UNIVERSIDADE POSITIVO
MESTRADO PROFISSIONAL EM ODONTOLOGIA CLÍNICA

A DEFICIÊNCIA DO ESTRÓGENO NO DESENVOLVIMENTO ESQUELÉTICO CRANIOFACIAL E NA EXPRESSÃO GÊNICA DE RANK, RANKL E OPG NO PERÍODO PUBERAL DE RATAS

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RESUMO

O estrogênio desempenha uma função importante no controle da aceleração e fusão óssea. O objetivo deste estudo *in vivo* foi investigar se o efeito de baixos níveis de estrogênio no período pré-púberal em ratas ovariectomizadas interfere nas medidas ósseas craniofaciais no período pós-puberal, e na expressão gênica de RANK, RANKL e OPG nos locais de crescimento ósseo. Cirurgias de placebo (Sham) e ovariectomia (OVX) foram realizadas em ratas Wistar com 21 dias de idade. Parte dos animais foram eutanasiados aos quarenta e cinco dias de idade (período puberal) para quantificar a expressão gênica de RANK, RANKL e OPG. A outra parte dos animais foram eutanasiados aos sessenta e três dias de vida (período pós-puberal) e seus crânios foram submetidos a microtomografia computadorizada para obtenção de medidas craniofaciais. As medidas bidimensionais e tridimensionais foram coletadas com base em imagens de microtomografia, utilizando os softwares ImageJ® e VGSTUDIO MAX® 3.3.2. A normalidade da amostra foi testada pelo teste de Shapiro-Wilk e a análise comparativa foi realizada pelo teste t-Student para as medidas craniofaciais. Para a comparação entre grupos da expressão gênica foi utilizado o teste Mann-Whitney. A significância estatística foi determinada para valores com probabilidade acima de 95% (alfa=0,05). Diferenças estatísticas significativas foram encontradas entre os grupos Sham e OVX, tanto em medidas mandibulares, quanto em medidas maxilares bidimensionais ($p > 0,05$). O volume condilar também foi aumentado no grupo OVX ($p > 0,05$). O grupo Sham apresentou maior nível de expressão de RANK na sutura palatina mediana ($p=0,036$), e os níveis de RANKL:OPG foram maiores no grupo OVX

(p=0,015). Os resultados sugerem que a deficiência de estrogênio durante o período pré-púbere pode causar aumento do crescimento ósseo maxilar e mandibular em ratas ovariectomizadas no período pós-puberal.

Palavras-chave: Estrogênios. Desenvolvimento ósseo. Expressão Gênica. Microtomografia por Raio-X.

Lara RM. The role of estrogen deficiency in craniofacial skeletal development and in the gene expression of RANK, RANKL and OPG during pubertal period of female rats [Master's Thesis]. Curitiba: Universidade Positivo; 2020.

ABSTRACT

Estrogen plays an important role in controlling bone acceleration and fusion. The aim of this *in vivo* study was to investigate whether the effect of low estrogen levels in the prepubertal period in ovariectomized rats interferes with craniofacial bone measurements in post pubertal stage and to quantify the gene expression of RANK, RANKL and OPG in the growth sites. Placebo (Sham) and ovariectomy (OVX) surgeries were performed on twenty-one-day-old Wistar rats. Part of the animals were euthanized at forty-five days old (pubertal stage) to quantify the gene expression of RANK, RANKL and OPG. The other part of the animals were euthanized at sixty-three days old (post-pubertal stage) and their skulls underwent to computed microtomography. Two-dimensional and three-dimensional measurements were collected based on microtomography images. The normality of the sample was tested by Shapiro-Wilk test and the comparative analysis was performed using t-Student test, for the measurements. For gene expression, comparison among the groups, Mann-Whitney U test was used. The statistical significance was determined for values with probability over 95% (alpha = 0.05). Significant statistical differences were found between Sham and OVX groups for mandibular, as well as maxillary measurements in the two-dimensional ($p<0.05$). The condylar volume was also bigger in OVX group ($p<0.05$). Sham group had a higher level of RANK expression in the midpalatal suture ($p=0.036$), and RANKL:OPG ratio levels were higher in OVX group ($p=0.015$). Results suggest that estrogen deficiency during prepubertal period can cause increased maxillary and mandibular bone length in ovariectomized rats.

Keywords: Estrogens. Bone Development. Gene Expression. X-Ray Microtomography.

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INTRODUÇÃO

O estrogênio (estradiol, estriol e estrona) é um hormônio sexual produzido principalmente pelos ovários nas mulheres. Entretanto, outros tecidos e células também produzem estrógeno. Nos homens, a maior produção desse hormonio é proveniente do tecido adiposo e do cérebro. Estudos populacionais têm indicado claramente que o estrogênio é necessário para a regulação da homeostase esquelética das mulheres e dos homens (Khosla *et al.*, 2001; Khosla *et al.*, 2002; Gennari *et al.*, 2003). Este hormônio possui como alvo diversas células do organismo, inclusive células presentes na maxila e na mandíbula (Gennari *et al.*, 2005).

Aceita-se que o estrogênio desempenhe um papel importante no controle da aceleração e fusão da placa de crescimento. Sua ação no desenvolvimento ósseo feminino é iniciada no estágio embrionário (Ben-Hur *et al.*, 1993) contínuo através do surto de crescimento puberal (Fujita *et al.*, 2004) e continua acontecendo para realizar a manutenção da homeostase óssea na idade adulta (Callewaert *et al.*, 2008). Esse hormônio induz citocinas a desempenhar duas funções opostas, durante duas fases distintas da puberdade: durante o período pré-púbere, a atividade do estrogênio é sistêmica e ocorre de acordo com o hormônio do crescimento (GH), causando alongamento ósseo e desenvolvimento (D´Angio *et al.*, 2003; Perry *et al.*, 2008). Após esse período de crescimento, ocorre o período conhecido como pós-puberal, quando o estrogênio atua no nível local e em menor quantidade, causando a fusão epifisária, resultando na maturação óssea (Perry *et al.*, 2008; Emons *et al.*, 2011).

