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The Impact of Testosterone Suppression on Dental Structures: An *In vivo* Study

Magali de Fátima Pereira Madureira [a], Caio Luiz Bitencourt Reis [b], Fabricio Fernandes Ferreira [c], Alessandra Esteves [d], Wagner Costa Rossi Júnior [d], Erika Calvano Küchler [e], Daniela Silva Barroso de Oliveira [c*] and Tomaz Henrique Araújo [a]

[a] Department of Structural Biology, Biomedical Sciences Institute, Federal University of Alfenas, Alfenas, Minas Gerais, Brazil.
[b] Department of Pediatric Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirao Preto, São Paulo, Brazil.
[c] School of Dentistry, Federal University of Alfenas, Alfenas, Minas Gerais, Brazil.
[d] Department of Anatomy, Biomedical Sciences Institute, Federal University of Alfenas, Alfenas, Minas Gerais, Brazil.
[e] Department of Orthodontics, University Hospital Bonn, Medical Faculty, Bonn, Germany.

Authors' contributions

This work was carried out in collaboration among all authors. Authors ECK and CLBR initiated and designed the study. Author MdFPM, DSBdO, AE, WCRJ and THA developed the project framework, engaged in the gathering of data, and played a pivotal role in the manuscript preparation. Author CLBR and FFF was instrumental in data acquisition, study's statistical analyses, and made substantial contributions to the manuscript's composition. Authors ECK and FFF meticulously reviewed and edited the manuscript, ensuring their approval of the final version. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: daniela.oliveira@unifal-mg.edu.br;*

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ABSTRACT

Aim: The aim of this study was to evaluate the impact of testosterone (T) suppression during puberty on the development of dental, periodontal and alveolar structures in rats.

Materials and Methods: Thirty-six Wistar rats were selected for this study. Orchiectomy (ORX) was performed on the animals of the experimental group (n=18) and sham surgery on the animals of the control group (n=18) on the 23rd day of life. The animals were allocated into 4 groups: an ORX group (n=9) and a sham group (n=9) euthanized at 45 days of age, and the other ORX group (n=9) and the other sham (n=9) euthanized at 73 days of age. After the experimental period, the animals were euthanized and the mandibles and maxillas were removed, dissected and fixed in 10% formalin, decalcified, cut at 7 µm and stained with hematoxylin and eosin and picrosirius red. Qualitative analysis of slides stained with Hematoxylin and Eosin were performed, while collagen synthesis obtained from slides stained with Red Silver was quantitatively evaluated using ImageJ software. Collagen synthesis was compared between groups using the student's t test using the IBM SPSS software.

Results: Histologically, the animals submitted to orchiectomy showed variations in the periodontal region, immature alveolar bone and periodontal ligament with the presence of atypical fat cells, in the dental structures, hyperemic pulp with calcification points (nodules) and variation in the arrangement and shape of the odontoblasts, with considerable significance when compared with the animals of the Sham group.

Conclusion: In conclusion, the testosterone suppression induces changes in the differentiation of cells that form the tissues of dental and alveolar structures, through the incidence of pulp alterations, presence of atypical cells in the periodontal ligament and delay in the neoformation of alveolar bone in rats during puberty.

Keywords: Testosterone; tooth; dentin; pulp; alveolar bone; periodontal ligament, collagen fibers.

1. INTRODUCTION

Testosterone is the predominant sex hormone in males and is responsible for the individual's sexual maturation during adolescence. Testosterone is involved in several physiological processes, mainly in bone metabolism [1-3]. Some conditions can unbalance plasma testosterone levels, such as congenital or acquired conditions, and lead to dysfunction in bone metabolism [4,5]. Studies in humans and animals have shown that decreases in testosterone concentrations can lead to decreased bone mineral density [6], decreased osteoblast differentiation, and collagen synthesis [7].

Previous studies showed that both dental and periodontal structures are sensitive to testosterone since cells in these regions have the Androgen Receptor in their plasma membranes [8-10]. In addition, the studies also show the direct role of testosterone on the differentiation of undifferentiated cells into osteoblasts, odontoblasts, and fibroblasts. Since testosterone acts directly on the differentiation of cells crucial for the development of dentoalveolar structures, it is plausible to assume that the development of these structures is significantly affected by testosterone dysfunctions. Some studies have already been carried out to evaluate the impact of testosterone on alveolar, periodontal, and dental structures [11-17]. However, several limitations of these studies did not allow a robust conclusion. Additionally only Roberts et al. [18], Gaethofs et al. [19] and Wang et al. [5] evaluated the impact of testosterone during puberty.

Thus, this study aimed to evaluate the effects of testosterone suppression during puberty on the development of dental and periodontal structures.

2. MATERIALS AND METHODS

This study is part of a broad line of research initiated by Reis et al. (2022). The PREPARE guideline (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) [20] was used for the design of this study, and the ARRIVE guideline (Animal Research: Reporting 60 *In Vivo* Experiments) was followed for the report [21].

This study was approved by the Ethics Committee on the Use of Animals of the Federal University of Alfenas (protocol: 024/2019). All recommendations from recognized institutions for animal welfare were followed.

Formula according to Miot [22] was used to calculate the sample size to compare a hypothesis in two independent groups. Cohen's D effect size was obtained from the results of Omori et al. [23]. Under an alpha = 0.05 and beta = 0.80, the result of 7 animals per group was obtained. After orchiectomy, the animals could suffer from hypothermia and infections resulting from the surgery, and about 20% of them could die from these complications [24]. Therefore, it was necessary to add two more animals per group, totaling 9 animals per group (n=36).

