Original Article

Estrogen-deficiency Effect on the Composition of Dental Enamel: A Pilot Study

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ABSTRACT

Background: Tooth enamel mineralization is assumed to be a target of endogenous estrogen imbalances. **Objective:** To evaluate the effect of estrogen deficiency during amelogenesis on the mineral composition of dental enamel. **Methods:** Ten female Wistar Hannover rats were randomly divided into two groups according to the intervention received: ovariectomy surgery (OVX, experimental) and fictitious surgery (SHAM, control). After 21 days, the rats of both groups were euthanized, and the upper incisors were extracted for analysis of the mineral composition by energy-dispersive X-ray fluorescence. The sensitivity of the enamel organ to estrogen was evaluated in both groups by immunohistochemical analysis of the odontogenic region of the lower incisors for the presence of estrogen receptors alpha and beta (ERα and ERβ, respectively) in ameloblasts in the maturation stage. Differences in the mineral composition between groups were compared using Student's *t*-test ($P < 0.05$). **Results:** No statistically significant difference was detected in the mineral composition between the OVX and SHAM groups ($P > 0.05$). ERα was immunostained in the ameloblasts of both groups. **Conclusion:** Although ameloblasts express ERα, estrogen deficiency during amelogenesis did not appear to affect the dental enamel composition in this murine model.

KEYWORDS: *Amelogenesis, dental enamel, estrogens*

BACKGROUND

The dental enamel, which protects the tooth from external damage, is a highly mineralized tissue

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consisting of 95–97% hydroxyapatite by weight.[1] Its main organic component, accounting for about 90% of the extracellular matrix, is amelogenin,[2] which is required for proper enamel development, the control of the crystal size and growth, the organization of the prismatic pattern, and the regulation of the enamel thickness.[3,4] Enamel formation occurs during the embryological and postnatal period,[5] when hydroxyapatite-like crystals are formed in the extracellular environment; this requires the presence of calcium and phosphate in the initial stages of crystal growth.[6]

Amelogenesis, which is the formation of tooth enamel, seems to be involved by the hormone called estrogen; however, there is still a gap in the literature.[7] Previous studies have reported that ameloblasts express estrogen receptors (ERs), and this raises the possibility that estrogen deficiency during amelogenesis could lead to alterations in the mineral content of tooth enamel, thereby affecting tooth development. Estrogen does not act solely as a female sex hormone for gonadal organ functions; it also has critical actions in extragonadal tissues in both genders, with increasing evidence for an involvement in both tissue-specific and cell-specific synthesis and signaling.[7-10] Moreover, estrogen regulates many physiological processes, including cell growth, reproduction, differentiation, and development.[8-12] Cellular estrogen signaling is mediated through estrogen receptors alpha and beta (ERα and ERβ, respectively).[13] Estrogen is present both during embryological life (at every gestational stage) and postnatal life.

Dental enamel has also been reported as an additional target for endocrine disrupters, such as bisphenol A, which has estrogenic effects and may be a causal agent of molar-incisor hypomineralization.[14,15] Several studies have demonstrated the expression of ERs in the tooth, specifically in odontoblasts,[13] the dental pulp,[16,17] and ameloblasts.[7,18] ERα expression has been shown in preameloblast proliferative cells in rats.[7]

The aim of this pilot study was to assess (i) the expression of ERs in ameloblasts and (ii) whether estrogen deficiency during the amelogenesis period affects the enamel mineral content in a murine model.

Materials and Methods

Ethical aspects

The study was conducted and reported according to the ARRIVE guidelines[19] and was approved by the Ethical Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil (#2018.1.40.58.3).

Experimental design

Ten female prepuberal Wistar Hannover rats (with 20 upper and 20 lower incisors), at 21 days of postuterine life, were selected for this pilot study. The animals originated from a previous study on tooth eruption rate.[20] The sample size was calculated using the G Power® 3.1.9.2 software (Düsseldorf, Germany), as reported in our previous study.[20] A β of 0.20, an α of 0.05, and an allocation rate of 1:1 were used. The animals were housed in the Bioterium II of the FORP/USP, with a controlled temperature environment and a 12-h light–dark cycle, with feed (Labina Purina®/Agribrands do Brasil LTDA, Paulínia, Brazil) and filtered water provided *ad libitum*.