O estrogênio atua em seus receptores, receptor alfa de estrogênio (ER-α) e receptor beta de estrogênio (ER-β), mantendo o equilíbrio entre as atividades de osteoblastos e osteoclastos (Cutler, 1997; Perry *et al.*, 2008). Sugere-se que o estrogênio possa desempenhar um papel importante na homeostase esquelética, pois esse hormônio pode influenciar e regular as citocinas envolvidas no processo de desenvolvimento, modelagem e remodelação óssea.

Mais especificamente, o estrogênio pode exercer seus efeitos anti-reabsorção no osso, estimulando os receptores de alta afinidade (ERs) nos osteoblastos (Bord *et al.*, 2003; Streicher *et al.*, 2017).

Baixos níveis de estrogênio em mulheres podem ser causados por diferentes fatores e os efeitos causados por essa deficiência dependem da idade, saúde geral e outros fatores. É sabido que baixos níveis de estrogênio podem levar a alterações na microarquitetura óssea em fêmures e mandíbulas de ratos (Hendrijanti *et al.*, 2019; Hsu *et al.*, 2016; Tanaka *et al.*, 2003; Tanaka *et al.*, 2002; Yang *et al.*, 2005) osteoporose (Ejiri *et al.*, 2008) e instabilidade na homeostase esquelética (Hernández *et al.*, 2011; Zaidi *et al.*, 2018). Também foram relatados casos de deficiência de aromatase em meninas pré-puberal e deficiência de estrogênio devido a mutações no gene da aromatase (CYP19), que poderiam afetar o desenvolvimento esquelético (Belgorosky & Rivarola, 2004; Belgorosky *et al.*, 2009) diminuição da densidade mineral nos ossos e retardam a maturação epifisária (Mullis *et al.*, 1997).

Além disso, já está bem estabelecido na literatura que os hormônios sexuais, incluindo o estrogênio, regulam a tríade RANK (receptor ativador do fator nuclear kB), RANKL (receptor ativador do fator nuclear ligante kB) e OPG (osteoprotegerina), que são membros da superfamília dos fatores de necrose tumoral. O RANKL é fundamental para a diferenciação, ativação e sobrevivência dos osteoclastos. O OPG é um receptor chamariz solúvel para RANKL, ligando-se no mesmo e inibindo a osteoclastogênese (Lacey et al., 1998; Kong et al., 1999). O RANKL atua através de seu receptor RANK, que é expresso na membrana celular dos osteoclastos e células precursoras dos osteoclastos (Li et al., 2000). (Figura 1)

Alguns estudos usando modelos animais foram realizados para descrever como a deficiência de estrogênio afeta o desenvolvimento de estruturas craniofaciais. Observou-se crescimento mandibular afetado quando injetado antagonista do Erβ em camundongos recém-nascidos (Hernández *et al.*, 2011), redução do crescimento do osso nasomaxilar e mandíbula quando realizada uma ovariectomia em camundongos com cinco dias de idade (Fujita *et al.*,

2004) e inferiores fração de volume ósseo e espessura trabecular em mandíbulas de ratos ovariectomizados com 20 semanas de idade (Hsu *et al.*, 2016). Por outro lado, medidas maiores da largura do côndilo foram encontradas em camundongos quando a deficiência de estrogênio foi criada em camundongos com oito semanas de idade (Fujita *et al.*, 2001), embora a largura normal, baixo volume ósseo trabecular do côndilo e redução na densidade mineral óssea também foram encontrados (Fujita *et al.*, 2006; Ejiri *et al.*, 2008; Hsu *et al.*, 2016).

Desta forma, o presente estudo tem como objetivo avaliar os efeitos da deficiência de estrogênio no período pré-puberal nas dimensões da maxila e mandíbula na fase adulta, tendo em vista que os estudos presentes até então não trazem unanimidade acerca do assunto e a microtomografia computadorizada traz melhor qualidade para avaliação do desenvolvimento ósseo se comparado a radiografia convencional.