Heterogenic male rats of the species Rattus norvegicus of the Wistar strain obtained at the Center for Bioterism of the Federal University of Alfenas (UNIFAL- MG) were obtained on the day of weaning (21 days). Three animals remained in each polypropylene box measuring 49x34x16, lined with wood shavings. The animals had free access to food and filtered water at a controlled temperature between 21º to 23º Celsius with air exhaustion and within a 12-hour light-dark cycle. Animals were not randomly housed to avoid losses from bite wounds [25-27].

The animals were randomly allocated by a researcher who did not perform the surgeries into two large groups: Orchiectomy (ORX – Group 1) and Sham Surgery (Sham – Group 2). Both groups were divided into two more groups: a group that would be euthanized at 45 days of life (pubertal outbreak period -Subgroup B) and another group that would be euthanized at 73 days of life (post-pubertal period – Subgroup A).

For the induction of testosterone suppression, the procedure in the experimental group was orchiectomy, which is the removal of both testes and epididymis. Orchiectomy is the safest and most effective method for testosterone suppression, which has been shown to directly affect serum levels of this hormone during puberty [26,28,29]. The animals were subjected to anesthesia by intraperitoneal injection with 10% Ketamine Hydrochloride (55 mg/Kg of body weight) and 2% Xylazine (5 mg/Kg of body weight). Orchiectomy was performed as protocoled by Idris [24]. The control group received sham surgery. This surgery reproduced anesthesia, surgical stress, incision, and soft tissue synthesis to adjust for confounding factors. A single operator previously trained by a veterinarian and who did not participate in the animal allocation process performed the procedures.

The animals were euthanized by isoflurane inhalation. The death of the animal was confirmed after the interruption of respiratory and muscular movements.

The maxillary and mandibular central incisor regions were carefully sectioned and separated for his technical processing, fixed by immersion in 10% buffered formalin at room temperature for 24 hours. After this period, the pieces were washed in running water for 4 hours and subjected to the demineralization process, through immersion in a 4.13% EDTA-based solution (pH 7 – 7.4). After this procedure, the pieces were submitted to routine his technical processing and underwent dehydration processes in alcohol of increasing concentrations (70%, 80%, and 95% for 45 minutes each and 3 changes of 100% alcohol for 45 minutes each), alcohol/xylene for 30 minutes, diaphanization in xylol (xylol I, II and III for 40 minutes each) and embedding in paraffin. Blocks containing the tissues were cut longitudinally on a microtome (Leica RM2145; Leica Microsystems GmbH, Wetzlar, Germany). Semi-serial cuts of 7µm were obtained along the entire length of the piece. The sections of each group were stained with hematoxylin and eosin (HE) and Picro Sirius Red and analyzed under conventional optical microscopy, using the Axio Imager.M1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), with an AxioCam MRc5 camera attached. (Carl Zeiss MicroImaging GmbH, Göttingen, Germany, to describe the characteristics of dental and periodontal tissues. A polarized light filter was inserted into the microscope for picrosirius images. Images were captured using an objective of up to 40X.

A descriptive and quantitative analysis of the histological slides stained with Hematoxylin and Eosin was performed to verify possible alterations in periodontal tissues and dental structures.

For quantitative analysis of collagen synthesis, slices comprising the total length of the lower incisor root were selected. Images under a 40x objective were obtained in the middle region of the root of the lower teeth, with a band of cementum as a reference. Quantitative analysis of collagen synthesis was performed using ImageJ software. After recognizing the areas of high 60 birefringence in the images under polarized light, which correspond to collagen, the software quantified the area of collagen synthesized in pixels and indicated the

percentage concerning the total area of the image. As it is compositional data, the percentages of collagen areas in the images were submitted to log10 transformation and, thus, compared by the student's t-test using the IBM SPSS software, and p values < 0.05 were considered statistically significant.

3. RESULTS

3.1 Dentin

The dentin of both groups and experimental times were observed with normality patterns with dentinal tubules symmetrically arranged along with the entire structure. In the entire extension of the dentin, a thin non-mineralized region was found, attached to the odontoblasts called pre-dentin.

At the dentin interface of the mandibular incisors, the ORX-1.A group presented a historically smaller mineralization front in terms of quantity and thickness when compared to the Sham-2.A group. However, no differences were observed between the ORX-1.B and Sham-2.B groups (Fig. 1).

The red arrows indicate a layer of non-mineralized matrix present throughout the dentin, adhered to the odontoblasts, and called pre-dentin.

No differences were observed in the dentin structure of the maxillary incisors. Both groups had normal-appearing dentin, dentinal tubules arranged in parallel along the length of the dentin, and numerous odontoblasts arranged along the presenting. Both groups present ameloblasts in large numbers and with normal morphological appearance (Fig. 2).

3.2 Pulp

The dental pulps of the lower incisors in the ORX-1 group presented a lesser degree of blood vessel formation, but with a larger caliber. The odontoblasts of the same group were arranged along the entire peripheral part of the pulp with a "pulled" arrangement, presenting different degrees of maturation and length, little active functional action, with an elongated shape, basal nucleus, and cytoplasm with few basophils. Differently from the odontoblasts observed in the Sham-2. A group, in which the odontoblasts presented larger and elongated cells, well-defined and basophilic cytoplasm, and a less stained nucleus, characterizing cells in an active function stage (Fig. 3).

