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The impact of hypoestrogenism and occlusal function on MMP1, MMP8 and MMP13 expression in the odontogenic region in rats

Impacto do hipoestrogenismo e da função oclusal na expressão de MMP1, MMP8 e MMP13 na região odontogênica em ratos

El impacto del hipoestrogenismo y la función oclusal en la expresión de MMP1, MMP8 y MMP13 en la región odontogénica en ratas

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Isabela Ribeiro Madalena
ORCID: https://orcid.org/0000-0002-4486-1318
Positivo University, Brazil
E-mail: isabelarmadalena@hotmail.com
Cláudia Salete Judachesci
ORCID: https://orcid.org/0000-0002-5580-7958
Positivo University, Brazil
E-mail: claudia_judachesci@hotmail.com
Erika Calvano Küchler
ORCID: https://orcid.org/0000-0001-5351-2526
Positivo University, Brazil
E-mail: erikacalvano@gmail.com
Paulo Nelson-Filho
ORCID: https://orcid.org/0000-0001-8802-6480
University of São Paulo, Brazil
E-mail: nelson@forp.usp.br
Lucas Alexandre Ramazzoto
ORCID: https://orcid.org/0000-0002-4579-4073
Federal University of São Carlos, Brazil
University of São Paulo, Brazil
E-mail: lucasramazzotto@gmail.com
Flares Baratto-Filho
ORCID: https://orcid.org/0000-0002-5649-7234
University of the Region of Joinville, Brazil
E-mail: fbaratto1@gmail.com
Kesly Mary Ribeiro Andrades
ORCID: https://orcid.org/0000-0002-5718-7211
University of the Region of Joinville, Brazil
E-mail: keslyribeiro@hotmail.com
Sabrina Schulze
ORCID: https://orcid.org/0000-0002-0122-5688
Synlab, Fresing, Germany
E-mail: saschulze2@gmail.com
João César Zielak
ORCID: https://orcid.org/0000-0003-3393-3491
Positivo University, Brazil
E-mail: jzielak2@gmail.com
João Armando Brancher
ORCID: https://orcid.org/0000-0002-8914-702X
Positivo University, Brazil
E-mail: brancher.a@gmail.com

Abstract

*Background***:** The impact of estrogen deficiency and occlusion in the matrix metalloproteinases (MMPs) expression in dental tissues has not yet been elucidated. *Objective***:** To evaluate the influence of estrogen deficiency and occlusal hypofunction and hyperfunction on the gene expression of MMP1, MMP8 and MMP13 in the odontogenic region of teeth in continuous growth, in the murine model. *Material and methods***:** Rats (Wistar Hannover lineage) were divided into two groups according to the intervention received: Hypoestrogenism Group - ovariectomy surgery and Control Group - fictitious surgery. Occlusal hypofunction and hyperfunction conditions were also established in all animals (each animal presented both conditions). After euthanasia, the hemimandibles were removed to evaluate the gene expression through real time PCR. T-test was used to compare the mean differences between groups ($P<0.05$). *Results***:**

There was no statistically significant difference of the relative gene expression of MMP1, MMP8 and MMP13 between the hypoestrogenism and control groups (*P>0.05*). A statistically significant difference of the relative MMP13 expression between the occlusal hypofunction and hyperfunction tooth was observed (*P=0.03*). In the hypoestrogenism group, MMP13 was overexpressed in hypofunction tooth (*P=0.045*). *Conclusion***:** Occlusal function affects MMP13 expression in the odontogenic region, in murine model.
Keywords: Dental sac; Estrogens; Matrix metalloproteinases; Osteogenesis.

