Testosterone Increases Fibroblast Proliferation *in vitro* Through Androgen and Estrogen Receptor Activation

Luis Carlos Leal Santana[1], Luis Carlos Spolidorio[2,] Jonleno Coutinho Paiva Pitombo[1], Fernanda Gonçalves Basso[2], Gabriel Guidio Guarenghi[3], Rodolfo Coelho Prates[4], João Paulo Steffens[3]

[1]Department of Diagnosis and Surgery, Universidade Estadual Paulista (UNESP), Faculdade de Odontologia, Araraquara, SP, Brazil. [2]Department of Physiology and Pathology, Universidade Estadual Paulista (UNESP), Faculdade de Odontologia, Araraquara, SP, Brazil. [3]Department of Stomatology, Universidade Federal do Paraná - UFPR, Curitiba, PR, Brazil. [4]Postgraduate Program in Health and Environment, Universidade da Região de Joinville – UNIVILLE, Joinville, SC, Brazil.

Abstract

Background: Skin-related disorders and periodontitis are distinct diseases that have been associated with altered levels of testosterone. Understanding the mechanisms through which testosterone mediates gingival enlargement in animals and humans is crucial for preventing or treating this condition. In this study, we investigated the impact of different doses of androgens, the role of aromatase inhibition, and the effects of testosterone association with sex hormone receptor antagonists or aromatase inhibitors on human gingival fibroblast proliferation and migration *in vitro*.

Methods: Fibroblasts were cultivated in Dulbecco's Modified Eagle's Medium in a humidified atmosphere and treated with different doses of testosterone or dihydrotestosterone, and testosterone in association with: aromatase inhibitor - anastrozole; antagonist of androgen receptors - flutamide; and antagonist of estrogen receptors - fulvestrant.

Results: Low (1nM) and high (1μM) doses of testosterone significantly increased cell migration, but the higher dose did not alter cell proliferation. Those effects were related to both androgen and estrogen receptors activation, as evidenced by the dihydrotestosterone and drug interaction groups.

Conclusions: Testosterone association with sex hormone receptor antagonists flutamide and fulvestrant suggests that not only androgen receptors, but also estrogen receptors, may take part in fibroblast cell proliferation and migration *in vitro*.

Keywords: Testosterone; Dihydrotestosterone; Androgen Receptor Antagonists; Fibroblasts.

Introduction

Steroid sex hormones are cholesterol-derived molecules known to influence craniofacial growth and development (Wang *et al.*, 2015), tooth dimension (Ribeiro *et al.*, 2013) and also play an essential role in bone metabolism (Shiau *et al.*, 2014). Testosterone is one of the most potent androgenic hormones and can be enzymatically converted by 5α-reductase to produce its most powerful metabolite, dihydrotestosterone. Dihydrotestosterone has a high affinity with the androgen receptor (Mawhinney and Mariotti, 2013). Likewise, some target cells such as skin fibroblasts (Harada, 1992) are prone to aromatize testosterone, but not dihydrotestosterone, into estradiol through aromatase activity (Mariotti and Mawhinney, 2013). In both men and women, testosterone aromatization is one of main extragonadal sources of estradiol (Mariotti and Mawhinney, 2013) and therefore, it is

Correspondence to: Prof. João Paulo Steffens. Rua Pref. Lothário Meissner, 632. Curitiba – PR – Brazil. Telephone: +55 41 3360-4032. E-mail joao.steffens@ufpr.br

reasonable to assume that many of the effects of testosterone may occur through the activation of estrogen receptors instead of androgen receptor (Callewaert *et al*., 2010).

Several diseases such as diabetes and metabolic syndrome (Svartberg, 2007), cardiovascular and renal diseases (Rogers *et al*., 2007), as well as skin disorders (i.e acne vulgaris, alopecia, and hirsutism) (Slominski *et al*., 2013) have been associated with altered levels of sex hormones. Testosterone supplementation has been shown to prevent loss of tibial mass and improve femoral stiffness in male and female rats (Yarrow *et al*., 2008b), and also retard mucosal wound healing processes (Engeland *et al*., 2008), which suggests its influence on both hard and soft tissue cells. More specifically, androgens and androgen receptors regulate wound healing processes through modulating inflammatory cytokines such as transforming growth factor beta (TGF-β), interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) (Gilliver *et al*., 2006).

Estrogen receptors and one sex hormone precursor, dehydroepiandrosterone, have been shown to dampen pro-inflammatory cytokines (Mills *et al*., 2005), whereby cutaneous wound healing is facilitated by estrogen receptor activation (Romana-Souza *et al*., 2014). Moreover, a recent study reported the stimulatory effects of estradiol, dehydroepiandrosterone, and dehydroepiandrosterone-sulfate on dermal fibroblasts and epithelial cells migration (Pomari *et al*., 2015).

Our previous work showed that both sub and supraphysiological levels of testosterone were related to gingival enlargement in male rats (Steffens *et al*., 2012). These results suggested a potential role for androgens and their receptors in modulating biological responses of connective tissue cells of the periodontium. In humans, anabolic androgenic steroid abuse in athletes has also been associated with gingival enlargement (Ozcelik *et al*., 2006). More recently, we demonstrated that pharmacological blockage of androgen or estrogen receptors affect gingival tissue expression of several cytokines and growth factors during periodontal disease progression (Steffens *et al*., 2019) and repair (Steffens *et al*., 2018), suggesting an interplay between androgens and both sex hormone receptors. Therefore, understanding the mechanisms through which testosterone mediates gingival enlargement are crucial for preventing or treating the clinical manifestation of this condition in men or women under androgen treatment. Thus, the purpose of the present study was to investigate the impact of different doses of androgens, the role of aromatase inhibition, and the effects of testosterone association with sex hormone receptor antagonists or aromatase inhibitors on human gingival fibroblast proliferation and migration *in vitro*.