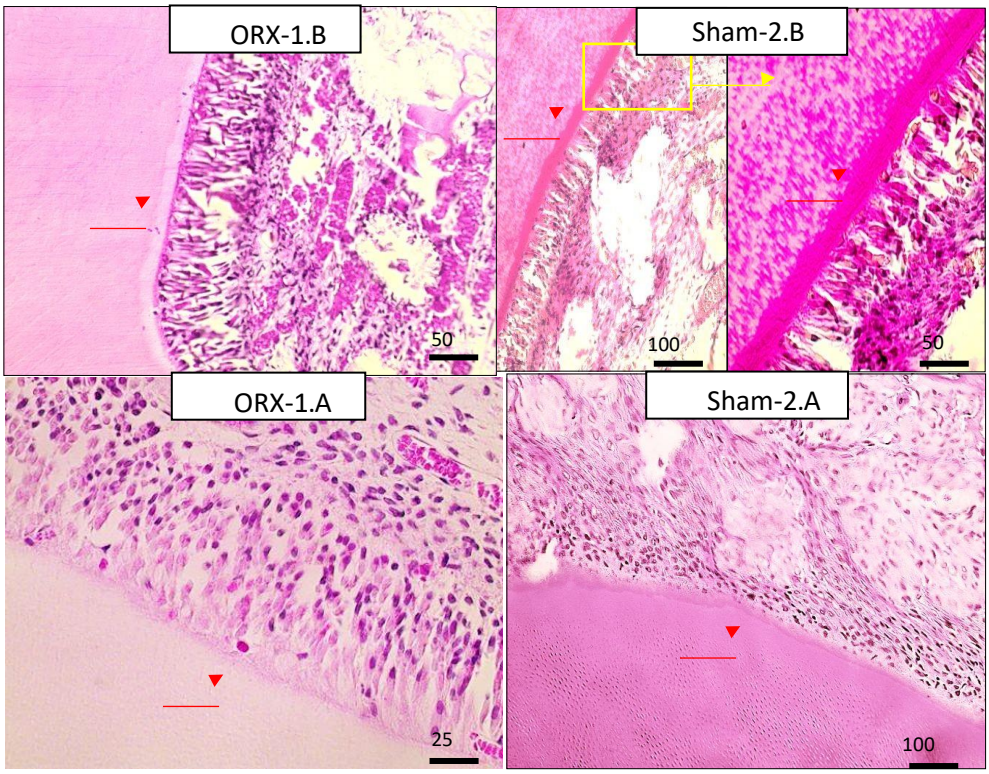

Fig. 1. Photomicrographs of the dentin region of the lower incisors

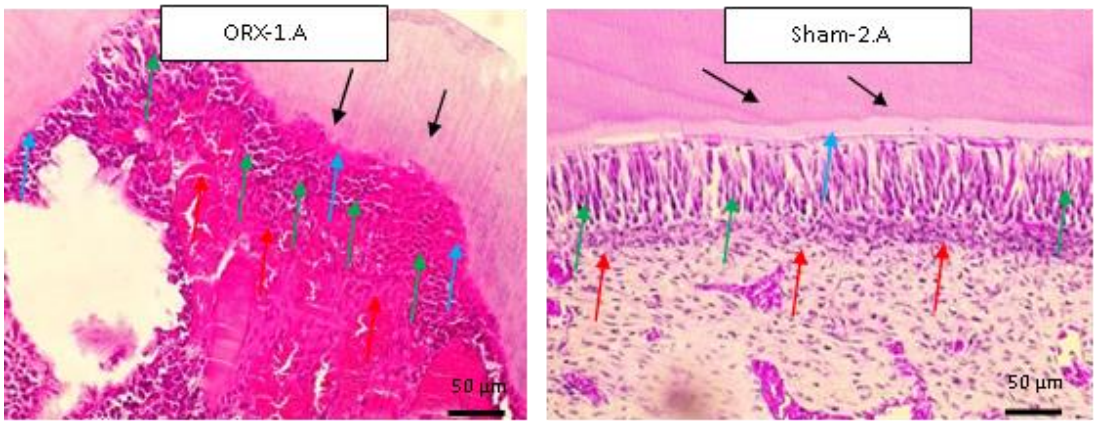

Fig. 2. Photomicrographs of the dentin region of the maxillary incisors. Longitudinal striations along the entire length of the dentin (black arrows). A layer of non-mineralized matrix present throughout the dentin, adhered to the differentiated odontoblasts (green arrows), called pre-dentin (blue arrow) indicating a linear mineralization front. Red arrows indicate preodontoblasts

