects of testosterone on bone. However, local cytokine expression suggests that these two models of testosterone action deprivation work through different pathways. IL-1β was not increased in flutamide-treated animals as it was for the orchiectomized ones. Additionally, IL-6 was significantly decreased in flutamide-treated animals. A possible explanation is that IL-6, besides its bone resorption-related effects, also displays anti-inflammatory and immunosuppressive effects, contributing to the resolution of acute and chronic inflammation [49].

In vitro, we show that androgen receptor expression is enhanced with increasing offer of testosterone. The presence of androgen receptor in mice osteoclast-like cells had already been demonstrated [50, 51], as well as the increase in androgen receptor expression and specific binding with increasing doses of androgens in osteoblastic cells [52]. Flutamide was able to reverse the significant androgen-derived decrease in osteoclast number, which is in accordance with previous studies that showed an antagonizing effect for both osteoclast number [32] and activity [33, 34]. However, the impact of high doses of testosterone in TNF and RANTES seem not to be related with the androgen receptor. As a matter of fact, the aromatase enzyme that converts testosterone into estradiol is expressed in osteoclast and osteoclast precursors cell culture, and therefore could be implicated in these findings [53].

Our study was designed to represent three possible clinical conditions of testosterone: low, normal and high serum concentrations. Orchiectomy was also performed to the testosterone-treated animals so they could serve as controls for the orchiectomized-only group. The choice of 30-day period before placing ligature was due to the induction of osteopenia/osteoporosis that follows long periods of low testosterone levels [54] and could insert other variables to the study other than testosterone levels.

Taken together, our results indicate that testosterone modulates host response to periodontal disease, interfering in the progression of periodontitis. This action is mediated, at

least in part, via the androgen receptor. Additionally, regulation of osteoclastogenesis is one of the mechanisms involved. Future studies should be performed to evaluate if nonphysiological testosterone levels, and also if endogenous variations in testosterone levels, could contribute to spontaneous initiation of periodontal disease. Additionally, it should be tested if treatment outcomes are modified by varying levels of testosterone.

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REFERENCES

1. Zhang, G., et al., *Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and GnRH.* Nature, 2013. **497**(7448): p. 211-6.
2. Lopez-Otin, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
3. Franceschi, C., et al., *Inflamm-aging. An evolutionary perspective on immunosenescence.* Ann N Y Acad Sci, 2000. **908**: p. 244-54.

4. de Magalhaes, J.P., J. Curado, and G.M. Church, *Meta-analysis of age-related gene expression profiles identifies common signatures of aging.* Bioinformatics, 2009. **25**(7): p. 875-81.

5. Singh, T. and A.B. Newman, *Inflammatory markers in population studies of aging.* Ageing Res Rev, 2011. **10**(3): p. 319-29.

6. Salminen, A., et al., *Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging.* Ageing Res Rev, 2008. **7**(2): p. 83-105.

7. Adler, A.S., et al., *Motif module map reveals enforcement of aging by continual NF-kappaB activity.* Genes Dev, 2007. **21**(24): p. 3244-57.

8. Salminen, A., K. Kaarniranta, and A. Kauppinen, *Inflammaging: disturbed interplay between autophagy and inflammasomes.* Aging (Albany NY), 2012. **4**(3): p. 166-75.

9. Mawhinney, M. and A. Mariotti, *Physiology, pathology and pharmacology of the male reproductive system.* Periodontol 2000, 2013. **61**(1): p. 232-51.

10. Maggio, M., et al., *Correlation between testosterone and the inflammatory marker soluble interleukin-6 receptor in older men.* J Clin Endocrinol Metab, 2006. **91**(1): p. 345-7.

11. Khosla, S., et al., *Effect of estrogen versus testosterone on circulating osteoprotegerin and other cytokine levels in normal elderly men.* J Clin Endocrinol Metab, 2002. **87**(4): p. 1550-4.

12. Malkin, C.J., et al., *The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men.* J Clin Endocrinol Metab, 2004. **89**(7): p. 3313-8.

13. Bobjer, J., et al., *Negative association between testosterone concentration and inflammatory markers in young men: a nested cross-sectional study.* PLoS One, 2013. **8**(4): p. e61466.

14. Kamis, A.B. and J.B. Ibrahim, *Effects of testosterone on blood leukocytes in plasmodium berghei-infected mice.* Parasitol Res, 1989. **75**(8): p. 611-3.

15. Yao, G., et al., *In vivo modulation of the circulating lymphocyte subsets and monocytes by androgen.* Int Immunopharmacol, 2003. **3**(13-14): p. 1853-60.

16. Graves, D.T., T. Oates, and G.P. Garlet, *Review of osteoimmunology and the host response in endodontic and periodontal lesions.* J Oral Microbiol, 2011. **3**.

17. Van Dyke, T.E., *Cellular and molecular susceptibility determinants for periodontitis.* Periodontol 2000, 2007. **45**: p. 10-3.

18. Kurling-Kailanto, S., et al., *Blockade of androgen or estrogen receptors reduces nandrolone's ability to modulate acute reward-related neurochemical effects of amphetamine in rat brain.* Pharmacol Biochem Behav, 2010. **95**(4): p. 422-7.

19. ElAttar, T.M., H.S. Lin, and D.E. Tira, *Testosterone inhibits prostaglandin formation by human gingival connective tissue: relationship to 14C-arachidonic acid metabolism.* Prostaglandins Leukot Med, 1982. **9**(1): p. 25-34.

20. Vittek, J., et al., *Concentration of circulating hormones and metabolism of androgens by human gingiva.* J Periodontol, 1979. **50**(5): p. 254-64.

21. Parkar, M., et al., *IL-6 expression by oral fibroblasts is regulated by androgen.* Cytokine, 1998. **10**(8): p. 613-9.

22. Gornstein, R.A., et al., *Androgens modulate interleukin-6 production by gingival fibroblasts in vitro.* J Periodontol, 1999. **70**(6): p. 604-9.

23. Coletta, R.D., et al., *Testosterone stimulates proliferation and inhibits interleukin-6 production of normal and hereditary gingival fibromatosis fibroblasts.* Oral Microbiol Immunol, 2002. **17**(3): p. 186-92.

24. Steffens, J.P., et al., *The effect of supra- and subphysiologic testosterone levels on ligature-induced bone loss in rats--a radiographic and histologic pilot study.* J Periodontol, 2012. **83**(11): p. 1432-9.

25. Mariotti, A., *The ambit of periodontal reproductive endocrinology.* Periodontol 2000, 2013. **61**(1): p. 7-15.

26. Nassar, C.A., et al., *Effect of cyclosporin A on alveolar bone homeostasis in a rat periodontitis model.* J Periodontal Res, 2004. **39**(3): p. 143-8.

27. Goes, P., et al., *Effect of alendronate on bone-specific alkaline phosphatase on periodontal bone loss in rats.* Arch Oral Biol, 2012. **57**(11): p. 1537-44.

28. Hock, J.M., et al., *Human parathyroid hormone-(1-34) increases bone mass in ovariectomized and orchidectomized rats.* Endocrinology, 1988. **122**(6): p. 2899-904.

29. Bu, S.Y., et al., *Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats.* Osteoporos Int, 2007. **18**(7): p. 931-42.

30. Wishart, J.M., et al., *Effect of age on bone density and bone turnover in men.* Clin Endocrinol (Oxf), 1995. **42**(2): p. 141-6.

31. Huber, D.M., et al., *Androgens suppress osteoclast formation induced by RANKL and macrophage-colony stimulating factor.* Endocrinology, 2001. **142**(9): p. 3800-8.

32. Chen, Q., et al., *Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor.* FEBS Lett, 2001. **491**(1-2): p. 91-3.

33. Michael, H., et al., *Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption.* J Bone Miner Res, 2005. **20**(12): p. 2224-32.

34. Pederson, L., et al., *Androgens regulate bone resorption activity of isolated osteoclasts in vitro.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 505-10.

35. Mazur, A., *The age-testosterone relationship in black, white, and Mexican-American men, and reasons for ethnic differences.* Aging Male, 2009. **12**(2-3): p. 66-76.

36. Rohrmann, S., et al., *The prevalence of low sex steroid hormone concentrations in men in the Third National Health and Nutrition Examination Survey (NHANES III).* Clin Endocrinol (Oxf), 2011. **75**(2): p. 232-9.

37. Ozcelik, O., M.C. Haytac, and G. Seydaoglu, *The effects of anabolic androgenic steroid abuse on gingival tissues.* J Periodontol, 2006. **77**(7): p. 1104-9.

38. Yarrow, J.F., et al., *Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration.* Am J Physiol Endocrinol Metab, 2010. **299**(5): p. E841-7.

39. Bellido, T., et al., *Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor.* J Clin Invest, 1995. **95**(6): p. 2886-95.

40. Asadullah, K., W. Sterry, and H.D. Volk, *Interleukin-10 therapy--review of a new approach.* Pharmacol Rev, 2003. **55**(2): p. 241-69.

41. Mohamed, S.G., et al., *Interleukin-10 inhibits RANKL-mediated expression of NFATc1 in part via suppression of c-Fos and c-Jun in RAW264.7 cells and mouse bone marrow cells.* Bone, 2007. **41**(4): p. 592-602.

42. Liva, S.M. and R.R. Voskuhl, *Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production.* J Immunol, 2001. **167**(4): p. 2060-7.

43. Kim, M.S., et al., *Induction of chemokines and chemokine receptors CCR2b and CCR4 in authentic human osteoclasts differentiated with RANKL and osteoclast like cells differentiated by MCP-1 and RANTES.* J Cell Biochem, 2006. **97**(3): p. 512-8.

44. Wintges, K., et al., *Impaired bone formation and increased osteoclastogenesis in mice lacking chemokine (C-C motif) ligand 5 (Ccl5).* J Bone Miner Res, 2013.

45. Boyce, B.F., et al., *TNF-alpha and pathologic bone resorption.* Keio J Med, 2005. **54**(3): p. 127-31.

46. Maggio, M., et al., *Circulating inflammatory cytokine expression in men with prostate cancer undergoing androgen deprivation therapy.* J Androl, 2006. **27**(6): p. 725-8.

47. Yialamas, M.A., et al., *Acute sex steroid withdrawal reduces insulin sensitivity in healthy men with idiopathic hypogonadotropic hypogonadism.* J Clin Endocrinol Metab, 2007. **92**(11): p. 4254-9.

48. Vandenput, L. and C. Ohlsson, *Sex steroid metabolism in the regulation of bone health in men.* J Steroid Biochem Mol Biol, 2010. **121**(3-5): p. 582-8.

49. Tilg, H., C.A. Dinarello, and J.W. Mier, *IL-6 and APPs: anti-inflammatory and immunosuppressive mediators.* Immunol Today, 1997. **18**(9): p. 428-32.

50. Mizuno, Y., et al., *Immunocytochemical identification of androgen receptor in mouse osteoclast-like multinucleated cells.* Calcif Tissue Int, 1994. **54**(4): p. 325-6.

51. Turner, A.G., et al., *Androgen Receptor Expression and Function in Osteoclasts.* The Open Physiology Journal, 2008. **1**(1): p. 28-33.

52. Wiren, K., et al., *Homologous androgen receptor up-regulation in osteoblastic cells may be associated with enhanced functional androgen responsiveness.* Endocrinology, 1999. **140**(7): p. 3114-24.

53. Jakob, F., et al., *Local estradiol metabolism in osteoblast- and osteoclast-like cells.* J Steroid Biochem Mol Biol, 1997. **61**(3-6): p. 167-74.

54. Gunness, M. and E. Orwoll, *Early induction of alterations in cancellous and cortical bone histology after orchiectomy in mature rats.* J Bone Miner Res, 1995. **10**(11): p. 1735-44.

Figure 1: Schematic representation of the *in vivo* study design.