The animals were coded by a strip on the tail and randomly divided into two groups according to the type of intervention: ovariectomy surgery (OVX, experimental) and fictitious surgery (SHAM, control). The OVX group underwent bilateral excision of the ovaries to cause an endogenous reduction in the circulating estrogen levels,[21-23] whereas the SHAM group underwent fictitious surgery without any damage to the ovaries.[24]

The animals were monitored throughout the pubertal period and were euthanized after 56 days of life. Puberty in rats of this lineage begins on the 35th to the 55th day of postuterine life, with a peak release of estrogen occurring in this time interval.[25] The success of the ovariectomy was confirmed by a gradual increase in body weight during the trial period and by weighing the atrophic uterus of the rats, as previously described by Chen, *et al.*[26] The decrease in endogenous estrogen release caused by ovariectomy causes significant differences in variables related to body weight and uterine weight. The OVX group, therefore, tended to present an increase in body weight and a decrease in the weight of the uterus when compared to the SHAM group.[26] Other animal studies have also used this method.[23,26,27] Animals that failed the surgical procedures or that died before reaching 56 days of life were excluded from the study.

After euthanasia, both upper incisors were extracted from each rat to assess the mineral content. The expression of ERs in ameloblasts was identified by isolating the odontogenic regions of both lower incisors for immunohistochemistry analysis.

Analysis of the enamel mineral content

The upper incisors were carefully extracted and embedded in acrylic resin (longitudinally). The tooth surface immersed in acrylic was polished until the opaque enamel boundary was visualized, keeping the same region for all teeth. The polishing was performed

with water and sandpaper of descending granulation levels (600–4000). The samples were analyzed for the maturation stages of the enamel by energy-dispersive x-ray fluorescence (ED-XRF, JEOL JMI4000; Peabody, MA, USA). ED-XRF is a nondestructive trace elemental microanalysis technique based on conventional ED-XRF and has the ability to probe extremely small sample amounts, making it a superior analytical technique for tooth samples.[28]

The percentages of the following mineral components were determined: calcium (Ca), phosphorus (P), carbon (C), oxygen (O), sulfur (S), chlorine (Cl), iron (Fe), and zinc (Zn). Other components observed were discarded from the results.

Analysis of ERα and ERβ in ameloblasts

The odontogenic region of the lower incisor was removed and fixed in 10% formalin for 24 h. The specimens were then decalcified in a 4.13% ethylenediamine tetraacetic acid (EDTA) solution (pH: 7.0–7.4) for a period of 30 days. The samples then underwent routine histochemical processing for embedding in paraffin. The paraffin blocks containing the specimens were cut with a microtome (Leica RM2145; Leica Microsystems®, Wetzlar, Germany) in the longitudinal and anteroposterior direction, semi-serrated, at a thickness of 3 µm. Thirty histological slides containing three or two sections each were obtained from each block.

Immunohistochemistry was performed by placing the sequential sections on slides coated with organo-silane (StarFrost®, Lowestoft, UK). The immunohistochemical reactions were performed using the immunoperoxidase technique. The slides were incubated overnight with the following primary antibodies (diluted in 1% BSA): ERα (clone 2Q418, 1:200 dilution; Santa Cruz Biotechnology, California, USA) and ERβ (clone B-1, 1:200 dilution; Santa Cruz Biotechnology, California, USA). After returning to room temperature and washing, the slides were incubated with biotinylated secondary antibodies (IgG2a, 1:200 dilution; Santa Cruz Biotechnology, California, USA) for 1 h at room temperature. The streptavidin-biotin-peroxidase complex reaction was then run for 30 min, followed by the addition of the chromogen 3,3′ diaminobenzidine tetrahydrochloride hydrate (Dako Products®, Glostrup, Denmark), along with 3% hydrogen peroxide in phosphate buffered saline (PBS) for 1 min. The slides were counterstained with Carazzi's hematoxylin. Negative control specimens were also run by replacing the primary antibody with isotype-specific serum.

Microscopy analysis (AXIO IMAGER.M1; Carl Zeiss, Jena, Germany) was performed on a digital camera (AXIOCAM MRc5; Carl Zeiss, Jena, Germany) by a previously trained evaluator. The results were evaluated by the same precalibrated blind examiner as the presence or absence of the immunomarkers.

Statistical analysis

The differences between the groups were compared using Student's *t*-test. The significance level was set at 5%.

Results

The success of the ovariectomy was confirmed by adequate survival of the animals and a gradual increase in body weight during the trial period, as well as by the weight of the atrophic uterus. The gain in body weight was greater in the OVX group than in the SHAM group ($P = 0.002$). After euthanasia, the OVX group showed significant uterine atrophy when compared to the SHAM group ($P \leq 0.0001$).

Table 1 shows the mean and standard deviation (SD) of the tooth mineral content of both groups (OVX and SHAM). The mean distribution did not show any statistically significant difference between the groups ($P > 0.05$).

