

Epithelial rests of Malassez in experimental animals at different ages

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ABSTRACT

Epithelial rests of Malassez (ERMs) are fragments of Hertwig's sheath in the periodontal ligament. There is extensive knowledge of their role in the etiology of pathological processes and current evidence links them to maintenance of periodontal homeostasis. The aim of this study was to assess the behavior of ERMs with relation to the changes in periodontal tissues during growth in an experimental model with Wistar rats. Mesiodistal sections were made of the first lower molars from Wistar rats aged 1 month (n=7), 3 months (n=7) and 5 months (n=6). Sections were stained with H&E to evaluate number of ERMs/mm, size/area of ERMs (μm^2), height of periodontal ligament (PL.h) (μm), area of cement in the furcation zone (C.Ar) (μm^2) and bone area related to the ERM zone (BAr/TAr)(%). Post-hoc Bonferroni and ANOVA were applied for statistical analysis of results. Number of ERMs/mm declined significantly with age (1m: 4.34 ± 1.51 , 3m: 1.48 ± 0.89 , 5m: 0.27 ± 0.50 , $p \leq 0.05$), and there was great variability in size. There was significant

increase in C.Ar (1m: 3418.96 ± 905.88 , 3m: 19365.76 ± 5500.52 , 5m: 32182.76 ± 7114.51 , $p \leq 0.05$) and interradicular (BAr/TAr) (1m: 25.26 ± 2.37 , 3m: 44.70 ± 3.95 , 5m: 46.81 ± 7.80 , $p \leq 0.05$: 1 vs 3, 1 vs 5). There was significant decline in PLh at 5 months (1m: 141.42 ± 29.25 , 3m: 162.06 ± 28.21 , 5m: 124.72 ± 18.67 , $p \leq 0.05$: 1 vs. 3, 3 vs. 5). The decline in number of ERMs as animal age increases may be related to the significant increase in C.Ar and reduction in PL.h. It remains to ascertain its relationship with the increase observed in BAr/TAr. Further studies are needed to learn more about the role of ERMs and their relationship with periodontal tissues when confronted with different normal and pathological stimuli.

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Keywords: epithelial rests of Malassez - periodontal ligament - alveolar bone - periodontium - histomorphometry.

Restos epiteliales de Malassez en animales experimentales a diferentes edades

RESUMEN

Los restos epiteliales de Malassez (ERMs) son fragmentos de la vaina de Hertwig, en el ligamento periodontal. Se conoce extensamente su rol en la etiología de procesos patológicos y actualmente las evidencias los vinculan al mantenimiento de la homeostasis periodontal. El objetivo del presente trabajo fue evaluar el comportamiento de los ERMs en relación a los cambios de los tejidos periodontales durante el crecimiento en un modelo experimental de ratas Wistar. Se obtuvieron cortes mesio-distales del 1er molar inferior de ratas Wistar de 1 mes (n=7), 3 meses (n=7) y 5 meses (n=6) de edad. En cortes coloreados con H&E, se evaluó: N° de ERMs/mm, tamaño/área de ERMs (μm^2), altura del ligamento periodontal (PL.h) (μm), área de cemento en la zona de furcación (C.Ar) (μm^2) y área del hueso relacionado con la zona de los ERMs (BAr/TAr) (%). Los resultados se analizaron estadísticamente mediante ANOVA y Bonferroni post-hoc. El N° de ERMs/mm disminuyó significativamente con la edad (1m: 4.34 ± 1.51 , 3m: 1.48 ± 0.89 , 5m: 0.27 ± 0.50 , $p \leq 0.05$), observándose una gran variabilidad