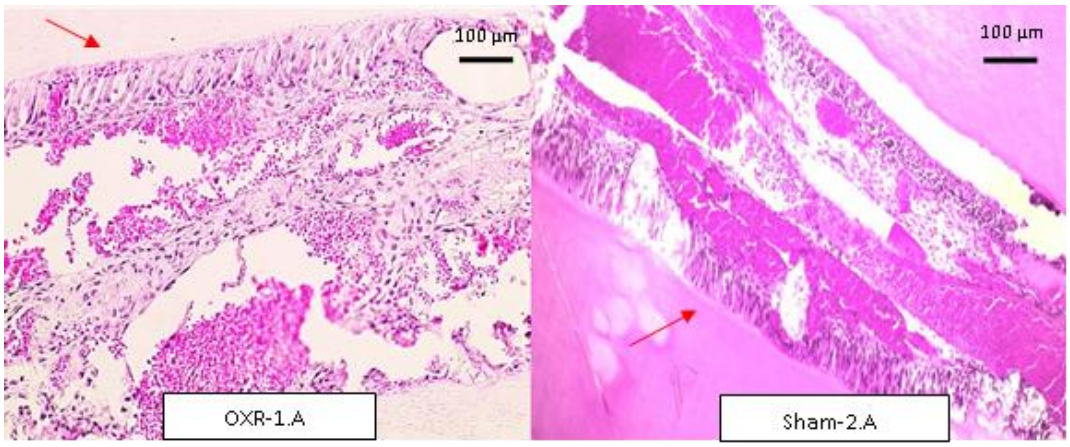

Fig. 3. Photomicrographs of the pulp region of the lower incisors. Odontoblasts are indicated by the red arrow

The dental pulps of the maxillary central incisors of the ORX-1.B group present a high degree of vascularization, large blood vessels, characterizing a pulpal hyperemia when compared to the Sham 2.B group. The ORX-1.A group presented less vascularized dental pulps than the Sham-2. A group, which presented intense vascularization, presence of decalcified structures in its central region and large and abundant vessels, a large number of odontoblasts close to the periphery, forming the pre-dentin with an aspect of normality in terms of arrangement and structure (Fig. 4).

3.3 Alveolar Bone

The alveolar bone of Group ORX-1.B in the lower incisor region showed immature bone formation, with a greater presence of early ossification processes characterized by young bone cells, spongy tissue, large trabeculae, presence of blood vessels, and with thin cortical plates significantly more evident when compared to Sham-2.A and B groups (Fig. 5).

In the maxillary incisor region, there is a relatively greater number of osteocytes and bone trabeculae in the ossification zone of the Sham groups compared to the ORX groups in the analysis of alveolar bone around all the roots. The ORX-1 groups present large gaps, evident angiogenesis process, neoformation characteristic of immature bone with vascularization present in the bone of the ORX-1 groups greater than that presented in the Sham-2.A and B groups and more evident in the ORX-1.B group, when compared to the ORX-1.A group (Fig. 6).

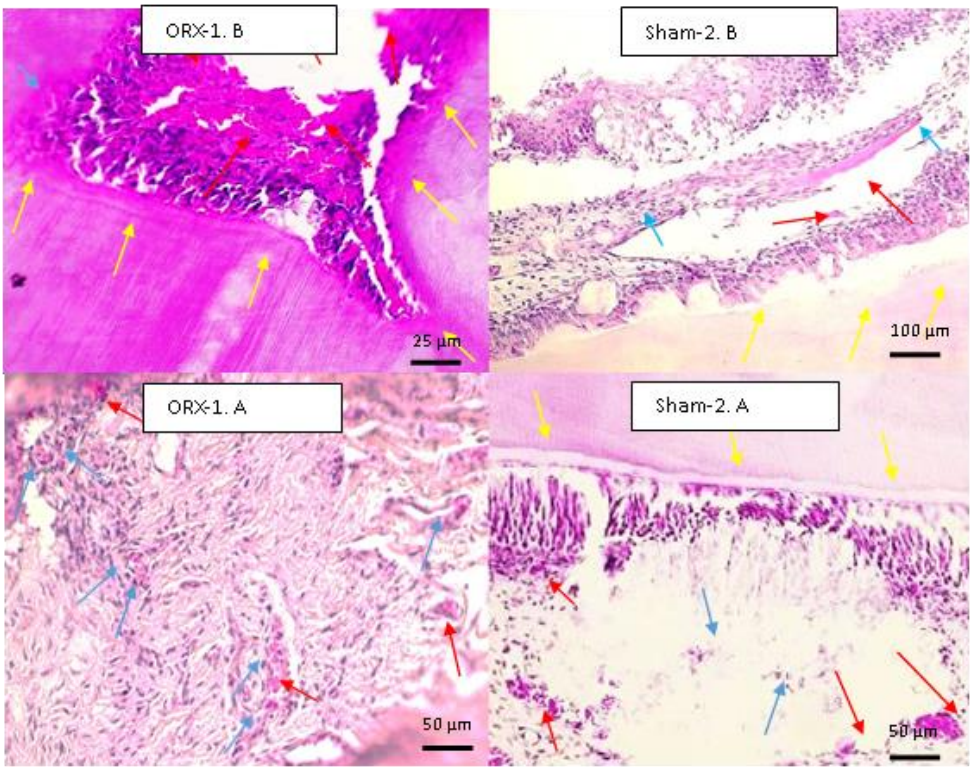

Fig. 4. Photomicrographs of the pulp region of the upper incisors. Red arrows indicate large-caliber vessels and blue arrows indicate smaller-caliber vessels. The yellow arrows indicate the pre-dentin/dentin interface layer

