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Androgen receptors and experimental bone loss – An in vivo and in vitro study

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Abstract

Testosterone is a sex hormone that exhibits many functions beyond reproduction; one such function is the regulation of bone metabolism. The role played by androgen receptors during testosterone-mediated biological processes associated with bone metabolism is largely unknown. This study aims to use a periodontal disease model *in vivo* in order to assess the involvement of androgen receptors on microbial-induced inflammation and alveolar bone resorption in experimental bone loss. The impact of hormone deprivation was tested through both orchietomy and chemical blockage of androgen receptor using flutamide (FLU). Additionally, the direct effect of exogenous testosterone, and the role of the androgen receptor, on osteoclastogenesis were investigated. Thirty male adult rats (n=10/group) were subjected to: 1- orchietomy (OCX); 2- OCX sham-surgery; or 3- OCX sham-surgery plus FLU, four weeks before the induction of experimental bone loss. Ten OCX sham-operated rats were not subjected to experimental bone loss and served as healthy controls. The rats were euthanized two weeks later, so as to assess bone resorption and the production of inflammatory cytokines in the gingival tissue and serum. In order

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Disclosure

All authors state that they have no conflicts of interest.

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to study the *in vitro* impact of testosterone, osteoclasts were differentiated from RAW264.7 cells and testosterone was added at increasing concentrations. Both OCX and FLU increased bone resorption, but OCX alone was observed to increase osteoclast count. IL-1 β production was increased only in the gingival tissue of OCX animals, whereas FLU-treated animals presented a decreased expression of IL-6. Testosterone reduced the osteoclast formation in a dose-dependent manner, and significantly impacted the production of TNF- α ; FLU partially reversed these actions. When taken together, our results indicate that testosterone modulates experimental bone loss, and that this action is mediated, at least in part, via the androgen receptor.

Keywords

Testosterone; Androgens; Receptors; Androgen; Androgen Receptor Antagonists; Periodontitis; Bone; Bones

1. Introduction

Hypothalamic GnRH is responsible for regulating the release of interstitial cell-stimulating hormone in men, which in turn stimulates the production of testosterone [1]. Production of GnRH declines with age, lowering testosterone levels and indicating the closure of an individual's reproductive period [2]. Male hypogonadism, a condition associated with a reduced libido, loss of body hair, small or shrinking testes, low bone mineral density and other ill effects, is more likely to happen secondary to low serum total testosterone levels (generally less than 300ng/dL) [3]. The prevalence of hypogonadism is variable; prevalence varies between 2.1–18.4%, with higher values for older individuals [4, 5]. This suggests that aging has an impact on testosterone levels.

Declines in testosterone have consequences that extend beyond reproduction. Cross-sectional and prospective studies suggest that low testosterone levels are related to increased serum sIL-6r in older men; however, there remains some controversy about the effect of IL-6 on testosterone [6, 7]. Testosterone replacement therapy in hypogonadal men has been seen to significantly decrease serum pro-inflammatory cytokines of IL-1 β and TNF [8]. A cross-sectional study demonstrated that MIP-1 α , MIP-1 β and TNF are negatively associated with total testosterone in young men, suggesting that low testosterone levels may also promote a pro-inflammatory status independent of aging [9]. These findings highlight the role male hormones play in inflammatory responses.

Research suggests that testosterone may impact bone tissues and cells [10–14]. Testosterone can act directly, through binding to the androgen receptor, or indirectly, by its conversion to a more potent hormone, DHT. Testosterone can also be converted to estradiol by the aromatase enzyme. Many of the effects testosterone has on bone tissues have been attributed to its conversion to estradiol, which acts through estrogen receptors α and β , and possibly GPR30 [15–17]. Other studies, however, support the direct role of the androgen receptor, or an estrogen-independent participation of testosterone on bone turnover and regulation [10, 18, 19].

