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ORIGINAL ARTICLE

Effects of ovariectomy and aging on tooth attachment in female mice assessed by morphometric analysis

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Abstract

Objective. Non-human primates, dogs, rats, hamsters and ferrets, have frequently been used as laboratory animals in periodontal biology and pathology. In the past, mice have been used less for this purpose, but nowadays attract a lot of interest because gene knockout and transgenic technologies utilize mice primarily. In this study, we investigate the effects of ovariectomy and aging on tooth attachment in female mice. *Material and methods.* Eight-week-old mice ($n=15$) were divided into three experimental groups (control, $n=5$; sham-operated, $n=5$; ovariectomy, $n=5$) and ovaries removed bilaterally. Attachment level, assessed by measuring alveolar bone height and apical termination of the junctional epithelium, was determined 6 weeks post-ovariectomy by digital morphometric analysis in sagittal sections of the mandible. The plasma level of the inflammation marker serum amyloid A (SAA) was determined by ELISA. In another series of experiments, tooth attachment was determined in female mice ($n=7$) at 8–26 weeks of age. *Results.* Withdrawal of female sex hormone production by ovariectomy had no effect on alveolar bone height and apical termination of the junctional epithelium. The SAA level in plasma was unaffected by removal of the ovaries, suggesting that systemic inflammation is not induced by ovariectomy. Bone height was similar in mice sacrificed at 8–26 weeks of age and apical termination of the junctional epithelium was at the cemento-enamel junction at all ages. *Conclusions.* Removal of ovarian production of female sex hormones by ovariectomy has no influence on tooth attachment, and further tooth attachment is preserved with age in female mice.

Key Words: *Morphometry, mouse, ovariectomy, serum amyloid A, tooth attachment*

Introduction

Gene knockout technology developed in mice has attracted a great deal of interest and focus on the mouse as a laboratory animal. Knockout of genes in mice, important in periodontal biology and pathology, requires a phenotypic characterization of mouse periodontal tissues. In periodontology, animal studies have been performed mostly in non-human primates, dogs and rats, while fewer studies have been conducted in mice [1].

It has been suggested that female sex hormones influence periodontal tissues and the development of periodontal disease [2]. During pregnancy, which is associated with high levels of estrogen and progesterone in plasma, many women develop gingivitis and after menopause, which is associated with low levels of estrogen and progesterone, changes in periodontal tissues that may affect tooth attachment have been reported [3–6]. A higher frequency of gingival bleeding has been reported in estrogen-deficient women compared to women with normal levels of estrogens [5,7]. Post-menopausal women subjected to estrogen supplementation therapy have been reported to develop less clinical attachment loss over a 6 years period than those not on estrogen supplementation therapy [8], suggesting that estrogen may have a protecting effect. Ovariectomy is a well-established experimental technique in rodents inhibiting endogenous ovarian production of estrogens in order to obtain an experimental model with a hormonal status similar to that observed in post-menopausal women.

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Estrogen is a well-known regulator of bone metabolism and is assumed to act by inhibiting osteoclast formation and by reducing the bone-resorbing activity of terminally differentiated osteoclasts [9]. Estrogen deficiency thus causes elevated osteoclast activity and subsequently may lead to osteoporosis affecting tooth attachment. Elevated osteoclast formation and activity is mediated via increased receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio and estrogen deficiency may either directly or indirectly, via elevated cytokine expression, increase this ratio [9].

Estrogen is assumed to exert both anti- and pro-inflammatory effects [10]. Autoimmune diseases such as rheumatoid arthritis are more common in women than in men suggesting that female sex hormones are pro-inflammatory, but, on the other hand, rheumatoid arthritis often improves during pregnancy, suggesting that the female sex hormones also have a beneficial anti-inflammatory effect [11]. Estrogen has been reported to reduce expression of cytokine and adhesion molecule expression in the brain and in the vasculature, thereby acting as an anti-inflammatory agent [12–14]. We and others have reported that human periodontal ligament cells express estrogen receptors, proposing that estrogen affects periodontal tissue function [15,16]. It can therefore be hypothesized that withdrawal of female sex hormone production by ovariectomy may affect the inflammatory and immunological responses, which in turn influence tooth attachment level. Changes in systemic inflammation response can be demonstrated by measuring the plasma level of the inflammation marker SAA.