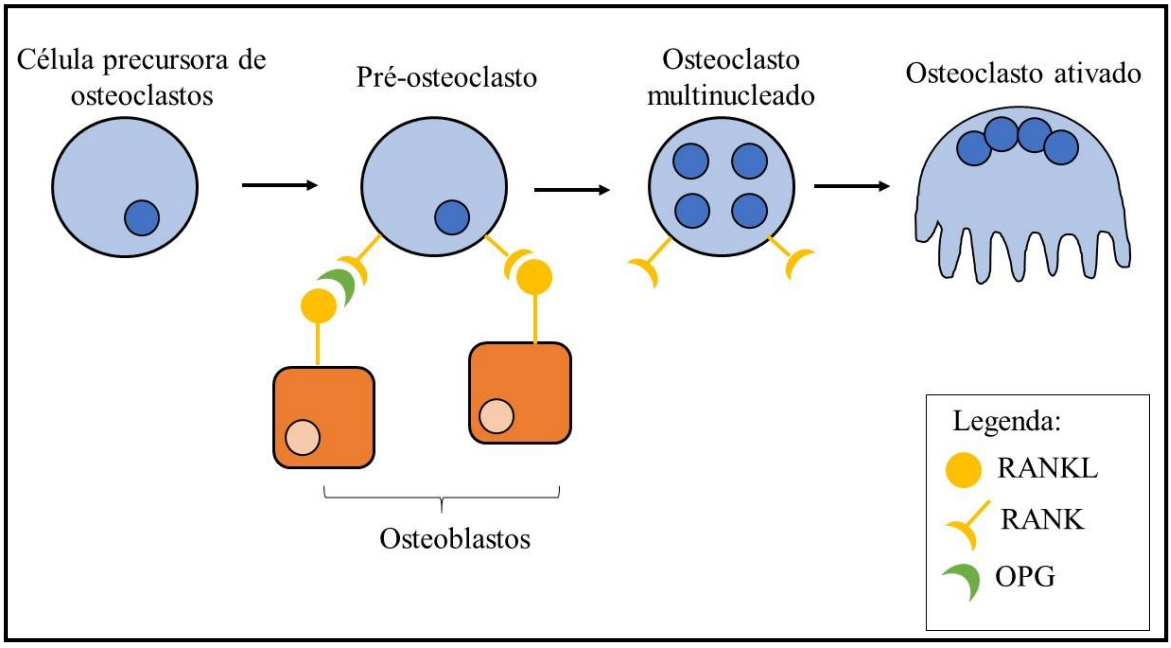

Figura 1. Esquema ilustrado sobre o mecanismo de ação da tríade RANK, RANKL e OPG.

PROPOSIÇÃO

Investigar se a deficiência de estrogênio pré-puberal em ratas ovariectomizadas afeta as medidas ósseas craniofaciais na idade adulta e quantificar a expressão gênica de RANK, RANKL e OPG nos sítios de crescimento das ratas com deficiência de estrógeno durante a puberdade.

MANUSCRITO

Estrogen deficiency in craniofacial skeletal development and in gene expression of RANK, RANKL and OPG during the pubertal period of female rats [1]

Abstract

The aim of this *in vivo* study was to investigate whether the effect of low estrogen levels in the prepubertal period in ovariectomized rats affects the craniofacial measurements in post pubertal stage and to quantify the gene expression of RANK, RANKL and OPG in the growth sites of estrogen deficient rats during puberty. Placebo (Sham) and ovariectomy (OVX) surgeries were performed on twenty-one-days-old Wistar rats. The animals euthanized at forty-five days old (pubertal stage) were used for gene expression analysis of RANK, RANKL and OPG. The animals euthanized at sixty-three days old (post-pubertal stage) were used for the craniofacial measurements using microtomography. Two-dimensional and three-dimensional measurements were collected based on microtomography images. The normality of the sample was tested by Shapiro-Wilk test and the comparative analysis was performed using t-Student test, for the measurements. For gene expression, comparison among the groups, Mann-Whitney U test was used. The statistical significance was determined for values with probability over 95% (alpha = 0.05). Significant statistical differences were found in the measurements between Sham and OVX groups in the two-dimensional analysis of both arches (mandible and maxilla) ($p<0.05$). Condylar volume was also statistically different among groups ($p<0.05$). Sham group had a higher level of RANK expression in the midpalatal suture ($p=0.036$), and RANKL:OPG

[1] Manuscrito será submetido ao periódico: *Plos One.* Manuscrito formatado de acordo com as normas específicas do periódico (acessado em: 19/03/2020).

ratio levels were higher in OVX group (p=0.015). Results suggest that estrogen deficiency during prepubertal period can cause increased maxillary and mandibular bone length in ovariectomized rats.

Introduction

Estrogen is an important hormone involved in the skeletal homeostasis that regulates different aspects of bone metabolism, and bone development, modeling and remodeling. The endogenous levels of estrogen change according to age and gender [1]. During puberty there is an increase in estrogen levels leading to the development of secondary sexual characteristics and a significant increase in growth rate [2, 3]. During the pubertal growth spurt, this hormone plays an important role controlling growth plate acceleration and fusion [4-6].

It is well established that sex steroids, including estrogen, regulate the RANK (receptor activator of nuclear factor-κB), RANKL (receptor activator of nuclear factor-κB ligand) and OPG (osteoprotegerin) axis, members of the tumor necrosis factor superfamily. One of the most important downstream mediators of the action of estrogen on bone is the RANKL [7]. RANKL is crucial for osteoclast differentiation, activation, and survival. OPG is a soluble decoy receptor for RANKL which binds RANKL, inhibiting osteoclastogenesis [8, 9]. RANKL acts through the receptor RANK which is expressed in the cell membrane of osteoclasts and osteoclast precursor cells [10].

Some studies using animal models, both mice and rats, were conducted to describe how estrogen affects the growth and development of the craniofacial structures [11-18] and a variety of outcomes were observed among the studies. Some studies demonstrated a growth inhibition of craniofacial complex in estrogen deficient newborn mice [11, 13, 17] while, bigger condyle dimensions were observed when the estrogen deficiency is performed in 8 weeks old mice [12]. In a study performed in rats with an estrogen deficiency created at 30 days old, no alterations in the craniofacial growth were observed [1]. On the other hand, in our previous study, in which

the estrogen deficiency was performed in the prepubertal stage (21 days old), estrogen deficient animals presented higher both, maxilla and mandible measurements [18].

In our previous study the radiographic analysis suggested that estrogen deficiency from the prepubertal stage affects the dimensions of the maxilla and mandible in female rats [18]. Therefore, in this study, we used high-resolution micro-computed tomography (μCT) to analyze in details the facial skeletal dimension of adult female rats, who suffered estrogen deficiency during the prepubertal stage, considering that computed microtomography brings superior image quality compared to conventional radiography, and also because there is no unanimity around the low levels of estrogen on bone development during pre-pubertal period. We also evaluated if estrogen deficiency affects the RANK, RANKL and OPG expression at growth sites of both maxilla and mandible at puberty.

