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THE INFLUENCE OF NANDROLONE DECANOATE IN ALVEOLAR BONE LOSS INDUCED BY EXPERIMENTAL PERIODONTITIS: A STUDY IN RATS

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Abstract: Background: Supraphysiological doses of nandrolone decanoate (N) increase proinflammatory cytokines and could increase the severity of periodontitis. The aim of this study was to evaluate the effect of N on experimental periodontitis (P). **Methods:** Thirty rats (60 teeth) were randomly divided into 3 groups and each group was subdivided into 2 subgroups: P + N (n = 10): intramuscular injections of N (5mg/Kg), 2x/week for 30 days, and concomitantly received subgingival ligature in one lower 1st molar; Health + N (n = 10): contralateral molars (H + N); P + Propylene Glycol (Pgl) (n = 10): intramuscular injections of Pgl (0.2mL/kg) as P + N protocol; H + Pgl (n = 10): contralateral molars; P (n = 10): the animals received only subgingival ligature in one lower 1st molar; H (n = 10): contralateral teeth were collected. The animals were euthanized after 30 days. **Results:** P + N had greater bone loss (BL) compared to P + Pgl (P <0.05) and no differences were observed in BL when P + N and P + Pgl were compared to P (P> 0.05). H + N had greater periodontal ligament thickness (PLT) compared to H (P < 0.05), and no significant differences were observed between H + N and H + Pgl (P> 0.05). No significant differences in tartrate-resistant acid phosphatase (TRAP) positive cells/mm were detected within the P subgroups (P > 0.05). H + Pgl had a higher number of TRAP-positive cells/mm compared to H (P <0.05). **Conclusion:** Nandrolone decanoate increased PLT in health and BL area in experimental periodontitis.

Keywords: Anabolic agents. Nandrolone decanoate. Periodontitis. Alveolar bone loss.

INTRODUCTION

Periodontitis is a set of diseases of multifactorial etiology resulting from the interaction between the periodontopathogenic bacterial biofilm, recognized as the primary etiological factor of periodontal diseases that lead to disruption of tissue homeostasis, and the immunoinflammatory response of the host[1, 2]. The onset, expression and severity of periodontal diseases can be modulated by genetic factors, habits, environmental, and systemic factors[1, 3, 4]. The result of this interaction is the destruction of the periodontium (supporting structure of the teeth), i.e., root cementum, periodontal ligament, and alveolar bone, which can lead to loss of teeth[5].

Anabolic Androgenic Steroids (AAS) are synthetic drugs that mimic the action of the male sex hormone, testosterone, which is an endogenous hormone produced by the testes[6]. These synthetic hormones were initially developed for therapeutic purposes, such as the treatment of cachexia in androgen deficiency syndromes, catabolic states, and chronic diseases associated with cancer and renal and hepatic failure[7]. However, these anabolic steroids are currently used mainly to acquire greater resistance during physical exercise due to their anabolic effect and also for aesthetic purposes, since these drugs increase cellular protein synthesis, leading to growth of skeletal muscles[8].

Nandrolone decanoate or Deca-Durabolin® was introduced in 1962 by the Oregon laboratory. It is an AAS in which nandrolone is the active substance, and it is commercially available as an injectable drug with systemic and tissue action lasting up to 3 weeks after intramuscular administration[9]. Following its administration, nandrolone decanoate is gradually released and hydrolyzed into nandrolone that exhibits greater anabolic action and less androgenic activity than testosterone, which is desirable in an AAS[10] (Wilson, 1996). Therefore, this drug is one of the most widely consumed AAS[9].

In the United States, there are records of the indiscriminate and endemic use of anabolic drugs among elite athletes, such as American football players (95%) and bodybuilders (88-99%), and it is estimated that these drugs are used by 4% - 6% of young Americans[11]. The most commonly encountered adverse effects of the consumption of these drugs include hepatic, endocrine, cardiovascular, musculoskeletal, immunological, reproductive and psychological abnormalities; hypertension, masculinizing effects in women, and altered bone growth;[12, 13] these effects depend on the gender and age of the user, as well as the duration of drug use, dose, and type of steroid used[11].