Resumo

*Introdução***:** O impacto da deficiência de estrógeno e da oclusão na expressão das metaloproteinases da matriz (MMPs) nos tecidos dentários ainda não foi elucidado. *Objetivo***:** Avaliar a influência da deficiência de estrógeno e da hipofunção e hiperfunção oclusal na expressão gênica de MMP1, MMP8 e MMP13 na região odontogênica de dentes em crescimento contínuo, no modelo murino. *Material e métodos***:** Ratas (linhagem Wistar Hannover) foram divididas em dois grupos de acordo com a intervenção recebida: Grupo Hipoestrogenismo - cirurgia de ovariectomia e Grupo Controle - cirurgia fictícia. Condições de hipofunção e hiperfunção oclusal também foram estabelecidas em todos os animais (cada animal apresentava ambas as condições). Após a eutanásia, as hemimandíbulas foram retiradas para avaliação da expressão gênica por meio de PCR em tempo real. O teste t foi usado para comparar as diferenças médias entre os grupos (*P<0,05*). *Resultados*: Não houve diferença estatisticamente significativa da expressão gênica relativa de MMP1, MMP8 e MMP13 entre os grupos de hipoestrogenismo e controle (*P>0,05*). Foi observada uma diferença estatisticamente significativa da expressão relativa de MMP13 entre a hipofunção oclusal e a hiperfunção dentária (*P=0,03*). No grupo de hipoestrogenismo, MMP13 foi superexpresso no dente com hipofunção (*P=0,045*). Conclusão: A função oclusal afeta a expressão de MMP13 na região odontogênica, em modelo murino.
Palavras-chave: Saco dentário; Estrógeno; Metaloproteinases da matriz; Osteogenese.

Resumen

*Antecedentes***:** El impacto de la deficiencia de estrógeno y oclusión dentaria sobre la expresión de metaloproteinasas de la matriz (MMPs) en los tejidos dentales aún no ha sido suficientemente estudiada. *Objetivo***:** Evaluar la influencia de la deficiencia de estrógeno y función oclusal (hipo- e hiperfunción) sobre la expresión génica de MMP1, MMP8 y MMP13 en la región odontogénica de dientes con erupción continua, usando un modelo murino. *Materiales y métodos***:** Ratas del linaje Wistar-Hannover fueron divididas en dos grupos de acuerdo a la intervención recibida: grupo con hipoestrogenismo - cirugía de ovariectomía, y grupo control - cirugía ficticia. Fueron creadas condiciones de hipo- e hiperfunción oclusal en los animales (cada animal presentó ambas condiciones). Una vez realizada la eutanasia, las hemimandíbulas fueron separadas para evaluar la expresión génica por medio de PCR en tiempo real. Se utilizó la prueba t para comparación entre los grupos (*P=0,05*). *Resultados***:** No hubo diferencia estadísticamente significativa en la expresión genética relativa de MMP1, MMP8 y MMP13 entre el grupo con hipoestrogenismo y el grupo control (*P>0,05*). Se observó una diferencia estatísticamente significativa en la expresión de MMP13 entre los grupos con hipo- e hiperfunción oclusal (*P=0,03*). MMP13 fue sobre expresada en los dientes con hipofunción del grupo de animales con hipoestrogenismo (*P=0,045*). *Conclusión*: La función oclusal afecta la expresión de MMP13 en la región odontogénica, en modelo murino.
Palabras clave: Saco dental; Estrógenos; Metaloproteinasas de la matriz; Osteogénesis.

1. Introduction

Estrogen is a steroidal hormone with lifelong production in the human body. In addition to the well-established relationship in male and female reproduction and in several other systems of the human body, estrogen has also been associated with several conditions, such as obesity, metabolic disorder, cancers, osteoporosis, lupus erythematosus, endometriosis, and uterine fibroids (Patel et al., 2018). The classic pathway by which estrogen performs its function is mediated by its intracellular receptors, the two most studied types are estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Paterni et al., 2014). Recent research shows that estrogen receptors are expressed in oral tissues, especially in dental cells with great potential for differentiation, such as dental pulp cells and the odontogenic region of teeth in continuous growth (Alhodhodi et al., 2017; Madalena, 2020; Manokawinchoke et al., 2016).