Periodontitis is a microbial-mediated chronic inflammatory disease affecting the supporting structures of teeth. Periodontitis is characterized by an overproduction of innate immune cytokines, such as IL-1 β , IL-6, and TNF [20]. This overproduction leads to tissue breakdown and the consequent resorption of the alveolar process, a bone structure consisting of outer cortical plates of compact bone, a central spongiosa (trabecular bone), and bone lining the alveolus (alveolar bone) [21]. Our previous studies suggest that both low and high testosterone levels regulate experimental bone loss in rats [13, 14]. This study tests the hypothesis that testosterone suppression increases inflammation-induced bone loss through the reduction of androgen receptor activation. This hypothesis is tested through the use of a model of experimental periodontitis in rats. The objectives of this study were as follows: to compare the impact of orchiectomy with that of chemical inhibition of androgen receptor *in vivo* through the assessment of associated bone markers and cytokine expression, and to investigate the impact of testosterone and its associated androgen receptor activation on osteoclastogenesis *in vitro*.

2. Materials and Methods

2.1 Animals

Forty male adult Holtzman rats weighing 300–400g (approximately 3 months old) were kept in cages under similar conditions (controlled temperature 23 $\pm$ 2°C, humidity 65–75% and 12-hour light-dark cycles). Food and water were provided *ad libitum*. Randomization of animals was performed using a raffle 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 (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

2.2 Androgen deprivation model

After 1 week of acclimatization, ten 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 b.w.) and xylazine (0.4 mL/kg) under sterile conditions. The rats were given acetaminophen (300 mg/kg; orally) for postoperative pain relief and an intramuscular dose of penicillin and streptomycin (1 mL/kg). After the procedure, the animals were kept in separate cages for recovery for 7 days. Thirty other rats received the same surgical procedure, except for the testicular removal (sham operation).

2.3 Chemical inhibition of androgen receptors

Ten sham-operated rats received flutamide (Sigma-Aldrich, Saint Louis, MO, USA; 50mg/kg), an androgen receptor antagonist, which was administered intragastrically every other day, until sacrifice. Flutamide was diluted to 25mg/mL in distilled water and Tween-20. The treatment was started three days following sham surgery [22].

2.4 Induction of Experimental Periodontal Bone Loss

Four weeks after orchietomy (or sham surgery), 30 rats (n=10/group) were anesthetized as described above. A 3.0 cotton ligature was placed in a subgingival position around the lower first molar tooth to enable bacterial accumulation, leading to inflammation and bone loss. The other 10 sham-operated animals did not receive ligatures and served as healthy controls. The ligatures were maintained for 2 weeks at which time all rats were euthanized.

2.5 Assessment of testosterone and local/systemic markers

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 20 minutes at 20,000×g to obtain serum. Each serum sample was analyzed for total testosterone levels using a chemiluminescence-based immunoassay (Immulite 2000, Diagnostic Products Corporation, Gwynedd, UK); and for the detection of estradiol, IL-1 β and IL-6 using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

The mucogingival tissues around the first molars of 5 animals per group were removed and processed for concentrations of IL-1 β and IL-6 using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions. Total protein was extracted in T-Per lysis buffer (Pierce, Thermo Scientific, Rockford, IL, USA) supplemented with proteinase inhibitors (cOmplete, Roche Diagnostics, Mannheim, Germany) and determined using the Bradford method. The results were used for data normalization.

2.6 microCT analyses

For quantitative three-dimensional analysis of the alveolar bone, mandibles of 5 animals per group were scanned using a microCT system (Skyscan, Aartselaar, Belgium). The specimens were scanned at a resolution of 18 μ m in all three spatial dimensions. CTan/CTvol software (Skyscan) was used for imaging and analysis. A standardized rectangular ROI measuring 1.13×0.97 mm was positioned at the furcation area (region among the roots of the teeth), in a slice-based method. The histogram settings were standardized at 90–130 and 101 serial slices were selected in each sample. Bone volume fraction was analyzed by the CT-scan software [14].

The linear distance between CEJ, an anatomical reference on the tooth, and alveolar bone crest was measured in the mesial surface of the first molars using software (Dataviewer 1.4.3, Skyscan). The measurement was performed three times by a calibrated individual (JPS) who was blinded to the treatment groups under the same background conditions. The mean of all three measurements was considered one sample and used for statistical analysis [16].

2.7 Histologic Analysis

After the rats were euthanized, 5 hemi-mandibles per group were used for histologic analysis. The samples were fixed in 10% formalin for 48 hours and demineralized for 10 hours using a rapid demineralization chemical agent containing EDTA, sodium and potassium tartrate, chloric acid and deionized water (Alkimia, Campinas, SP, Brazil). Serial

sections (5 μm) were obtained in a buccal-lingual orientation and the slides were stained using hematoxylin and eosin or TRAP-staining kit (Sigma-Aldrich), according to the manufacturer's instructions.