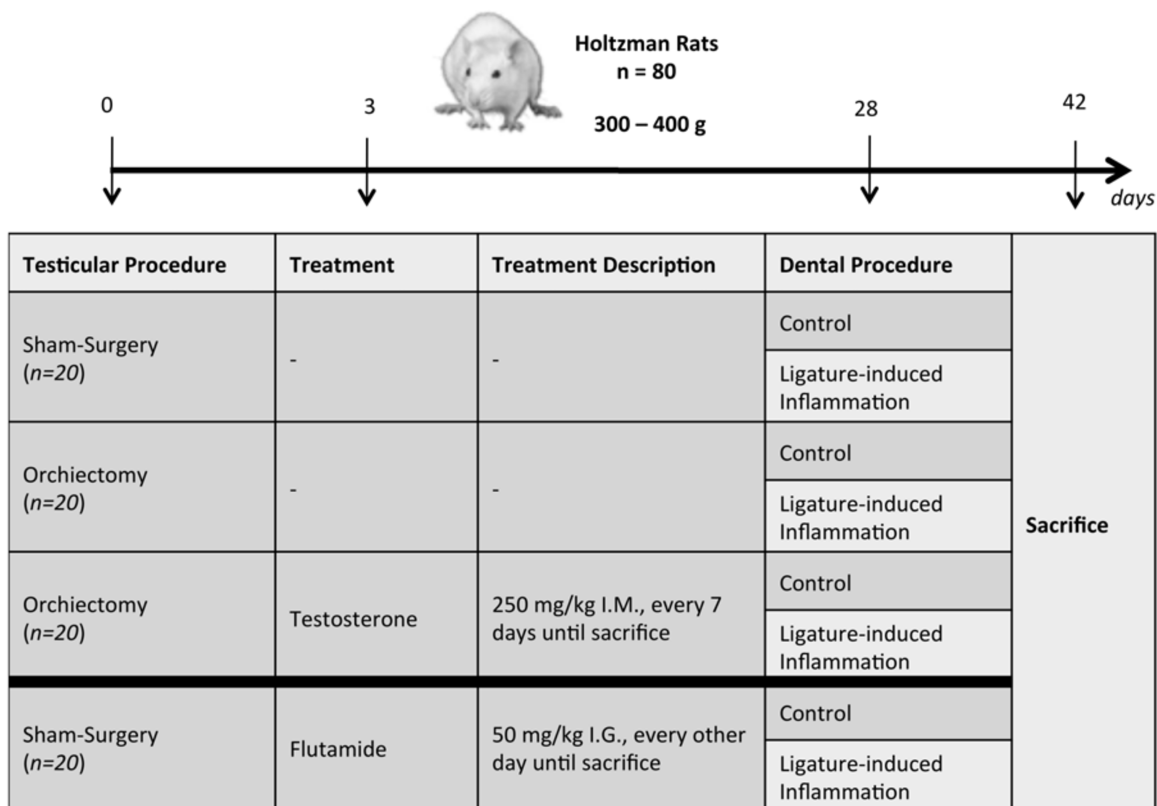
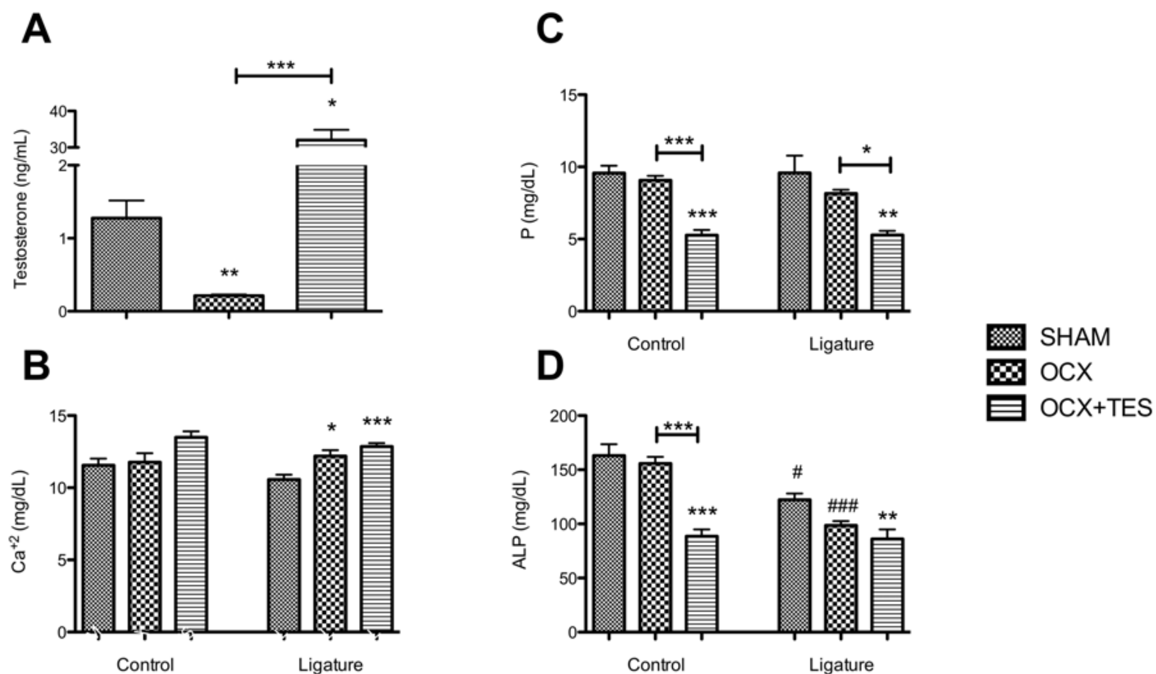
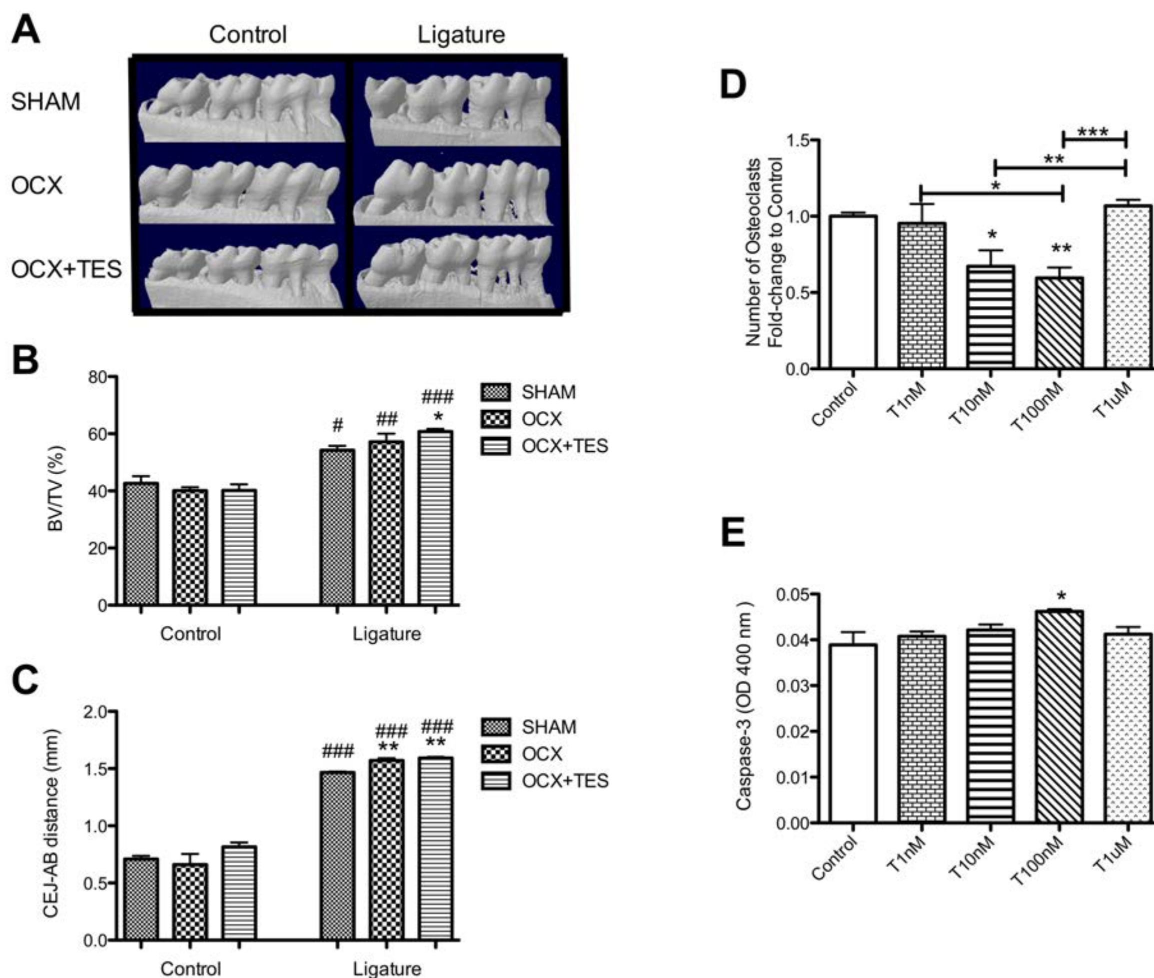


Figure 2: Impact of testosterone levels modulation on serum concentration of *A)* total testosterone; *B)* Calcium (Ca^{+2}); *C)* Phosphorus (P); *D)* Alkaline Phosphatase (ALP).



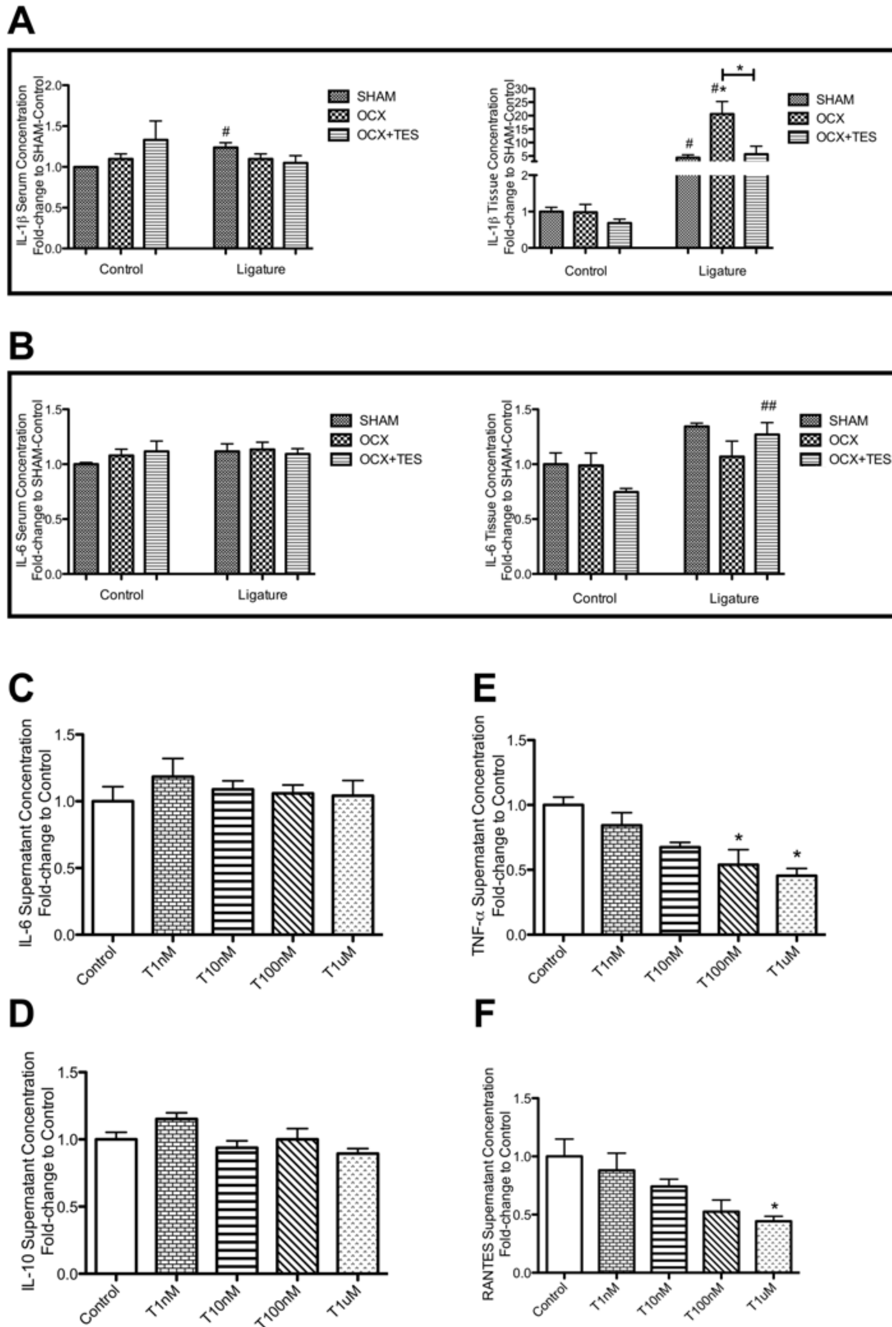
* p<0.05; ** p<0.01; *** p<0.001 when compared to the respective Control or Ligature SHAM group, unless otherwise connected.
# p<0.05; ## p<0.01; ### p<0.001 when compared to the same treatment Control group.

Figure 3: Testosterone modulation affects bone metabolism *in vivo* and *in vitro*. *A)* Representative µCT images; *B)* Bone volume fraction (BV/TV); and *C)* linear distance between cemento-enamel junction (CEJ) and alveolar bone (AB) on the mesial surface of the first molars of each experimental group in each experimental condition. *D)* Fold-change in the number of RAW264.7 and RANKL-derived TRAP-positive osteoclasts with 3 or more nuclei; and *E)* Colorimetric detection of caspase-3 in the presence of increasing doses of testosterone.



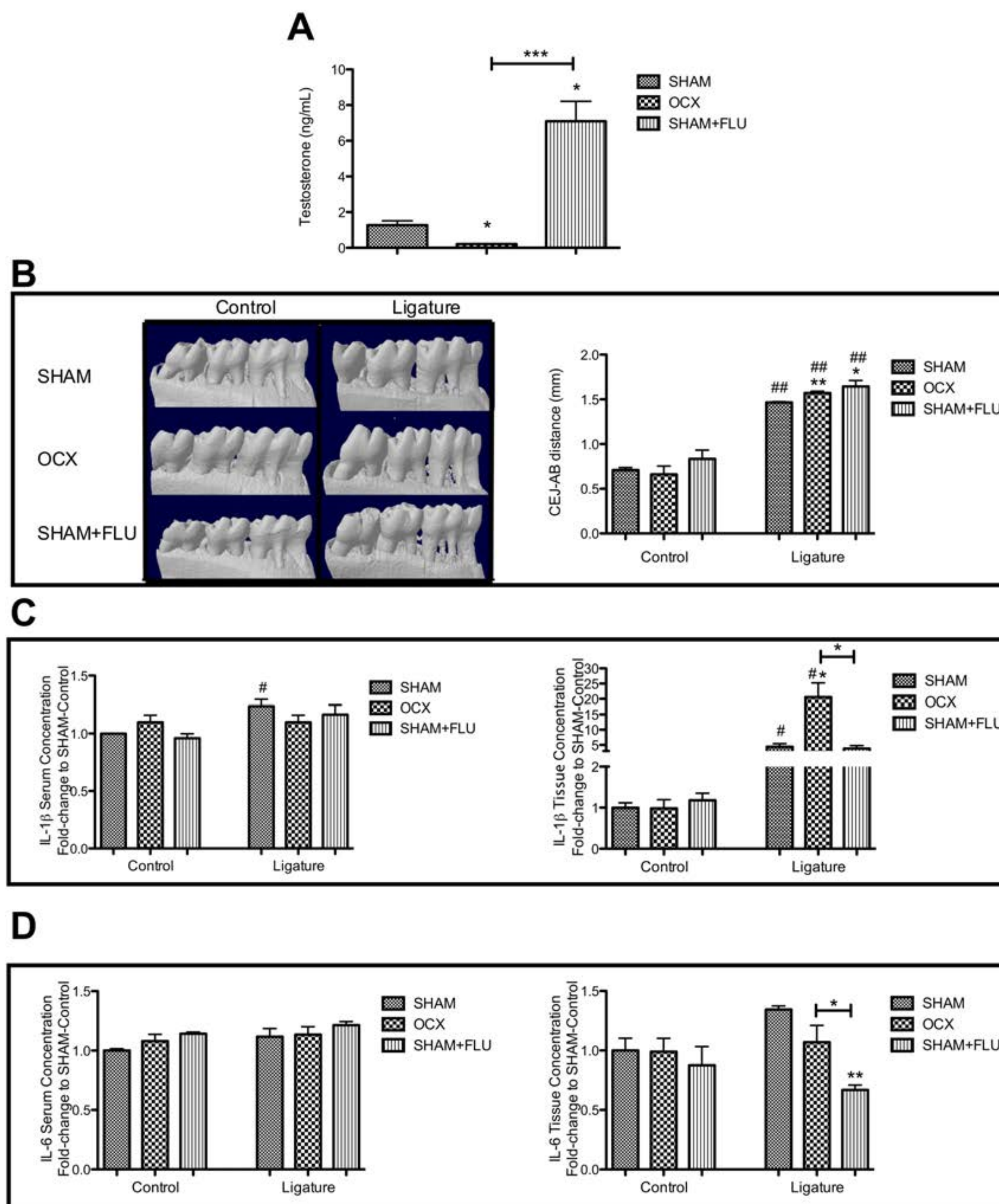
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to the respective Control or Ligature SHAM group, unless otherwise connected.
# $p<0.05$; ## $p<0.01$; ### $p<0.001$ when compared to the same treatment Control group.