Sex hormones and their byproducts appear to play a key role in the pathogenesis of periodontal diseases, as they can be metabolized by gingival fibroblasts. The expression of dihydroxytestosterone, the major metabolite of testosterone, is significantly increased in inflamed gingival tissues[14, 15]. In addition, sex hormones can affect wound healing because of their ability to modulate individual inflammatory response[16], which could be closely related to the inflammatory response in periodontal diseases, altering the phenotype and progression of the disease, as well as the healing of periodontal sites after basic or surgical therapy procedures. A recent study has shown that men who use anabolic steroids are at an increased risk of developing periodontitis, and of having higher rates of true periodontal pathogens[17]. Therefore, the literature indicates that the use of anabolic steroids may interfere with the inflammatory response and alter the microbiota of the periodontal biofilm. Thus, it can be hypothesized that the use of AAS affects the expression of periodontal diseases. However, uncertainty regarding the action of anabolic drugs on the periodontium still remains, and there are few records in the literature of the influence of anabolic steroids on the severity and extent of periodontal diseases, especially regarding the interference of their use at onset and over the course of periodontitis.

Accordingly, the aim of this study was to evaluate by histometry and histochemistry the influence of nandrolone decanoate on alveolar bone resorption promoted by experimental periodontitis and on the thickness of the periodontal ligament of periodontally healthy teeth.

MATERIAL AND METHODS

ANIMALS

We used thirty adult male Wistar (Rattus norvegicus albinus, Wistar) rats aged 16 weeks and weighing between 350 g and 400 g. The animals were housed in the vivarium of the Universidade do Sagrado Coração, kept in plastic cages with access to food and water ad libitum. Prior to the procedures, the animals underwent a 5-day laboratory environment acclimatization period, at a temperature of 22º-24ºC. This study was approved by the Animal Research Ethics Committee, under protocol number 2330-1.

EXPERIMENTAL DESIGN

The experiment was of the split-mouth type, i.e., the first molars on both sides were used. Thus, 30 animals, totaling 60 teeth, were randomly divided into one of the Nadrolone (N), Sham Propylene Glycol (Pgl), and Negative Control (NC) groups, and each 1st molar was allocated to one of the periodontitis (P) or health (H) subgroups, with a total number of 10 animals per group and 10 teeth per subgroup:

I. Nandrolone (N) Group

a. P + N (n=10): the animals received intramuscular injections of nandrolone decanoate (5mg/Kg)[18, 19], twice a week for 30 days, and simultaneously with the

first administration, received a segment of cotton thread applied unilaterally to a lower 1st molar randomly selected to experimentally develop periodontitis through mechanical retention of biofilm during the 30 days of the experiment.

b. H + N (n=10): the animals received intramuscular injections of nandrolone decanoate (5mg/Kg)[18, 19] twice a week for 30 days, and had the contralateral lower 1st molar randomly selected with the purpose of assessing the effect of nandrolone decanoate on the healthy periodontium.

II. Propylene Glycol (Pgl) Sham Group

a. P + Pgl (n=10): the animals received intramuscular injections of a propylene glycol vehicle (0.2ml/Kg)[18, 19] twice a week for 30 days, and simultaneously with the first administration, received a segment of cotton thread applied unilaterally to a randomly chosen 1st molar for the purpose of developing experimental periodontitis through mechanical retention of biofilm during the 30 days of the experiment.

b. H + Pgl (n=10): the animals received intramuscular injections of a propylene glycol vehicle (0.2ml/Kg)[18, 19] twice a week for 30 days, and had the contralateral lower 1st molar randomly chosen with the purpose of assessing the effect of the vehicle used as placebo on the healthy periodontium.

III. Negative Control (NC) Group

a. P (n=10): the animals received unilateral insertion of a segment of cotton thread on a lower 1st molar randomly chosen with the purpose of obtaining initial data on the influence of the ligature insertion on the animals' periodontium through mechanical retention of biofilm during the 30 days of the experiment.

b. H (n=10): the contralateral teeth of the animals in the NC group received no interference. This subgroup was designed to obtain initial data on the size of the periodontal ligament.