In order to perform stereometric analysis, a 1,000 $\times$ 500 μm grid was positioned vertically upon the connective tissue in histologic images (200 $\times$ magnification), with its lower border in contact with the alveolar bone crest and the vertical border in contact with the root cementum. The grid consisted of 19 $\times$ 9 squares making 200 intersection points. The structure in each intersection point was morphologically recognized and recorded as 'inflammatory cell', 'other structure', or 'out of the region of interest'. The intersection points reported as 'out of the region of interest' were discarded and the remaining points were considered to be the total number of possible points (100%) [23].

In order to perform osteoclast quantification using TRAP-staining, images of the alveolar bone were obtained under 400 $\times$ magnification. An area of interest was set from the bone crest to 500 μm below it and osteoclasts were considered to be multinucleated TRAP-positive cells on the surface of the bone structure [24]. For both analyses, the identification of the structures was performed in one section per slide, two slides per animal, by a single individual who was blinded to the grouping of each slide.

2.8 Impact of testosterone and androgen receptor on osteoclastogenesis

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% FBS and 1% penicillin/streptomycin until approximately 90% cell confluence, with media replacement every three days. Cells were seeded at 2.5×10^4 cells/mL in 96-well plates (200 μL /well) in the same media, with the addition of 50 ng/mL recombinant murine sRANKL (PeproTech, Rocky Hill, NJ, USA) to induce osteoclastic differentiation.

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 1 nM, 10 nM and 100 nM. 0.1% DMSO was added to each control well. Flutamide (Sigma-Aldrich), 1 μM [25], was used in addition to the testosterone dose that caused the highest impact for each variable, so as 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 non-denaturing conditions for the analysis of IL-1 β , IL-6, IL-10, TNF and 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 with 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 40 $\times$ 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.

2.9 Statistical Analysis

Analysis was performed to test for homogeneity of variance and normal distribution. If data satisfied both criteria, one-way 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 $\pm$ SD, unless otherwise stated.

3. Results

Interventions of testosterone metabolism in rats significantly altered serum concentration of testosterone. While orchietomy decreased testosterone levels below the detection point of the assay (0.21 ± 0.05 ng/mL), flutamide treatment resulted in a 4-fold increase in the hormone concentration (7.1 ± 4.04 ng/mL) when compared with control animals (1.28 ± 0.83 ng/mL). The differences in serum concentrations of testosterone were statistically significant both when the test groups were compared to controls ($p < 0.05$) and when compared between test groups ($p < 0.001$). Serum concentration of estradiol, however, was observed at similar levels in all groups (Figure 1).

Three-dimensional analysis of bone volume fraction demonstrated that ligature successfully induced periodontal bone loss. Linear analysis in the mesial surface of the first molars showed that the distance between CEJ and alveolar bone was increased for both orchietomized and flutamide-treated animals, when compared to ligated controls (Figure 2).

Descriptive histologic analysis showed that ligature placement led to loss of epithelial attachment, apical migration of the alveolar bone crest, and modification of the connective tissue structure, including increased inflammatory infiltrate. This finding was also supported by stereometric analysis. The number of osteoclasts was observed to increase in this inflammatory model. Among the ligated animals, it was possible to observe new epithelial structure on the top of the connective tissue, but only in orchietomized and flutamide-treated animals. The number of osteoclasts in the orchietomy group was significantly higher than in the sham or flutamide-treated groups, while the inflammatory cell infiltrate was similar among all groups (Figure 3).

Orchietomized animals showed significantly higher gingival IL-1 β levels when compared to ligated controls and flutamide-treated animals. Flutamide treatment was observed to promote decreased gingival levels of IL-6 when compared to ligated controls and orchietomized animals. There was no difference among the ligated groups in serum levels of IL-1 β or IL-6 (Figure 4).

In vitro, the number of osteoclasts was significantly diminished in the presence of 10nM ($p < 0.01$) and 100nM ($p < 0.001$) testosterone. The lowest tested dose of testosterone (1nM)

showed no difference when compared to the control. Flutamide treatment antagonized the impact of 100nM dose of testosterone (Figure 5).