de tamaño. Se observó un aumento significativo del C.Ar (1m: 3418.96 ± 905.88 , 3m: 19365.76 ± 5500.52 , 5m: 32182.76 ± 7114.51 , $p \leq 0.05$) y del (BAr/TAr). interradicular (1m: 25.26 ± 2.37 , 3m: 44.70 ± 3.95 , 5m: 46.81 ± 7.80 , $p \leq 0.05$: 1 vs 3, 1 vs 5). Además, se halló una disminución significativa en PLh a los 5 meses (1m: 141.42 ± 29.25 , 3m: 162.06 ± 28.21 , 5m: 124.72 ± 18.67 , $p \leq 0.05$: 1 vs 3, 3 vs 5). La disminución hallada del número de ERMs conforme aumenta la edad del animal podría vincularse con el aumento significativo del C.Ar y la reducción del PL.h. Resta por dilucidar su relación con el aumento de BAr/TAr. observado. Se requieren más estudios para profundizar sobre el rol de los ERMs y su relación con los tejidos periodontales ante distintos estímulos normales y patológicos.

Palabras clave: restos epiteliales de Malassez - ligamento periodontal - hueso alveolar - periodontium - histomorfometría.

INTRODUCTION

The insertion periodontium components that make up the topographic and functional unit are root cementum, periodontal ligament and alveolar bone. These structures evolve in an interrelated, coordinated manner over the life of the tooth, adapting continuously to changes that take place in the mouth. The periodontal ligament is located between the cementum and the alveolar bone. It is a fibrous connective tissue comprising a wide variety of cells: fibroblasts, osteoblasts, cementoblasts, osteoclasts, macrophages, mastocytes, undifferentiated ectomesenchymal cells and epithelial rests of Malassez (ERMs). Cell activity is mediated by several signaling factors that regulate the complex growth and regeneration machinery of periodontal structures¹. After root formation, remnants of Hertwig's sheath persist as ERMs throughout the life of the tooth^{2,3}. Although their etiological role in the formation of odontogenic tumors and cysts is widely known, ERMs are currently a subject of interest as structures that take part in the histophysiology of the periodontal ligament. Histologically, ERMs are identified as small groups of epithelial cells within the periodontal ligament, in close contact with the cementum surface, becoming increasingly distant from the cementum surface towards the coronal third of the root⁴. Oblique sections of the periodontal ligament have shown ERMs to form part of mesh around the root⁵. ERMs have also been shown to have an irregular nucleus with dense heterochromatin and a small, scarcely distinguishable peripheral halo of cytoplasm⁶. ERM ultrastructure in sections of rat periodontal ligament is similar to that in humans and other animals^{7,8}. A basal lamina separates the ERM cell islands from the connective tissue⁹, and there are tight junctions between them and hemidesmosomes, and tonofilaments within the cytoplasm. ERMs can produce different proteins and macromolecules, including cytokeratins^{10,11} and neuropeptides¹². The expression of CK 17 could be a marker used for identification¹³. Other studies^{14,15} also report the expression of cell surface proteins such as epidermal growth factor receptors. It has been observed that proteins typical of the enamel matrix such as amelogenin and ameloblastin are expressed from samples of human periodontal ligament^{16,17}. It has been suggested that these two proteins may act as growth factors and participate in adhesiveness, proliferation, migration and differentiation of

periodontal ligament fibroblasts^{18,19}. Moreover, it has been found that disruption of periodontal integrity induced early expression of APINA (another protein of the enamel matrix), forming part of a cascade of events possibly leading to the activation of the ERMs during periodontal healing and regeneration²⁰.

Despite the ectodermal origin and epithelial nature of these cell groups, they can synthesize components that are frequently associated to cells of mesenchymal origin, such as glycosaminoglycans, hyaluronic acid, dermatan sulfate and chondroitin sulfate²¹, as well as osteopontin (OPN), bone sialoprotein (BSP) and osteoprotegerin (OPG)^{4,22}. They can also degrade collagen by synthesis of collagenases and proteinases^{23,24}. It has therefore been suggested that they may contribute to periodontal regeneration by synthesizing a series of proteins related to the cementum and the bone tissues²⁵. Current scientific evidence suggests that the possible role of ERMs in adult periodontal ligament is related to maintaining homeostasis of the periodontal space, thereby preventing ankylosis, inhibiting root resorption and contributing to cementum repair^{26,27}.