Figure 4: Testosterone modulation affects cytokines expression *in vivo* and *in vitro*. Fold-change in the serum and tissue concentration of *A)* Interleukin (IL)-1β; and *B)* IL-6 for each experimental group in each experimental condition. Fold-change in the osteoclast-derived supernatant concentration of *C)* IL-6; *D)* IL-10; *E)* Tumor Necrosis Factor (TNF); and *F)* Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) in the presence of increasing doses of testosterone.



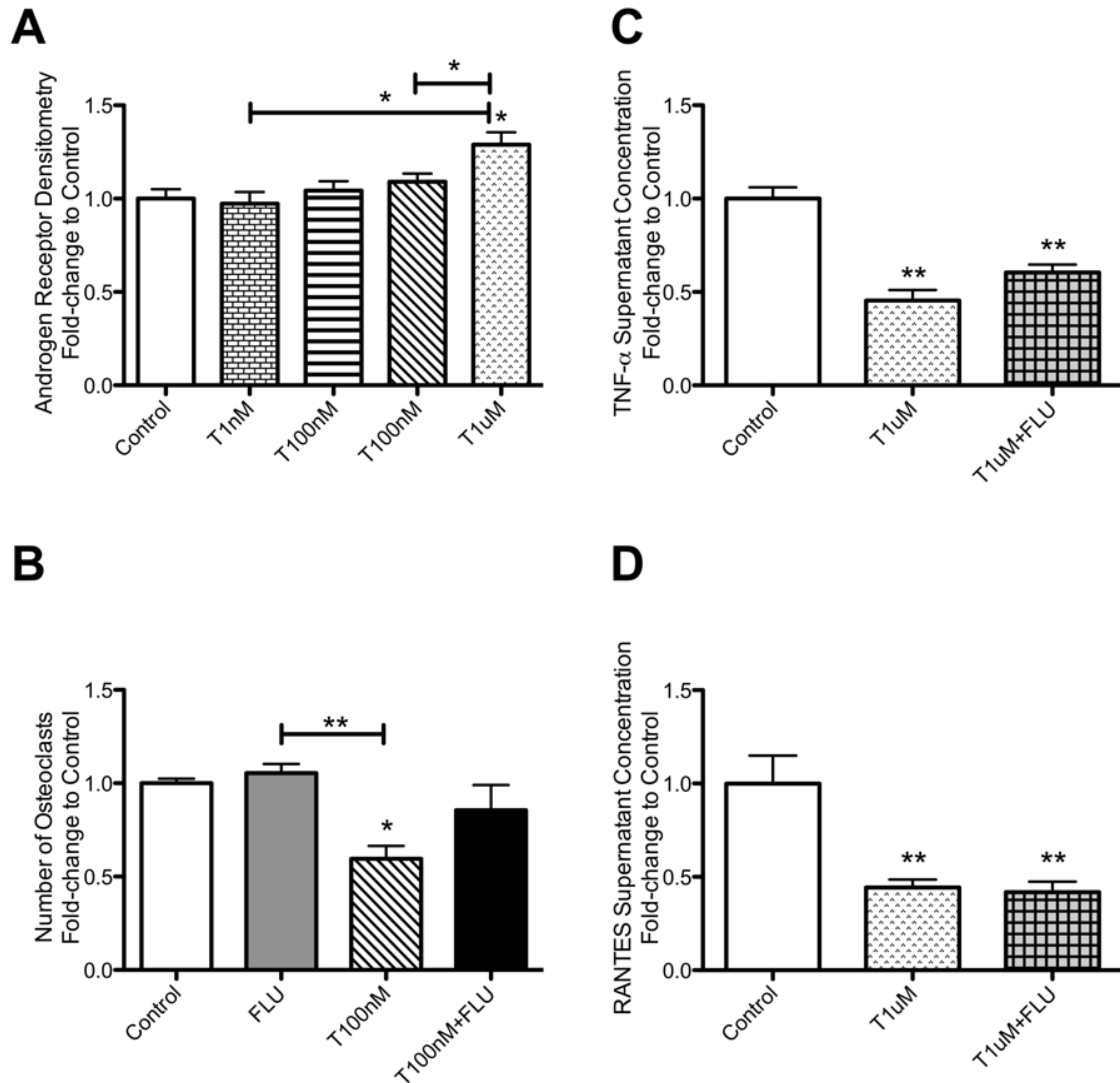
* p<0.05; ** p<0.01; *** p<0.001 when compared to the respective Control or Ligature SHAM group, unless otherwise connected.
# p<0.05; ## p<0.01; ### p<0.001 when compared to the same treatment Control group.

Figure 5: Androgen receptor blockade using flutamide also affects bone metabolism *in vivo*, but through different mechanisms. *A)* Serum total testosterone levels; *B)* Representative µCT images and linear distance between cemento-enamel junction (CEJ) and alveolar bone (AB) on the mesial surface of the first molars. Fold-change in the serum and tissue concentration of *C)* Interleukin (IL)-1β; and *D)* IL-6 for each experimental group in each experimental condition.



* p<0.05; ** p<0.01; *** p<0.001 when compared to the respective Control or Ligature SHAM group, unless otherwise connected.
# p<0.05; ## p<0.01; ### p<0.001 when compared to the same treatment Control group.

Figure 6: Androgen receptor (AR) expression and involvement in testosterone-derived responses *in vitro*. A) Fold-change in AR densitometry with increasing doses of testosterone. Fold-change in *B)* the number of RAW264.7 and RANKL-derived TRAP-positive osteoclasts with 3 or more nuclei; *C)* TNF; and *D)* RANTES when flutamide was added to the testosterone concentration that had a significant impact on each variable.



* p<0.05; ** p<0.01; *** p<0.001 when compared to the respective Control or Ligature SHAM group, unless otherwise connected.

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Title: Resolvin D2 ameliorates down-regulation of osteocalcin and osteoprotegerin by testosterone in primary murine osteoblasts

Short Running Title: Resolvin and testosterone on osteoblasts

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For Peer Review

Abstract:

The objective of this study was to evaluate the impact of different testosterone doses on osteoblasts using markers of osteoblast activity and osteoclast differentiation in primary murine cell culture. Additionally, we assessed the reversibility of those actions using the pro-resolving mediator resolvin (Rv) D2 (10 nM). Osteoblasts were differentiated from neonatal mice calvariae for 10 days and then treated with testosterone 10 nM (physiologic), a low (1 nM) or a high dose (100 nM) for 48 hours. The cell lysates and conditioned media were used for the determination of alkaline phosphatase, osteocalcin, RANKL and osteoprotegerin (OPG) protein concentrations. Physiologic testosterone dose significantly decreased osteocalcin, RANKL and OPG levels, while the high dose significantly increased the RANKL:OPG ratio. RvD2 partially reversed the negative impact of 10 nM testosterone on osteocalcin and OPG. These findings suggest that physiologic testosterone levels are protective against osteoclastogenesis, but that impact is weakened by the disadvantageous down-regulation of osteocalcin and OPG. The pro-resolving mediator RvD2 could partially reverse those findings.

Keywords: Testosterone; Osteoblasts; Docosahexaenoic Acids; RANK Ligand; Osteocalcin; Osteoprotegerin.

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Introduction

Testosterone is the primary sex hormone in men with actions related to the development and maintenance of muscle mass, stimulus of erythropoiesis, increased brain perfusion, influence of mood and cognition, and bone health [1]. Low levels of testosterone are associated with a decrease in muscle mass and strength, an increase in central body fat, and a decrease in bone mass and osteoporosis [2]. Similarly, anabolic-androgenic steroid abuse that results in high levels of testosterone has adverse effects, such as alterations in the cardiovascular, central nervous, and endocrine systems [3]. We have previously demonstrated bimodal actions of testosterone; both low and high testosterone levels increase bone resorption in an inflammatory dental model in rats [4].

From these clinical and experimental observations, it is clear that testosterone plays a role in inflammation and bone metabolism. Several *in vitro* studies demonstrated that androgens (mainly testosterone and 5α-dihydrotestosterone - DHT, a non-aromatizable androgen) can directly decrease osteoclast differentiation or activity in avian, mouse, rat and human cells [5-7]. However, it has also been suggested that some of the impact of androgens on osteoclastogenesis can be mediated through cells of the osteoblast lineage [7, 8].

Androgens have several functions, including inhibition of PTH- and IL-1α-derived Prostaglandin E_2 production in cultured neonatal mouse calvariae [9]; suppression of IL-6 production, a cytokine related to osteoclastogenesis and bone loss, in both cultured bone marrow stromal cells and osteoblasts [10-12]; and increase Transforming Growth Factor β (TGF-β), which is a mitogen for osteoblasts, in a murine

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calvarial cell system. TGF-β mRNA is up-regulated in normal human osteoblastic cells [13] and in osteoblast-like human osteosarcoma cells (HOS TE85) [14], but not in normal human osteoblast-like cells (hOB) [15]. Overall, androgens stimulate proliferation of osteoblast progenitors and differentiation of mature osteoblasts while inhibiting apoptosis of osteoblasts [8].

Osteoblasts can produce and release receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG), which play important roles in osteoclast differentiation; binding of RANKL to RANK on osteoclast precursors induces differentiation. OPG is a scavenger receptor for RANKL; hence, the RANKL:OPG ratio is critical in osteoclastogenesis [16]. The actions of androgens in RANKL:OPG regulation are controversial. OPG was found to be significantly upregulated by androgens in primary murine cells, osteoblast-like cells, and in a co-culture system of human cells [7, 17], but dose-dependently down-regulated in human cells [18]. Yet, other reports suggest that RANKL mRNA has not been consistently detected and/or has been described as not regulated by androgens [17, 18].

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Inflammation plays a critical role in bone pathology and endogenous control of inflammation through agonists of resolution of inflammation play a critical role in maintaining bone homeostasis [19]. Once an inflammatory response is initiated by trauma or infection, a cascade of pro-inflammatory events aimed at elimination of the noxious stimuli is set into action. A basic tenet of modern medicine is that upon neutralization of the stimulus, inflammation resolves due to catabolism of 'pro-inflammatory' mediators. The resolution of inflammation is a highly coordinated and active process. The process, similar to 'pro-inflammatory' mechanisms, utilizes cells and

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various messenger molecules generated by cells to provide ‘stop signals’ that lead to shutdown and clearance of inflammatory cells [20]. Thus, inflammation includes both pro-inflammatory and resolving mechanisms inherent to the body where the host attempts to confine and/or eliminate the invaders and, when accomplished, actively resolves the response to limit damage to self [21]. Hence, the capacity to actively control inflammation is endogenously programmed.

Resolution programs require the local biosynthesis of endogenous specialized pro-resolving lipid mediators. These mediators include the lipoxins, resolvins, protectins and maresins [19], which are enzymatically synthesized via sequential steps involving lipoxygenases and cyclooxygenases. These mediators are dual functioning because they limit neutrophil accumulation and stimulate non-phlogistic activation of macrophages *in vivo* [22]. Resolvin (Rv) D2 was originally identified in resolving exudates, and is considered to be a potent endogenous regulator of excessive inflammatory responses that acts via multiple cellular targets to stimulate resolution and preserve immune vigilance [23]. However, its impact on bone cells is yet to be determined.

Due to the important role played by osteoblasts in osteoclastogenesis and consequent bone turnover, the objective of this study was to evaluate the impact of testosterone on osteoblasts using markers of osteoblast activity (alkaline phosphatase and osteocalcin) and osteoclast differentiation (RANKL, OPG and RANKL:OPG ratio) in primary murine cell culture. Additionally, we demonstrate that RvD2 reverses the negative impact of testosterone on the expression of osteocalcin and OPG by osteoblasts.

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Materials and Methods

Materials

BalbC mice were obtained from Charles River Laboratories (Wilmington, MA) and bred in house. Minimal Essential Medium (MEM)-α was purchased from Life Technologies (Grand Island, NY), Fetal Bovine Serum (FBS) from ATCC (Manassas, VA), Penicillin/Streptomycin from Corning (Corning, NY), and testosterone was purchased from Sigma-Aldrich Co. (St. Louis, MO). A single plex OPG and multiplex RANKL-Osteocalcin Milliplex Map kits were purchased from EMD Millipore (Billerica, MA). Minimum detectable concentration (MinDC) for the OPG single plex defined as 2.3 pg/mL. The RANKL-Osteocalcin multiplex kit defined a MinDC of 2.7 pg/mL for RANKL and 4.7 pg/mL for Osteocalcin. Milliplex assays were read on a Bio-Plex 200 from Bio-Rad Laboratories Inc. (Hercules, CA). Alkaline Phosphatase (ALP) Enzyme-Linked Immunosorbent Assay (ELISA) kit was purchased from Life Sciences Advanced Technologies Inc. (Saint Petersburg, FL). All protocols were performed according to the manufacturers' instructions.

Primary Murine Osteoblast Culture

Neonatal mouse calvarial osteoblasts were isolated from litters (7-8 mice) by dissection of the scalp skin and removal of the calvariae. Calvariae were incubated in α-MEM medium supplemented with 10% FBS and 10,000 IU penicillin, 10,000μg/mL streptomycin and then incubated for 10 min at 37°C in a 4mM Na_2 EDTA solution 3 times in order to demineralize the extracellular matrix; the supernatants were discarded.