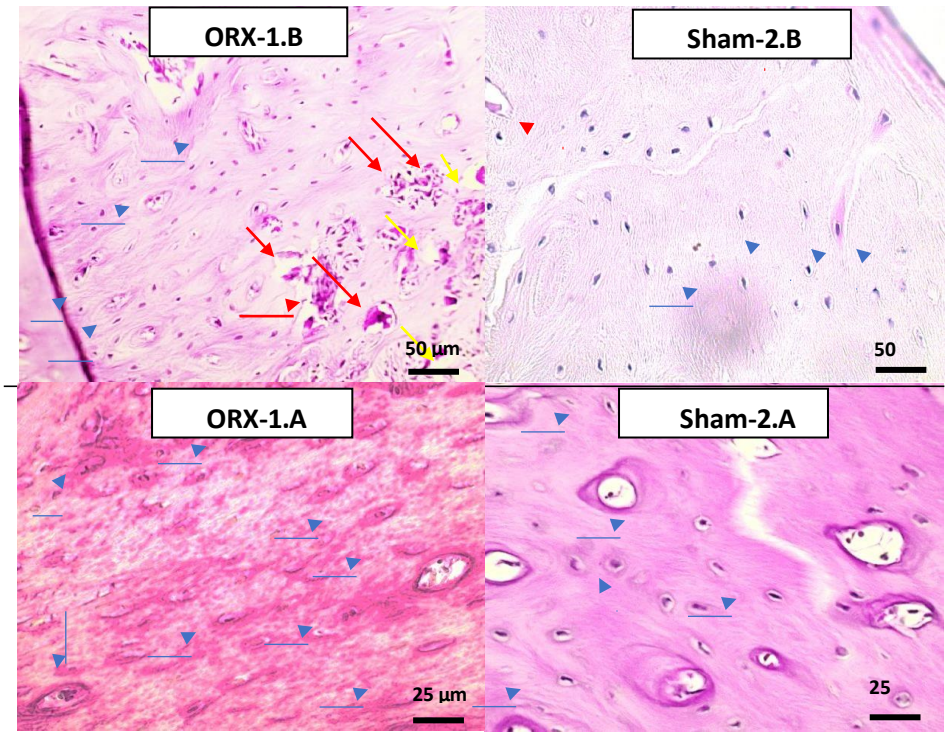

Fig. 5. Photomicrograph of mandibular alveolar bone section. The inferior ligament and globular areas (red arrows) are seen in contact with the alveolar bone. The osteocyte shown by the blue arrowhead is parallel

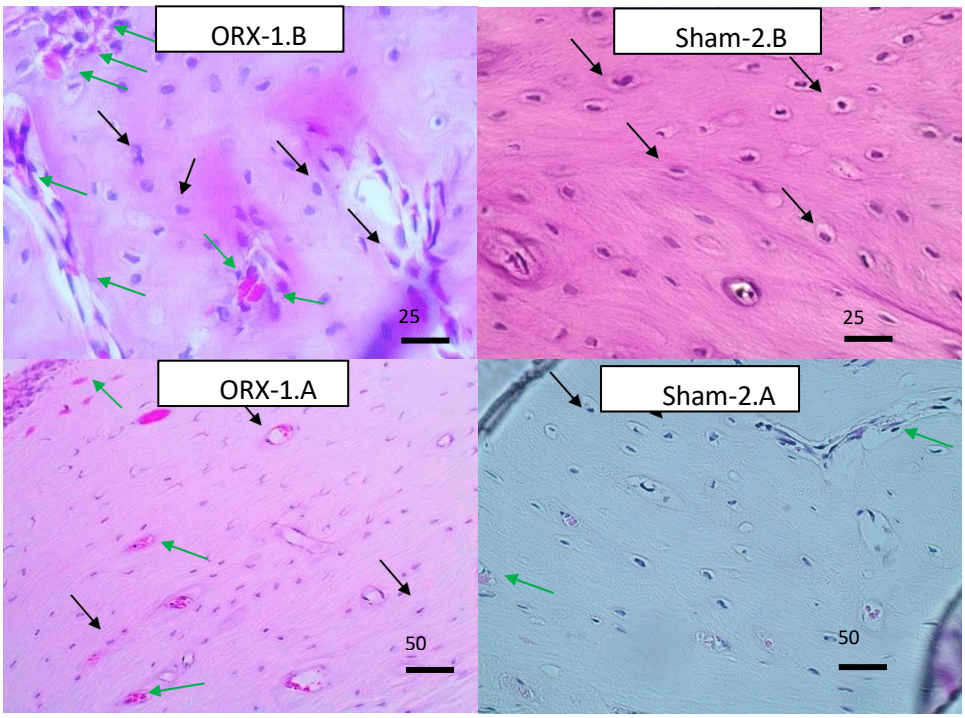

Fig. 6. Photomicrograph of the alveolar bone in the anterior region of the maxilla. Concentric lamellae (white arrows) involving vascularized and innervated channels and parallel lamellae (green arrow) can be observed. The osteocyte shown by the black arrow is parallel