Materials and Methods

Cell Culture

Primary human gingival fibroblasts were cultivated according to a protocol approved by the Human Ethics Committee of São Paulo State University (UNESP) - School of Dentistry at Araraquara, São Paulo, Brazil (CAAE 48882715.4.0000.5416). An established protocol was used for gingival tissue collection (Basso *et al*., 2016a). Briefly, after specimen collection, the samples were immersed in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA). Subsequently, fibroblasts were isolated by enzymatic digestion using 3 mg/ml of type I collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA), as described elsewhere (Basso *et al*., 2016a; Basso *et al*., 2016b). Thereafter, fibroblasts were cultured in 75 cm^2 flasks with DMEM (with phenol red, high glucose and L-glutamine) supplemented with 10% of fetal bovine serum (FBS; Gibco) and antibiotics (10.000 U/ml of penicillin and streptomycin) at 37ºC, 95% relative humidity and 5% CO_2 (Thermo Scientific REVCO, Waltham, MA, USA). The medium was replaced every 2-3 days with supplemented DMEM until cells reached 80% to 90% of confluence (approximately 5 days). Cells were trypsinized using 5 ml of 0.25% trypsin/EDTA (Gibco) for 10 min, and fibroblasts between 4th and 8th passages were used. Groups used in fibroblasts according to its pharmacological concentration were: control (Ethanol ≤0.1%), testosterone (1 nM; 10 nM; 100 nM; 1 μM; Durateston, MSD, Campinas, SP, Brazil), dihydrotestosterone (1 nM; 10 nM; 100 nM; 1 μM; Sigma-Aldrich, St. Lous, MO), anastrozole (ANA; 10 nM; Sigma-Aldrich, St. Louis, MO), flutamide (FLU; 10 nM; Sigma-Aldrich, St. Louis, MO, USA), fulvestrant (FUL; 10 nM; Sigma-Aldrich, St. Louis, MO), testosterone+anastrozole (1 nM + 10 nM), testosterone+flutamide (1 nM + 10 nM) and testosterone+fulvestrant (1 nM + 10 nM).

Cell Migration

Cell migration was assessed based on *in vitro* wound healing protocols (Lackler *et al*., 2000; Mumford *et al*., 2001). Fibroblasts cells were seeded into 24-well plates (8×10^4 cell/well) and grown in a humidified atmosphere for 24h. After 24h of serum starvation (Sobral *et al*., 2012), an *in vitro* scratch wound was created by scraping the cell monolayer with a sterile 5 ml pipette tip (Basso *et al*., 2012), which is sufficient to visualize the gap in the cell monolayers. Subsequently, the cells were washed twice with PBS and the pharmacological treatments were performed for 72h to allow migration into the scratch (Basso *et al*., 2012; Kirker *et al*., 2012). Thereafter, fibroblasts cells were fixed with 70% ethanol and stained with 0.1% crystal violet for 15 min. The scratch wound

images were taken on a digital camera (Leica DFC295; Leica Microsystems, Germany) coupled to a stereomicroscope (Leica MZ6) with a 2.5x magnification. The margins of cell monolayers were outlined using the polygonal tool of Photoshop CC 2015 (Adobe Systems Inc., San Jose, CA, USA) and the scratch wound area was measured at three random points of each well using ImageJ (National Institutes of Health, United States). The areas were then calculated to represent fold-change to the respective control group.

Cell Proliferation

Fibroblast proliferation was determined using Alamar-Blue (Gibco) assay (Bhardwaj and Kundu, 2012). Initially, fibroblasts cells were cultured in 24-well plates (3×10^4 cells/well) in humidified atmosphere (37°C and 5% CO_2) for 24h. After replacing the culture medium with serum-free DMEM, the pharmacological treatments were performed for 72h. Afterwards, cells were incubated with 10% AlamarBlue at 37°C and 5% CO_2, for 4h. The AlamarBlue supernatant was transferred (100 µl) into 96-well plates and samples were analyzed by a fluorimeter (Synergy H1, BioTek, Winooski, VT, USA) at 570/600 nm.

Statistical Analysis

Software (GraphPad Prism 5.0, La Jolla, CA, USA) was used to perform the statistical analysis of cell proliferation, adhesion, and cell migration. The normal distribution was tested using the Kolmogorov-Smirnov normality test. If normality criteria were met, significant differences among the different doses of testosterone or dihydrotestosterone were verified by one-way analysis of variance (One-way ANOVA) and Tukey's post-hoc test. The non-parametric Kruskal-Wallis and Dunn's multiple comparison tests were performed otherwise. For the analysis of interactions between testosterone and flutamide, fulvestrant or anastrozole, 2-way ANOVA with Tukey correction for multiple comparisons was performed. Statistically significant differences were considered when $p<0.05$.

Results

Response to androgen treatment with/without antagonists of sex hormone receptors

Photomicrographs illustrated a large cell-free area in the control group, whereas a reduced cell-free area can be observed in some treated groups (Fig. 1A). All tested dihydrotestosteronedoses statistically increased fibroblast migration ($p<0.01$), as evidenced by the lower scratch area. On the other hand, only the lower (1nM) and higher (1µM) doses of testosterone had the same significant effect ($p<0.01$). Testosterone+flutamide and testosterone+fulvestrant increased by 45% and 57% fibroblasts cell repopulation, respectively ($p<0.0001$), which was significantly greater than cell migration promoted by testosterone alone ($p<0.01$). Those observations seem to be derived from the impact of flutamide or fulvestrant in the medium, which significantly increased cell migration ($p<0.01$; Fig. 1B).