INDUCTION OF THE EXPERIMENTAL PERIODONTITIS

The animals were anesthetized by intramuscular administration of ketamine (1 mL/kg) (Dopalen®, Vetbrands LTDA, Jacareí, SP, Brazil) and xylasine hydrochloride (0.3 mL/kg) (Virbaxil®; Virbac from Brazil Indústria e Comércio LTDA, Roseira, SP, Brazil). The jaws were opened using the modified Doku apparatus and the teeth were gently spaced with a modified endodontic file. This instrument was inserted between the lower 1st and 2nd molars to provide the necessary space for the insertion of the cotton ligatures (No. 10 kite line from Pinheiro, Coats Corrente LTDA, São Paulo, SP, Brazil). The ligatures were positioned unilaterally around one of the lower first molars randomly selected among all the animals, so as to be subgingivally positioned. Three single knots were made and locked. The ligatures were fixed so that the knots were positioned on the mesial surface of the lower first molar.

EUTHANASIA OF THE ANIMALS AND HISTOLOGICAL PROCESSING

The animals were given general anesthesia via intramuscular injection of 1 mL/kg ketamine (Dopalen, Vetbrands LTDA, Jacareí, SP, Brazil) and 0.3 mL/kg xylazine hydrochloride (Virbaxil, Virbac from Brazil Indústria e Comércio LTDA, Roseira, SP, Brazil). The anesthesia was deepened and the animals were subsequently decapitated and

had their mandibles were removed and hemi-sectioned along the symphisis. The material obtained was immersed in 10% formalin with phosphate buffer (pH 7.0) for 24 hours. Then the hemi-mandibles were immersed in PBS and the solution was changed three times, with the last renewed solution being maintained for another 24 hours. The hemi-mandibles were then decalcified with 20% EDTA, with weekly changes of solution for 45 days, at room temperature. After demineralization, the specimens were dehydrated in increasing concentrations of ethanol, diaphanized in xylol, and embedded in paraffin. Longitudinal mesiodistal sections of 6-µm thickness will be obtained with the use of a microtome (Leica RM 2145, Germany). The slides will be stained with hematoxylin and eosin according to the laboratory's routine protocol and the images of the furcation region will be captured by a 50X optical microscope (5X objective and 10X ocular, Nikon Eclipse 80i microscope, Nikon Instruments INC, Melville, NY, USA; QIMAGING Micropublisher 3.3 Cooled camera, RTV, Surrey, BC, Canada). Bone resorption and bone density parameters will be evaluated using the Image-Pro® software (Media Cybernetics, Silver Spring, MD, USA).

Interradicular alveolar bone resorption

Serial sections with a thickness of 6 µm were obtained from the buccal bone plate and the sections in which the bifurcation region was identified were then separated. The first section was rejected for volume estimation and the other sections were selected equidistantly according to the total number of histological sections obtained per tooth. Thus, ten histological sections were scanned and evaluated per tooth. Scans were performed to include radicular dentin, cementum, periodontal ligament, and interradicular alveolar bone. The quantification of the area of connective tissue present in the interradicular region as a result of bone loss was performed using the Image Pro® software (Image-Pro®, Media Cybernetics, Silver Spring, MD, USA) according to the previously described methodology[20].

STAINING FOR TARTRATE RESISTANT ACID PHOSPHATASE - TRAP

Mesiodistal sections with a thickness of 6 µm were deparaffinized and then incubated at 37 °C for 15 min with a mixture of 4 mg naphthol AS-BI phosphate (Sigma Chemical Co., St. Louis, MO, USA) as substrate, and 24 mg red violet salt (Sigma Chemical Co., St. Louis, MO, USA) diluted in 30 mL of acetate buffer (pH 5.2) containing 0.3 mmol/L tartrate (Sigma), pH 5.0. The substrate was omitted in the slides of the negative control of the reaction. Sections were contrasted with hematoxylin (Sigma Chemical Co., St. Louis, MO, USA). Quantitative analysis of the number of TRAP-positive cells was performed on the linear surface of the bone crest, just below the roof of the furcation of the first molars, using an optical microscope (Nikon Eclipse 80i, Nikon Instruments INC, Melville, NY, USA; QIMAGING Micropublisher 3.3 Cooled camera, RTV, Surrey, BC, Canada), with 400X magnification (40X objective and 10X ocular). The results were expressed as number of TRAP-positive cells/mm.