Increasing evidence also shows the involvement of estrogen in matrix metalloproteinases (MMPs) activity (Jung et al., 2010; Lu et al., 2006). The influence of estrogen and estrogen receptors on the expression of MMPs has already been described in chondrocytes (Lee et al., 2003), osteoblasts (Schiltz et al., 2008), fibroblasts of epithelial tissue (Philips & Devaney, 2003), stromal endometrial cells (Kokorine et al., 1996), and endothelial cells involved in breast carcinogenesis (Nilsson et al., 2007),

among others. The increase in the MMPs levels has also been described before under estrogen deficiency condition, also known as hypoestrogenism (Lee et al., 2003).

The family of MMPs is divided into six main groups of proteases (Birkedal-Hansen et al., 1993). In special, the collagenases, composed of interstitial collagenase (MMP1), neutrophilic collagenase (MMP8), and collagenase-3 (MMP13), stimulate cells such as fibroblasts and osteoblasts to cause tissue damage (Souza & Line, 2001). The expression of MMP1, MMP8, and MMP13 has already been associated with collagen remodeling during periodontal tissue destruction (Al-Majid et al., 2018; Hernandez et al., 2006; Zhang et al., 2011). These MMPs were also involved tooth eruption process (Pizzol-Júnior et al., 2018; Tsubota et al., 2002) and during mechanical force overlapping the periodontal ligament in dysfunctional occlusive conditions (Huang et al., 2008).

Therefore, it is possible to hypothesize that hypoestrogenism, as well as occlusal condition (hypofunction and hyperfunction) may affect the expression of MMP1, MMP8, and MMP13 in the odontogenic region. In occlusive stress condition, represented by occlusal hypofunction or hyperfunction, there is an increase in collagen synthesis, due to the increased remodeling of the extracellular matrix (Kanoza et al., 1980; Omar et al., 2018). Thus, the present study aimed to evaluate the influence of hypoestrogenism and occlusal function on MMP1, MMP8, and MMP13 genes expression, in the odontogenic region, in rats.

2. Methodology

2.1 Ethical Aspects

This research was performed and reported according to the ARRIVE guidelines (Kilkenny et al., 2010). The Ethical Committee in Animal Experimentation from the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil, approved this study (#2018.40.58.3). All efforts were made to minimize animal suffering.

2.2 Experimental design

Samples from Wistar Hannover lineage rats were divided from two groups, Hypoestrogenism Group (n=10) and Control Group (n=10) were used in this study (Madalena, 2020). The animals came from the Central Bioterium of the University of São Paulo - Ribeirão Preto Campus and were requested with 21 days of post-uterine life, corresponding to the pre-pubertal period (Ojeda et al., 1976). The mean weight of the animals was 62.04 grams (SD=14.31). The animals were stored in collective cages (4 animals per cage), in a controlled temperature environment and a 12-hour light-dark cycle, with free demand for the feed (Labina Purina®/Agribrands do Brasil LTDA , Paulínia, BR) and filtered water *ad libitum*.

2.3 Hypoestrogenism - Estrogen deficiency model

To create estrogen deficiency (decrease the endogenous production of estrogen), a bilateral surgical excision of the ovaries (ovariectomy) was performed in the hypoestrogenism group. While the control group was submitted to fictitious surgery, in which the ovaries were moved and returned to their initial position, as previously described in Omori et al. (2020).