Fluorimeter analysis showed that, except for testosterone 1µM, all treatments statistically increased fibroblast cell proliferation ($p<0.05$; Fig. 2).

Response to aromatase inhibitor with/without testosterone administration

Aromatase inhibitor anastrozole was used to prevent the aromatization of testosterone to estradiol *in vitro*. Anastrozole and testosterone+anastrozole significantly increased in 30% ($p<0.0001$) and 39% ($p<0.0001$) gingival fibroblasts migration, respectively. Those effects were significantly greater than those observed when testosterone 1nM was used alone ($p<0.05$; Fig. 3).

Fluorimeter analysis showed that anastrozole and testosterone+anastrozole significantly increased fibroblasts proliferation in approximately 19% ($p<0.01$) and 14% ($p<0.05$), respectively (Fig. 4).

Discussion

Sex steroids are often described as major regulators of wound healing (Gilliver *et al.*, 2006; Romana-Souza *et al.*, 2014), bone turnover (Shiau *et al.*, 2014), skin immunity (Slominski *et al.*, 2013), and ultimately may influence periodontal disease progression and repair (Steffens *et al.*, 2015; Steffens *et al.*, 2018; Steffens *et al.*, 2019). Testosterone replacement therapy in late onset hypogonadism has been shown to be beneficial (Bhasin *et al.*, 2018), although this treatment has not been evaluated for clinical or radiographic parameters of periodontitis (Chaves *et al.*, 2019). Anabolic androgenic steroid abuse in athletes, with or without medical prescription, has been found to be associated with gingival enlargement (Ozcelik *et al.*, 2006). Results of previous investigations by the present research team demonstrated that androgens levels significantly increased the gingival tissue mass (i.e., connective tissue area) in a ligature-induced model of periodontitis in rats (Steffens *et al.*, 2012), suggesting that androgens could modulate fibroblast proliferation and activity. More recently, we used androgen receptor blockage to understand the role of androgens in periodontal repair in female rats (Steffens *et al.*, 2018). The results from these studies suggested that both androgen and estrogen receptors can modulate periodontal hard and soft tissues, but they seem to act through different pathways (Steffens *et al.*, 2018). In the presentr study, designed to better understand our previous findings, we investigated the influence of different concentrations of testosterone and dihydrotestosterone (a non-aromatizable