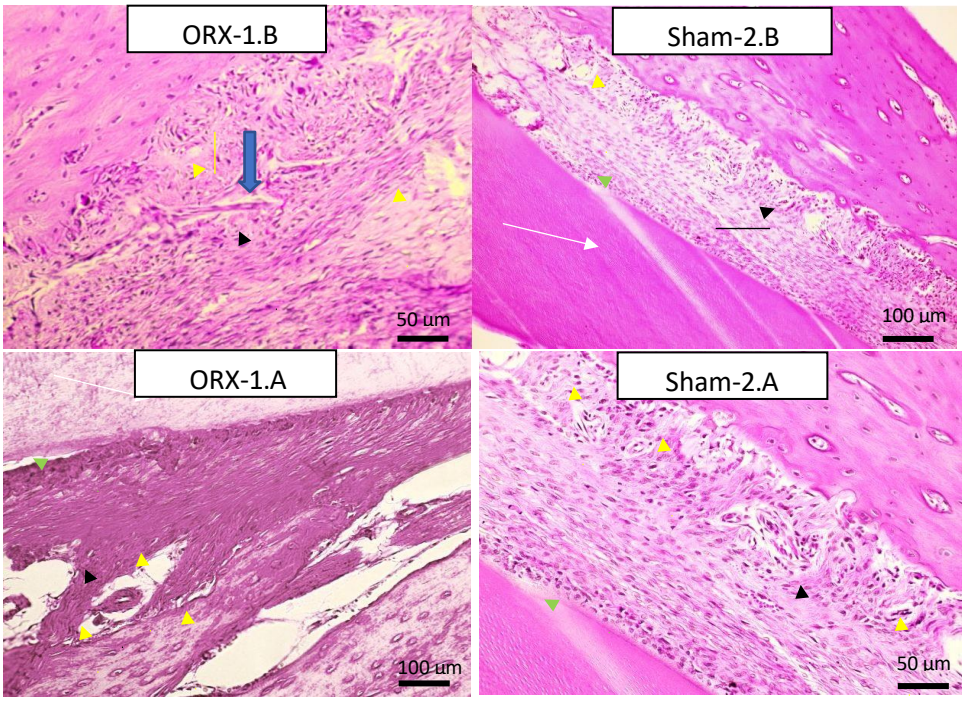

Fig. 7. Photomicrograph of the lower incisor periodontal ligament region. Note the dentin (white arrow), the space that would be occupied by enamel and cementum (green arrow). The inferior ligament and globular areas (black arrows) are highly vascularized and innervated. There is also a medium-sized vein (wide arrow). Sharpey's fibers, in the communication of the ligament with the bone, are indicated by the yellow arrow

3.4 Periodontal Ligament

The periodontal ligament of the region of the lower incisors of the ORX-1. A Group presents oblique collagen fibers, with normal thickness, considering teeth in masticatory function, in addition to the presence of macrophages and cementoblasts included in an amorphous material, but in smaller quantities when compared to the specimens of the ORX-1. B group. The evidence of periodontal ligament formation, differentiation, and function is more evident when the ORX-1 and Sham-2 groups are compared since in the Sham-2 groups the arrangements, the number of cementoblasts, and the differentiation are more evident and are in line with the normal aspects described in the literature, considering the age of the animals (Fig. 7).

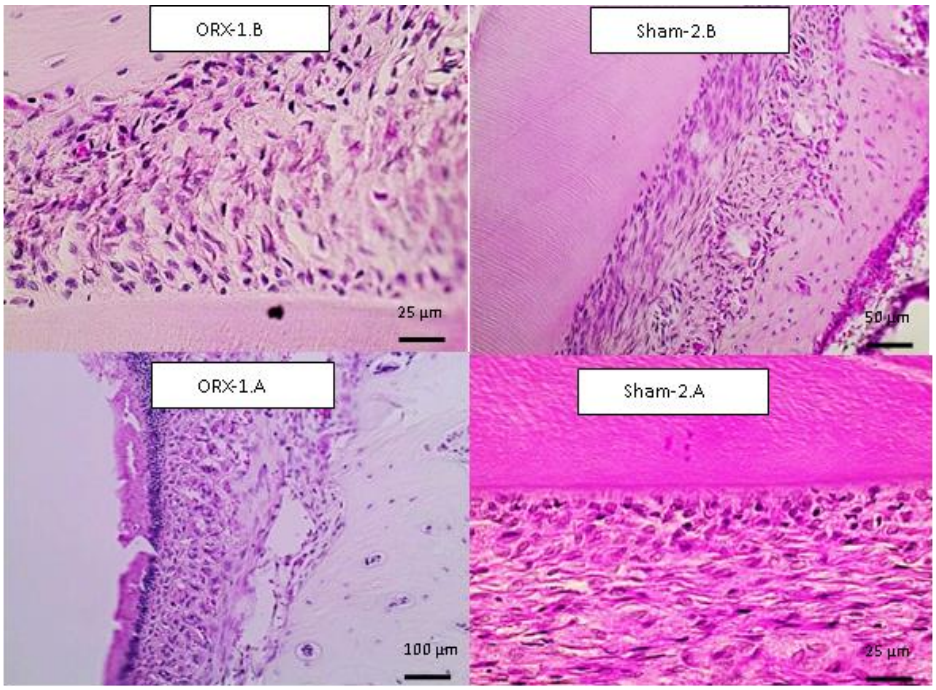

Fig. 8. Photomicrograph of the alveolar process of the anterior region of the maxilla. Ligament fibers were reduced in number and density in ORX when compared to Sham.

Table 1. Structural changes in the dentoalveolar tissues of the ORX and Sham groups

Structure	ORX Group	Sham Group
Dentin	Smaller mineralization front in terms of quantity and thickness in 73 days.	Normality patterns.
Pulp	Lesser degree of blood vessel formation, but with a larger caliber. Odontoblasts arranged along the entire peripheral part of the pulp with a "pulled" arrangement, presenting different degrees of maturation and length, little active functional action, with an elongated shape, basal nucleus, and cytoplasm with few basophils.	Normality patterns.
Alveolar Bone	Immature formation, with a greater presence of early ossification processes characterized by young bone cells, spongy tissue, large trabeculae, presence of blood vessels, and with thin cortical plates significantly more evidente.	Normality patterns.
Periodontal Ligament	The ligament fibers were reduced in number and density	Normality patterns.