In previous papers, our group has reported that in an experimental periodontitis model, ERMs exhibited cell hypertrophy when there was root resorption²⁸. Other authors have evaluated ERM cell response to *in vivo* mechanical stimuli, observing cell proliferation and hypertrophy²⁹. Regarding this point, our group studied ERMs in an experimental model of root resorption mediated by orthodontic forces in rats, finding no significant morphological change that would highlight their role in root resorption induced by orthodontic forces³⁰. Other studies have described the expression of several proteins^{27,31} that may contribute to the maintenance of cementogenesis and osteogenesis. Among these proteins, the expression of HSP 70 (Heat Shock Protein) by ERMs may provide protection against different forms of attack, including oxidizing agents, inflammation, hypoxia, hyperthermia and mechanical stimuli such as orthodontic forces³².

The **aim** of this study was to assess the behavior of periodontal tissues with relation to ERMs in healthy Wistar rats at different ages.

MATERIALS AND METHODS

We used 20 healthy male Wistar rats aged 1 month (n=7), 3 months (n=7) and 5 months (n=6). All

animals had “ad libitum” access to balanced feed and water. They were housed in galvanized wire cages containing not more than 5 animals each, with temperature 21 °C to 24 °C; Humidity 52% to 56%; light/dark cycles: 12 hours/12 hours.

The experimental protocol was approved by the Ethics Committee of the School of Dentistry 012/2016 CICUAL-ODONTO-FOUBA of Buenos Aires, Argentina, and is in keeping with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Histology and Histomorphometry

Following euthanasia at the relevant experimental times, lower jaws were extracted and fixed in 10% buffered formalin (pH 7.4). Samples were desiccated and decalcified in 10% EDTA solution, pH 7.0, for 25 days.

The section of each hemimandible corresponding to the three lower molars was embedded in paraffin to prepare mesiodistal histological sections of the lower first molar. Sections were stained with hematoxylin-eosin. The section with best orientation from each hemimandible, showing mesial and distal roots up to the apices, was selected.

The following parameters were evaluated at the furcation zone:

- **Number of ERMs/mm:** number of ERMs in the periodontal ligament in the perimeter limited by the beginning of cellular cementum, expressed with relation to root surface (Fig. 1).
- **Size/Area of ERMs (μm^2):** Area of ERMs
- **Height of periodontal ligament (PL.h) (μm):** average of five equidistant linear measurements between the cementum surface and the beginning of the bone tissue (Fig. 2).
- **Area of cementum in the furcation zone: C.Ar (μm^2):** measurement of the area of cementum in the zone limited by the projection of two lines, on the mesial and distal root surfaces (Fig. 3).

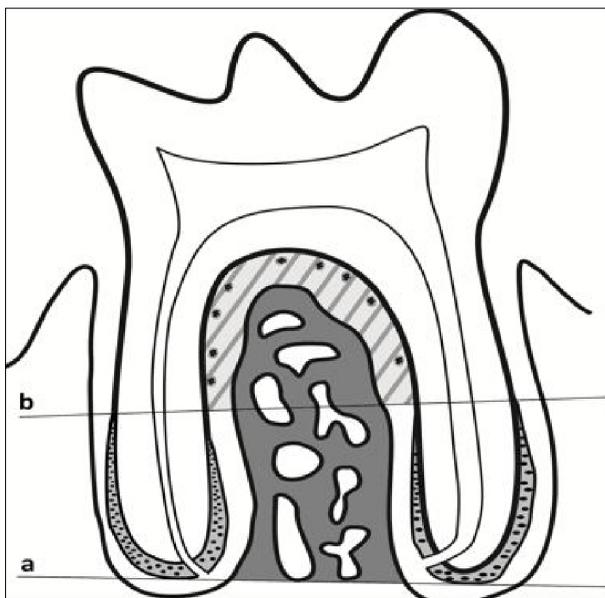


Fig. 1: Diagram of rat molar in situ. Line “a” joins the two apices and limits the total interradicular space in which B.Ar/T.Ar (%) was evaluated. Line “b”, drawn at the beginning of the cellular cementum, limits the periodontal area (shaded and lined) where ERMs were evaluated.