The supernatant levels of IL-1 β , and IL-10 were not modulated by the different doses of testosterone. IL-6 and RANTES were dose-dependently down-regulated, although no statistical significant difference could be observed. Concentration of TNF was also down-regulated, with the 100 nM dose promoting a statistically significant difference when compared to control and the 1 nM dose. Flutamide addition to 100 nM testosterone-treated groups partially reversed the impact of testosterone; this can be seen through the similarity between the flutamide treated group and the control group (Figure 6).

4. Discussion

Modulating host response is one of the many functions sex hormones perform in the body. Many *in vitro* studies assess the role of androgens on different periodontal soft tissue cells: in gingival connective tissue testosterone inhibits prostaglandin formation [26], in inflamed gingiva androgens increase 5-alpha-reductase activity [27]; and in fibroblasts, increasing concentrations of androgens inhibit IL-6 production [28, 29] and increase cell proliferation [30]. Previous studies have demonstrated that low and high testosterone levels in rats increase both gingival and connective tissue areas [13], increase periodontal bone loss; and impact the release of RANKL and osteoprotegerin by murine osteoblasts [14].

In this study, orchietomy was observed to successfully decrease serum testosterone levels in adult rats. Flutamide treatment, however, was observed to significantly increase serum levels of testosterone. This may indicate a feedback mechanism, due to the absence of androgen receptor activation. Flutamide was used to isolate the impact of low testosterone levels (which could also imply modifications on interconversion to other sex hormones, including estrogen) from effects related to androgen receptor activation. Estrogen is a key regulator of bone turnover, and its impact in men has been documented [15]. However, contrary to what may be expected, estradiol levels were not significantly reduced after orchietomy. This finding is in accordance with a previously published study [31]. Thusly, the serum estradiol levels cannot account for the differences in cytokine release and osteoclast count observed between the orchietomy and flutamide groups, and should be further investigated. The understanding of sex hormones' actions as deriving solely from their serum levels may then be misleading: intraskeletal reservoirs of sex hormones are only partially influenced by the circulation, and orchietomy can maintain or increase the concentration of testosterone and DHT locally [31].

This study suggests a localized impact of testosterone suppression on periodontal bone loss, as inferred from the linear analysis of the teeth mesial surfaces. Although the two approaches seem to act through different pathways, the similarity of bone loss in both orchietomized and flutamide-treated groups suggests that androgen receptor activation is necessary for the beneficial impact of testosterone on bone. As has been previously shown, linear analysis in the mesial area is more sensitive than volumetric evaluation in the detection of differences in bone loss under this experimental model [14]. This greater

sensitivity can be understood as mainly deriving from the extension of resorption promoted by 14 days of ligature and that the knot is placed in this region.

The osteoclast number was also shown to significantly increase in orchietomized animals in the *in vivo* model; accordingly, testosterone decreases osteoclast formation from RANKL-stimulated RAW264.7 cells *in vitro* in a dose-dependent manner. A similar study has shown that this decrease occurs through the selective regulation of *c-Jun*, a key transcription factor that is essential for osteoclast formation [32]. Other studies have shown that androgens decrease osteoclastogenesis from stimulated mouse unfractionated bone cell culture [25] and human peripheral blood CD14⁺ monocytes [10], as well as decreasing bone resorption by primary avian cell culture, primary murine cell culture, and human osteoclasts from giant cell tumors [33]. Flutamide was shown to reverse the significant androgen-derived decrease in osteoclast number (at 100nM dose). This finding is in accordance with previous studies showing an antagonizing effect for both osteoclast number [25] and activity [10, 33].

Several studies have used knockout animal models to understand the role played by testosterone and androgen receptor activation in bone metabolism [34–37]. Androgen receptor knockout mice present lower trabecular and cortical bone volume due to high bone turnover with increased bone resorption, related to increased RANKL expression in primary osteoblasts obtained from these animals [36, 37]. A recent study using this particular animal model suggests that, while androgens act on cancellous bone through androgen receptor signaling in osteoblasts, cortical bone is unaffected by direct androgen receptor signaling [38]. Hence, stromal cells seem to be an androgen receptor target for this model [39]. However, it is important to note that primary cultures deriving from knockout mice seem to yield different responses when compared to wild type cell cultures [10, 25].