Table 2. Collagen synthesis comparisons between groups

	Groups 45 days (SD)		*p-value*	Groups 73 days (SD)		*p-value*
	Sham (2.B)	ORX (1.B)		*Sham (*2.A)	ORX (1.A)	
Collagen area (Log10)	1.32 (0.10)	1.27 (0.31)	0.696	1.43 (0.14)	1.34 (0.22)	0.349

Note: SD means standard deviaton.

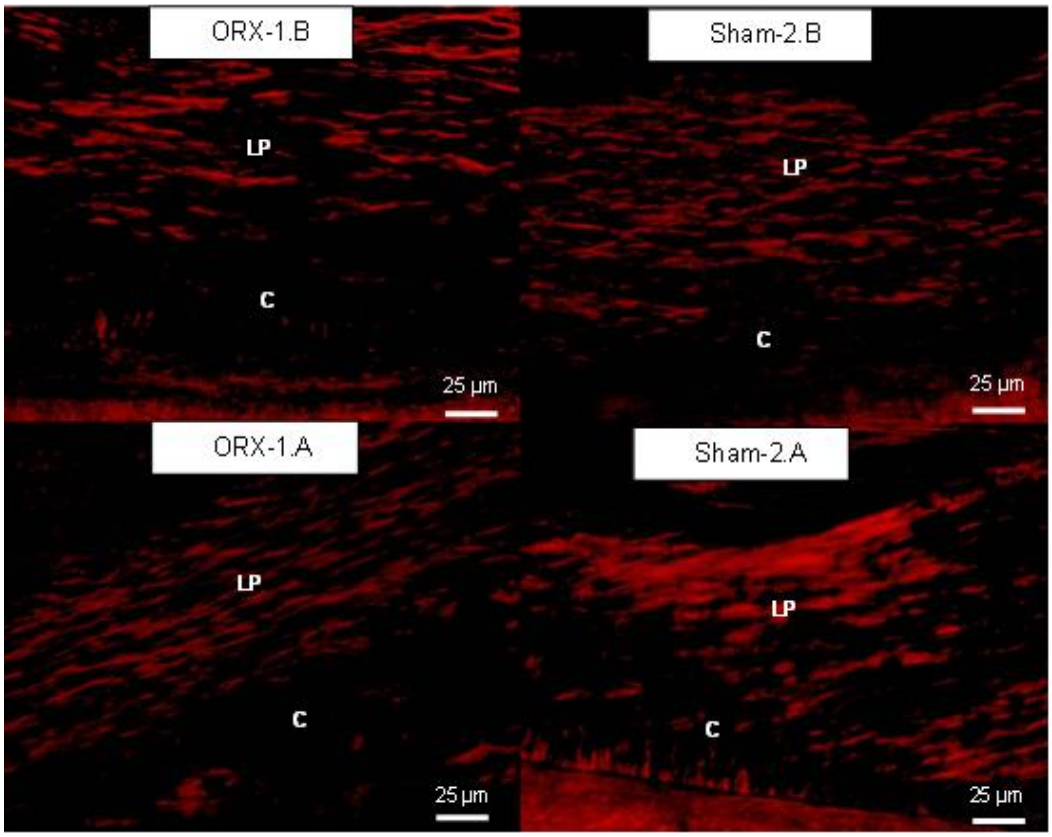

Fig. 9. Photomicrograph of the periodontal ligament under polarized lightLP: Periodontal *Ligament. C: Cement*

In the maxillary incisor region, the ligament fibers were reduced in number and density in the ORX-1 group when compared to the Sham-2 group. Alveolar bone showed horizontally arranged bone trabeculae, with increased medullary spaces and formation of new immature trabeculae more evidently in the ORX -1.A group compared to the Sham-2.A and 2.B groups. Continuous cemental line and without the presence of inflammatory infiltrate was observed in the Sham- 2.A group when compared to the ORX-1. A group, also presents the collagen fibers in normal position and insertion. The Sham- 2.B group presented a more intense vascularization in the apex region when compared to the ORX -1. B group (Fig. 8).

The changes that characterize the structural differences between the ORX-1 and Sham-2 groups are described in Table 1.

3.5 Cuts Stained with Prikrosirius

In the analysis of collagen quantification in the periodontal ligament, there was no statistically significant difference between the ORX-1 and SHAM-2 groups at any of the experimental times (Table 2) (Fig. 9).

4.DISCUSSION

The results of this work allows to confirm the initial hypothesis that testosterone suppression impacts the differentiation and metabolism of cells that form dental structures. The absence of this hormone considerably reduced the formation of tertiary dentin of the lower incisors, with impact, more specifically, on the dentin mineralization front. Some studies corroborate with our results, as follows: Wang et al. [5] demonstrated that the canines of ovariectomized monkeys had a smaller area compared to

monkeys in the control group; the human studies by Gaethofs et al. [19] and Roberts et al. [18] demonstrate a direct impact of testosterone on adolescent dental age during puberty; regarding mineralization. Reis et al. [7] demonstrated that in the endochondral growth area of the mandible there is a delay in the mineralization of the trabecular bone in ovariectomized animals, a process similar to the mineralization of dentin. To the best of our knowledge, no study has evaluated the dentin structure of ovariectomized animals in histological sections. We hypothesize that testosterone directly affects the mineralization process, and reduced levels of this hormone decrease both odontoblast differentiation and function. This impact can affect tooth size and decrease the quality of dentin, which could be associated with more susceptible to dental caries and developmental defects of enamel.