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The calvariae were then incubated twice at 37°C in a freshly prepared collagenase type 2 solution (Worthington, LS004176) in 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4 x H_2O on an orbital shaker to separate the cells from the extracellular matrix; the supernatants were discarded. The digests from five sequential incubations with collagenase were pooled, centrifuged, and resuspended in α-MEM media supplemented with 10% FBS, 10,000 IU penicillin, 10,000 µg/mL streptomycin. Cells were plated at a density of 5 x 10^5 cells in 75 ml flasks and upon reaching 90% confluence, cells were passaged into 24-well plates at a density of 2x 10^4 cells/mL (1mL/well). Ascorbic acid (50 µg/mL) and β-glycerophosphate (10 mM) were added to the medium every other day for 10 days to allow osteoblast differentiation.

Testosterone Treatment

Testosterone was diluted in DMSO to a stock solution of 100 mM. A 0.1% DMSO solution was used as control and further testosterone dilutions were performed using culture medium. After 10 days of osteoblast differentiation with ascorbic acid and β-glycerophosphate, cells were treated with 10–fold increasing concentrations of testosterone (1-100 nM). ‘Physiologic’ testosterone dose was considered to be 10 nM. All experiments were performed in quadruplicate and repeated at least 3 times.

Resolvin Treatment

To assess the reversibility of the negative impact of testosterone on osteoblast expression of osteocalcin and OPG by pro-resolving mediators, RvD2 (10 nM) was

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simultaneously added to 10 nM testosterone groups. Data were normalized to the control group (value 1) and are shown as fold-change.

Biomarker Analyses

Two days after testosterone (with or without RvD2) treatment, the conditioned media and cell lysates were collected. Cells were recovered using lysis buffer containing 1% phosphatase inhibitor. Samples were stored at -80°C until analysis. Conditioned media was used for the detection of ALP, Osteocalcin and OPG, while RANKL analyses were performed in cell lysates. The RANKL:OPG ratio was obtained by dividing the RANKL levels by OPG levels in each sample.

Statistical Analysis

One-way analysis of variance (ANOVA) was used for group comparisons. Tukey post-hoc test or an unpaired t test was used for pairwise comparisons. All data were analyzed using GraphPad Prism version 5.0 for Mac OS X software, San Diego, California, USA. The significance level was set at $\alpha=0.05$. All data are expressed as mean ± SEM.

Results

Impact of testosterone on bone markers

ALP concentration in the control group was 0.58 ± 0.03 ng/mL and testosterone treatment did not significantly alter its production at any concentration (p=0.7).

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Osteocalcin concentration was significantly decreased by 10 nM testosterone when compared to the control (375.90 ± 69.75 pg/mL) or 1 nM groups ($p<0.05$).

Cell lysates contained a 17.34 ± 2.31 pg/mL concentration of RANKL in the control group, which was significantly reduced by 10 nM testosterone treatment ($p<0.05$). The difference was also significant when comparing 10 and 100 nM doses ($p<0.05$). All testosterone-treated groups exhibited decreased levels of OPG when compared to the control group (1361 ± 132.4 pg/mL), although the difference was statistically significant only for the 10 ($p<0.01$) and 100 nM ($p<0.05$) groups. Physiologic levels of testosterone (10 nM) protected against osteoclastogenesis as observed by the significantly lower RANKL:OPG ratio when compared to the control group ($p<0.05$). It should also be noted that the highest dose, 100 nM, significantly increased the RANKL:OPG ratio when compared to control, 1 nM ($p<0.01$) and 10 nM doses ($p<0.001$).

The impact of testosterone concentration on osteoblast expression of ALP, osteocalcin, RANKL, OPG and RANKL:OPG ratio are shown in figure 1.

Impact of RvD2 on 10 nM testosterone-derived down-regulation of osteocalcin and OPG

RvD2 rescued osteocalcin expression inhibited by 10 nM testosterone by 235% ($p>0.05$). When OPG levels were evaluated, RvD2 resulted in a more modest 49% increase, which was statistically significant ($p<0.01$). The expression of osteocalcin and OPG in testosterone-treated cells with or without RvD2 is illustrated in figure 2.

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Discussion and Conclusions

In this report, the impact of testosterone on bone homeostasis is demonstrated through direct actions of low and high levels of testosterone on osteoblasts. The proresolution agonist, RvD2, apparently binds to osteoblasts reversing the actions of testosterone and increasing osteocalcin and OPG secretion.

Testosterone levels in men may vary greatly among individuals and in different race/ethnical populations [24]. A nationally representative sample of Americans, for example, revealed a total testosterone concentration mean of 20.68 nM (IC 95%: 19.54-21.93) in 20-29 year olds, declining to 15.89 nM (IC 95%: 14.85-17.00) in late adulthood (50-59 years) [25]. In rats, we have shown that mean serum concentration of the hormone is approximately 7 nM and a 10-fold reduction in these levels was obtained following orchiectomy as a model of late onset hypogonadism [4].

People who abuse anabolic androgenic steroids, such as bodybuilders and professional athletes, generally use these drugs in a concentration 10-100 times higher than those used for medical conditions [26]. However, it is possible that the concentration of the hormone in bone is higher than in serum [27]. In this study, we tested 3 different testosterone concentrations in an attempt to simulate the possible clinical presentations of testosterone levels.

We observed no difference in ALP levels after 48 hours of testosterone treatment. Accordingly, Vaishnav et al. [28] used human bone cells treated with vitamin D to demonstrate that stanozolol, an anabolic steroid, did not affect ALP concentration after 48h culture, and only the 10 nM dose (of the 4 doses tested) significantly increased ALP levels after 24h culture. Kasperk et al. [29] reported that ALP

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concentration was increased with DHT treatment in human cells as observed by cell staining. They later attributed the increase in ALP to the higher TGF-β levels observed in testosterone-treated osteoblasts [30]. Chen et al. [31] also showed that 100 pg/mL testosterone increased proliferation, ALP activity, accelerated cell cycle and reduced apoptosis in chicken osteoblasts. Hofbauer et al. [32] observed that 10nM testosterone decreased ALP levels in human cell lysates additionally treated with vitamin D. The same authors demonstrated that osteocalcin is not significantly altered by testosterone treatment using a competitive immunoassay. In stanozolol-treated cells, lower doses produced the highest osteocalcin secretion, with decreasing osteocalcin with increasing stanozolol concentration, for both 24 and 48h incubations [28]. Additionally, it was recently demonstrated that 24-hour supernatants from primary murine osteoblasts enhanced the production of testosterone by testis explants as well as Leydig cells, and this effect was attributed to osteocalcin [33]. Interestingly, our results demonstrated that osteocalcin expression is diminished in the presence the 'physiologic' 10 nM testosterone concentrations. This could represent a feedback loop to regulate testosterone production *in vivo*.

If testosterone demonstrates a dose-dependent inhibition in osteoclast culture, it would be reasonable to hypothesize that if this impact was to be maintained *in vivo*, there should be down-regulation of RANKL, an upregulation of OPG, or both, resulting in a down-regulation of the RANKL:OPG ratio. Our results evaluating protein levels are in accordance with this hypothesis: both RANKL and OPG are reduced by 10 nM testosterone, but RANKL reduction is greater, leading to a decreased ratio. Previously, RANKL mRNA had not been consistently detected and/or was described as not

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regulated by androgens [18], but we were able to detect RANKL expression in cell lysates but not on cell supernatants. OPG mRNA was significantly increased in the presence of 10 nM testosterone in primary murine cells and osteoblast-like cells [17], and the protein was increased in a co-culture system of human cells [7], but significantly and dose-dependently decreased when human cells were treated with DHT [18]. Our study is consistent with these findings. Those authors suggest that these observations partially explain the weaker anti-resorptive effects of testosterone as compared to estrogens. Most importantly, the RANKL:OPG ratio was significantly diminished with the 'physiologic' 10 nM dose, meaning that this dose is protective against excessive osteoclastogenesis and favors bone homeostasis. The 'superphysiologic' 100 nM dose was demonstrated to be inductive for osteoclastogenesis, which can explain, at least partially, our *in vivo* results showing that high testosterone levels lead to increased bone resorption using an inflammatory dental model in rats [4].

Previous studies from our group have already shown that osteoclast differentiation, activity and thus, bone remodeling, can be modulated by RvE1 with direct actions on bone, rescuing OPG production and restoring a favorable RANKL:OPG ratio [34, 35]. Here, we test the hypothesis that RvD2 reverses the undesirable testosterone-derived down-regulation of osteocalcin and OPG at 'physiologic' levels. Similar to RvE1, RvD2 also had a positive impact on osteoblasts, either by bringing osteocalcin levels back to control levels or by significantly increasing OPG levels. This partial rescue can be explained by the simultaneous addition of the two treatments to the media, so that cells have a receptor agonist stimulus to respond to.

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Taken together, these findings suggest that 'physiologic' testosterone levels in osteoblasts down-regulate osteocalcin production, but are also protective against osteoclastogenesis as observed by the lower RANKL and RANKL:OPG ratio levels. High doses of testosterone increase the RANKL:OPG ratio and can stimulate osteoclastogenesis. At the same time, the down-regulation of OPG may be an explanation why testosterone is weaker than estrogens regulating bone metabolism. Additionally, the pro-resolving mediator RvD2 can ameliorate testosterone-derived down-regulation of osteocalcin and OPG by primary murine osteoblasts.

For Peer Review

References

1. Bain, J., *Testosterone and the aging male: to treat or not to treat?* Maturitas, 2010. **66**(1): p. 16-22.
2. Bassil, N., S. Alkaade, and J.E. Morley, *The benefits and risks of testosterone replacement therapy: a review.* Ther Clin Risk Manag, 2009. **5**(3): p. 427-48.
3. Basaria, S., *Androgen abuse in athletes: detection and consequences.* J Clin Endocrinol Metab, 2010. **95**(4): p. 1533-43.
4. Steffens, J.P., et al., *The effect of supra- and subphysiologic testosterone levels on ligature-induced bone loss in rats--a radiographic and histologic pilot study.* J Periodontol, 2012. **83**(11): p. 1432-9.
5. Pederson, L., et al., *Androgens regulate bone resorption activity of isolated osteoclasts in vitro.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 505-10.

6. Huber, D.M., et al., *Androgens suppress osteoclast formation induced by RANKL and macrophage-colony stimulating factor.* Endocrinology, 2001. **142**(9): p. 3800-8.

7. Michael, H., et al., *Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption.* J Bone Miner Res, 2005. **20**(12): p. 2224-32.

8. Vanderschueren, D., et al., *Androgens and bone.* Endocr Rev, 2004. **25**(3): p. 389-425.

9. Pilbeam, C.C. and L.G. Raisz, *Effects of androgens on parathyroid hormone and interleukin-1-stimulated prostaglandin production in cultured neonatal mouse calvariae.* J Bone Miner Res, 1990. **5**(11): p. 1183-8.

10. Hofbauer, L.C., R.M. Ten, and S. Khosla, *The anti-androgen hydroxyflutamide and androgens inhibit interleukin-6 production by an androgen-responsive human osteoblastic cell line.* J Bone Miner Res, 1999. **14**(8): p. 1330-7.

11. Bellido, T., et al., *Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor.* J Clin Invest, 1995. **95**(6): p. 2886-95.

12. Manolagas, S.C., S. Kousteni, and R.L. Jilka, *Sex steroids and bone.* Recent Prog Horm Res, 2002. **57**: p. 385-409.

13. Kasperk, C., et al., *Studies of the mechanism by which androgens enhance mitogenesis and differentiation in bone cells.* J Clin Endocrinol Metab, 1990. **71**(5): p. 1322-9.

For Peer Review

14. Benz, D.J., et al., *High-affinity androgen binding and androgenic regulation of alpha 1(I)-procollagen and transforming growth factor-beta steady state messenger ribonucleic acid levels in human osteoblast-like osteosarcoma cells.* Endocrinology, 1991. **128**(6): p. 2723-30.

15. Bodine, P.V., B.L. Riggs, and T.C. Spelsberg, *Regulation of c-fos expression and TGF-beta production by gonadal and adrenal androgens in normal human osteoblastic cells.* J Steroid Biochem Mol Biol, 1995. **52**(2): p. 149-58.

16. Nelson, C.A., et al., *RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor.* Structure, 2012. **20**(11): p. 1971-82.

17. Chen, Q., et al., *Testosterone increases osteoprotegerin mRNA expression in mouse osteoblast cells.* Horm Metab Res, 2004. **36**(10): p. 674-8.

18. Hofbauer, L.C., et al., *Regulation of osteoprotegerin production by androgens and anti-androgens in human osteoblastic lineage cells.* Eur J Endocrinol, 2002. **147**(2): p. 269-73.

19. Serhan, C.N., *Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways.* Annu Rev Immunol, 2007. **25**: p. 101-37.

20. Serhan, C.N., *A search for endogenous mechanisms of anti-inflammation uncovers novel chemical mediators: missing links to resolution.* Histochem Cell Biol, 2004. **122**(4): p. 305-21.

21. Van Dyke, T.E. and C.N. Serhan, *Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases.* J Dent Res, 2003. **82**(2): p. 82-90.

For Peer Review

22. Schwab, J.M., et al., *Resolvin E1 and protectin D1 activate inflammation-resolution programmes.* Nature, 2007. **447**(7146): p. 869-74.

23. Spite, M., et al., *Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis.* Nature, 2009. **461**(7268): p. 1287-91.

24. Mazur, A., *The age-testosterone relationship in black, white, and Mexican-American men, and reasons for ethnic differences.* Aging Male, 2009. **12**(2-3): p. 66-76.

25. Rohrmann, S., et al., *The prevalence of low sex steroid hormone concentrations in men in the Third National Health and Nutrition Examination Survey (NHANES III).* Clin Endocrinol (Oxf), 2011. **75**(2): p. 232-9.

26. Ozcelik, O., M.C. Haytac, and G. Seydaoglu, *The effects of anabolic androgenic steroid abuse on gingival tissues.* J Periodontol, 2006. **77**(7): p. 1104-9.

27. Yarrow, J.F., et al., *Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration.* Am J Physiol Endocrinol Metab, 2010. **299**(5): p. E841-7.