STATISTICAL ANALYSIS

The data from the measurements were organized in an Excel spreadsheet (Microsoft Office Excel, Redmond, WA, United States). The SigmaPlot software (Sigma Plot, San Jose, CA, USA) version 13.0 was used and data were analyzed for normal distribution

(Shapiro-Wilk test and equality of variance). A significance level of 5% was set for the analyses.

The Holm-Sidak test was used for the bone resorption/periodontal ligament thickness parameter, when there was normal distribution of the means; the Kruskal-Wallis test and confirmation with application of the Tukey test were used for the subgroups with periodontal health when there was no normal distribution of the means.

The TRAP-positive cell parameter did not exhibit normal distribution of means. The Kruskal-Wallis test was used to detect the differences. Confirmation was performed using the Tukey test in the periodontally healthy subgroups and the Friedman aligned-ranks test as used when no differences were detected.

EXAMINER CALIBRATION

Twenty resorption-related and twenty TRAP-related sections were randomly selected and their measurements were taken at baseline and repeated three weeks after the first measurement. The high value of the correlation coefficient of bone resorption and TRAP data (r = 0.9908 and r = 0.9999 respectively, 95% confidence interval) shows consistency in the reproducibility of both readings, showing excellent examiner calibration.

RESULTS

The comparative analyses of alveolar bone resorption or periodontal ligament thickness and of TRAP-positive cells were performed between the subgroups with experimental periodontitis and between the periodontally healthy subgroups.

In the periodontally healthy subgroup, a significant increase in the thickness of the periodontal ligament (PL) was observed when the H + N group (0.42 ± 0.07) and H group (0.32 ± 0.04) were compared (P = 0.009). No statistical differences were detected when comparing the mean PL thickness in the H + Pgl group with that in the H + N group (P = 0.384) and in the H group (P = 0.235) (Table 1).

In the subgroups in which experimental periodontitis was developed, a significant increase in bone resorption was observed in the P + N group (1.343 ± 0.281 mm2) when compared to the P + Pgl group (0.979 ± 0.167 mm^2) (P = 0.003), and when compared to the P group (1.046 ± 0.123 mm^2) (P = 0.009). No differences were detected in bone resorption means between the P + Pgl (0.979 ± 0.167 mm2) and P (1.046 ± 0.123 mm^2) (P = 0.475) groups (Figures 1 and 2).

Histochemical evaluation was performed based on the count of TRAP-positive cells present on the alveolar bone crest immediately below the roof of the furcation The analysis of the periodontally healthy subgroups showed no significant difference between the mean of the S + N group (4.75 ± 0.92) and those of the S + Pgl group (12.22 ± 4.07) (P = 0.99) and S group (1.5 ± 1.37) (P = 0.137). There was a significantly higher number of TRAP-positive cells in the S + Pgl group (12.22 ± 4.07) than in the S (1.5 ± 1.37) group (P <0.01) (Figures 3 and 4).

The comparison of the number of TRAP-positive cells between the subgroups with experimental periodontitis showed no significant difference between P + N (11.74 ± 6.99) and P + Pgl (14.24 ± 4.71) (P > 0.05), between P + N (11.74 ± 6.99) and P (12.63 ± 3.33)(P > 0.05), and between P + Pgl (14.24 ± 4.71) and P (12.63 ± 3.33)(P > 0.05) (Table 2).

DISCUSSION

Nandrolone decanoate is an AAS that has a greater anabolic action, less androgenic activity, and a sustained effect for up to 3 weeks[10]. In this study, a supraphysiologic dose

Subgroup	Thickness mm² (Mean ± SD)	Median	25%	75%
H + N	0.42 ± 0.07 A	0.422	0.358	0.476
H + Pgl	0.39 ± 0.15 AB	0.355	0.309	0.461
H	0.32 ± 0.04 B	0.316	0.295	0.350
H	8.714			
*P*Kruskall-Wallis	0.013			

H = health; N = nandrolone decanoate; Pgl = propylene glycol; Intergroup: Means followed by different upper case letters in the column differ in the Tukey test (P = 0.009).

Table 1- Distribution of means (mm²) of the thickness of the periodontal ligament followed by their respective standard deviations (SD), medians and quartiles in the periodontally healthy subgroups.