Materials and methods

Ethics aspects

The present study was conducted and reported according to the ARRIVE guidelines [19]. The Ethical Committee in Animal Experimentation from the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil, approved the protocols of this study (2014.1.721.58.7).

Sample selection

The sample size was defined based on our previous results from two-dimensional imaging analysis reported in Omori et al. (2020) (α=0.05, β=0.2, data not shown). A total of 36 female rats were equally divided into OVX and Sham-operated control groups (6 rats for gene expression and 12 rats for morphometric analysis per group). Briefly, the animals were anesthetized using intraperitoneal injection of 10% ketamine hydrochloride (55 mg/kg of gross body weight) and 2% xylazine hydrochloride (10 mg/kg of gross body weight). At 21 days old (prepubertal stage), bilateral ovariectomy was performed in the OVX group and sham

surgery was performed in Sham group. At an age of 45 days (pubertal stage) animals

were euthanized for gene expression analyses. At an age of 63 days (post-pubertal stage) the remained rats were euthanized for morphometric analysis. The success of ovariectomy was confirmed by weighing the uterus of the rats as previously described by Chen et al. (2014) and Omori et al. (2020). Body and uterus weight were significantly higher in the OVX animals than in Sham animals at 63 day-old ($p<0.05$).

Morphometric analysis

All scans were performed on the micro-CT system phoenix v|tome|x s 240/180 research edition from GE Sensing & Inspection Technologies GmbH (software phoenix datos|x 2 acquisition 2.4.0). Scanning parameters for the rat upper jaws were as follows: 60 kV voltage, 800 µA current, 333 ms time, 1500 images, voxel size 55 µm, fast scan; for the rat lowerjaws: 60 kV voltage, 600 µA current, 333 ms time, 1500 images, voxel size 37 µm, fast scan. Reconstructed volumes were processed using the respective manufacturer's software phoenix datos|x 2 reconstruction 2.4.0.

Images were analyzed using VGSTUDIO MAX 3.3 – Voxel Data Analysis and Visualization (Volume Graphics GmbH, Germany, Heidelberg) and Image J software (National Institutes of Health, Bethesda, MD, USA). To perform the maxilla and mandible morphometric measurements, the images obtained by the µCT were settled in the dorsal, lateral and ventral views positions using the VGSTUDIO MAX 3.3 software, and standardized images were performed in the same scale for all rats. The landmarks and linear measurements for maxilla and mandible are demonstrated in Figure 1 and described in Table 1. The morphometric analysis was performed based on Wei et al. (2017), Fujita *et al.* (2004), Kiliaridis et al. (1985). The evaluated dimensions are described in the Table 2. Image J software was used to measure the linear dimensions (mm) and the angular dimensions.

Table 1. The description of the maxillary and mandibular landmarkers used.

Landmarker	***Description***
Maxilla	
1	Intersection of nasal bones
2	Posterior point of the suture between palatines and the anterior border of the mesopterygoid fossa
3	The most prominent lateral point on the buccal surface of the upper first molar
4	Anterior limit of upper first molar
5	Anterior notch on zygomatic process
6	Anterior most point of alveolus upper incisor
7	The most prominent point between the incisal edges of the incisors
Mandible	
1	Most posterior point of mandibular body base
2	Lowest point of alveolar bone around lower incisor
3	Highest point of alveolar bone around lower incisor
4	Lateral face of condylar process
5	Medial face of condylar process
6	Top of coronoid process
7	Distal face of third lower molar
8	Posterior limit of lower molars
9	Vestibular face of mesiobuccal cusp of first lower molar
10	Lateral face of both sides mentum bone
11	Highest point of alveolar bone around lower incisor
12	Most prominent point between incisal edges of lower incisor
13	The topmost point of condylar process
14	The most prominent lateral point on the buccal surface of the lower first molar
15	Mental foramen
16	The point located at the gonial angle of the mandible
17	Most superior point of the condyle
18	Posterior-most point of condyle
19	Tip of mandibular angle
20	Point on most inferior contour of angular process of mandible
21	Point in deepest part of antegonial notch curvature
22	Inferior point on mandibular symphysis
23	Inferior rim point on lower incisor alveolus
24	Most prominent point between incisal edges of lower incisor
25	Superior rim point on lower incisor alveolus
26	Point on intersection between the mandibular alveolar bone and mesial surface of first molar

Table 2. Linear and angular measurements evaluated in this study.

Landmarkers	***Description***	***References***
Maxilla		
1-2	Maxillary arch length	-
3-3	Maxillary intermolar distance	-
1-4	Maxillary diastema length	-
2-4	Maxillary posterior segment length	-
6-7	Maxillary central incisor length	-
Mandible		
18-19	Mandibular height – from most posterior point of condyle to most posterior point of mandible's angle	Wei *et al.*, 2017
18-25	Upper mandibular length	Wei *et al.*, 2017
19-22	Lower mandibular length	Wei *et al.*, 2017
20-22	Mandibular plane length	Wei *et al.*, 2017; Fujita *et al.*, 2004
17-22	Diagonal mandibular length	Wei *et al.*, 2017; Fujita *et al.*, 2004
17-19	Distance between the most superior point of the condyle to mandible angle	Wei *et al.*, 2017; Fujita *et al.*, 2004
23-24	Mandibular central incisor length	Wei *et al.*, 2017; Fujita *et al.*, 2004
25-26	Distance between first lower molar mesial face to lower central incisor buccal face	Wei *et al.*, 2017; Fujita *et al.*, 2004
17-20	Mandibular height – from most superior point of the condyle to mandible base	Wei *et al.*, 2017
17-19-20-22	Mandibular angle composed between the lines 17-19 and 20-22	Fujita *et al.*, 2004
20-22-24	Mandibular angle composed by the landmarkers 20, 22 and 24	Fujita *et al.*, 2004
17-20-22	Mandibular angle composed by the landmarkers 17, 20 and 22	Fujita *et al.*, 2004
7-7	Mandibular arch width	Corte *et al.*, 2019
9-9	Mandibular intermolar distance	Corte *et al.*, 2019
8-12	Mandibular arch length	Corte *et al.*, 2019
8-9	Mandibular posterior segment length	Corte *et al.*, 2019
9-11	Mandibular diastema length	Corte *et al.*, 2019
10-10	Mandibular interdiastemal breadth	Corte *et al.*, 2019
6-6	Intercoronoidal breadth	Corte *et al.*, 2019
4-5	Thickness of condylar process	Wang *et al.*, 2016
1-2	Inferior mandibular body length	Corte *et al.*, 2019
2-3	Anterior mentum height	Corte *et al.*, 2019
13-13	Mandibular superior third width	Wei *et al.*, 2017
14-14	Mandibular middle third width	Perilo *et al.*, 2014
16-16	Mandibular inferior third width	Perilo *et al.*, 2014
15-15	Distance between mentum foramens	Corte *et al.*, 2019