The objectives of the present study were to determine the effects of withdrawal of sex hormone production by ovariectomy and aging on tooth attachment, i.e. alveolar bone height and connective tissue attachment, in female mice by using morphometric analysis, since it can be hypothesized that estrogen deficiency influences tooth attachment.

Material and methods

Animals and experimental procedures

Adult female mice of the NMRI strain (8-week-old littermates, 22 mice in total) were purchased from Taconic (Tornbjerg, Denmark) and kept under standardized conditions in a regular 12 h light/dark cycle. The mice were anesthetized with pentobarbital sodium (75 mg/kg, i.p.) and the ovaries were removed bilaterally in accordance with standard procedures. The ovaries were identified and carefully removed. In sham-operated animals, the abdominal wall was opened and the ovaries exposed. Ovariectomy was performed in 5 mice, and 5 mice were sham-operated. Five animals served as non-operated controls. Immediately after closing the abdominal wall, the mice were placed on a heated (37°C) pad to control body temperature and they recovered rapidly (within 6–8 h) from the operation. After a 6-week period with food (normal mouse chow) and water *ad libitum*, the mice were killed by cervical dislocation. Bodyweight was determined before operation and at the time of sacrifice. Blood samples were immediately collected in heparin pretreated tubes by aspiration from the heart. The mandible was removed and freed from muscle, fat and connective tissue, fixed in 4% paraformaldehyde (in phosphate buffered saline, PBS) for 2 days at 4°C, and then washed carefully in PBS. In another series of experiments, the effects of aging were determined. These mice ($n=7$) were littermates and kept together under standardized conditions. They were killed at 8, 14, 20, and 26 weeks of age. The mandibles were removed and processed as described above. The experiments were approved by the Animal Ethics Committee at Lund University (M210-06, October 2, 2006).

Determination of connective tissue attachment and alveolar bone height by morphometric analysis

The specimens were demineralized in citrate-buffered 22% formic acid for one week and subsequently dehydrated in graded series of ethanol and xylen. The mandibles were then divided between the incisors into right and left halves, embedded in paraffin and serially sectioned in 4 μm sagittal sections. The sections were stained with hematoxylin-eosin and analyzed using a light microscope (Nikon Eclipse 80i; Nikon Instr., Amstelveen, The Netherlands) equipped with a digital camera and image analysis software (Nikon DS-2Mv and DS-L1). Connective tissue attachment and alveolar bone height were determined morphometrically on digital images of the area between the 1st and 2nd molars. The connective tissue attachment level was determined by measuring the distance between the cemento-enamel junction and the apical termination of the junctional epithelium. The bone height was determined by measuring the distance between the cemento-enamel junctions at the distal surface of the 1st molar and the mesial surface on the 2nd molar, respectively, and the highest point on the alveolar crest (Figure 1). For every right and left half of the mandible, approximately 30 serial sections were analyzed and a mean value for each sample was computed. Morphometric analysis was performed in a blinded fashion.

Determination of serum amyloid A

Blood plasma levels of serum amyloid A were determined by ELISA using a kit from Tridelta Development Ltd., Kildane, Ireland. The samples were analysed in duplicate and serum amyloid A

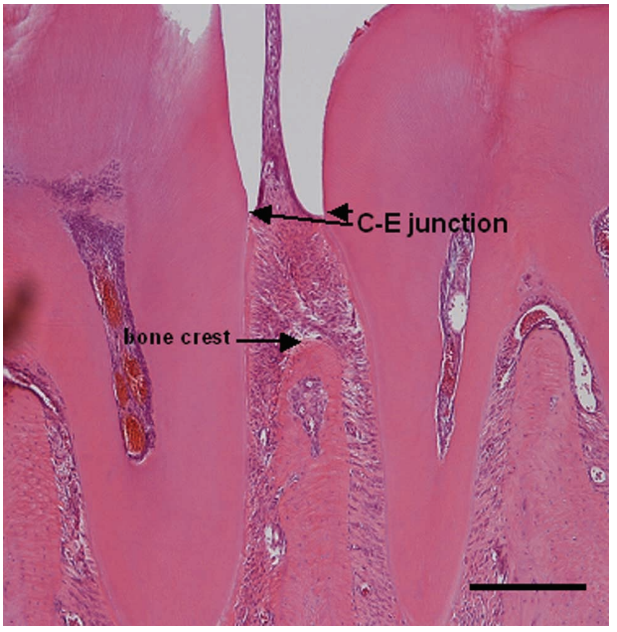

Figure 1. Alveolar bone height of the mouse mandible was determined by measuring the distance between the cemento-enamel (C-E) junction of the distal surface of the 1st molar and the mesial surface of the 2nd molar, respectively, and the highest point on the alveolar crest in longitudinal sections of mandibles stained with hematoxylin-eosin. This is a representative section from an unoperated control mouse 8 weeks of age. Bar represents 100 μm.

determined according to the instructions of the manufacturer.