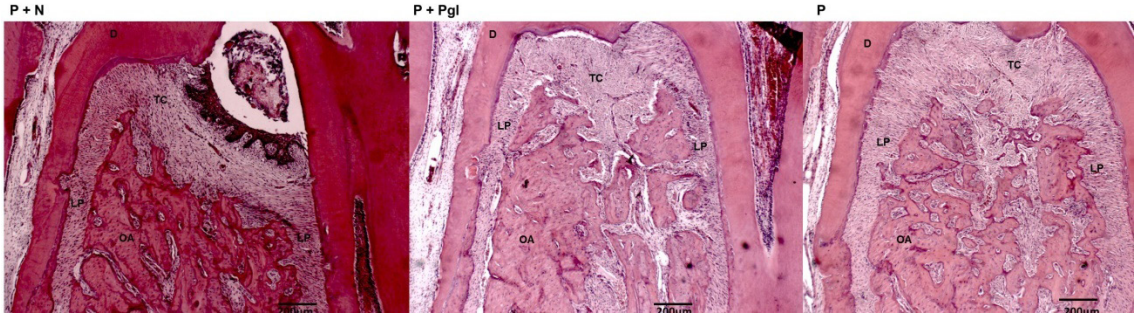

Figure 1 - Photomicrographs showing alveolar bone resorption in the furcation region of the 1st molars under 50x magnification (5x objective and 10x ocular) in the subgroups with experimental periodontitis. P + N = periodontitis + nandrolone decanoate; P + Pgl = periodontitis + propylene glycol; P = periodontitis; D = dentin; TC = filling connective tissue found in the area of bone resorption; OA = interarticular alveolar bone; Bar = 200 **µm.**

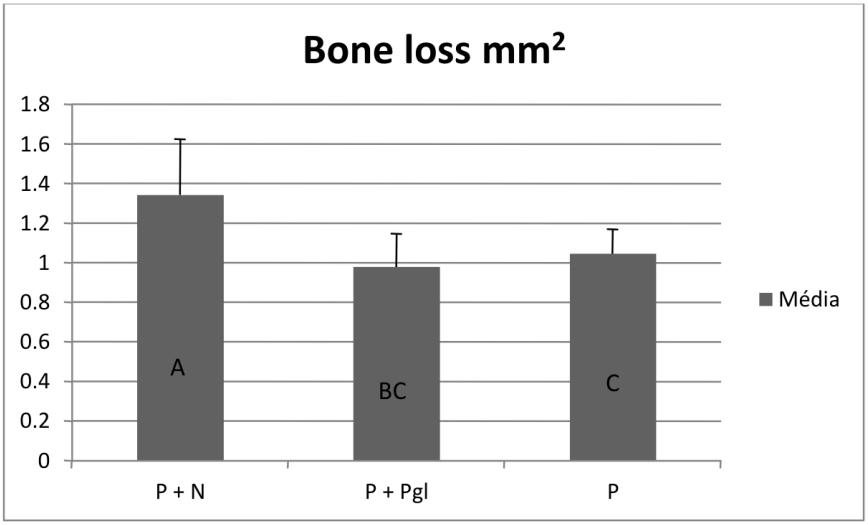

Figure 2 - Graph representing the distribution of means and standard deviations of alveolar bone resorption (mm²) in the subgroups with periodontitis. Values followed by different upper case letters in the columns are significantly different (*P* <0.05). P + N = periodontitis + nandrolone decanoate; P + Pgl = periodontitis + propylene glycol; P = periodontitis.