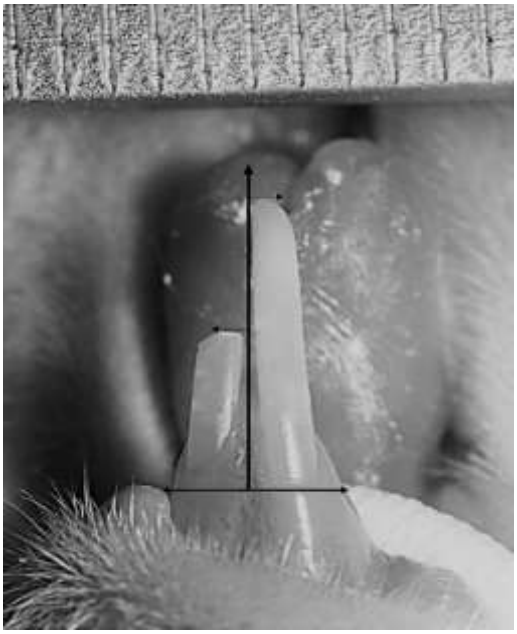
The success of the surgical procedure was confirmed by the animals' survival, gradual increase in body weight during the experimentation period and by the uterine atrophy after euthanasia in the experimental group (Omori et al., 2020). The decrease in endogenous estrogen release, caused by ovariectomy, provides significant differences in the body weight and uterine weight (Chen et al., 2014). Thus, the hypoestrogenism group presented an increase in body weight and a decrease in the uterus weight when compared to the control group ($p<0.05$).

2.4 Procedures for simulating the occlusal hypofunction and hyperfunction conditions of teeth with continuous growth

The occlusal hypofunction condition was simulated adjusting the incisal edge, at the level of the gingival papilla, in the

lower right incisor (Lee et al., 2002). Consequently, the contra-lateral side presents a hyper occlusal function, and thus the occlusal hyperfunction condition was establishes in the lower left incisor. These are demonstrated in the Figure 1.

Figure 1. Occlusal hypofunction and hyperfunction condition performed on the lower incisors during the pubertal period. The hypofunction condition was performed on the right lower incisor by adjusting the incisal edge at the level of the gingival papillae. The adjustment was performed with a double-sided diamond disk 7011 (KG Sorensen®, Cotia, BR) 0.18mm thick.



Source: Authors.

Both conditions were reestablished every 48 and 72 hours due to the continuous growth of the incisors of the murine model. The animals were sedated intramuscularly to cut their teeth. After 21 consecutive days, the animals were euthanized.

2.5 Euthanasia and preparation of specimens

Euthanasia was carried out following the guidelines of the National Council for the Control of Animal Experimentation - CONCEA. Anesthetic overdose was performed through the combination of ketamine hydrochloride (300mg/Kg of weight) and xylazine hydrochloride (30mg/Kg of weight), which are also available on the Ethics Committee for Animals Use website at www.forp.usp br. Additionally, as suggested by the Ethics Committee for Animals Use to ensure the optimization of procedures, decapitation was also adopted. Therefore, the hemimandibles were removed, dissected, and sectioned to isolate the odontogenic region for gene expression analysis (Figure 2).

Figure 2. Schematic illustration of the intact hemimandibula and isolated odontogenic region. Note in (A) the intact hemimandible. The black circle delimits the odontogenic region that will be isolated. In (B), the delimitation is noted. The condyle region, coronoid process, mandible branch, and anterior region were removed to avoid increasing the relative expression of MMP1, MMP8, and MMP13 in these regions. In (C), the sectioned odontogenic region is noted.

Source: Authors.

2.6 Analysis of MMP1, MMP8 e MMP13 in odontogenic region – RT-qPCR

The specimens were kept in RNAlater (Life Technologies Corporation - Carlsbad®, Canada, USA) and frozen at -80ºC until the day of processing. The mirVana ™ miRNA Isolation kit (Thermo Fischer Scientific, Carlsbad, USA) was used to extract total RNA. Complementary DNA (cDNA) was synthesized by reverse-transcription with a Hight Capacity Kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was carried out on a StepOnePlus™ sequence detection system (Applied Biosystems™, Foster City, CA, USA) using TaqMan® primers and probes (Thermo Fisher Scientific, MA, USA) for MMP1 (Rn01486634-m1), MMP8 (Rn00573646-m1) and MMP13 (Rn01448194-m1). GAPDH (Rn01462661-g1) and ACTB (Rn01412977-g1) were used as endogenous controls. The relative levels of mRNA expression were determined by the $2^{-\Delta\Delta}$ Cycle Threshold ($2^{-\Delta\Delta CT}$) method (Omori et al., 2020). Both, GAPDH and ACTB, genes were used for sample normalization to calculate the relative quantification. All procedures were performed following the respective manufacturer's instructions and according to established protocols.