Longitudinal sections of the odontogenic region showed nuclear immunoexpression of ERα in ameloblasts of both groups [Figure 1]. ERβ can show nuclear and cytoplasmic immunoexpression, but cytoplasmic immunoexpression of ERβ was absent in the ameloblasts of both groups [Figure 2].

Discussion

Dental enamel is one of the most remarkable examples of matrix-mediated biomineralization. Enamel crystals form in an extracellular environment, producing complex microstructural patterns through a process orchestrated by ameloblast cells.[6] The main goal of this study was to assess whether estrogen deficiency

Table 1: Influence of the experimental model (OVX and SHAM) on the mineral composition of dental enamel

Mineral composition of dental enamel	OVX group, mean (SD)	SHAM group, mean (SD)	*P value*
C	8.47 (4.07)	8.59 (4.57)	0.95
O	45.01 (3.11)	44.99 (2.87)	0.99
P	17.38 (1.05)	17.13 (1.19)	0.37
S	0.13 (0.05)	0.14 (0.07)	0.56
Cl	0.09 (0.08)	0.07 (0.09)	0.46
Ca	28.58 (0.89)	28.72 (1.26)	0.78
Fe	0.03 (0.02)	0.03 (0.05)	0.55
Zn	0.31 (0.28)	0.32 (0.27)	0.89

affects amelogenesis. This is an important question, as estrogen strongly influences the mineralization process and could impact primary and permanent tooth formation.

Dental enamel containing hydroxyapatite-like crystals is considered to function as a nonstoichiometric carbonated Ca^{2+} hydroxyapatite, so it also incorporates ions, such as Na^{+}, Mg^{2+}, Cl^{-}, and Fe^{3+}. These other ions compete for space in the crystal, thereby influencing the properties of the dental enamel.[6] Therefore, in this study, we evaluated some elements from the dental enamel (Ca, P, C, O, S, Cl, Fe, and Zn) and assessed the possible influence of estrogen on the tooth mineral content. However, the results obtained did not indicate that estrogen deficiency affected the enamel mineral content, in contrast to a previous study showing that the enamel microhardness was significantly reduced in animals with estrogen deficiency.[29]

The animals in this study were estrogen-deficient because of the ovariectomy procedure; however, other cells, such as those from nongonadal organs (liver, heart, skin, and brain), continue to produce estrogen.[30] In addition, ERs can be modulated by other hormones such as testosterone and growth hormone.[31] Therefore, if estrogen plays only a small role in dental enamel mineralization, its reduction might not be detected in the OVX animal models used, suggesting caution in the interpretation of the results. The presence of ERα and the absence of ERβ were confirmed by the immunohistochemical analysis, suggesting that estrogen