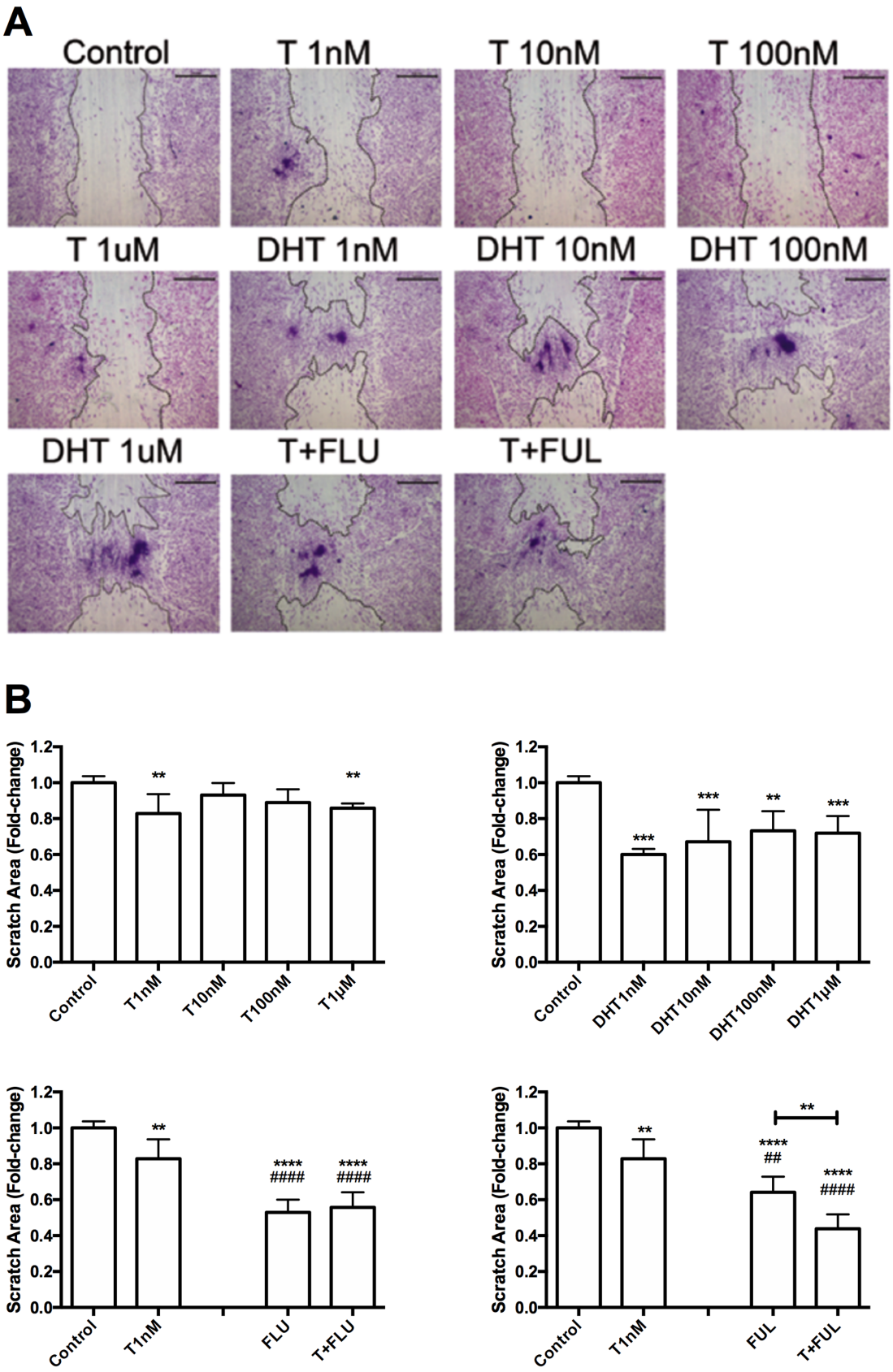

Fig. 1: Cell migration after dihydrotestosterone (DHT) and testosterone (T) association to 10nM flutamide (T+FLU) or fulvestrant (T+FUL) on HGF scratch wound. (A) photomicrographs after staining with 0.1% crystal violet. Dotted lines indicate the edge of the scratch wound after cell exposure to T, DHT, T+FLU or T+FUL, for 72 h (Scale bar: 1.0 mm). (B) Quantification of the cell migration assay (fold-change). Lower scratch areas represent increased cell migration. The results of three separate experiments are presented as mean (SD) fold-change to control group. Control group refers to ethanol ≤1%. n=7/group; **P<0.01 ***P<0.001 ****P<0.0001 compared to control group, unless otherwise connected; ##P<0.01 ####P<0.0001 compared to T1nM.

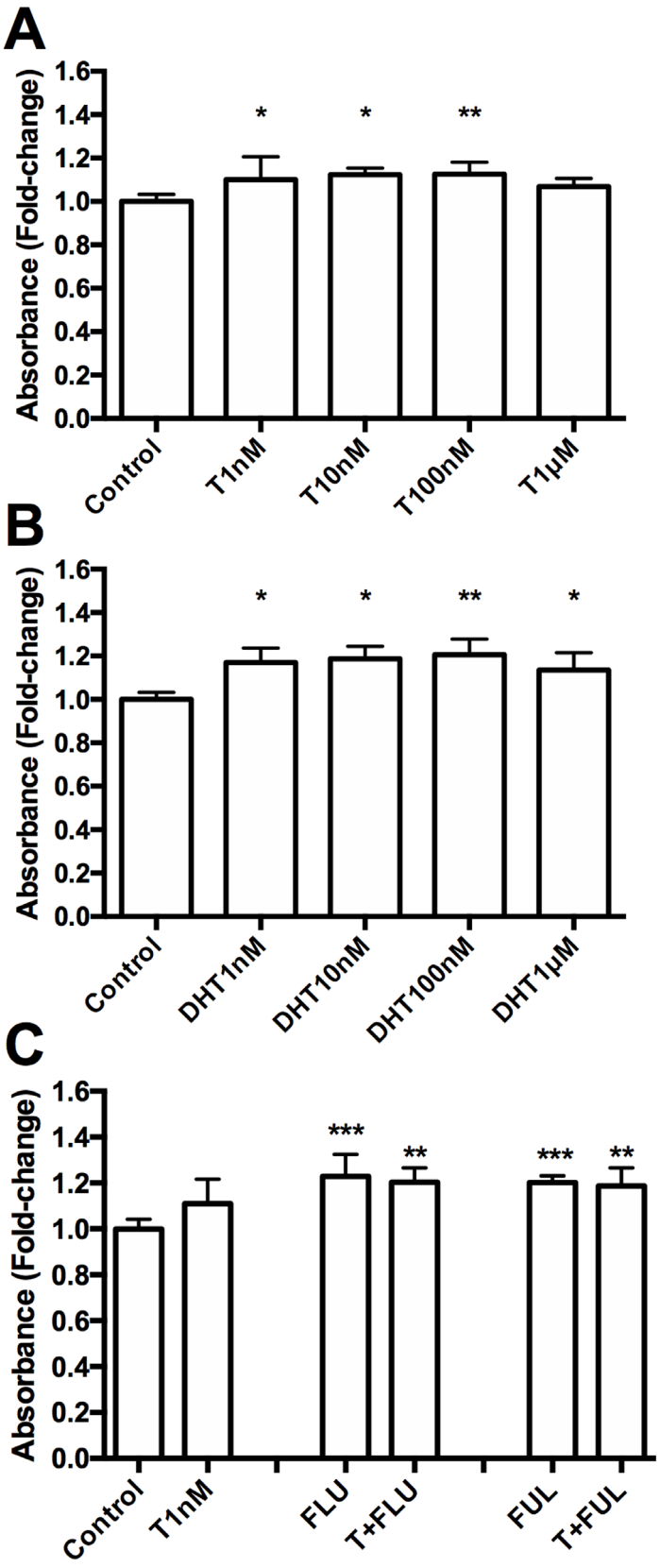

*Fig. 2: The absorbance values at 570 nm indicate cell proliferation following HGF exposure to (A) testosterone (T), (B) DHT, and (C) T association to 10nM flutamide (T+FLU) or fulvestrant (T+FUL), for 72h. The results of three separate experiments are presented as mean (SD) fold-change to control group. Control group refers to ethanol ≤ 1%. n=7/group; *P<0.05 compared to control; **P<0.01 ***P<0.001 compared to control.*