Statistics

Summarized data are presented as means and SD. Statistical significance was calculated using Student's two-tailed *t*-test for unpaired comparisons. *P*-values <0.05 were regarded as denoting statistical significance. Bonferroni analysis was used for multiple comparisons as appropriate.

Results

Bodyweights and plasma SAA levels

The mice recovered within 6–8 h from the anesthesia and operation. As shown in Table I, no differences in bodyweights were observed between operated and control mice, i.e. neither before ovariectomy nor at the end of the 6-week post-ovariectomy observation period. In both ovariectomized (OVX) and control mice, bodyweights were increased by about 7% during the observation period. The plasma level of SAA was determined 6 weeks post-ovariectomy. No difference in SAA was observed between OVX and control mice (19.4 (SD 1.6) μg/mL in OVX mice vs. 23.1 (SD 11.4) μg/mL in control mice; $n=3$–5), showing that ovariectomy caused no chronic systemic inflammatory response within the observation period.

Table I. Bodyweights (g) in ovariectomized (OVX) and control mice before ovariectomy (B) and at the end of the 6-week observation period (E). Each mouse was weighed before operation and 6 weeks post-ovariectomy. Values are presented as means and SD of 5 observations in each group

	B	E
Control	31.2 (1.0)	33.5 (0.6)**
OVX	30.2 (2.2)	33.1 (2.0)*

*$P<0.05$ and **$p<0.01$ compared to bodyweights before ovariectomy.

Alveolar bone height and connective tissue attachment level

The alveolar bone height, computed as described in Figure 1, was similar in OVX, sham-operated and control mice killed 6 weeks post-ovariectomy. Alveolar bone heights in OVX, sham-operated and control mice were 94.0 (SD 23.6) μm, 95.2 (SD 36.1) μm, and 115.3 (SD 47.1) μm; $n=8$–12. The apical termination of the junctional epithelium was at the cemento-enamel junction in every individual animal in all three experimental groups, showing that the connective tissue attachment level was unaffected by the ovariectomy (Figure 2). Bone morphology, assessed in the hematoxylin-eosin stained sections, was similar in OVX, sham-operated and control animals (Figure 2). Lacunae with osteocytes were observed in both OVX and control mice.

In one set of experiments, we assessed the effects of aging on alveolar bone height and apical termination of the junctional epithelium in non-ovariectomized female littermate mice. Between 8, 14, 20, and 26 weeks of age, the bodyweights increased as the mice became older. The mice weighed about 60% more at 26 weeks of age than at 8 weeks of age (31.2 (SD 0.9) g at 8 weeks vs. 51.2 (SD 9.0) g at 26 weeks; $p<0.01$, $n=3$–5). The distance between the cemento-enamel junction and the alveolar crest was similar at all ages. The mean distance between the cemento-enamel junction and the alveolar crest at 8, 14, 20, and 26 weeks of age was 132.7 (SD 29.2) μm ($n=8$). The apical termination of the junctional epithelium was at the cemento-enamel junction in every individual animal at all ages (not shown).

Discussion

Here, we demonstrate morphometrically that tooth attachment in mice, assessed by measuring alveolar bone height and apical termination of the junctional epithelium, is not affected by removal of ovaries and aging between 8 and 26 weeks, suggesting that mouse periodontium is unaffected by periodontal pathogenic factors and systemic factors such as ovarian sex hormones within this time-frame. In the senescence-accelerated SAM mouse, changes in periodontal tissues, i.e. apical migration of the