28. Vaishnav, R., et al., *Effects of the anabolic steroid stanozolol on cells derived from human bone.* Clin Sci (Lond), 1988. **74**(5): p. 455-60.

29. Kasperk, C.H., et al., *Androgens directly stimulate proliferation of bone cells in vitro.* Endocrinology, 1989. **124**(3): p. 1576-8.

30. Kasperk, C.H., et al., *Gonadal and adrenal androgens are potent regulators of human bone cell metabolism in vitro.* J Bone Miner Res, 1997. **12**(3): p. 464-71.

For Peer Review

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31. Chen, X., et al., *17beta-estradiol combined with testosterone promotes chicken osteoblast proliferation and differentiation by accelerating the cell cycle and inhibiting apoptosis in vitro.* Vet Res Commun, 2010. **34**(2): p. 143-52.

32. Hofbauer, L.C., K.C. Hicok, and S. Khosla, *Effects of gonadal and adrenal androgens in a novel androgen-responsive human osteoblastic cell line.* J Cell Biochem, 1998. **71**(1): p. 96-108.

33. Oury, F., et al., *Endocrine regulation of male fertility by the skeleton.* Cell, 2011. **144**(5): p. 796-809.

34. Herrera, B.S., et al., *An endogenous regulator of inflammation, resolvin E1, modulates osteoclast differentiation and bone resorption.* Br J Pharmacol, 2008. **155**(8): p. 1214-23.

35. Gao, L., et al., *Resolvin E1 and chemokine-like receptor 1 mediate bone preservation.* J Immunol, 2013. **190**(2): p. 689-94.

For Peer Review

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Figure 1: Concentration (mean±SEM) of Alkaline Phosphatase, Osteocalcin, RANKL, OPG and RANKL:OPG ratio expressed by primary murine osteoblasts with increasing doses of testosterone.

* ANOVA; $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to control, unless otherwise connected.

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Figure 2: Fold-change of the concentration (mean±SEM) of Osteocalcin and OPG expressed by primary murine osteoblasts upon testosterone-treated cells (10 nM) with or without RvD2 (10 nM).

* t test; p<0.05; ** p<0.01 when compared to control, unless otherwise connected.

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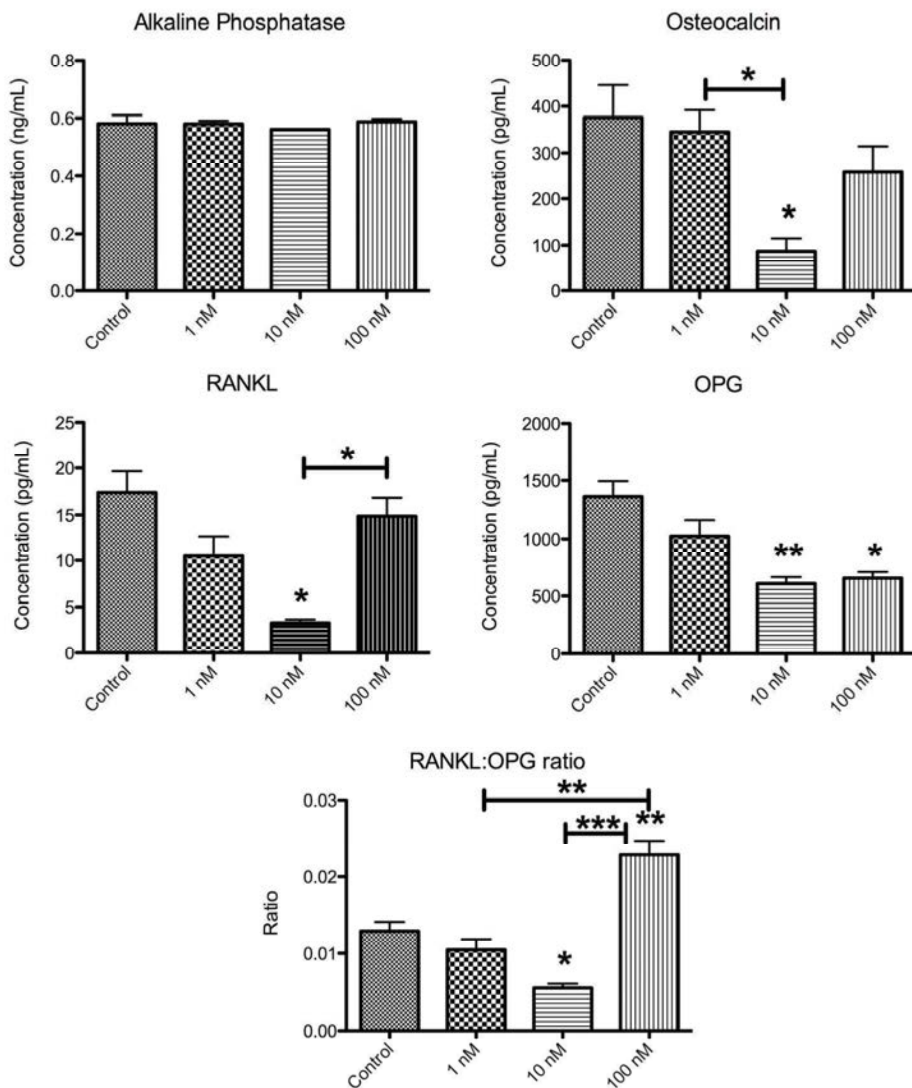

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* ANOVA; $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to control, unless otherwise connected.

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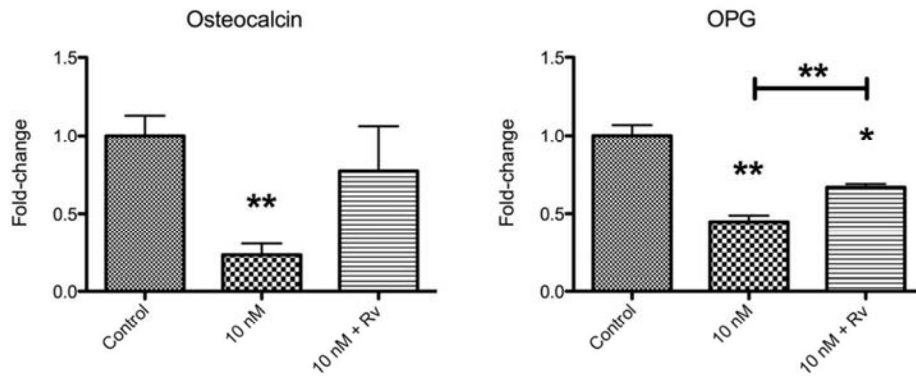

Figure 2: Fold-change of the concentration (mean±SEM) of Osteocalcin and OPG expressed by primary murine osteoblasts upon testosterone-treated cells (10 nM) with or without RvD2 (10 nM).

* t test; $p<0.05$; ** $p<0.01$ when compared to control, unless otherwise connected.

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CAPÍTULO 5

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Review

Telomere length and its relationship with chronic diseases – New perspectives for periodontal research

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ABSTRACT

Objective: The ageing process is accompanied by a variety of cellular modifications, and telomere shortening is a common finding. Large epidemiological studies have reported an association between shorter telomere length in peripheral leukocytes and several inflammatory diseases of the elderly including diabetes, atherosclerosis and, recently, periodontitis. The primary aim of this study was to critically discuss available evidence regarding the potential mechanisms relating shorter telomeres to periodontitis.
Design: A narrative literature review was performed to report evidence relating shorter telomeres to the ageing process and inflammation. Then, we searched MEDLINE (1950 to May 2012) and ISI WEB OF SCIENCE (1950 to May 2012) databases for the combination of the terms 'telomere' and 'periodontitis'.
Results: Although these associations suggest a possible role of telomere attrition in the onset or evolution of chronic inflammatory diseases, only two studies addressed the relationship between telomere length and periodontitis.
Conclusion: We suggest that the chronic inflammatory burden observed in people with chronic periodontitis could represent the driver of telomere shortening. However, further evidence is needed to confirm whether inflammation is the cause or the consequence of the shorter leukocyte telomere length observed in people with periodontitis.
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1. Introduction

Telomeres are heterochromatic domains composed of repetitive DNA (TTAGGG repeats) bound to an array of specialized proteins. They cap the ends of chromosomes, protecting them from unscheduled DNA repair and degradation.[1,2] Telomere sequences end in a 100–200 bp nucleotide 3′ single strand overhang forming a loop structure.[3,4] This sequence is similar for mammalians, but the length and exact sequence of the repeat may vary from species to species.[2]

As telomeres shorten with each cell division they chronicle the replicative history of somatic cells. However, in progenitor and stem cells telomere length is partially maintained by the activity of a ribonucleoprotein enzyme: the telomerase. A recent study with highly purified telomerase extracts has demonstrated that the telomerase enzyme contains two molecules each of the telomerase reverse transcriptase subunit (Tert) and the telomerase-associated RNA molecule (Terc), as well as one molecule of dyskerin,[5] a protein known to stabilize the telomerase complex.[6]

It has been suggested that telomere length inversely correlates with lifespan for many mammals.[7] In humans, the ability of leukocyte telomere length (LTL) to predict future mortality has been extensively investigated.[8–10] In somatic cells, telomeres undergo erosion at each cell replication. The loss of telomere length is due to the inability of DNA polymerase to replicate the lagging DNA strand at its terminus, a phenomenon called the 'end-replication problem'.[1] Therefore, telomere length acts as a mitotic clock for somatic cells, potentially providing information about the number of cell replications undertaken by each cell during its lifespan. However, cell divisions may occur to a certain extent when telomere reaches a length that cannot be further reduced. This point, when cell enters senescence and cannot go under another replication, is known as the Hayflick limit.[11] Thus, it is not surprising that several epidemiological reports have described a strong association between LTL and chronological age because cells from older individuals have likely undergone more replication cycles compared to cells from younger individuals. Fig. 1 illustrates the telomere structure and describes the factors known to induce telomere shortening.

Recent evidence suggests that chronic inflammatory diseases, such as type 2 diabetes mellitus,[12,13] atherosclerosis,[14] obesity[15] and rheumatoid arthritis[16] are associated with shorter LTL, independent of an individuals' age. Interestingly, these chronic diseases are associated with an increased risk of premature mortality, suggesting that the shorter LTL could reflect a premature ageing process. However, given that older people may be more susceptible to chronic inflammatory diseases, a reverse causality cannot be excluded.[17]

Despite the large amount of evidence on the role of telomeres in the pathogenesis of a number of chronic diseases,[12,18] studies investigating the relationship between periodontal diseases and telomere shortening are scarce and inconclusive. Therefore, the aims of this review were to (i) present background knowledge regarding the telomere structure and its relationship with ageing, inflammation and chronic diseases in general and (ii) assess and discuss studies that investigated the relationship between telomere length and periodontitis.

2. Literature review

2.1. *Ageing, telomere shortening, and inflammation*

As a matter of fact, an increase in the mean age of a population is a worldwide phenomenon and is one of the most important epidemiological changes of the 20th century. According to the World Health Organization, the proportion of people aged 60 or over is growing faster than any other age group. Between 1970 and 2025, approximately 223% growth in the number of older persons is expected.[19] Increased life expectancy and diminished fertility rates contribute to this new epidemiologic scenario. The challenges of an ageing population have already begun to present themselves; over the past decades, the

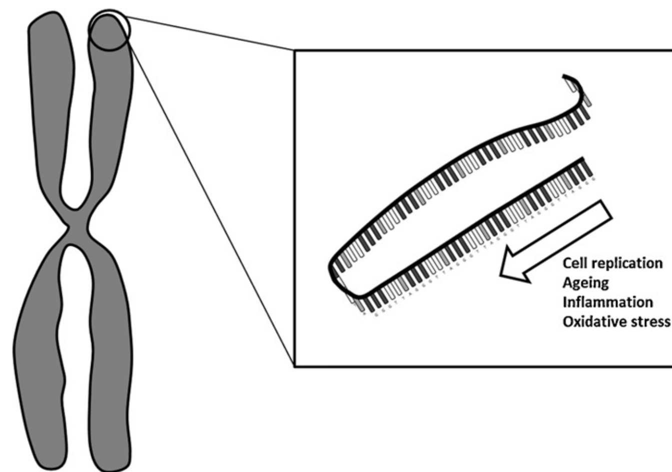

Fig. 1 – The telomere structure. Eukaryotic chromosome-end tandem DNA (TTAGGG) repeats, ending in a 100–200 base-pair nucleotide 3′ single strand overhang that preserves genome integrity, ensures complete replication and prevents chromosomes from erosion and end-to-end fusion.[1,2,4]

oldest-old group (aged > 85 years) has been the most rapidly expanding segment of the population in developed countries.[20] Because the elderly population represents the most susceptible age group for the development of diseases and disabilities, the promotion of healthy ageing represents a major challenge for populations of developed countries. Research on ageing has the potential to identify specific biological pathways that could be targeted to more effectively reduce the ageing-related disease burden.