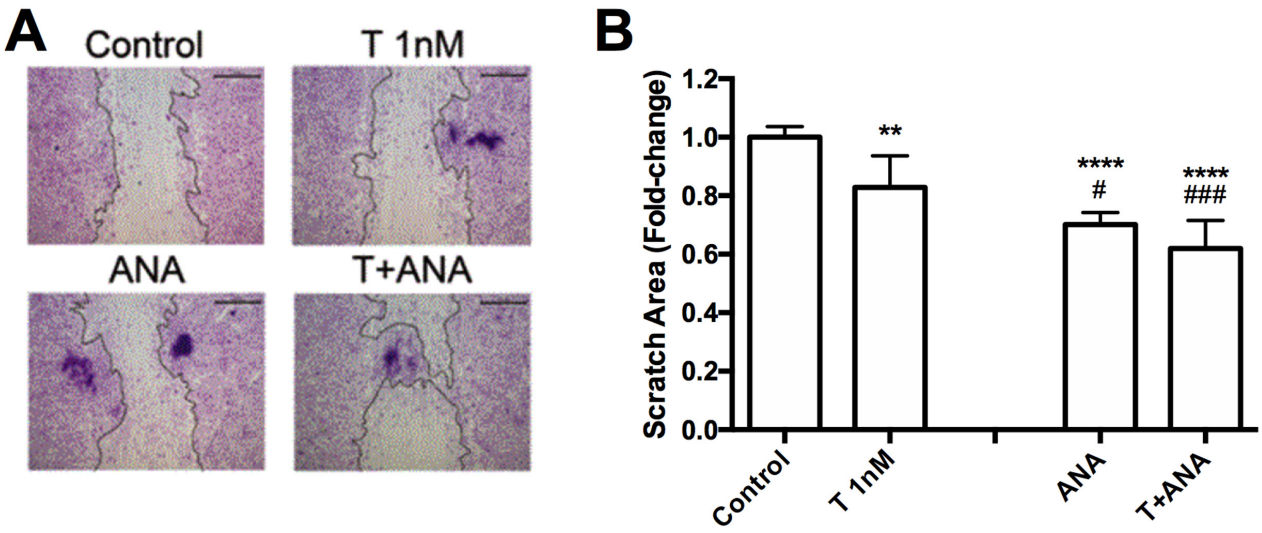

Fig. 3: Cell migration after 10nM anastrozol (ANA) or testosterone association to ANA (T+ANA) on HGF scratch wound (A). Dotted lines in photomicrographs indicate the edge of the scratch wound after cell exposure to testosterone (T), ANA, or T+ANA, for 72 h (Scale bar: 1.0 mm). (B) Quantification of the cell migration assay (fold-change). Lower scratch areas represent increased cell migration. The results of three separate experiments are presented as mean (SD) fold-change to control group. Control group refers to ethanol ≤1%. n=7/group; **P<0.01*P<0.0001 compared to control; #P<0.05 ###P<0.001 compared to T1nM.***

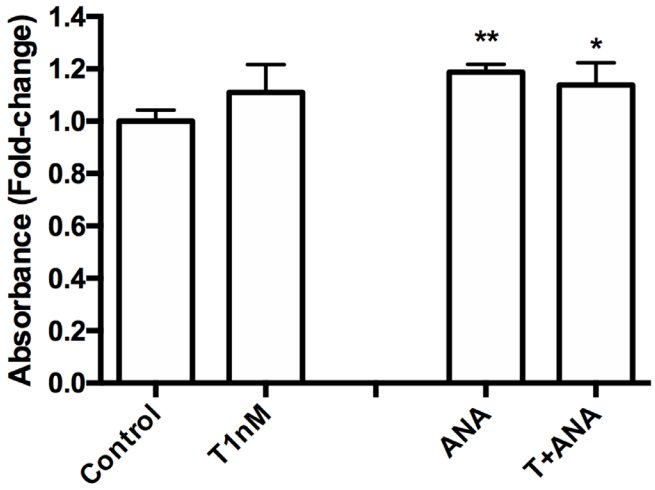

Fig. 4: The absorbance values at 570 nm indicate cell proliferation after HGF exposure to testosterone (T), 10nM anastrozole (ANA), and T association to ANA (T+ANA). The results of three separate experiments are presented as mean (SD) fold-change to control group. Control group refers to ethanol ≤1%. n=7/group; *P<0.05 **P<0.01 compared to control.

androgen) on gingival fibroblast cells proliferation, adhesion and migration *in vitro*. The rationale for the use of different doses of androgens is that lower and higher levels of serum testosterone were found to be related to significant alveolar bone loss in a rat model of ligature-induced periodontal disease (Steffens *et al*., 2014). Since many cells express the encoding gene for aromatase and 5α-reductase enzymes (Simpson, 2003; Aggarwal *et al*., 2010), we also evaluated the impact of testosterone association with androgen receptor antagonist flutamide, estrogen receptor antagonist fulvestrant, or aromatase inhibitor anastrozole. Furthermore, as testosterone and 17β-estradiol effects were abrogated by a 10-fold higher concentration of antagonists of sex hormone receptors (Stamatiou *et al*., 2011; Campelo *et al*., 2012), a 10 nM dose of flutamide, fulvestrant or anastrozole

was used in association with testosterone 1 nM. To our knowledge, there is no previous work that used such a comprehensive methodology to include these hormones and receptor modulators to study hormone interplay in those target cells.

It has been documented that many drugs can modulate androgens metabolism and 5α-reductase activity in fibroblasts *in vitro* (Soory and Tilakaratne, 2003). Yet, androgens effects on human fibroblasts vary across the different studies. Our results confirmed previous findings that reported the proliferative effects of androgens on fibroblasts (Coletta *et al.*, 2002; Almeida *et al.*, 2005). We also showed that testosterone and dihydrotestosterone treatments increased fibroblast migration. These results are in accordance with earlier studies that reported the stimulatory effects of androgens on embryonic (Castoria *et al.*, 2011) and dermal (Pomari *et al.*, 2015) fibroblast migration.