In this study, orchietomy was observed to upregulate IL-1 β production at the site of inflammation, but not systematically, and orchietomy displayed no effect on IL-6 either locally or at the system level. In flutamide-treated animals, however, IL-6 was observed to significantly decrease. *In vitro*, IL-6 release was dose-dependently downregulated, although no statistical significant difference was observed. IL-1 β , on the other hand, was not affected by testosterone treatment. Both IL-1 β and IL-6 are important cytokines related to bone loss through their regulation of osteoclast number and activity [20]. Additionally, concentrations of TNF were significantly downregulated in the 100 nM testosterone dose, showing a statistically significant difference in TNF concentrations compared to control and the 1 nM dose. A cause and effect relationship between TNF and bone loss in several chronic inflammatory diseases has been previously demonstrated [40].

Some studies show controversial results involving IL-6, depending on the target cells: IL-6 was downregulated in macrophages from androgen receptor knockout mice and upregulated with increasing testosterone, or in aromatase knockout mice in the presence of inflammation [34, 35, 41, 42]. Meanwhile, testosterone and DHT have been shown to inhibit IL-6 production by bone marrow-derived stromal cells *in vitro*; and orchietomy could not alter osteoclast number or activity in IL-6 deficient mice [43]. A possible explanation for the lower IL-6 levels observed in flutamide-treated animals is that, besides its bone resorption-related effects, it also displays antiinflammatory and immunosuppressive effects. These

particular characteristics contribute to the resolution of acute and chronic inflammation [44]. IL-6 induces synthesis of TIMPs, suppresses LPS-induced TNF and IL-1 β production and stimulates release of the anti-inflammatory cytokines IL-10 and IL-1ra [45, 46]. Thusly, a decrease in IL-6 may amplify the inflammatory response. Our findings are not in accordance with experiments in male mice and murine bone marrow-derived cells, in which testosterone was observed to decrease the expression of IL-6 gene, which itself was observed to mediate the upregulation of osteoclastogenesis and bone loss [43].

Similarly to IL-6, TNF was inhibited in macrophages from androgen receptor-knockout mice [34]; and increased in the serum of LPS-induced and metabolic syndrome aromatase deficient mice [35, 42]. TNF was also shown to be reduced in healing wounds of orchiectomized and flutamide-treated animals, suggesting that androgen receptors are critical for wound healing suppression [47, 48]. Interestingly, these results support the hypothesis that the histological finding of epithelial formation on top of the connective tissue could represent an earlier initiation of the repair process for the orchiectomized and flutamide-treated animals [13].

Neutrophil counts were significantly decreased in androgen receptor knockout mice, but orchiectomy showed only a moderate impact on neutrophils in blood and bone marrow [49]. Since neutrophils are important sources of inflammatory cytokines, this may represent an important difference between the two research models [47, 50]. Thus, any labeling of testosterone as a 'pro-inflammatory' or 'anti-inflammatory' hormone is overly simplistic; testosterone presents many different actions on different cell types and tissues. Therefore, our results using this inflammatory model of periodontal disease – which involves a close intertalk between periodontal tissue cells (namely epithelial cells, connective tissue cells and bone cells, as well as those cells from the inflammatory infiltrate) - should be understood in the light of these peculiarities and not directly compared with studies performed using different techniques.

It is also important to note that the action of sex hormones, and the cellular expression of hormone receptors, may vary according to the type of osseous tissue (cortical or trabecular bone) and the species studied. Androgen receptors have been identified in osteoclasts of avian [33], mouse [51] and rat origin [52], but conflicting results have been found for human osteoclasts [10, 53, 54]. There is considerable evidence that estrogen receptor activation plays a very important role on bone metabolism in male humans, but these findings are often not corroborated by animal studies [17]. In this study, we have compared the inhibition of androgen receptors with testosterone deprivation through orchiectomy, and not with the inhibition of estrogen receptors. Our results should thusly be interpreted carefully with consideration of the utilized experimental model and species.

Testosterone-mediated action on inflammatory cytokines and regulation of bone loss seems to be only partially related with the androgen receptor. A possible explanation may be that both testosterone and the androgen receptor antagonist were simultaneously added to the cell culture. Another possibility is that the aromatase enzyme - which converts testosterone into estradiol - is expressed in osteoclast and osteoclast precursors cell culture, and therefore may be implicated in these findings [55].