There are several theories for cell ageing and senescence mechanisms, which are cellular events that accompany the ageing process, and environmental and intrinsic factors may contribute to this process.[21] Short telomeres are usually detected in aged individuals but their lengthening may be highly variable depending on heritable factors, cell lineage and disease/conditions.[22] For example, individuals with dyskeratosis congenital, an inherited bone marrow failure syndrome that is characterized by a triad of dysplastic nails, lacy reticular skin pigmentation, and oral leukoplakia, is related with abnormally short telomeres due to lower telomerase activity. These biological features result in clinical signs of early ageing phenotypes and increased cancer risk.[2,23–25]

Additionally, inflammation has been shown to account for a faster rate of LTL attrition. It is associated with an increased number of circulating leukocytes and increased levels of leukocyte turnover. The continuous demand to maintain high numbers of leukocytes may promote an increased number of cell replications, which in turn may be reflected in an increased rate of telomere shortening due to the 'end-replication problem'.[1] Furthermore, inflammatory responses are often associated with increased production of reactive oxygen species (ROS). Oxidative stress is thought to strongly impact the standard telomere sequence.[3,26–28] Indeed, guanine (G) bases on genomic DNA are highly sensitive to hydroxyl radicals, which can produce single-strand breaks either directly or as an intermediate step in the repair of oxidative base modifications. The telomeric sequence contains GGG triplets and, compared to the rest of the genome, has been reported to be deficient in the repair of single-strand breaks. Thus, increased oxidative stress could result in a longer stretch of telomeres being lost with each cell replication.[29]

Mitochondrial dysfunction is also commonly observed in aged individuals and the resulting increase in intracellular oxidative stress levels may be linked with shortened telomeres. It has been proposed that mitochondrial production of reactive oxygen species could be considered a determinant of telomere-dependent senescence and that could explain the heterogeneity of the replicative lifespan of human cells.[30] More recently, Sahin et al. have demonstrated an interaction between telomere dysfunction, p53 expression and mitochondrial dysfunction.[18] According to their data, telomere dysfunction induces p53 expression which may promote cell-growth arrest, apoptosis or PGC-1 downregulation. PGC-1 is a family of master regulators of mitochondrial function, and its reduced activity may result in mitochondrial-derived accumulated reactive oxygen species, which in turn damage mitochondrial DNA.[18,31] Consequently, this process may lead to several age-related conditions and diseases such as muscle atrophy, progressive loss of vigour, diabetes, and cardiomyopathy.[32–35]

2.2. *Telomere shortening and chronic diseases*

Several chronic, inflammatory and age-related diseases may dramatically impact on telomere length. The prevalence of type 2 diabetes mellitus increases in the elderly and in vivo studies demonstrated that telomere deficiency could affect β-cell metabolism. Furthermore, TERC-deficient mice presented impaired insulin secretion and glucose intolerance, although these features do not seem to be related with a reduced beta cell number.[12,13] Additionally, the upregulation on gluconeogenesis observed in quiescent and highly proliferative organs of telomerase deficient mice may potentially contribute to the increased circulating glucose levels.[18] Taken together these findings suggest that diabetes mellitus could be an age-related disease. Indeed, case–control studies found an association between shortened LTL and the presence of type 2 diabetes, even in non-elderly individuals.[36,37] This association may be attributed to the higher levels of oxidative stress recorded in diabetic individuals rather than to a rate of cellular replication in diabetic individuals.

The association between short telomere length and clinical signs of atherosclerosis is one of the best characterized. A recent study evaluated telomere length in patients presenting with atherosclerosis who underwent a surgical procedure; patients presenting with atherosclerosis who did not receive surgery; and healthy controls. The results demonstrated that arterial tissues with atherosclerosis presented shorter telomeres than tissues without the disease.[14] These findings also suggest a relationship between atherosclerosis and telomere length independently from age. Additionally, an in vitro experiment observed that CD14+ and CD16+ monocytes with shorter telomeres shows higher expression of chemokine receptors and increased adhesion to endothelial cells.[38] In mice, severe telomerase dysfunction led to left ventricular diameter increase and thinning of left ventricular wall, which are signs of end-stage cardiomyopathy.[18] In humans, the association between telomere length and heart diseases has been proposed[39,40] but is still controversial,[41,42] since this involves several variables, such as age, sex, cigarette smoking, body mass index and other coexisting diseases.

Obesity is another chronic disease characterized by a high inflammatory burden which has been associated with shorter telomeres. A case–control study with 793 French children and young people aged 2–17 years old demonstrated that obese individuals presented telomeres 24% shorter than nonobese controls.[15] A great concern about that result is that, interestingly, another study found that former obese individuals present shorter telomeres in adipose tissue cells when compared to control individuals, which means that alteration in telomere length may be an irreversible phenomenon.[43] However, the relationship between adipocytes and leucocyte telomere length still needs further investigation.

Other diseases/conditions related to telomere length include rheumatoid arthritis,[16] major depressive disorder,[44] alcohol consumption,[45] osteosarcoma[46] and periodontitis.[47] Furthermore, while it is not a subject of this review, telomeres dynamic and telomerase activity play an important role in cancer biology.[48]

2.3. *Telomere shortening and periodontitis*

We searched MEDLINE (1950 to January 2012) and ISI WEB OF SCIENCE (1950 to January 2012) databases for the combination of the MeSH terms 'telomere' and 'periodontitis'. Only full-text articles were considered for inclusion. The search retrieved two results[46,48] and their findings are summarized and discussed below.

In 2004, Takahashi et al. investigated LTL in peripheral blood and also assessed telomere length in gingival fibroblasts established by cellular outgrowth from explants of patients suffering from aggressive periodontitis (AgP) and compared telomere length in these patients to age-matched controls without periodontal disease. The results were consistent with a difference in LTL between AgP and controls that was not statistically significant. The small sample size of this study and the high degree of variability in LTL between individuals could help to explain these findings. Nevertheless, the authors confirmed that there was no association between LTL and gingival fibroblast telomere length (at passage number 3). This led to the conclusion that patients with AgP do not show telomere attrition in circulating cells or in gingival fibroblasts.[49]

More recently, Masi et al. reported a larger case–control study on LTL in people with chronic periodontitis (CP), AgP and healthy controls. The authors measured LTL and correlated these measures with biomarkers of systemic inflammation and cumulative markers of systemic oxidative stress. Their findings are consistent with shorter LTL (compared to controls) in individuals with CP, but not those with AgP. This effect appeared to be independent of age differences, since controls and CP patients had similar mean ages. Furthermore, LTL was negatively correlated with age, measures of oxidative stress and the severity of periodontitis. The authors suggested that the local and systemic effects of oxidative stress in people with periodontitis could be the main causes of LTL shortening via a state of chronic inflammation.[47]

3. Discussion

Senescent cells are involved in metabolic changes and increased susceptibility to many disorders.[1] Aged cells present shorter telomeres, mitochondrial dysfunction and limited replicative capacity.[18] Oxidative stress and inflammation may promote premature ageing, with early telomere shortening.[26] Some disabilities and diseases have been related with telomere shortening, including muscle atrophy, progressive loss of vigour, type 2 diabetes, cardiomyopathy, atherosclerosis and obesity.[14,32–35,43]

Many studies have investigated the associations between short telomeres and systemic chronic diseases. However, the vast majority presents an observational approach (mostly case–control studies), and the direct cause–effect relationship remains to be determined. In fact, age is a very important confounding factor when telomeres and chronic diseases are assessed, even though the studies that report an association between these two variables perform statistical adjustments.

Only two studies evaluated the relationship between LTL and periodontitis. Although one study observed no impact of periodontitis on telomere length,[49] the other demonstrated that loss of periodontal attachment was negatively correlated with LTL.[47] A number of factors could account for the different results reported by these two studies: sample size (71 vs. 563); inclusion criteria (AgP only vs. AgP and CP); LTL measurement method (Southern blotting vs. qPCR). However, neither study reported an association between AgP and shorter LTL. Authors speculated that CP, but not AgP, is associated with shorter LTL because of the longer inflammatory exposure that characterizes the chronic evolution of CP.[47] Although this could represent a potential explanation, it should be noted that the exact age at which AgP and CP developed was unknown and, although patients with AgP were significantly younger than those with CP, the AgP group included subjects who were 40 years of age or older.[47] This means that patients in the AgP group could have had an even longer exposure to the chronic disease if one considers its usual early onset. Furthermore, as stated before, case–control studies provide limited evidence on the temporal association between these two factors and therefore do not confirm causality. This means that there are three hypotheses which could account for the relationship between short LTL and CP: 1 – CP promotes telomere shortening; 2 – shorter telomeres increase susceptibility to CP; or 3 – CP and telomere shortening are predisposed/induced by similar mechanisms, but they occur independently (Fig. 2).

First, CP could promote telomere shortening as suggested by Masi et al. via increased systemic inflammation and oxidative stress, both of which are known to induce telomere shortening.[26,47] Previous studies have reported an association between periodontitis, higher oxidative stress[50] and increased levels of systemic inflammatory markers, such as C-reactive protein.[51,52] Exposure to a chronic inflammatory environment,

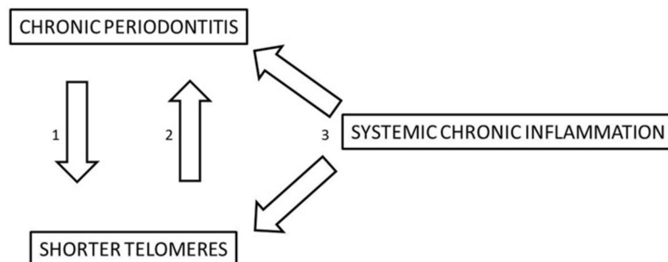

Fig. 2 – Schematic representation of the three hypotheses for the relationship between shorter telomere length and CP; CP promotes telomere shortening; shorter telomeres increase susceptibility to CP; CP and telomere shortening are predisposed/induced by similar mechanisms but they occur independently.

as the one provided by periodontitis, may accelerate telomere attrition and shorter LTL appears as a result. This hypothesis is in accordance with the findings of Masi et al.[47]

An alternative hypothesis is that shorter telomeres increase patient susceptibility to periodontitis. This is supported by the percentage of individuals presenting with 3 mm or more of periodontal attachment loss increasing with age.[53] However, including age as a risk factor for periodontitis is controversial. Borrell and Papapanou argued that increased age may reflect the cumulative exposure to true risk factors and that predisposed individuals will develop periodontitis during adolescence or adulthood.[54] In spite of this possibility, ageing could be a risk factor for periodontitis, albeit one without well-designed studies addressing its relationship to periodontitis.[55] It is also possible that younger individuals with periodontitis have premature cell ageing (i.e., shorter telomeres), predisposing these individuals to chronic diseases. As telomere length becomes critically shortened, many cellular activities become dysfunctional. This dysfunctional phenotype usually precedes the irreversible arrest of the replication machinery, a feature of senescent cells. Therefore, in individuals with CP, short telomeres recorded in immune cells could reflect dysfunction in the activity of the immune system, promoting the proliferation of local gingival bacteria and favouring the evolution of oral disease. In this model, local tissues could also have a role in the amplification of the local inflammatory response. Indeed, a recent report by Rodier et al. demonstrated that human fibroblasts with persistent activation of DNA damage response due to short telomere length start to produce high levels of IL-6.[56] These findings support the idea that the increased susceptibility to periodontitis in CP patients could be related to shorter telomere length. The set point of telomere length in mice is determined by the parents' telomere length.[57] Telomere length may also be inherited in humans.[58–62] It is therefore possible that, even without any significant differences in patient age among the groups in the study performed by Masi et al., individuals born with shorter LTL could be more likely to develop periodontitis.

Finally, we could hypothesize that both CP and telomere shortening are induced by similar mechanisms but occur independently. Even if periodontitis itself does not induce telomere shortening, or vice-versa, there are other possible pathways underlying this relationship. As previously reported, several systemic chronic diseases, including diabetes, may be correlated with telomere shortening.[12,13] At the same time, extensive studies have been performed to establish interrelationships between periodontal diseases and diabetes, and some findings suggest that this relationship may be bidirectional.[55,63] This could be an indication that other systemic chronic diseases, such as diabetes, could possibly play an important role in the relationship between periodontitis and shorter telomeres. Interestingly, other conditions related to shorter telomeres, such as atherosclerosis, smoking and obesity, are also associated with periodontitis.[64–66]

Nevertheless, it is important to notice that the methods for telomere measurement can vary and that makes comparisons among studies more difficult. Although highly specific methods that can reliably quantify telomere length do not exist yet, southern blot has been shown to be more accurate than qPCR, although it is more time-consuming.[67] Additionally, studies demonstrating differences in telomere length tend to be more rapidly accepted than studies that find the contrary and this publication bias should be considered.

4. Conclusion

CP, but not AgP, is associated with shorter LTL. This association may represent a mechanism of premature ageing in periodontitis patients, a cellular basis for including age (and its consequent shorter telomeres) as a risk factor for periodontitis, or a new pathway for the correlation between periodontitis and systemic inflammatory diseases. Because cellular ageing is an emerging multidisciplinary research field that may clarify the current understanding of the pathogenesis of periodontitis and other chronic diseases, new longitudinal and experimental studies are necessary to better understand this issue.

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Competing interests

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable.

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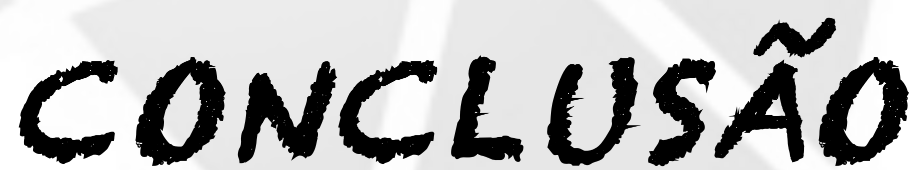
REFERENCES