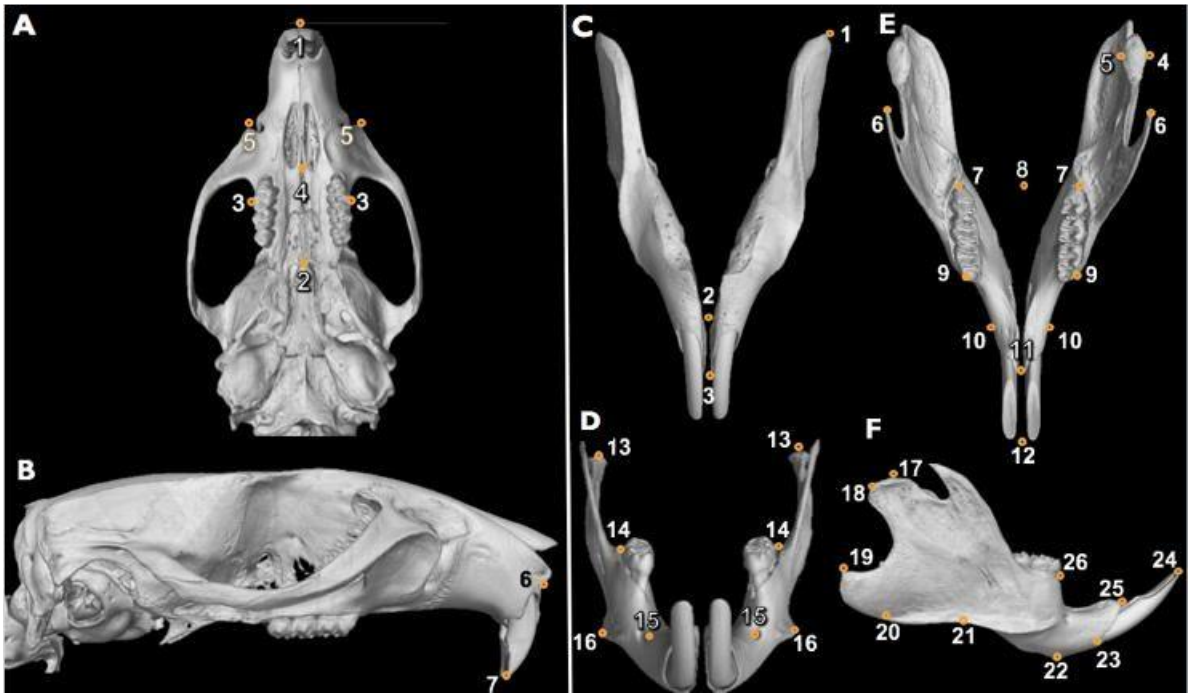

Fig 1. Land markers used for craniofacial measurements.
(A) inferior view of maxilla. (B) lateral view of skull. (C) inferior view of mandible. (D) frontal view of mandible. (E) superior view of mandible. (F) lateral view of mandible.

The volume of the condyles was obtained using the VGSTUDIO MAX 3.3. All mandibles were settled in a lateral view of the right condyle, and the condyle was separated from the mandible using a specific tool from the software to measure the volume (mm^3). The amount of bone selected to measure the volume was standardized by positioning a pre sized square over the condyle, in a way that the superior edge of the square matched with the most superior border of the condyle, and the left edge of the square matched with the most posterior border of the condyle in order to select the region of interest (ROI) (Figure 2).