However, when the maxillary incisors are evaluated, it is noted that there are no differences between the experimental and control groups. Studies indicate that the morphogenesis of each type of tooth is differently regulated by different growth factors [30-32]. Thus, it is reasonable to hypothesize that testosterone suppression may affect a specific type or group of teeth [33-35].

During 45 to 73 days, a decrease in the downward trend of new dentin formation and odontoblast differentiation was observed. During this period, there is also a drop in serum levels of testosterone, GHR, and IGF-1 in healthy rats, which indicates the animal's hormonal peak and corroborates the hypothesis that testosterone would have a greater impact on the animal's growth during the period from 40 to 50 days of life, which is the period of peak hormones [36-38].

The amount and morphology of ameloblasts were similar between groups regardless of the experimental time. Since ameloblasts are sensitive to testosterone through Androgen Receptors [9], it was expected that there would be an impact on the number or morphology of these cells. However, the literature has not yet shown whether testosterone impacts the differentiation of mesenchymal cells into mature ameloblasts, despite having already demonstrated an impact on the differentiation of odontoblasts and fibroblasts [8,39]. More studies are needed to identify the possible effects of testosterone on ameloblasts and, consequently, enamel.

The results demonstrate that the dental pulp of ovariectomized animals has vascularization and vasodilation more accentuated when compared to the control group, characterizing pulpal hyperemia. This hyperemia may be due to the impact of testosterone on the levels of inflammatory mediators, which modify angiogenesis in the pulp in the face of chemical or physical aggression [30]. Machado et al. [16] did not find any pulpal changes when performing the orthodontic movement in ovariectomized animals compared to animals in the placebo group. However, the different ages of the animals between the studies may explain this divergence.

Testosterone influences the development of rats from pregnancy onwards [40]. However, Verdonck et al. [26] demonstrate that testosterone would have an impact on craniofacial growth only between 40 to 50 days, and not during the animal's childhood. The period between 40 to 50 days corresponds to the peak of testosterone, GH, and IGF-1 in healthy animals [37-39]. Thus, testosterone suppression would only have an impact at the time of the peak of these hormones, and orchiectomy before puberty would be the most appropriate time to assess its effects on the development of dentoalveolar structures, as shown by the results found in our study.

The findings in the alveolar bone region agree with Schour [41], Shapiro and Shklar [8], Shklar et al. [11], Mohamad et al. [29], Gonçalves et al. [15], Steffens et al. [12-13] and Reis et al. [7]. These previous studies demonstrated that there is an impact of testosterone suppression on the proliferation of precursor cells in the periosteum, which results in a lower number of osteoblasts, osteocytes, and immature bone. However, at the end of puberty, testosterone ceases to influence this proliferation, suggesting, again, that the hormone has greater impacts only during the pubertal peak. The impact of testosterone on dentoalveolar development may occur indirectly by regulating angiogenesis. Angiogenesis is a physiological process that allows for bone turnover and remodeling. Testosterone has already been used as a therapeutic process in bone lesions in rats and one of its main effects is the promotion of angiogenesis at the lesion site [41,42]. Angiogenesis is necessary for the renewal and stimulation of intramembranous growth, in addition to being responsible for the arrival of odontoblasts in the formation zone [43,44].

In the qualitative analysis of the periodontal ligament, the number and density of collagen fibers were smaller. However, in the quantitative analysis, no statistical differences were observed between the groups at any experimental time. Due to the influence of testosterone on fibroblasts, it was expected that there would be a direct impact on collagen production, as demonstrated by Reis et al. [7] and Arai et al. [45]. We hypothesize that, in this study, it was possible to evaluate only the middle portion of the lower incisor root, and not the periodontal ligament as a whole, which can be considered a limitation of this study.

The evaluation of the influence of testosterone suppression on dental and periodontal structures makes this study relevant, as it corroborates the advance in the understanding of the etiology of hormonal deficiencies in dental and periodontal physiological changes. In addition, this study also suggests that further research in humans be carried out to measure serum testosterone levels during puberty and correlate with the repair response of dentoalveolar structures. This study also highlights the importance of serological analysis of the patient before starting orthodontic and periodontal treatment to identify factors that may be significant during growth and during orthodontic movements, such as low testosterone levels, which may directly influence the host response to treatment.

The unfeasibility of performing statistical analysis of all histological observations is a limitation of this study. However, it is possible to infer that the analyzed structures are affected by testosterone suppression only in qualitative comparisons between groups. Another limitation was the absence of serum testosterone measurements, but orchiectomy is a procedure accepted and effective in the literature for the reduction of testosterone levels [45].

5. CONCLUSION

Testosterone suppression induces changes in the differentiation of cells that form the tissues of dental and alveolar structures, through the incidence of pulp changes, presence of atypical cells in the periodontal ligament and delay in the neoformation of alveolar bone in rats during puberty. Future studies should assess the impact of testosterone suppression in humans, as well as whether supplementation has the potential to reverse the changes observed. The results of this study are relevant to clinical practice in that they awaken health professionals to the need to investigate androgen levels in patients and identify changes in the development of dentoalveolar structures resulting from dysfunction.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The procedures involving animal models were previously subjected to Ethics Committee on the Use of Animals of the Federal University of Alfenas, which approved this project under protocol number 024/2019. All experiments have been examined and approved by the appropriate ethics committee.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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