Regarding the role of sex hormones receptors in modulating cellular biological response, previous studies reported that testosterone mediated inhibitory activity on human fibroblasts (Chung *et al.*, 2014) was suppressed by androgen receptors antagonists flutamide and nilutamide, respectively. Besides, tamoxifen, a selective estrogen receptor modulator, inhibited dihydrotestosterone synthesis in response to phenytoin or estradiol in fibroblasts (Soory and Tilakaratne, 2003). The inhibitory effects of fulvestrant were described on mammary fibroblast aromatase activity (Long *et al.*, 1998) and on transfected human mammary epithelial cells (Duss *et al.*, 2007). To the best of our knowledge, this is the first study showing that testosterone association to a 10-fold higher concentration of flutamide, fulvestrant or anastrozole resulted in increased fibroblast cell proliferation and migration. Our results also suggested that those modulators (flutamide, fulvestrant or anastrozole) alone are capable of significantly altering cell migration and proliferation. It is possible that the stimulatory effects of testosterone+flutamide on fibroblasts might have occurred due to testosterone aromatization to estradiol (Simpson, 2003) and activation of estrogen receptors (Stevenson *et al.*, 2009; Pomari *et al.*, 2015), whereas testosterone+fulvestrant and testosterone+anastrozole enhanced androgens effects, which might have occurred through androgen receptors activation (Almeida *et al.*, 2005). This hypothesis is consistent with previous results in which both testosterone and estradiol increased dermal fibroblast and epidermal keratinocyte viability in a dose-dependent manner (Pomari *et al.*, 2015).

Our results should be interpreted understanding that in the present study, fibroblast cell cultures were established with phenol red-medium, which is known to be a weak agonist of estrogen receptor (Berthois *et al.*, 1986; Welshons *et al.*, 1988). In the culture of tumor cells, phenol red significantly stimulates cell proliferation in a dose-dependent manner (Berthois *et al.*, 1986). Therefore, during serum starvation and pharmacological treatments, it is reasonable to assume that phenol red may have interacted with estrogen receptors and influenced the mechanisms involved in the interplay between testosterone and sex hormone receptors. Nevertheless, both control and treatment groups were treated under the same conditions, which allows us to make direct comparisons between groups. Also, it is rather unlikely that phenol red had fully inhibited testosterone conversion into dihydrotestosterone (Mäkelä *et al.*, 1990). Besides, the estrogenic activity of phenol red may vary due to impurities introduced during the manufacturing process of cell culture medium (Welshons *et al.*, 1988), which may affect its final concentration in the medium (Souza Santos *et al.*, 2017).

We have used anastrozole to prevent testosterone conversion to estradiol, therefore isolating aromatase effect *in vitro*. The impact of testosterone+anastrozole was comparable to that of dihydrotestosterone, as expected. However, anastrozole treatment alone also increased the ability of fibroblasts to repopulate *in vitro* wound area, apart from increasing fibroblast cell proliferation. It was previously demonstrated that letrozole, a nonsteroidal aromatase inhibitor, induced papillomatous gingival growth of pregnant baboons (Reynolds *et al.*, 2004). Although a recent study reported the reverse effects of anastrozole on dermal fibroblasts (Pomari *et al.*, 2015), we shall not exclude the hypothesis that anastrozole might have the potential to trigger cell signaling pathways in fibroblast cell migration and proliferation. In fact, the precise nature of anastrozole interactions with aromatase enzyme is not fully understood (Hong *et al.*, 2011) and requires further investigation.

In summary, our results suggest that the gingival enlargement previously observed (Steffens *et al.*, 2012) may be due to androgens ability to modulate fibroblast cells proliferation and migration, which occurs through both activation of androgen and estrogen receptors. Some limitations of our study should be acknowledged and taken into consideration in future research: mechanistic studies should be developed to better understand anastrozole, flutamide and fulvestrant stimulatory effects on cells proliferation and fibroblast migration *in vitro*– for example, should they involve JNK MAP kinase and/or WNT signaling like in drug-induced gingival overgrowth? (Trackman & Kantarci, 2015); pharmacological modulation of cells *in vitro* may be less effective than cell biology techniques of protein expression modulation; and the choice of the 'right dose' for further experiments are highly subjective, since concentrations vary to produce different biological responses. Furthermore, it should be noted that this is an *in vitro* study, using cell culture and, therefore, all the results should be interpreted with the caution of all *in vitro* studies.

We concluded that testosterone and dihydrotestosterone treatments promoted gingival fibroblast proliferation and migration, and that those actions are mediated by both androgen and estrogen receptors.

Acknowledgments

This study was supported by a grant from the São Paulo Research Foundation – FAPESP, São Paulo, SP, Brazil (Grant # 2013/12014-6). We also would like to thank FAPESP for the scholarship provided to JPS (#2013/07833-8) and FGB (#2012/17947-8). LCLS was the recipient of a scholarship from the Amazon State Research Foundation – FAPEAM, Manaus, AM, Brazil (# 19894.483.36583.10102014). LCS is recipient of a fellowship from the National Council for Scientific and Technological Development (CNPq, Brasília, DF, Brazil).The authors would also like to thank the Academic Publishing Advisory Center (Centro de Assessoria de Publicação Acadêmica, *CAPA* - www.capa.ufpr.br) of the Federal University of Paraná for assistance with English language editing and Dr. Alpdogan Kantarci, from The Forsyth Institute, Cambridge, US, for critically reviewing the manuscript.