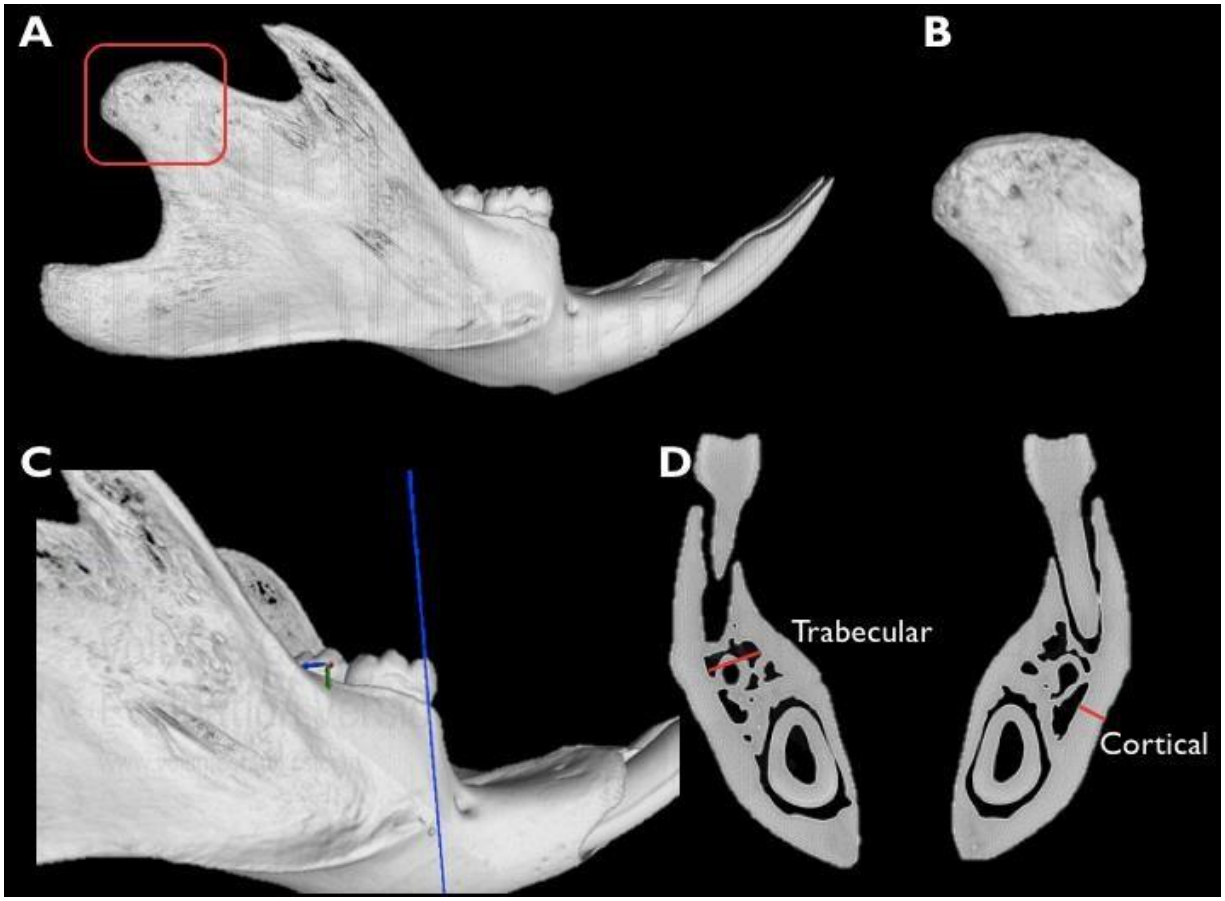

Fig 2. Three-dimension view of mandible for measurements.
(A) Selection of the condyle volume. (B) Creation of a new ROI. (C) Selection of the mandible area to be analyzed. (D) Trabecular and cortical bones analyzed.

Gene expression of RANK, RANKL and OPG

Gene expression was performed to in growth sites of the maxilla (midpalatal suture) and mandible (condyle, mandibular angle, symphysis/parasymphysis and coronoid process) at the pubertal stage. Bone samples were dissected after euthanasia at the age of 45 days and stored in RNAlater (Life Technologies Corporation – Carlsbad, CA, USA) at -80ºC until processing. Total RNA was extracted using the mirVana™ miRNA Isolation kit (Ambion/Life Technologies™, USA). Complementary DNA (cDNA) was synthesized by reverse-transcription with a High Capacity kit (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using a StepOnePlusTM sequence detection system (Applied Biosystems™, Foster City, CA, USA) using TaqMan® primers and probes (Thermo Fisher Scientific, MA, EUA) for RANK, RANKL and OPG. GAPDH (Rn01462661-g1) and ACTB (Rn01412977-g1) were used as endogenous controls and confirmed to be stably expressed. The relative levels of mRNA expression were determined by the 2-ΔΔ Cycle Threshold (2-ΔΔCT) method. Both, GAPDH and ACTB, genes were used for sample normalization according to Livak and Schmittgen (2001), to calculate the relative quantitation. All procedures were performed following the respective manufacturer's instructions and according to established protocols.

Statistical analysis

The sample normality test was analyzed by Shapiro-Wilk test. The comparative analysis was performed initially by the Student t test to verify the difference between the measurements among OVX and Sham groups. For the gene expression comparison among the groups, Mann-Whitney U test was used. The statistical significance was determined for values with probability over 95% (alpha = 0.05). All analyses were performed using the Prism 8 software (Graph Pad Software Inc., San Diego, California, United States of America).

Results

The overall dimensions in millimeter (mm) of the reconstructed rats' maxilla and mandible μCT images are demonstrated in the Table 3. The maxillary posterior segment length was smaller in the OVX group (p=0.012), while the maxillary central incisor length was bigger in the OVX group (p=0.010). In the mandibular sagittal plane, the mandibular height (p=0.006), the upper mandibular length (p=0.037), the mandibular plane length (p=0.019), the diagonal mandibular length (p=0.014), the distance between the condyle to mandible angle (p=0.025)

and the mandibular height (p=0.004) were bigger in the OVX group. Also, the mandibular angle composed by the lines intersecting the landmarkers 17-19 and 20-22 was higher in the OVX group (p=0.009). In the mandibular transversal plane the mandibular interdiastemal breadth (p=0.002) and the thickness of condylar process (p<0.0001) were larger in the OVX group.