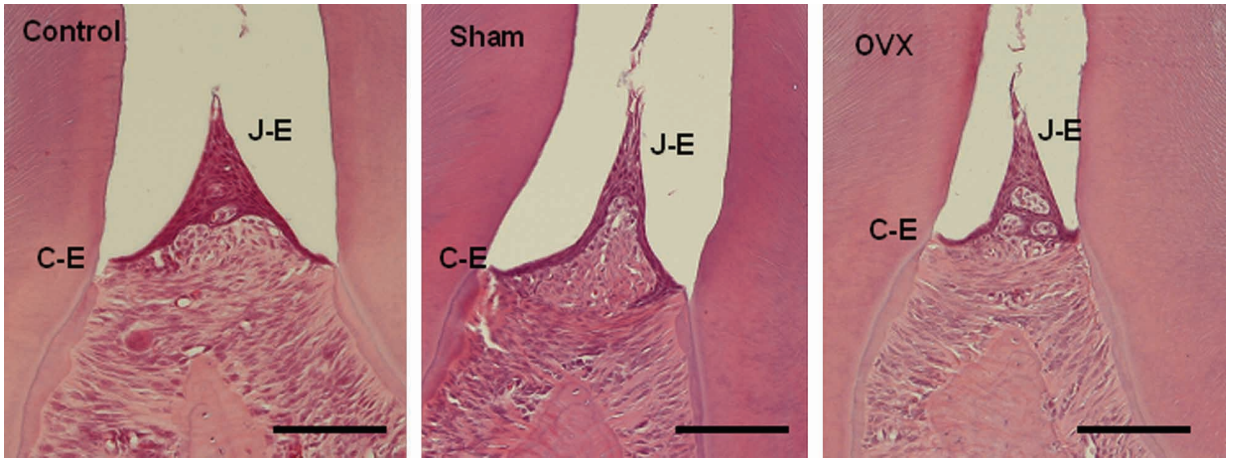

Figure 2. Assessment of the apical termination of the junctional epithelium in control, sham-operated (sham) and OVX mice. In each individual mouse, the apical termination of the junctional epithelium (J-E) was localized to the C-E junction in control, sham-operated, and in OVX mice. Bars represent 100 μm.

junctional epithelium and a decrease in bone height, have been reported [17,18], suggesting that periodontal tissues are affected in pre-term senescence but not normal mice. Laboratory mice are normally used for biomedical investigations and experiments between 2 and 6 months of age [19], i.e. at the same age as used by us in the present study. Our data show that tooth attachment is unaltered by age and lack of ovaries within this time-frame, leading to the conclusion that tooth attachment is stable and robust and that changes in attachment do not occur without provocation in the female mouse. Our conclusions are based on robust and conclusive data, although we have to admit that the animal number in each experimental group is small.

The SAA protein is a major acute-phase reactant in all vertebrates produced mainly by the liver in response to inflammation promoters and cytokines/chemokines [20]. During inflammation, the *in vivo* concentration of SAA may increase by as much as 1000 times. The SAA protein shows the same response speed and sensitivity as C-reactive protein (CRP) [20,21]. In the present study, we show that the ovariectomy induces no change in the basal production of inflammation markers as demonstrated by unaltered plasma SAA level in OVX compared to control mice. These data suggest that withdrawal of endogenous female sex hormone production in mice has no impact on the systemic inflammatory response. In humans divergent effects of female sex hormones on CRP have been reported. In post-menopausal women receiving hormone replacement therapy both increased and unaltered level of CRP have been reported [22,23].

In the present study, we report that removal of endogenous sex hormone production by ovariectomy in mice has no effect on connective tissue attachment level and alveolar bone height determined 6 weeks after ovariectomy. It might be argued that 6 weeks is too short an observation time. However, in rats, a decrease in mandibular alveolar bone height 6 weeks post-ovariectomy, i.e. at the same time-point after ovariectomy at which we observed no effect in mice, has been reported by Cao et al. [24], suggesting that there is an important difference between rats and mice in their alveolar bone response to removal of the ovaries. Osteoclastogenesis has been reported to be induced in the OVX rat periodontium, suggesting that alveolar bone decomposition observed in the OVX rat is caused by elevated activity of osteoclasts [25]. We cannot rule out the possibility that ovariectomy in mice would affect periodontal tissues after, for example, ligation of teeth or treatment with inflammation promoters, but nevertheless rats seem to be more sensitive than mice to removal of the ovaries.

Characterization of mouse periodontal tissues and tooth attachment at different ages and hormonal status, as performed by us in the present study, has an impact on the future evaluation of periodontal tissue phenotype in transgenic and gene knockout mouse models. The numbers of transgenic and gene knockout mouse models are increasing successively and thus more and more attention will be drawn to the mouse as laboratory animal, which will undoubtedly have an impact on future periodontal research.

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Declaration of interest: The authors reports no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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