When taken together, our results indicate that testosterone modulates microbial-induced inflammatory alveolar bone loss, interfering in the progression of the disease. This action is mediated, at least in part, via the androgen receptor. Regulation of osteoclastogenesis through androgen receptor activation is a mechanism involved in this process. Future studies should be performed to evaluate if subphysiologic testosterone levels, and endogenous variations in testosterone levels, could contribute to bone-related chronic inflammatory diseases.

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Abbreviations

GnRH	Gonadotropin-releasing hormone
sIL6r	Soluble interleukin-6 receptor
IL-6	Interleukin-6
IL-1β	Interleukin-1 β
TNF	Tumor necrosis factor
MIP	Macrophage inflammatory protein
DHT	Dihydrotestosterone
GPR30	G-protein coupled receptor-30
b.w.	body weight
ELISA	Enzyme-linked immunosorbent assays
RANKL	Receptor Activator of nuclear factor kappa-B ligand
microCT	Micro-computed tomography
ROI	region of interest
BV/TV	Bone volume fraction
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
RANTES	Regulated on activation normal T cells expressed and secreted
CEJ	Cement-enamel junction
TRAP	Tartrate-resistant acid phosphatase
TIMPs	Tissue inhibitor of metalloproteinases
LPS	Lipopolysaccharyde

IL-1ra Interleukin-1 receptor antagonist

REFERENCES

1. Mawhinney M, Mariotti A. Physiology, pathology and pharmacology of the male reproductive system. *Periodontol* 2000. 2013; 61(1):232–251. [PubMed: 23240952]
2. Zhang G, et al. Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and GnRH. *Nature*. 2013; 497(7448):211–216. [PubMed: 23636330]
3. Bhasin S, et al. Testosterone therapy in men with androgen deficiency syndromes: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2010; 95(6):2536–2559. [PubMed: 20525905]
4. Araujo AB, et al. Prevalence of symptomatic androgen deficiency in men. *J Clin Endocrinol Metab*. 2007; 92(11):4241–4247. [PubMed: 17698901]
5. Tajar A, et al. Characteristics of androgen deficiency in late-onset hypogonadism: results from the European Male Aging Study (EMAS). *J Clin Endocrinol Metab*. 2012; 97(5):1508–1516. [PubMed: 22419720]
6. 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):345–347. [PubMed: 16263825]
7. 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):1550–1554. [PubMed: 11932280]
8. Malkin CJ, et al. The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men. *J Clin Endocrinol Metab*. 2004; 89(7):3313–3318. [PubMed: 15240608]
9. 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):e61466. [PubMed: 23637840]
10. Michael H, et al. Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption. *J Bone Miner Res*. 2005; 20(12):2224–2232. [PubMed: 16294275]
11. Gunness M, Orwoll E. Early induction of alterations in cancellous and cortical bone histology after orchietomy in mature rats. *J Bone Miner Res*. 1995; 10(11):1735–1744. [PubMed: 8592951]
12. Wiren KM, et al. Androgen prevents hypogonadal bone loss via inhibition of resorption mediated by mature osteoblasts/osteocytes. *Bone*. 2012; 51(5):835–846. [PubMed: 22910580]
13. Steffens JP, 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):1432–1439. [PubMed: 22309177]
14. Steffens JP, et al. Testosterone regulates bone response to inflammation. *Horm Metab Res*. 2014; 46(3):193–200. [PubMed: 24526374]
15. Vandenput L, Ohlsson C. Sex steroid metabolism in the regulation of bone health in men. *J Steroid Biochem Mol Biol*. 2010; 121(3–5):582–588. [PubMed: 20362055]
16. Ohlsson C, Vandenput L. The role of estrogens for male bone health. *Eur J Endocrinol*. 2009; 160(6):883–889. [PubMed: 19304870]
17. Khosla S. Commentary: New Insights Into Androgen and Estrogen Receptor Regulation of the Male Skeleton. *J Bone Miner Res*. 2015
18. Scopacasa F, et al. The relation between bone density, free androgen index, and estradiol in men 60 to 70 years old. *Bone*. 2000; 27(1):145–149. [PubMed: 10865222]
19. Ucer S, et al. The effects of androgens on murine cortical bone do not require AR or ERalpha signaling in osteoblasts and osteoclasts. *J Bone Miner Res*. 2015
20. Graves DT, Oates T, Garlet GP. Review of osteoimmunology and the host response in endodontic and periodontal lesions. *J Oral Microbiol*. 2011; 3