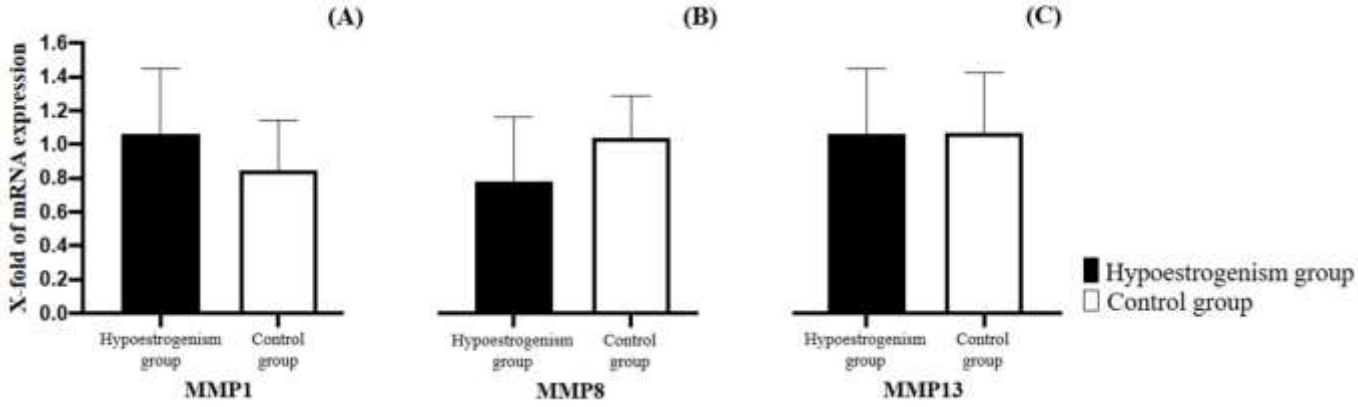
2.7 Statistical analysis

The data were evaluated using the GraphPad Prism 7.04 software (GraphPad Software®, La Jolla, USA), with a significance of 5%. Normality was tested using the Shapiro-Wilk test. Student's t test was used to compare the means between the hypoestrogenism and control groups. Paired student's t test was used to compare the means between the sides with occlusal hypofunction or hyperfunction.

3. Results

The analysis was performed in the survival animals: Hypoestrogenism Group (n=8) and Control Group (n=9). The difference in MMP1, MMP8 and MMP13 expression in the odontogenic region of the lower incisors of both groups (hypoestrogenism and control), are shown in Figure 3. The mean and standard deviation (SD) of MMP1 relative gene expression level was 1.06 (SD=0.39) for the hypoestrogenism group and 0.85 (SD=0.29) for the control group. For MMP8, the mean relative gene expression level was 0.78 (SD=0.38) for the hypoestrogenism group and 1.03 (SD=0.25) for the control group. For MMP13, the mean relative gene expression level was 1.06 (SD=0.38) for the hypoestrogenism group and 1.06 (SD=0.36) for the control group. There were no statistically significant differences between the groups ($P=0.33$, $P=0.12$ and $P=0.97$, respectively).