Table 3. Mean and standard deviation of 2D and 3D measurement points

Measurements	Groups		p Value
	OVX Mean (SD)	Sham Mean (SD)	
Maxilla			
Arch length (1-2)	30.21 (0.67)	30.53 (1.26)	0.533
Intermolar distance (3-3)	9.77 (0.39)	9.87 (0.50)	0.672
Diastema length (1-4)	17.56 (0.50)	17.50 (0.93)	0.858
Posterior segment length (2-4)	9.78 (0.38)	10.36 (0.47)	**0.012***
Maxillary central incisor length (6-7)	11.11 (0.23)	10.50 (0.60)	**0.010***
Mandible			
Mandibular height (18-19)	10.96 (0.66)	10.05 (0.61)	**0.006***
Upper mandibular length (18-25)	35.47 (1.75)	33.55 (1.88)	**0.037***
Lower mandibular length (19-22)	29.90 (1.81)	28.25 (1.76)	0.063
Mandibular plane length (20-22)	23.04 (1.40)	21.26 (1.52)	**0.019***
Diagonal mandibular length (17-22)	29.93 (1.12)	28.15 (1.56)	**0.014***
Distance between the condyle to mandible angle (17-19)	14.35 (0.52)	14.07 (0.46)	**0.025***
Mandibular central incisor length (23-24)	12.67 (1.03)	13.22 (0.52)	0.142
Distance between first molar to central incisor (25-26)	8.72 (0.35)	8.41 (0.60)	0.213
Mandibular height (17-20)	17.66 (1.09)	16.16 (0.90)	**0.004***
Angle composed between the lines 17-19 and 20-22	114.21 (2.71)	109.88 (3.77)	**0.009***
Angle composed by the landmarkers 20, 22 and 24	135.33 (1.62)	134.49 (1.75)	0.305
Angle composed by the landmarkers 17, 20 and 22	99.47 (5.9)	100.21 (2.44)	0.599
Arch width (7-7)	12.40 (0.94)	12.28 (0.76)	0.748
Intermolar distance (9-9)	11.39 (1.10)	11.15 (0.57)	0.543
Arch length (8-12)	25.72 (2.06)	25.71 (0.89)	0.988
Posterior segment length (8-9)	10.13 (0.86)	10.02 (0.43)	0.725
Diastema length (9-11)	9.35 (1.25)	8.99 (0.33)	0.365
Mandibular interdiastemal breadth (10-10)	6.00 (0.32)	5.49 (0.28)	**0.002***
Intercoronoidal breadth (6-6)	27.20 (2.08)	26.62 (2.03)	0.558
Thickness of condylar process (4-5)	2.29 (0.15)	1.94 (0.12)	**<0.0001***
Inferior mandibular body length (1-2)	39.01 (0.96)	38.23 (2.43)	0.405
Anterior mentum height (2-3)	5.63 (0.68)	5.42 (0.59)	0.479
Mandibular superior third width (13-13)	19.69 (1.03)	19.95 (1.47)	0.670
Mandibular middle third width (14-14)	14.60 (0.75)	14.86 (1.19)	0.595
Mandibular inferior third width (16-16)	18.18 (1.06)	17.77 (1.24)	0.466
Distance between mentum foramens (15-15)	10.32 (0.68)	10.38 (0.84)	0.873

**indicates statistical significance difference (p<0.05). The dimensions were measured in millimeter (mm)*

The condyle volume (mm3) was significantly bigger in the OVX group (mean=10.10; S.D=0.54) than in the Sham group (mean=8.91; S.D=1.15) (p=0.016).

The mandibular cortical bone thickness was smaller in OVX group (mean=1.52; S.D=0.09) than in the Sham group (mean=1.71; S.D=0.20) (p=0.037). However, the mandibular Trabecular bone thickness was not statistically significant different between OVX group (mean=0.52; S.D=0.06) and Sham group (mean=0.48; S.D=0.06) (p=0.242).

The gene expression of RANK, RANKL, OPG and RANKL:OPG ratio is presented in the Table 4. Sham group had a higher level of RANK expression in the midpalatal suture (p=0.036). The RANKL:OPG ratio levels were higher in OVX group (p=0.015).

Table 4. Gene expression in the growth center.

Growth sites	*Groups*	RANK		RANKL		OPG		RANKL:OPG	
		Mean (SD)	p-value	Mean (SD)	p-value	Mean (SD)	p-value	Mean (SD)	p-value
Midpalatal suture	***OVX***	0.40 (0.08)	0.036*	1.22 (0.54)	0.239	0.85 (0.18)	0.306	1.40 (0.96)	0.313
	Sham	0.67 (0.02)		0.65 (0.05)		0.68 (0.06)		0.96 (0.16)	
Condyle	***OVX***	1.80 (1.48)	0.174	1.57 (0.84)	0.061	0.35 (0.13)	0.868	4.48 (1.61)	0.015*
	Sham	0.44 (0.07)		0.40 (0.14)		0.37 (0.16)		1.17 (0.43)	
Mandibular angle	***OVX***	0.43 (0.13)	0.191	0.41 (0.03)	0.209	0.41 (0.11)	0.136	1.02 (0.19)	0.588
	Sham	0.27 (0.16)		0.28 (0.18)		0.28 (0.10)		0.93 (0.24)	
Symphysis / parasymphysis	***OVX***	0.62 (0.56)	0.806	1.00 (0.56)	0.451	1.05 (0.36)	0.453	0.92 (0.40)	0.804
	Sham	0.35 (0.43)		0.65 (0.06)		0.77 (0.44)		1.04 (0.68)	
Coronoid process	***OVX***	0.43 (0.14)	0.412	0.39 (0.14)	0.904	0.29 (0.09)	0.703	1.37 (0.34)	0.285
	Sham	0.33 (0.09)		0.34 (0.11)		0.32 (0.06)		1.06 (0.33)	

Note: *means statically significant difference (p<0.05).