Conflicts of interest statement*:*

No conflicts of interest.

References

Aggarwal S, Thareja S, Verma A, Bhardwaj T and Kumar M. An overview on 5α-reductase inhibitors. *Steroids* 2010; **75**:109-53.

Almeida JP, Coletta RD, Silva SD, *et al.* Proliferation of fibroblasts cultured from normal gingiva and hereditary gingival fibromatosis is dependent on fatty acid synthase activity. *Journal of Periodontology* 2005; **76**:272-278.

Basso FG, Pansani TN, Turrioni AP, Bagnato VS, Hebling J and de Souza Costa CA. *In vitro* wound healing improvement by low-level laser therapy application in cultured gingival fibroblasts. *International Journal of Dentistry* 2012; **2012**:719452.

Basso FG, Pansini TN, Turrioni AP, Soares DG, De Souza Costa CA and Hebling J. Tumor Necrosis Factor-Alpha And Interleukin (Il)-1beta, Il-6, And Il-8 Impair *In Vitro* Migration And Induce Apoptosis Of Gingival Fibroblasts And Epithelial Cells, Delaying Wound Healing. *Journal of Periodontology* 2016a; **87**:990-996.

Basso FG, Soares DG, De Costa C and Hebling J. Low-level laser therapy in 3d cell culture model using gingival fibroblasts. *Lasers in Medical Science* 2016b; **31**:973-978.

Berthois Y, Katzenellenbogen JA and Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America* 1986; **83**:2496-2500.

Bhardwaj N and Kundu SC. Chondrogenic differentiation of rat mesenchymal stem cells on porous scaffolds of silk fibroin/chitosan blends. *Biomaterials* 2012; **33**:2848-2857.

Bhasin S, Brito JP, Cunningham GR, *et al.*Testosterone therapy in men with hypogonadism: an endocrine society clinical practice guideline. *The Journal of Clinical Endocrinology and Metabolism* 2018; **103**:1715-1744.

Callewaert F, Boonen S and Vanderschueren D. Sex steroids and the male skeleton: a tale of two hormones. *Trends in Endocrinology & Metabolism* 2010; **21**:89-95.

Campelo AE, Cutini PH and Massheimer VI. Cellular actions of testosterone in vascular cells: mechanism independent of aromatization to estradiol. *Steroids* 2012 ;**77**:1033-1040.

Castoria G, D'amato L, Ciociola A, *et al.* Androgen-induced cell migration: role of androgen receptor/filamin a association. *Plos One* 2011; **6**:E17218.

Chaves JDP, Figueredo TFM, Warnavin SVSC, Pannuti CM and Steffens JP. Sex hormone replacement therapy in periodontology - a systematic review. *Oral Diseases* 2019 Doi:10.1111/Odi.13059.

Chung CC, Hsu RC, Kao YH, Liou JP, Lu Y and Chen YJ. Androgen attenuates cardiac fibroblasts activations through modulations of transforming growth factor-β and angiotensin ii signaling. *International Journal of Cardiology* 2014; **176**:386-393.

Coletta RD, Reynolds MA, Martelli-Junior H, Graner E, Almeida OP and Sauk JJ. Testosterone stimulates proliferation and inhibits interleukin - 6 production of normal and hereditary gingival fibromatosis fibroblasts. *Oral Microbiology and Immunology* 2002; **17**:186-192.

Duss S, Andre S, Nicoulaz AI, *et al.* An oestrogen-dependent model of breast cancer created by transformation of normal human mammary epithelial cells. *Breast Cancer Research* 2007; **9**:R38.

Engeland CG, Sabzehei B and Marucha PT. Sex hormones and mucosal wound healing.*Brain, Behavior and Immunity* 2008; **23**:629-635.

Gilliver SC, Ashworth JJ, Mills SJ, Hardman MJ and Ashcroft GS. Androgens modulate the inflammatory response during acute wound healing. *Journal of Cell Science* 2006; **119**:722-732.

Harada N. A unique aromatase (P-450arom) Mrna formed by alternative use of tissue-specific exons 1 in human skin fibroblasts. *Biochemical and Biophysical Research Communications* 1992; **189**:1001-1007.

Hong Y, Rashid R and Chen S. Binding features of steroidal and nonsteroidal inhibitors. *Steroids* 2011; **76**:802-806.

Kirker KR, James GA, Fleckman P, Olerud JE and Stewart PS. Differential effects of planktonic and biofilm MRSA on human fibroblasts. *Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society* 2012; **20**:253-261.

Lackler KP, Cochran DI, Hoang AM, Takacs V and Oates TW. Development of an *in vitro* wound healing model for periodontal cells. *Journal of Periodontology* 2000; **71**:226-237.

Long BJ, Tilghman SI, Yue W, Thiantanawat A, Grigoryev DN and Brodie A. The steroidal antiestrogen ici 182,780 is an inhibitor of cellular aromatase activity. *Journal of Steroid Biochemistry and Molecular Biology* 1998; **67**:293-304.

Mäkelä S, Santti R, Martikainen P, Nienstedt W and Paranko J. The influence of steroidal and nonsteroidal estrogens on the 5α-reduction of testosterone by the ventral prostate of the rat. *Journal of Steroid Biochemistry* 1990; **35**:249-256.

Mariotti A and Mawhinney M. Endocrinology of sex steroid hormones and cell dynamics in the periodontium. *Periodontology 2000* 2013; **61**:69-88.

Mawhinney M and Mariotti A. Physiology, pathology and pharmacology of the male reproductive system. *Periodontology 2000* 2013; **61**:232-251.