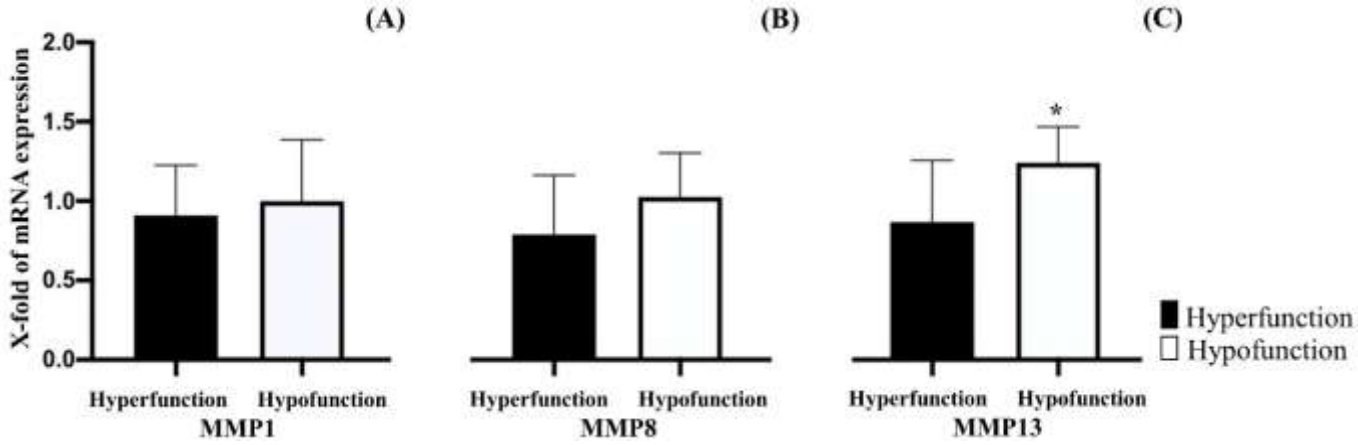
Figure 3. Relative gene expression of MMP1 (A), MMP8 (B) and MMP13 (C) in the odontogenic region of the hypoestrogenism and control groups. There was no statistically significant difference (*P>0.05*).



Source: Search data.

The difference in MMP1, MMP8 and MMP13 expression in the odontogenic region of the lower incisors of both conditions (occlusal hypofunction and hyperfunction), are shown in Figure 4. MMP1 and MMP8 expression were not statistically significant different between occlusal hypofunction and hyperfunction (*P=0.70* and *P=0.16*, respectively). The mean distribution of the relative gene expression of MMP13 showed a statistically significant difference between the occlusal hypofunction and hyperfunction teeth (*P=0.03*). In the hypoestrogenism group, MMP13 was overexpressed in hypofunction teeth (*P=0.045*).

Figure 4. Relative gene expression of MMP1, MMP8 and MMP13 in the odontogenic region in teeth with occlusal hypofunction and hyperfunction. MMP13 demonstrated a significant difference between hypofunction and hyperfunction conditions (*P=0.03*).



Source: Search data.

Table 1 shows the comparison between occlusal hypofunction and hyperfunction conditions.

Table 1. Gene expression of MMP1, MMP8 and MMP13 in the odontogenic region of the lower incisors of both groups, in teeth with occlusal hypofunction and hyperfunction.

Groups	**Hypoestrogenism**	***p-value***	**Control**	***p-value***
Mean (SD) – MMP1				
Hypofunction tooth	1.01 (0.47)	*0.21*	0.98 (0.33)	*0.19*
Hyperfunction tooth	1.16 (0.19)		0.65 (0.02)	
Mean (SD) – MMP8				
Hypofunction tooth	0.98 (0.43)	*0.16*	0.99 (0.39)	*0.33*
Hyperfunction tooth	0.58 (0.23)		1.06 (0.09)	
Mean (SD) - MMP13				
Hypofunction tooth	1.32 (0.29)	*0.045**	1.18 (0.16)	*0.48*
Hyperfunction tooth	0.80 (0.28)		0.93 (0.51)	

Source: Search data.

Source: Authors.

4. Discussion

Some physiological and/or pathological conditions in the human body affects the odontogenic region causing alterations in the development of dental tissues (Omar et al., 2018). In this study, we established as an initial hypothesis that hypoestrogenism affects the expression of MMP1, MMP8 and MMP13 in the odontogenic region. To test this hypothesis, we used teeth with continuous growth in a murine model- rats. In this study, we also evaluated if the occlusal condition affected the MMPs expression. Therefore, the null hypothesis was rejected.