Discussion

The postnatal craniofacial skeletogenesis is a unique process, in which many factors could affect its growth and development. Our study confirms that estrogen is one factor involved in the maxilla and mandible growth. Therefore, clinicians should be aware of the potential implications of estrogen deficiency to the facial complex development, once low estrogen levels in women and teenager girls can be caused by different factors and the effects caused by this deficiency depends on individuals' age and general health. It is well known that

estrogen deficiency can cause osteoporosis [14, 16, 24]. Estrogen deficient levels have been reported in syndromes and genetic conditions [25-28], menstrual disorders [29], primary ovarian insufficiencies [30], underweight [31], excessive exercise [32], and chemotherapy [33]. There were also reported cases of aromatase deficiency in pre pubertal girls and estrogen deficiency due to mutations in the aromatase gene (CYP19) affected the skeletal development [14, 34], decreased the mineral density in bones and delay the epiphyseal maturation [17].

In animal models, it is well known that low levels of estrogen can lead to changes in bone microarchitecture in rats' femurs and mandibles [2, 7, 35, 36] osteoporosis, [16] instability in the skeletal homeostasis [37, 38] and alterations in the craniofacial development [11-13, 17, 18]. In our study, we were able to confirm the observation reported in Omori et al. (2020), in which estrogen deficiency during puberty leads to alterations in maxilla and mandible, however, the fact that in the present study we used μCT, we were able to perform a more reliable analysis. In Omori et al. (2020), a radiograph two-dimensional cephalometric linear analysis was performed. Radiograph cephalometric linear and angular analysis of murine skulls has been developed long ago [39-41] and is very similar to that used in human, which has practical and successful clinical applications [21]. Although some landmarks are difficult to identify ontwo-dimensional radiograph of mouse and rat skull, radiograph-based cephalometry has been used successfully to identify morphometric changes in estrogen models of mouse [11, 17] and rats [18, 34]. However, μCT-based craniofacial measurement has the advantage of high resolution and the ability to determine the morphology and volume of the maxilla and mandible.

In our study, the thickness of condylar process, as well as the condyle volume were bigger in the OVX group. Larger measures of the condyle breadth were found in mice when the deficiency of estrogen was created in eight-week-old mice [11], although the normal width, low trabecular bone volume of the condyle and reduction in the bone mineral density were also found [13, 14, 16].

We were able to observe that although posterior segment length of the maxilla was smaller, the others dimensions were bigger in the OVX group. This arch size difference could reflect the clinical phenotype of prognathism in humans. An explanation for maxilla and mandible explanation could be the fact that estrogen performs two opposite functions, in two distinct phases of puberty. During the prepubertal period, the activity of estrogen is systemic in accordance with growth hormone (GH) causing bone elongation [42, 43]. After this period of growth, comes the period known as post pubertal, when estrogen acts at local level causing the epiphyseal fusion, resulting in the bone maturation [35, 43].

Estrogen is needed during bone growth and development for proper closure of epiphyseal growth plates both in females and in males. Also in young skeleton, estrogen deficiency leads to increased osteoclast formation and enhanced bone resorption [44]. It is important to emphasize that estrogen plays a role in bone through RANK/RANKL/OPG triad.

RANKL is a crucial for osteoclast differentiation, activation, and survival, while OPG is a soluble decoy receptor for RANKL which binds RANKL, inhibiting osteoclastogenesis, through the receptor RANK. RANKL, RANK, and OPG are essential, non-redundant factors for osteoclast biology [7]. During this process, the osteoclast precursor cells become mature osteoclasts, and as these mature osteoclasts also have the RANK receptor for their activation, once the RANKL binds to RANK the mature osteoblasts are activated and start the process of bone resorption. On the other hand, the inhibition of bone resorption is led by the OPG. This cytokine has the ability to bind to RANKL in the same way RANK does, but it causes the opposite effect, blocking the RANK connection with RANKL, it blocks the action of RANKL on the osteoclast precursor cells and on the mature osteoclasts, avoiding the bone resorption [45-47]. The RANKL:OPG ratio levels in the condyle was higher in the OVX group.

Conclusion

Ovariectomized rats with low levels of estrogen showed impact cranio-facial bones measurements, mainly in mandible. Additionally, estrogen deficiency influence the RANK and RANKL:OPG expression in the growth areas of the face.

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CONSIDERAÇÕES FINAIS

Ratos ovariectomizados com baixos níveis de estrogênio apresentaram medidas bidimensionais aumentadas em alguns ossos cranio-faciais, principalmente na mandíbula e no volume dos côndilos. Enquanto isso, a quantidade de osso trabecular no corpo mandibular foi maior no grupo Sham. Além disso, a expressão gênica de RANK foi maior no grupo Sham na sutura palatina mediana em relação ao grupo OVX.

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ANEXO

Parecer do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Ribeirão Preto - Universidade de São Paulo

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE RIBEIRÃO PRETO
CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE RIBEIRÃO PRETO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO CEUA – FORP/USP

Certificamos que o Protocolo nº 2014.1.721.58.7 sobre a pesquisa intitulada **"Avaliação do papel do estrógeno nas estruturas do complexo dento-facial"**, sob a responsabilidade do Prof. Dr. Paulo Nelson Filho, está de acordo com os Princípios Éticos na Experimentação Animal adotados pela Comissão de Ética no Uso de Animais da Faculdade de Odontologia de Ribeirão Preto, USP, foi APROVADO em reunião da CEUA de 29/08/2014 (totalizando 40 animais).

We hereby certify that the protocol nº 2014.1.721.58.7 regarding the research entitled **"Evaluation of the role of the estrogen in the dental-facial structures"**, under the responsibility of Prof. Dr. Paulo Nelson Filho, is in accordance with the Ethical principles in animal research adopted by the Animal Research Ethics Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil, and was approved in 29/08/2014 (totalizing 40 animals).

Ribeirão Preto, 29 de agosto de 2014.

Profa. Dra. Andiara De Rossi Daldegan
Coordenadora da CEUA – FORP/USP