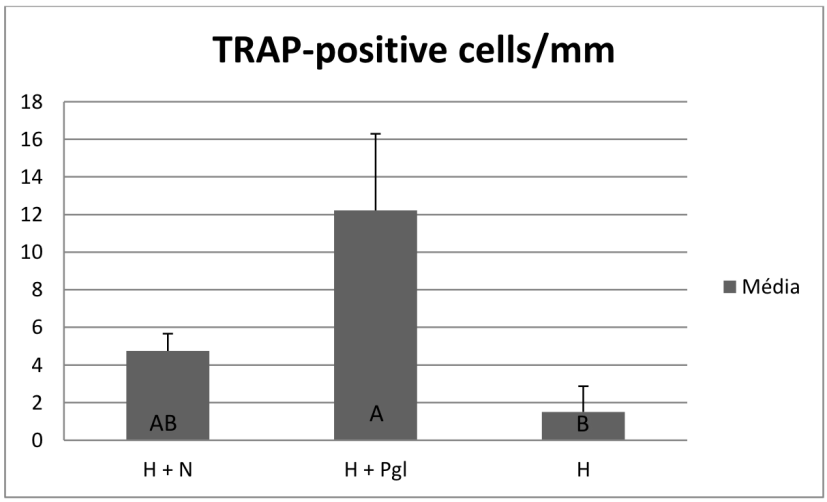

Figure 3- Graph representing the distribution of means and standard deviations of the number of TRAP-positive cells in the periodontally healthy subgroups. Different upper case letters in the columns represent presence of a statistically significant difference (P <0.05). H + N = periodontal health + nandrolone decanoate; H + Pgl = periodontal health + propylene glycol; H = health.

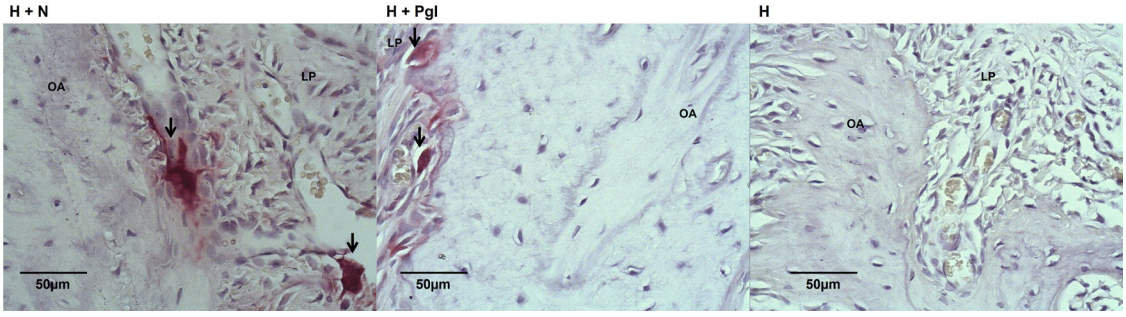

Figure 4- Photomicrographs showing TRAP-positive cells in the alveolar bone crest of the furcation region of the lower 1st molars under 400x magnification (40x objective and 10x ocular) in the periodontally healthy subgroup. H + N = periodontal health + nandrolone decanoate; H + Pgl = periodontal health + propylene glycol; H = health; Bar = 50 µm. Arrows represent TRAP-positive cells = osteoclasts; OA = alveolar bone of furcation; PL = periodontal ligament.

Subgroup	TRAP cells/mm (Mean ± SD)	Median	25%	75%
P + N	11.74 ± 6.99 A	8.608	5.417	14.792
P + Pgl	14.24 ± 4.71A	13.125	8.338	22.583
P	12.63 ± 3.33 A	13.467	6.958	16.458
*P*ANOVA	0.202			

P + N = experimental periodontitis + nandrolone decanoate; P + Pgl = experimental periodontitis + propylene glycol; P = periodontitis; Intergroup: Groups with equal upper case letters in the column did not differ from each other.

Table 2- Distribution of means of TRAP-positive cell counts/mm followed by their respective standard deviations (SD), medians and quartiles in the subgroups with experimental periodontitis.

of nandrolone decanoate totaling 10 mg/kg per week (2 injections of 5 mg/kg per week) was used to experimentally mimic the dose used by athletes, which is approximately 8 mg/kg per week[21]. We investigated an alternative hypothesis of negative interaction of the drug on the progression of periodontitis by increasing the recommended dose.

It is important to clarify that experimental models in rats are widely used in the field of dentistry due to the high reproducibility, ease of handling, knowledge of the animal's biological characteristics, low cost of the model, and because we are able to control systemic health, food, genetic and behavioral variables of these animals, thus obtaining a more homogeneous sample[22]. Moreover, one day in the life of an adult rat is comparable to 1 month in the life of a human being, and the results of 30 days of periodontitis chronification can be extrapolated to 30 months in humans[23, 24].