Estrogen receptors signaling pathways were associated with overexpression of MMPs (Jung et al., 2010; Lu et al., 2006), estrogen acts through its receptor and allowing the regulation of many genes. MMP1 has already been described as inversely related to the expression of sex steroid receptors in epithelial cells (Philips & Devaney, 2003). Both receptors, ERα and ERβ, were observed in cells in the odontogenic region. ERα expression was not affected under estrogen deficiency condition, while ERβ expression was increased in the odontogenic region in the hypoestrogenism group (Madalena, 2020). ERα can significantly increase the activity of the MMP13 promoter in particular, through the AP-1 activation site (Lu et al., 2006). ERβ signaling is more characterized by its performance in the nuclear signaling pathway, that is, the genomic signaling pathway (Hamilton et al., 2017). MMP8 (Orajarvi et al., 2011) and MMP13 were also differentially expressed in the mandibular condyle and tibia, respectively, of ovariectomized rats. However, in our present study, a statistical difference was not observed between hypoestrogenism and control groups, suggesting that the absence of estrogen is not an important factor in MMP1, MMP8, and MMP13 expression in the odontogenic region.

It is also important to report that the odontogenic region consisting basically of connective tissue and mineralized tissue. The increased expression of MMPs, especially the collagenases MMP1, MMP8, and MMP13, could influence collagen destruction in the periodontal ligament, affecting the occlusion. The first stages of remodeling of the periodontal ligament are marked by the action of collagenase, including MMP1, MMP8, MMP13, and MMP18, therefore, complete collagen degradation occurs through the action of MMPs gelatinase, MMP2, and MMP9 (Hui et al., 2001; Omar et al., 2018).

Initially, it was hypothesized that the mechanical force exerted in the hyperfunction tooth could increase the collagenases expression in the odontogenic region since, the express increase of some MMPs also seen in cells of the human periodontal ligament subjected to occlusal stress (Howard et al., 1988; Huang et al., 2008). The same hypothesis was applied in the hypoestrogenism group since an estrogen deficiency promotes fragility of the periodontal ligament (Wang & McCauley, 2016). However, our results demonstrated that MMP13 was overexpressed in the occlusal hypofunction, mainly in the hypoestrogenism group.

This interesting pattern was also observed in the experiment from Orajarvi et al. (2011) in which MMP8 positive cells was higher in the condylar cartilage of ovariectomized rats fed the soft diet than in non-ovariectomized control rats fed the soft diet. Also in their experiment, control rats fed with the normal diet had a higher MMP8 positive cells than control rats fed with the soft diet. Although the statistical difference was not observed for MMP8 in our study, it is possible this difference could be observed in an experiment with a larger sample.

In the study performed by Omar et al. (2018) also using rats' incisors as a model, they observed that the loss of occlusal contact, in rats submitted to hypofunctional eruption, increased MMP2 activity and eruption rate, but decreased MT1-MMP and TIMP-2 expression and disrupted collagen organization in the periodontal ligament. They concluded that occlusal contact may be an important factor for regulating the remodeling of the periodontal ligament against the continuous eruption process observed in rat incisors.

In our study, a statistical significant difference was observed only for MMP13 expression. MMP13 is highly important in collagen remodeling (Birkedal-Hansen et al., 1993; Souza & Line, 2002). Under occlusal hypofunction condition there is a constant bone resorption related to the periodontal ligament, which is three times faster than in teeth with hyperfunctional condition (Gerlach et al., 2002). In addition to estrogen deficiency, which also demonstrates a significant change in collagen, modifying bone porosity, as well as disorganization of the collagen fibers of the periodontal ligament (Zhang et al., 2011). These facts could explain the results observed here.

Finally, our result pointed some interesting factors related to the molecular mechanisms involved in the occlusion and how systemic condition affect the expression of important molecules in odontogenic region. More studies are necessary to evaluate different pathways.

5. Conclusion

Occlusal function affects MMP13 expression in the odontogenic region, in murine model.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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