1. Oeseburg H, de Boer RA, van Gilst WH, van der Harst P. Telomere biology in healthy aging and disease. *Pflugers Archiv European Journal of Physiology* 2010;**459**(2):259–68.
2. Songyang Z. Introduction to telomeres and telomerase. *Methods in Molecular Biology* 2011;**735**:1–11.
3. Brandl A, Meyer M, Bechmann V, Nerlich M, Angele P. Oxidative stress induces senescence in human mesenchymal stem cells. *Experimental Cell Research* 2011;**317**(11):1541–7.
4. Blackburn EH. Telomere states and cell fates. *Nature* 2000;**408**(6808):53–6.
5. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human

telomerase from immortal cells. *Science* 2007;**315**(5820):1850–3.
6. Collins K, Mitchell JR. Telomerase in the human organism. *Oncogene* 2002;**21**(4):564–79.
7. Gomes NM, Ryder OA, Houck ML, Charter SJ, Walker W, Forsyth NR, et al. Comparative biology of mammalian telomeres: hypotheses on ancestral states and the roles of telomeres in longevity determination. *Aging Cell* 2011;**10**(5):761–8.
8. Astrup AS, Tarnow L, Jorsal A, Lajer M, Nzietchueng R, Benetos A, et al. Telomere length predicts all-cause mortality in patients with type 1 diabetes. *Diabetologia* 2010;**53**(1):45–8.
9. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* 2003;**361**(9355):393–5.
10. Kimura M, Hjelmborg JV, Gardner JP, Bathum L, Brimacombe M, Lu X, et al. Telomere length and mortality: a study of leukocytes in elderly Danish twins. *American Journal of Epidemiology* 2008;**167**(7):799–806.
11. Hayflick L. The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research* 1965;**37**:614–36.
12. Guo N, Parry EM, Li LS, Kembou F, Lauder N, Hussain MA, et al. Short telomeres compromise beta-cell signaling and survival. *PLoS One* 2011;**6**(3):e17858.
13. Kuhlow D, Florian S, von Figura G, Weimer S, Schulz N, Petzke KJ, et al. Telomerase deficiency impairs glucose metabolism and insulin secretion. *Aging (Albany NY)* 2010;**2**(10):650–8.
14. Nzietchueng R, Elfarra M, Nloga J, Labat C, Carteaux JP, Maureira P, et al. Telomere length in vascular tissues from patients with atherosclerotic disease. *Journal of Nutrition Health and Aging* 2011;**15**(2):153–6.
15. Buxton JL, Walters RG, Visvikis-Siest S, Meyre D, Froguel P, Blakemore AI. Childhood obesity is associated with shorter leukocyte telomere length. *Journal of Clinical Endocrinology and Metabolism* 2011;**96**(5):1500–5.
16. Costenbader KH, Prescott J, Zee RY, De Vivo I. Immunosenescence and rheumatoid arthritis: does telomere shortening predict impending disease? *Autoimmunity Reviews* 2011;**10**(9):569–73.
17. Probst-Hensch NM. Chronic age-related diseases share risk factors: do they share pathophysiological mechanisms and why does that matter? *Swiss Medical Weekly* 2010;**140**:w13072.
18. Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 2011;**470**(7334):359–65.
19. World Health Organization. *Active ageing: a policy framework*. Geneva, Switzerland: WHO; 2002. Available at http://whqlibdoc.who.int/hq/2002/WHO_NMH_NPH_02.8.pdf.
20. Christensen K, Doblhammer G, Rau R, Vaupel JW. Ageing populations: the challenges ahead. *Lancet* 2009;**374**(9696):1196–208.
21. Weinert BT, Timiras PS. Invited review: theories of aging. *Journal of Applied Physiology* 2003;**95**(4):1706–16.
22. Takubo K, Izumiyama-Shimomura N, Honma N, Sawabe M, Arai T, Kato M, et al. Telomere lengths are characteristic in each human individual. *Experimental Gerontology* 2002;**37**(4):523–31.
23. Marrone A, Mason PJ. Dyskeratosis congenita. *Cellular and Molecular Life Sciences* 2003;**60**(3):507–17.
24. Vulliamy T, Marrone A, Goldman F, Dearlove A, Bessler M, Mason PJ, et al. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature* 2001;**413**(6854):432–5.
25. Atkinson JC, Harvey KE, Domingo DL, Trujillo MI, Guadagnini JP, Gollins S, et al. Oral and dental phenotype of dyskeratosis congenita. *Oral Diseases* 2008;**14**(5):419–27.
26. von Zglinicki T. Oxidative stress shortens telomeres. *Trends in Biochemical Sciences* 2002;**27**(7):339–44.
27. Brandl A, Hartmann A, Bechmann V, Graf B, Nerlich M, Angele P. Oxidative stress induces senescence in chondrocytes. *Journal of Orthopaedic Research* 2011;**29**(7):1114–20.
28. Tchirkov A, Lansdorp PM. Role of oxidative stress in telomere shortening in cultured fibroblasts from normal individuals and patients with ataxia-telangiectasia. *Human Molecular Genetics* 2003;**12**(3):227–32.
29. Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. Telomere length assessment: biomarker of chronic oxidative stress? *Free Radical Biology and Medicine* 2008;**44**(3):235–46.
30. Passos JF, Saretzki G, Ahmed S, Nelson G, Richter T, Peters H, et al. Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence. *PLoS Biology* 2007;**5**(5):e110.
31. Kelly DP. Cell biology: ageing theories unified. *Nature* 2011;**470**(7334):342–3.
32. Kirkwood TB. Understanding the odd science of aging. *Cell* 2005;**120**(4):437–47.
33. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annual Review of Genetics* 2005;**39**:359–407.
34. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell* 2005;**120**(4):483–95.
35. Conley KE, Marcinek DJ, Villarin J. Mitochondrial dysfunction and age. *Current Opinion in Clinical Nutrition and Metabolic Care* 2007;**10**(6):688–92.
36. Zee RY, Castonguay AJ, Barton NS, Germer S, Martin M. Mean leukocyte telomere length shortening and type 2 diabetes mellitus: a case–control study. *Translational Research* 2010;**155**(4):166–9.
37. Salpea KD, Talmud PJ, Cooper JA, Maubaret CG, Stephens JW, Abelak K, et al. Association of telomere length with type 2 diabetes, oxidative stress and UCP2 gene variation. *Atherosclerosis* 2010;**209**(1):42–50.
38. Merino A, Buendia P, Martin-Malo A, Aljama P, Ramirez R, Carracedo J. Senescent CD14+CD16+ monocytes exhibit proinflammatory and proatherosclerotic activity. *Journal of Immunology* 2011;**186**(3):1809–15.
39. Samani NJ, Boultby R, Butler R, Thompson JR, Goodall AH. Telomere shortening in atherosclerosis. *Lancet* 2001;**358**(9280):472–3.
40. Fitzpatrick AL, Kronmal RA, Gardner JP, Psaty BM, Jenny NS, Tracy RP, et al. Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. *American Journal of Epidemiology* 2007;**165**(1):14–21.
41. O'Donnell CJ, Demissie S, Kimura M, Levy D, Gardner JP, White C, et al. Leukocyte telomere length and carotid artery intimal medial thickness: the Framingham Heart Study. *Arteriosclerosis Thrombosis and Vascular Biology* 2008;**28**(6):1165–71.
42. Vasan RS, Demissie S, Kimura M, Cupples LA, White C, Gardner JP, et al. Association of leukocyte telomere length with echocardiographic left ventricular mass: the Framingham heart study. *Circulation* 2009;**120**(13):1195–202.
43. Moreno-Navarrete JM, Ortega F, Sabater M, Ricart W, Fernandez-Real JM. Telomere length of subcutaneous adipose tissue cells is shorter in obese and formerly obese subjects. *International Journal of Obesity* 2010;**34**(8):1345–8.
44. Wolkowitz OM, Mellon SH, Epel ES, Lin J, Dhabhar FS, Su Y, et al. Leukocyte telomere length in major depression: correlations with chronicity, inflammation and oxidative stress – preliminary findings. *PLoS One* 2011;**6**(3):e17837.

45. Aida J, Yokoyama A, Izumiyama N, Nakamura K, Ishikawa N, Poon SS, et al. Alcoholics show reduced telomere length in the oesophagus. *Journal of Pathology* 2011;**223**(3):410–6.
46. Mirabello L, Richards EG, Duong LM, Yu K, Wang Z, Cawthon R, et al. Telomere length and variation in telomere biology genes in individuals with osteosarcoma. *International Journal of Molecular Epidemiology and Genetics* 2011;**2**(1):19–29.
47. Masi S, Salpea KD, Li K, Parkar M, Nibali L, Donos N, et al. Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis. *Free Radical Biology and Medicine* 2011;**50**(6):730–5.
48. Cree IA. Cancer biology. *Methods in Molecular Biology* 2011;**731**:1–11.
49. Takahashi K, Nishida H, Takeda H, Shin K. Telomere length in leukocytes and cultured gingival fibroblasts from patients with aggressive periodontitis. *Journal of Periodontology* 2004;**75**(1):84–90.
50. D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J, Donos N. Oxidative stress, systemic inflammation, and severe periodontitis. *Journal of Dental Research* 2010;**89**(11):1241–6.
51. Gomes-Filho IS, Freitas Coelho JM, da Cruz SS, Passos JS, Teixeira de Freitas CO, Aragao Farias NS, et al. Chronic periodontitis and C-reactive protein levels. *Journal of Periodontology* 2011;**82**(7):969–78.
52. Paraskevas S, Huizinga JD, Loos BG. A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. *Journal of Clinical Periodontology* 2008;**35**(4): 277–90.
53. Albandar JM. Periodontal diseases in North America. *Periodontology 2000* 2002;**29**:31–69.
54. Borrell LN, Papapanou PN. Analytical epidemiology of periodontitis. *Journal of Clinical Periodontology* 2005;**32**(Suppl. 6):132–58.
55. Tonetti MS, Claffey N. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research Group C consensus report of the 5th European Workshop in Periodontology. *Journal of Clinical Periodontology* 2005;**32**(Suppl. 6):210–3.
56. Rodier F, Coppe JP, Patil CK, Hoeijmakers WA, Munoz DP, Raza SR, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nature Cell Biology* 2009;**11**(8):973–9.
57. Chiang YJ, Calado RT, Hathcock KS, Lansdorp PM, Young NS, Hodes RJ. Telomere length is inherited with resetting of the telomere set-point. *Proceedings of the National Academy of Sciences of the United States of America* 2010;**107**(22):10148–53.
58. Nawrot TS, Staessen JA, Gardner JP, Aviv A. Telomere length and possible link to X chromosome. *Lancet* 2004;**363**(9408):507–10.
59. Nordfjall K, Larefalk A, Lindgren P, Holmberg D, Roos G. Telomere length and heredity: indications of paternal inheritance. *Proceedings of the National Academy of Sciences of the United States of America* 2005;**102**(45):16374–8.
60. Bakaysa SL, Mucci LA, Slagboom PE, Boomsma DI, McClearn GE, Johansson B, et al. Telomere length predicts survival independent of genetic influences. *Aging Cell* 2007;**6**(6):769–74.
61. Hunt SC, Chen W, Gardner JP, Kimura M, Srinivasan SR, Eckfeldt JH, et al. Leukocyte telomeres are longer in African Americans than in whites: the National Heart Lung, and Blood Institute Family Heart Study and the Bogalusa Heart Study. *Aging Cell* 2008;**7**(4):451–8.
62. Nordfjall K, Svenson U, Norrback KF, Adolfsson R, Roos G. Large-scale parent–child comparison confirms a strong paternal influence on telomere length. *European Journal of Human Genetics* 2010;**18**(3):385–9.
63. Taylor GW. Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective. *Annals of Periodontology* 2001;**6**(1):99–112.
64. Scannapieco FA, Bush RB, Paju S. Associations between periodontal disease and risk for atherosclerosis, cardiovascular disease, and stroke. A systematic review. *Annals of Periodontology* 2003;**8**(1):38–53.
65. Suvan J, D'Aiuto F, Moles DR, Petrie A, Donos N. Association between overweight/obesity and periodontitis in adults. A systematic review. *Obesity Reviews* 2011;**12**(5):e381–404.
66. Morla M, Busquets X, Pons J, Sauleda J, MacNee W, Agusti AG. Telomere shortening in smokers with and without COPD. *European Respiratory Journal* 2006;**27**(3):525–8.
67. Aviv A, Hunt SC, Lin J, Cao X, Kimura M, Blackburn E. Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR. *Nucleic Acids Research* 2011;**39**(20):e134.


CONCLUSÃO

3 Conclusão

Avaliados em conjunto, nossos dados sugerem que os hormônios sexuais, em especial a testosterona, estão associados à presença e severidade de periodontite em homens, sendo que esta relação pode ser causal, uma vez que nossos estudos em animais demonstraram que a modulação dos níveis séricos de testosterona alteram a perda óssea alveolar e área gengival em um modelo de periodontite experimental em ratos. A regulação da expressão de Interleucina-1β local e da osteoclastogênese, diretamente ou através da regulação da expressão de RANKL e OPG por osteoblastos, parecem ser mecanismos envolvidos.

No entanto, não está claro se as variações nos níveis fisiológicos de testosterona participam também da iniciação da doença periodontal; se a testosterona é fator igualmente protetivo em fêmeas; ou ainda se a terapia de reposição hormonal favorece o tratamento periodontal.

REFERÊNCIAS*

1. Achar S, Rostamian A, Narayan SM. Cardiac and metabolic effects of anabolic-androgenic steroid abuse on lipids, blood pressure, left ventricular dimensions, and rhythm. Am J Cardiol. 2010; 106(6): 893-901.

2. Bain J. Testosterone and the aging male: to treat or not to treat? Maturitas. 2010; 66(1): 16-22.

3. Del Fabbro E, Hui D, Nooruddin ZI, Dalal S, Dev R, Freer G, et al. Associations among hypogonadism, C-reactive protein, symptom burden, and survival in male cancer patients with cachexia: a preliminary report. J Pain Symptom Manage. 2010; 39(6): 1016-24.

4. Instituto Brasileiro de Geografia e Estatística. Projeção da população do Brasil por sexo e idade - 1980-2050. Revisão 2008. Rio de Janeiro, Brasil: IBGE; 2008.

5. Kanayama G, Hudson JI, Pope HG Jr. Long-term psychiatric and medical consequences of anabolic-androgenic steroid abuse: a looming public health concern? Drug Alcohol Depend. 2008; 98(1-2): 1-12.

6. Laaksonen DE, Niskanen L, Punnonen K, Nyyssonen K, Tuomainen TP, Valkonen VP, et al. Testosterone and sex hormone-binding globulin predict the metabolic syndrome and diabetes in middle-aged men. Diabetes Care. 2004; 27(5): 1036-41.

7. Laughlin GA, Barrett-Connor E, Bergstrom J. Low serum testosterone and mortality in older men. J Clin Endocrinol Metab. 2008; 93(1): 68-75.

8. Maggio M, Basaria S. Welcoming low testosterone as a cardiovascular risk factor. Int J Impot Res. 2009; 21(4): 261-4.

9. Mariotti A, Mawhinney M. Endocrinology of sex steroid hormones and cell dynamics in the periodontium. Periodontol 2000. 2013; 61(1): 69-88.

10. Mariotti A. The ambit of periodontal reproductive endocrinology. Periodontol 2000. 2013; 61(1): 7-15.

11. Meier C, Nguyen TV, Handelsman DJ, Schindler C, Kushnir MM, Rockwood AL, et al. Endogenous sex hormones and incident fracture risk in older men: the Dubbo Osteoporosis Epidemiology Study. Arch Intern Med. 2008; 168(1): 47-54.

12. Mellstrom D, Johnell O, Ljunggren O, Eriksson AL, Lorentzon M, Mallmin H, et al. Free testosterone is an independent predictor of BMD and prevalent fractures in elderly men: MrOS Sweden. J Bone Miner Res. 2006; 21(4): 529-35.