21. Nanci A, Bosshardt DD. Structure of periodontal tissues in health and disease. *Periodontol* 2000. 2006; 40:11–28. [PubMed: 16398683]
22. 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):422–427. [PubMed: 20211643]
23. Guimaraes MR, et al. Potent anti-inflammatory effects of systemically administered curcumin modulate periodontal disease in vivo. *J Periodontal Res*. 2011; 46(2):269–279. [PubMed: 21306385]
24. Coimbra LS, et al. Clopidogrel enhances periodontal repair in rats through decreased inflammation. *J Clin Periodontol*. 2014; 41(3):295–302. [PubMed: 24433307]
25. Chen Q, et al. Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor. *FEBS Lett*. 2001; 491(1–2):91–93. [PubMed: 11226426]
26. 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(1):25–34. [PubMed: 6813875]
27. Vittek J, et al. Concentration of circulating hormones and metabolism of androgens by human gingiva. *J Periodontol*. 1979; 50(5):254–264. [PubMed: 287781]
28. Parkar M, et al. IL-6 expression by oral fibroblasts is regulated by androgen. *Cytokine*. 1998; 10(8):613–619. [PubMed: 9722934]
29. Gornstein RA, et al. Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *J Periodontol*. 1999; 70(6):604–609. [PubMed: 10397515]
30. Coletta RD, et al. Testosterone stimulates proliferation and inhibits interleukin-6 production of normal and hereditary gingival fibromatosis fibroblasts. *Oral Microbiol Immunol*. 2002; 17(3):186–192. [PubMed: 12030972]
31. Yarrow JF, 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):E841–E847. [PubMed: 20739509]
32. Huber DM, et al. Androgens suppress osteoclast formation induced by RANKL and macrophage-colony stimulating factor. *Endocrinology*. 2001; 142(9):3800–3808. [PubMed: 11517156]
33. Pederson L, et al. Androgens regulate bone resorption activity of isolated osteoclasts in vitro. *Proc Natl Acad Sci U S A*. 1999; 96(2):505–510. [PubMed: 9892663]
34. Huang CK, et al. New therapy via targeting androgen receptor in monocytes/macrophages to battle atherosclerosis. *Hypertension*. 2014; 63(6):1345–1353. [PubMed: 24688120]
35. Yang YH, et al. Endogenous estrogen regulation of inflammatory arthritis and cytokine expression in male mice, predominantly via estrogen receptor alpha. *Arthritis Rheum*. 2010; 62(4):1017–1025. [PubMed: 20131258]
36. Yeh S, et al. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci U S A*. 2002; 99(21):13498–13503. [PubMed: 12370412]
37. Kawano H, et al. Suppressive function of androgen receptor in bone resorption. *Proc Natl Acad Sci U S A*. 2003; 100(16):9416–9421. [PubMed: 12872002]
38. Ucer S, et al. The Effects of Androgens on Murine Cortical Bone Do Not Require AR or ERalpha Signaling in Osteoblasts and Osteoclasts. *J Bone Miner Res*. 2015; 30(7):1138–1149. [PubMed: 25704845]
39. Sinnesael M, et al. The androgen receptor has no direct antiresorptive actions in mouse osteoclasts. *Mol Cell Endocrinol*. 2015; 411:198–206. [PubMed: 25958043]
40. Boyce BF, et al. TNF-alpha and pathologic bone resorption. *Keio J Med*. 2005; 54(3):127–131. [PubMed: 16237274]
41. Gilliver SC, et al. Androgens modulate the inflammatory response during acute wound healing. *J Cell Sci*. 2006; 119(Pt 4):722–732. [PubMed: 16449322]
42. Scott NJ, et al. Generation and characterization of a mouse model of the metabolic syndrome: apolipoprotein E and aromatase double knockout mice. *Am J Physiol Endocrinol Metab*. 2012; 302(5):E576–E584. [PubMed: 22185842]