The use of the supraphysiological dose of nandrolone decanoate increased alveolar bone resorption promoted by experimental periodontitis, and increased the thickness of the periodontal ligament in the periodontally healthy subgroup, which showed a negative effect on the periodontium both in periodontal health and in established disease. Supraphysiological doses of nandrolone decanoate increase the production of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in cultures of human peripheral blood lymphocytes in vitro[25, 26]. IL-1β and TNF-α are proinflammatory cytokines that are related to the progression of periodontal diseases and the destruction of bone and collagen, and could partly explain the increase in bone resorption and ligament thickness in the subgroups with periodontitis and health, respectively[27, 28]. According to Page and Schroeder[29], early lesions describe clinically healthy gums, yet an increase in the neutrophil infiltrate is already detected histologically, as well as an increase in adhesion molecules. Therefore, there is a subclinical inflammatory process that, in the model used herein, may have been negatively affected by the administration of nandrolone decanoate. An increase in the thickness of the periodontal ligament probably occurs due to the disruption of tissue homeostasis in the presence of microbial challenge due to changes in the host's immunoinflammatory response.

Brusca et al.[17] ascertained that the standard of periodontal health was significantly higher in patients who did not use AAS than in patients using these drugs. In a clinical study, Ozcelik et al.[11] found a significant increase in the gingival thickness of anabolic drug users when compared to the group of non-users. It can be speculated that the increase in gingival thickness quantitatively affects biofilm deposition, acting as a retentive factor and altering the contact of the biofilm with the periodontal tissues and, consequently, the progression rate of the disease, which explains the findings in the healthy and experimental periodontitis subgroups. Brusca et al.[17] found a greater risk in the progression of periodontal diseases, as evidenced by the increase in the proportion of *A. actinomycetemcomitans, P. gingivalis, P. intermedia* and *Candida ssp*, in nonsmoking patients who were AAS users than in nonsmoking patients who were AAS nonusers. These findings help to understand the results observed in the present study with regard to the worsening of the assessed parameters in the groups with periodontal health and with experimental periodontitis in which nandrolone decanoate was administered.

In contrast, in the present study no differences were observed in the number of TRAP-positive cells/mm in the alveolar bone

crest of the furcation region in the group with experimental periodontitis. This is partly due to the fact that in this group, bone resorption and, therefore, osteoclast activation occurred in all specimens. This result should be treated with caution because osteoclast activation cannot be interpreted as amount of bone resorption, but merely resorption activity. However, the non-detection of differences between the P + Pgl and P + N groups may also be partly explained by a limitation of this type of evaluation, because the number of osteoclasts observed on bone surfaces is lower than that found in resorption lacunae. However, an evaluation on the remaining alveolar bone crest was performed because of the direct action of the subgingival ligature and the histometric parameter of resorbed area/periodontal ligament immediately below the roof of the furcation.

On the other hand, the group with periodontal health S + Pgl had more TRAP-positive cells/mm than the S group, possibly due to the stress generated by the propylene glycol injection. A study of a regeneration model in rats showed that there was an increase in the amount of TRAP-positive cells in animals subjected to chronic stress by immobilization [30]. The drug administration model is not used as an experimental model of stress induction, but in the present study the injection of the drug alone was able to increase the number of TRAP-positive cells in the alveolar bone crest, even though it did not entail an increase in the thickness of periodontal ligament area in the periodontally healthy subgroup. This shows the importance of the Sham group to control possible biases of the experimental model and to better understand its applicability and limitations.

It is important to recognize that there are limits to extrapolating experimental results to the clinical setting, because experimental animal models do not necessarily replicate human biology and masticatory dynamics. However, preclinical studies are essential for the evaluation and improvement of methods of prevention, diagnosis and treatment of the disease in humans, and are still important tools in science.

Further experimental studies should be conducted to better elucidate the inflammatory process exacerbated by the use of nandrolone decanoate from a cellular and molecular point of view. Clinical studies should also be carried out to assess periodontal clinical parameters and better understand this interaction.

CONCLUSIONS

Within the limitations of this study, it was concluded that the administration of nandrolone decanoate had a negative impact on the periodontal ligament, by increasing its thickness in periodontally healthy individuals and the area of bone resorption promoted by experimental periodontitis.

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