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* De acordo com o manual da FOAr/UNESP, adaptadas das normas Vancouver. Disponível no site: http://www.foar.unesp.br/#!/biblioteca/manual.

13. Mooradian AD, Morley JE, Korenman SG. Biological actions of androgens. Endocr Rev. 1987; 8(1): 1-28.

14. Nasri F. O envelhecimento populacional no Brasil. Einstein. 2008; 6(Supl. 1): S4-S6.

15. Selvin E, Feinleib M, Zhang L, Rohrmann S, Rifai N, Nelson WG, et al. Androgens and diabetes in men: results from the Third National Health and Nutrition Examination Survey (NHANES III). Diabetes Care. 2007; 30(2): 234-8.

16. Stellato RK, Feldman HA, Hamdy O, Horton ES, McKinlay JB. Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. Diabetes Care. 2000; 23(4): 490-4.

17. Traish AM, Guay A, Feeley R, Saad F. The dark side of testosterone deficiency: I. Metabolic syndrome and erectile dysfunction. J Androl. 2009; 30(1): 10-22.

18. Traish AM, Saad F, Guay A. The dark side of testosterone deficiency: II. Type 2 diabetes and insulin resistance. J Androl. 2009; 30(1): 23-32.

19. Traish AM, Saad F, Feeley RJ, Guay A. The dark side of testosterone deficiency: III. Cardiovascular disease. J Androl. 2009; 30(5): 477-94.

APÊNDICE A

MATERIAL E MÉTODOS

- EXPERIMENTO IN VIVO:

Foram utilizados 80 ratos (*Rattus norvergicus* albinos, Holtzman), machos, com peso médio de 300-400 g, provenientes do Biotério Central da UNESP, Campus de Araraquara. Os animais foram aleatoriamente separados em grupos de igual número e mantidos em gaiolas plásticas em um ambiente com temperatura controlada (23±2°C), umidade (65-75%) e ciclos de luz (12h claro-12h escuro) e alimentados com água e ração *ad libitum*. Os grupos e condições experimentais foram os seguintes:

- Sham - “controle” (n=10) e “periodontite” (n=10)
- Orquiectomia (OQX) - “controle” (n=10) e “periodontite” (n=10)
- Orquiectomia + Testosterona (OQX+T) - “controle” (n=10) e “periodontite” (n=10)
- Sham + Bloqueador de Receptor de Andrógeno (SHAM+BRA) - “controle” (n=10) e “periodontite” (n=10)

O protocolo experimental foi aprovado pelo Comitê de Ética de Experimentação Animal local e realizado de acordo com as normas da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL).

- Orquiectomia (OQX)

Os animais foram submetidos à anestesia geral através de injeção intra-peritoneal de 1 mL/kg de peso corporal de cetamina (Francotar, Virbac, São Paulo,

SP, Brasil) e 0,4 mL/kg de peso corporal de Cloridrato de Xilazina (Virbaxil, Virbac). Foi realizada a tricotomia na bolsa escrotal, no local da cirurgia e a seguir foi feita uma incisão com auxílio de um bisturi nº 15 expondo os testículos. Após a exposição ocorreu incisão na túnica albugínea e foi feita a ligadura do cordão espermático com fio de algodão, o que facilitou a retirada dos testículos com auxílio de uma lâmina de bisturi n° 15. Posteriormente, foi feita a sutura com fio reabsorvível e desinfecção com álcool iodado. Todos os processos cirúrgicos foram feitos sob condições estéreis, com o objetivo de evitar eventuais infecções pós-operatórias. Os animais foram colocados em caixas desinfectadas com álcool 70°, com maravalha e ração autoclavada, além da administração de amoxicilina (1 mL/kg) via subcutânea e em dose única, e da administração de acetaminofeno (dose única). Os animais foram mantidos durante 28 dias no biotério até a indução de doença periodontal.

- Tratamento Com Propionato de Testosterona

Três dias após a cirurgia, os ratos orquiectomizados pertencentes ao grupo OQX+T receberam 250 mg/kg de peso corporal de ésteres de testosterona (Durateston, MSD, Campinas, SP, Brasil), via intramuscular, dissolvido em 0,1 mL de veículo oleoso (óleo de milho), semanalmente, até o sacrifício.

- Tratamento Com Bloqueador De Receptor De Andrógeno (Flutamida)

A fim de se verificar o papel da conversão da testosterona em estrógeno, no grupo “SHAM+BRA” os receptores de andrógeno foram bloqueados através da utilização de flutamida oral (50 mg/kg), a cada 2 dias, dissolvida em 25 mg/mL de água destilada e Tween-20, até o sacrifício dos animais.

- Indução Da Doença Periodontal Experimental

No dia 28, após anestesia geral, os animais pertencentes aos grupos "periodontite" foram posicionados em mesa operatória apropriada, para permitir uma manutenção adequada de abertura bucal dos ratos, facilitando o acesso aos dentes da região posterior da mandíbula. Com o auxílio de instrumental adequado, foi colocado um fio de algodão estéril n° 30 ao redor dos primeiros molares inferiores dos lados direito e esquerdo. Esta ligadura atua como irritante gengival na indução da resposta inflamatória, favorecendo o acúmulo de biofilme dental e assim, representando de forma apropriada a situação clínica da doença periodontal em humanos. Todos os animais foram sacrificados 2 semanas após este procedimento.

- Impacto Dos Tratamentos Sobre Os Níveis Séricos De Testosterona

Após o período experimental, os animais foram submetidos à anestesia geral conforme descrito anteriormente e 3 mL de sangue foram coletados através de punção cardíaca com auxílio de uma seringa. Após a obtenção do soro por centrifugação, a concentração sérica de testosterona foi analisada utilizando-se ensaio de quimioluminescência (Immulite 2000, Diagnostic Products Corporation, Gwynedd, Reino Unido), no Hospital Estadual de Américo Brasiliense.

- Avaliação Bioquímica Dos Parâmetros Do Metabolismo Ósseo

Após a obtenção do soro por centrifugação, foram analisadas as concentrações de Ca^{2+} (Cálcio Arsenazo III), fósforo (UV Crystal), fosfatase alcalina óssea através de testes colorimétricos, utilizando Kit comercial (Bioclin, Quibasa, Belo Horizonte, MG, Brasil).

- Microtomografia Computadorizada (μCT) Das Mandíbulas

No momento do sacrifício dos animais, as hemi-mandíbulas do lado esquerdo de 5 animais por grupo foram removidas para análise por microtomografia computadorizada. Essas peças foram fixadas em formol 10% por 48 horas e transferidas para etanol 70%. O escaneamento das peças em microtomógrafo (Skyscan, Aartselaar, Bélgica) foi feito com as seguintes especificações: resolução 2000 x 1336, filtro AR 0,5 m, 50 kV, 495 μA, Skyscan 1176, 18 μm. Nas imagens tridimensionais das peças, incluindo os primeiros molares inferiores e tecidos adjacentes, foi delimitada uma região de interesse (ROI) em pontos de reparo anatômico padronizados. Com o auxílio do software que acompanha o equipamento (CTAn), as seguintes análises foram realizadas: fração de volume ósseo (BV/TV) e análise linear da distância entre junção amelocementária e crista óssea alveolar na mesial do primeiro molar.

- Avaliação Microscópica

No momento do sacrifício dos animais, as hemi-mandíbulas do lado esquerdo de 5 animais por grupo foram removidas para análise histológica. As peças foram fixadas em formol 10% durante 48 h, e então desmineralizadas em um descalcificador rápido (Allkimia, Campinas, SP, Brasil) durante 10 horas. Cortes seriados de 5 μm de espessura foram obtidos na direção vestíbulo-lingual, montados em lâminas de vidro e corados com Hematoxilina & Eosina (H&E) para análise descritiva do processo de doença periodontal. Foi realizada histometria dos tecidos conjuntivo e epitelial para determinar a área gengival, utilizando-se software (ImageJ, NIH, Bethesda, MD, Estados Unidos).

- **Avaliação Dos Efeitos Da Testosterona Na Modulação Da Resposta Inflamatória**

O papel da testosterona na modulação da resposta inflamatória foi avaliado por ensaios ELISA em amostras de soro e também de proteína total mucogengival extraída com o reagente T-Per (Pierce, Thermo Fisher) – 100µL - e quantificadas segundo o método de Lowry (DC protein assay, Bio-Rad). Foram utilizados kis de ELISA comercialmente disponíveis para detecção das citocinas IL-1β e IL-6 (R&D Systems). Amostras provenientes de quatro animais por grupo experimental (controle e periodontite) de cada condição (cirurgia sham, OQX, OQX+T, SHAM+BRA) foram analisadas em duplicata, sendo que a amostra mucogengival foi diluída 2 vezes, enquanto o soro não foi diluído. O procedimento foi realizado segundo instruções do fabricante, e as leituras foram realizadas em leitor de placas a 450 nm com correção a 570 nm.

- EXPERIMENTO IN VITRO

- Papel Da Testosterona Na Osteoclastogênese

A fim de se buscar mecanismos de ação para os achados do estudo in vivo, foi realizada esta parte in vitro. A linhagem de monócitos/macrófagos murinos Raw 264.7 foi utilizada como precursora de osteoclastos. Estas células foram cultivadas em α-MEM suplementado com 10% de soro fetal bovino inativado por calor e 100 UI/mL de penicilina e 100 µg/mL de estreptomicina em incubadora de CO_2. Estas células foram plaqueadas em placas de 96 poços, e cultivadas por 5 dias na presença de RANKL (50 ng/mL) com e sem adição de doses crescentes de testosterona múltiplas de 10 (1 nM - 1 µM). Flutamida (1µM) foi utilizada para avaliar

a reversão dos efeitos da testosterona. O grupo controle recebeu DMSO 1%, produto utilizado na diluição da testosterona. A identificação dos osteoclastos foi feita pela visualização, em microscopia óptica de luz comum, de células TRAP-positivas contendo ao menos 3 núcleos. A coloração TRAP foi realizada utilizando-se kit comercialmente disponível (Sigma-Aldrich, Saint Louis, MO, Estados Unidos) e a identificação de osteoclastos com o auxílio de software (Image J e Image Tool, UTHSCSA, San Antonio, TX, Estados unidos). Todos os experimentos foram realizados pelo menos em quadruplicata e por pelo menos 3 vezes.

O sobrenadante foi utilizado para avaliação da expressão das citocinas/quimiocinas Interleucina (IL)-6, IL-10, Fator de Necrose Tumoral (TNF) e *Reguated on Activation Normal T cell Expressed and Secreted* (RANTES), utilizando-se ensaio multiplex (Millipore, Billerica, MA, Estados Unidos).

A expressão de receptor de andrógeno nas células tratadas com testosterona foi avaliada através de Western Blot. Após realização do experimento conforme descrito acima, o lisado celular foi coletado utilizando-se RIPA buffer contendo inibidor de protease. O anticorpo primário utilizado (sc-816, Santa Cruz, Dallas, TX, Estados unidos) foi diluído 1:200 em solução 5% de leite/TBS-T e a incubação ocorreu overnight a 4°C. As bandas de β-actina serviram para normalização dos resultados no momento da densitometria.

A indução de apoptose pela testosterona em células precursoras e em osteoclastos foi avaliada pela detecção de caspase-3 por ensaio colorimétrico (Invitrogen, Carlsbad, CA, Estados Unidos). Em síntese, as células Raw 264.7 foram cultivadas conforme descrito anteriormente e o experimento foi terminado no dia 3. O lisado celular, extraído sobre gelo, foi utilizado para detecção do marcador, de acordo com as instruções do fabricante.

- Papel Da Testosterona Sobre Osteoblastos

Osteoblastos de calvária de camundongos neonatos (7-8 camundongos) foram incubados em meio de cultura α-MEM suplementado com 10% soro fetal bovino, 10.000 IU penicilina e 10.000 μg/mL de estreptomicina por 10 min a 37°C em solução 4mM Na_2 EDTA 3 vezes para desmineralização da matriz extracelular. Os sobrenadantes foram então descartados. As calvárias foram então encubadas por 2 vezes a 37°C em uma solução de colagenase tipo 2 para separação das células da matriz extracelular. Os sobrenadantes foram descartados. O remanescente das cinco incubações sequenciais foram adicionados em um *pool*, centrifugados, e resuspendidos em α-MEM suplementado. As células foram plaqueadas em uma densidade de $5x10^5$ e, ao alcançar confluência de 90%, transferidas para placas de 24 poços em densidade $2x10^4$ células/mL (1mL/poço). Ácido ascórbico (50 μg/mL) e β-glicerol-fosfato (10 mM) foram adicionados ao meio a cada 2 dias durante 10 dias para permitir diferenciação de osteoblastos.

Após os 10 dias, as concentrações utilizadas de testosterona foram de 1, 10 e 100 nM. Dois dias após o tratamento com testosterona, os sobrenadantes e lisados celulares foram coletados e analisados para detecção de osteocalcina, osteoprotegerina e RANKL utilizando-se Luminex (Millipore) e fosfatase alcalina por ELISA (Life Sciences Advanced Technologies Inc., Saint Petersburg, FL, Estados Unidos). Todos os protocolos foram realizados de acordo com as instruções dos fabricantes.

ANEXO A

APROVAÇÃO DO COMITÊ DE ÉTICA

UNIVERSIDADE ESTADUAL PAULISTA
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Câmpus de Araraquara

FACULDADE DE ODONTOLOGIA

Proc. CEEA nº 25/2010

Araraquara, 16 de setembro de 2010

Senhores Pesquisadores:

O Comitê de Ética em Experimentação Animal-CEEA desta Faculdade reunido em 15/09/2010, após a avaliação do projeto de sua responsabilidade intitulado "Efeito da redução dos níveis de testosterona na resposta imunoinflamatória associada à indução de doença periodontal e artrite reumatóide em ratos adultos" (Proc. CEEA nº 25/2010) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para SETEMBRO/2012.

Atenciosamente.

Profª Drª ELENY ZANELLA BALDUCCI
Coordenadora do CEEA

Ao
Prof. Dr. LUIS CARLOS SPOLIDÓRIO
DD. Pesquisador Responsável
a/c João Paulo Steffens
Departamento de Fisiologia e Patologia

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16 July 2013

Dr. João P. Steffens
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Dear Dr. Steffens,

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João Paulo Steffens