Mills SJ, Ashworth JJ, Gilliver SC, Hardman MJ and Ashcroft GS. The sex steroid precursor dhea accelerates cutaneous wound healing via the estrogen receptors. *Journal of Investigative Dermatology* 2005; **125**:1053-1062.

Mumford JH, Carnes DI, Cochran Dl and Oates TW. The effects of platelet-derived growth factor-bb on periodontal cells in an *in vitro* wound model. *Journal of Periodontology* 2001; **72**:331-340.

Ozcelik O, Haytac MC and Seydaoglu G. The effects of anabolic androgenic steroid abuse on gingival tissues. *Journal of Periodontology* 2006; **77**:1104-1109.

Pomari E, Valle L, Pertile P, Colombo L and Thornton JM. Intracrine sex steroid synthesis and signaling in human epidermal keratinocytes and dermal fibroblasts. *FASEB Journal* 2015; **29**:508-524.

Reynolds MA, Aberdeen GW, Pepe GJ, Sauk JJ and Albrecht ED. Estrogen suppression induces papillary gingival overgrowth in pregnant baboons. *Journal of Periodontology* 2004; **75**:693-701.

Ribeiro DC, Brook AH, Hughes TE, Sampson WJ and Townsend GC. Intrauterine hormone effects on tooth dimensions. *Journal of Dental Research* 2013; **92**:425-431.

Rogers JI, Mitchell AR, Maricc C, Sandberg K, Myers A and Mulroney SE. Effect of sex hormones on renal estrogen and angiotensin type 1 receptors in female and male rats. *American Journal of Physiology* 2007; **292**:794-799.

Romana-Souza B, De Brito Tl, Pereira GR and Monte-Alto-Costa A. Gonadal hormones differently modulate cutaneous wound healing of chronically stressed mice. *Brain, Behavior, and Immunity* 2014; **36**:101-110.

Shiau HJ, Aichelmann-Reidy ME and Reynolds MA. Influence of sex steroids on inflammation and bone metabolism. *Periodontology 2000* 2014; **64**:81-94.

Simpson ER. Sources of estrogen and their importance. *The Journal of Steroid Biochemistry and Molecular Biology* 2003; **86**:225-230.

Slominski A, Zbytek B, Nikolakis G, *et al.* Steroidogenesis in the skin: Implications for local immune functions. *Journal of Steroid Biochemistry and Molecular Biology* 2013; **137**:107-123.

Sobral LM, Aseredo F, Agostini M, *et al.* Molecular events associated with ciclosporin A-induced gingival overgrowth are attenuated by Smad7 overexpression in fibroblasts. *Journal of Periodontal Research* 2012; **47**:149-158.

Soory M and Tilakaratine A. Modulation of androgen metabolism by phenytoin, oestradiol and tamoxifen in human gingival fibroblasts. *Journal of Clinical Periodontology* 2003; **30**:556-561.

Souza Santos R, Frank AP, Clegg DJ. The impact of sex and sex hormones on cell function. *Steroids* 2017; **128**:72-74

Stamatiou R, ParaskeveE, Papagianni M, Molyvdas PA and Hatziefthimiou A. The mitogenic effect of testosterone and 17beta-estradiol on airway smooth muscle cells. *Steroids* 2011; **76**:400-408.

Steffens JP, Coimbra LS, Ramalho-Lucas PD, Rossa C and Spolidorio LC. The effect of supra- and subphysiologic testosterone levels on ligature-induced bone loss in rats — a radiographic and histologic pilot study. *Journal of Periodontology* 2012; **83**:1432-1439.

Steffens JP, Herrera BS, Coimbra LS, *et al.* Testosterone regulates bone response to inflammation. *Hormone and metabolic research* 2014; **46**:193-200.

Steffens J, Coimbra LS, Rossa C, Kantarci A, Van Dyke TE and Spolidorio LC. Androgen receptors and experimental bone loss – an *in vivo* and *in vitro* study. *Bone* 2015; **81**:683-690.

Steffens JP, Santana LCL, Pitombo JCP, *et al.* The role of androgens on periodontal repair in female rats. *Journal of Periodontology* 2018; **89**:486-495.

Steffens JP, Valenga HM, Santana LCL, Albaricci MCC, Kantarci A and Spolidorio LC. Role of testosterone and androgen receptor in periodontal disease progression in female rats. *Journal of Periodontology* 2019; https://Doi.Org/10.1002/Jper.19-0099.

Stevenson S, Sharpe DT and Thornton JM. Effects of oestrogen agonists on human dermal fibroblasts in an *in vitro* wounding assay. *Experimental Dermatology* 2009; **18**:988-990.

Svartberg J. Epidemiology: Testosterone And The Metabolic Syndrome. *International Journal of Impotence Research* 2007; **19**:124-128.

Trackman PC and Kantarci A. Molecular and clinical aspects of drug-induced gingival overgrowth. *Journal of Dental Research* 2015; **94**:540-546

Wang Q, Kessler MJ, Kensler TB and Dechow PC. The mandibles of castrated male rhesus macaques (macaca mulatta): The effects of orchidectomy on bone and teeth. *American Journal of Physical Anthropology* 2015; **159**:31-51.

Welshons WV, Wolf MF, Murphy CS, Jordan VC. Estrogenic activity of phenol red. *Molecular and Cellular Endocrinology* 1988; **57**:169-178.

Yarrow JF, Conover CF, Purandare AV, *et al*. Supraphysiological testosterone enanthate administration prevents bone loss and augments bone strength in gonadectomized male and female rats. *American Journal of Physiology Endocrinology and Metabolism* 2008; **295**:1213-1222.