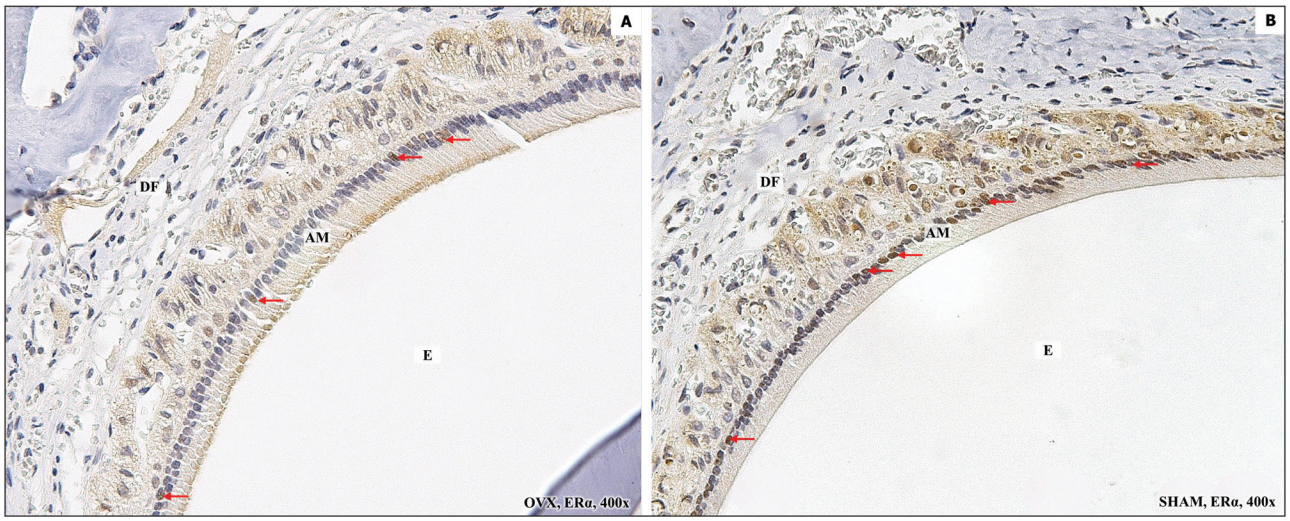

Figure 1: Photomicrographs of the portion referring to the ameloblast region following immunohistochemistry for ERα. ERα is immunoexpressed in both the SHAM (A) and OVX (B) groups, as indicated by the red arrows. AM = ameloblasts, DF = dental follicle, E = enamel

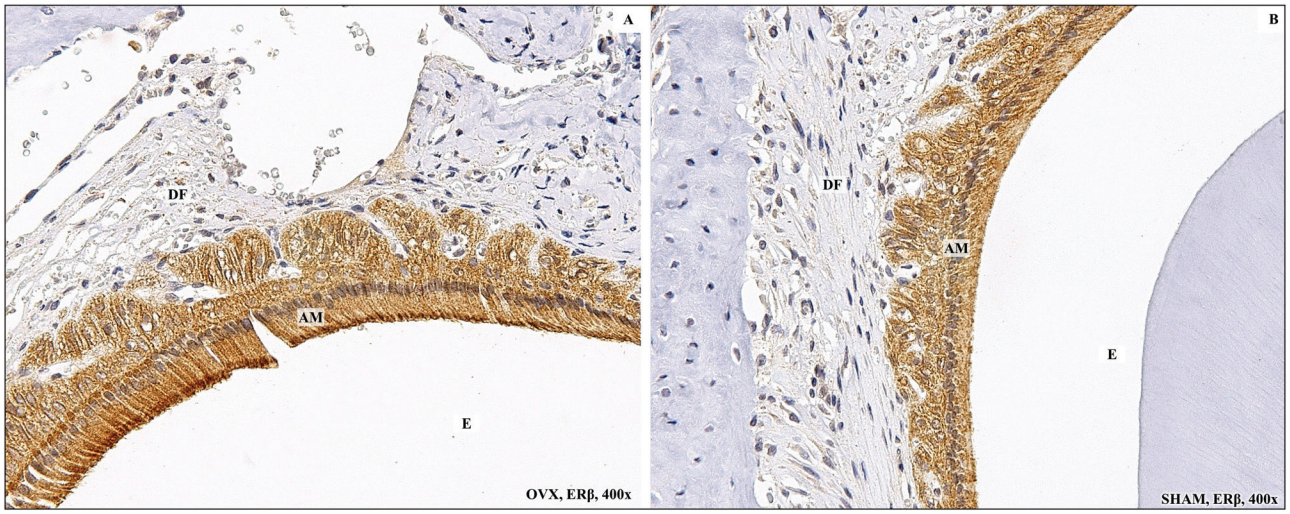

Figure 2: Photomicrographs of the ameloblast region following immunohistochemistry for ERβ. Erβ was not immunoexpressed in either the SHAM (A) or the OVX (B) group. AM = ameloblasts, DF = dental follicle, E = enamel

plays a role in enamel formation. One important point to emphasize that the estrogen hormone has a multifunctional role and might be involved in other aspects of amelogenesis. Interestingly, both males and females expressed similar levels of ERα[7,14,18] in the maturation-stage ameloblasts.

Scientific evidence is lacking regarding the real changes in the dental enamel caused by estrogen in animals. Thus, this study in female rats can be viewed as adding some new information to this field. Although we were not able to confirm a role for estrogen in dental enamel mineralization, our study findings suggest that estrogen deficiency during pregnancy and/or during childhood (in the phases where amelogenesis occurs) does not impact the mineral component of the dental enamel. The evaluation of uterine weight and atrophy is considered more humane than serological evaluation and vaginal smears; however, the absence of estrogen level measurements in our groups could be a limitation of our study. Evaluation of body and uterus weight is an established protocol,[26] but estrogen measurements could provide valuable additional results to our study. Other studies that address estrogen levels should be performed during the time when microhardness changes, as previously reported.[29] The small size sample in this study could also have led to false-positive results; therefore, studies with larger samples should be performed based on the results of this pilot study to establish better results.

Conclusion

Although ameloblasts showed ERα expression, estrogen deficiency during amelogenesis did not appear to affect the mineral component of the dental enamel in a murine model.

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Conflicts of interest

There are no conflicts of interest.

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