43. 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):2886–2895. [PubMed: 7769130]
44. Tilg H, Dinarello CA, Mier JW. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol Today*. 1997; 18(9):428–432. [PubMed: 9293158]
45. Barton BE. IL-6: insights into novel biological activities. *Clin Immunol Immunopathol*. 1997; 85(1):16–20. [PubMed: 9325064]
46. Lotz M, Guerne PA. Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). *J Biol Chem*. 1991; 266(4):2017–2020. [PubMed: 1846608]
47. Lai JJ, et al. Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. *Am J Pathol*. 2012; 181(5):1504–1512. [PubMed: 22959669]
48. Ashcroft GS, Mills SJ. Androgen receptor-mediated inhibition of cutaneous wound healing. *J Clin Invest*. 2002; 110(5):615–624. [PubMed: 12208862]
49. Chuang KH, et al. Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *J Exp Med*. 2009; 206(5):1181–1199. [PubMed: 19414555]
50. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol*. 2005; 15(11):599–607. [PubMed: 16202600]
51. Mizuno Y, et al. Immunocytochemical identification of androgen receptor in mouse osteoclast-like multinucleated cells. *Calcif Tissue Int*. 1994; 54(4):325–326. [PubMed: 8062148]
52. Van Der Eerden BC, et al. Sex steroid metabolism in the tibial growth plate of the rat. *Endocrinology*. 2002; 143(10):4048–4055. [PubMed: 12239116]
53. Abu EO, et al. The localization of androgen receptors in human bone. *J Clin Endocrinol Metab*. 1997; 82(10):3493–3497. [PubMed: 9329391]
54. Noble B, et al. Androgen receptors in bone-forming tissue. *Horm Res*. 1999; 51(1):31–36. [PubMed: 10095167]
55. Jakob F, et al. Local estradiol metabolism in osteoblast- and osteoclast-like cells. *J Steroid Biochem Mol Biol*. 1997; 61(3–6):167–174. [PubMed: 9365187]

Highlights

- Testosterone deprivation through orchiectomy increases bone resorption in an inflammatory environment using a model of microbial-mediated experimental alveolar bone loss;
- Pharmacological inhibition of androgen receptors promotes similar clinical effects;
- Cytokine release is differently regulated by orchiectomy and androgen receptors inhibition;
- *In vitro* osteoclastogenesis and cytokines expression can be modified by increasing testosterone doses;
- The modulation of cytokine release *in vitro* can be only partially attributed to androgen receptors.

Figure 1. Serum concentration of (A) testosterone and (B) estradiol in each experimental group. * $p < 0.05$ when compared to SHAM, *** $p < 0.001$

Figure 2.

Estimation of bone loss using micro-computed tomography analysis: A) representative images from each experimental group; B) bone volume fraction (BV/TV) in the furcation area (inter-root region) using an interpolated region of interest; and C) linear measurement between cement-enamel junction (CEJ) and alveolar bone (AB) in the mesial surface of the tooth. * $p < 0.05$ when compared to SHAM+L, ** $p < 0.01$ when compared to SHAM+L, #### $p < 0.0001$ when compared to SHAM

Figure 3.

Histologic analysis of the periodontal tissues: A) representative histologic images stained with hematoxylin and eosin; B) inflammatory cell infiltrate, and C) osteoclast number in the connective tissue in each experimental group.

OE – oral epithelium; JE – junctional epithelium; CEJ – cement-enamel junction; CT – connective tissue; T – tooth; AB – alveolar bone.

* $p < 0.05$ when compared to SHAM+L and FLU+L, *** $p < 0.001$ when compared to all other groups, **** $p < 0.0001$ when compared to all other groups.

A **B** **Figure 4.**

Expression of (A) IL-1 β and (B) IL-6 in the serum and mucogingival tissue in each experimental group. # p<0.05 when compared to SHAM, ## p<0.01 when compared to SHAM, * p<0.05 when compared to SHAM+L, unless otherwise connected, ** p<0.01 when compared to SHAM+L, unless otherwise connected

Figure 5.

Number of osteoclasts identified as TRAP-positive cells containing 3 or more nuclei derived from RAW264.7 precursors treated with RANKL in the presence of increasing doses of testosterone (1, 10 and 100 nM) and with flutamide (1 μ M). ** $p < 0.01$ when compared to Control, *** $p < 0.001$ when compared to Control

Figure 6.

Expression of IL-1 β , IL-6, IL-10, TNF and RANTES in the supernatant of osteoclasts treated with RANKL in the presence of increasing doses of testosterone (1, 10 and 100 nM) and with flutamide (1 μ M). ** $p < 0.01$ when compared to Control, unless otherwise connected