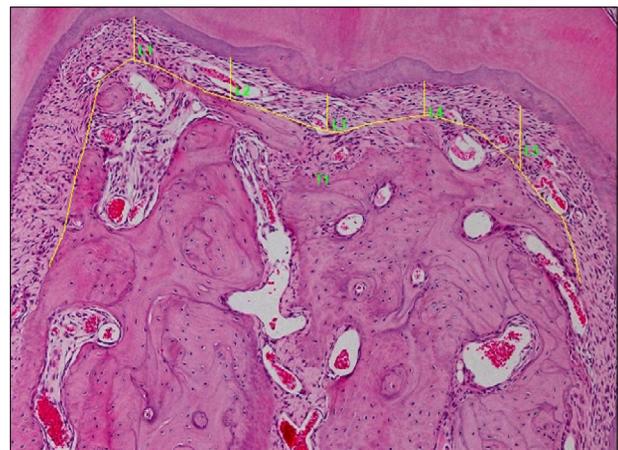


Fig. 2: Microphotograph of a mesiodistal section of lower molar showing the 5 lines used for measuring height of the periodontal ligament. H&E. 100X.

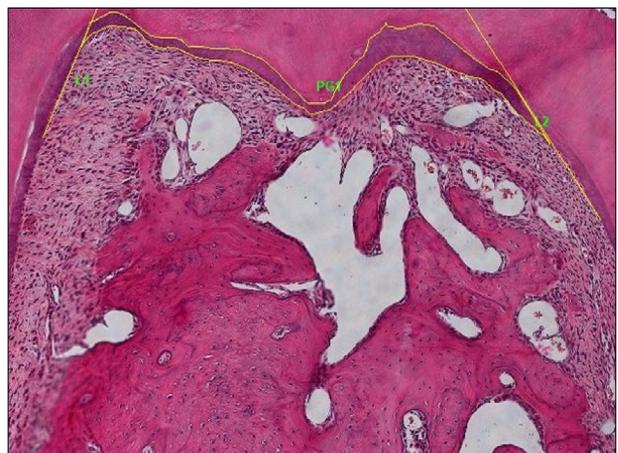


Fig. 3: Microphotograph of a mesiodistal section of lower molar. The two lines (L1 and L2) projected on the cementum surface demarcate the furcation zone where cementum area (C.Ar) was evaluated. H&E. 100X.

- **Bone area B.Ar/T.Ar:** Area of trabecular bone tissue in the zone selected for measuring ERMs, expressed as percentage of total area of interradicular bone (Fig. 1).

Number of ERMs was counted directly under 100x magnification with a Bausch & Lomb microscope. The rest of the histomorphometric measurements were taken using 100X digital microphotographs taken with a Nikon Eclipse photomicroscope and *Image Pro-Plus* Software.

Statistical analysis was performed with post-hoc Bonferroni and ANOVA, considering significance at $p < 0.05$.

RESULTS

The results show that number of ERMs/mm declined significantly with age of animals, with major variability and high standard deviations in size of ERMs found in each group. This variability is reflected by the high standard deviations obtained for this parameter. In the 5-month-old group, ERMs were only observed in 3 of the 6 animals. Moreover, there was a significant increase in cementum area and bone area in the interradicular zone and a significant decrease in the height of the periodontal ligament in the 5-month-old group in relation to the other groups (Table 1 and Figs 4 and 5). Interestingly, we observed nuclei compatible with ERMs associated with and even embedded in the cementum matrix in the furcation zone (Fig. 6).

Table 1: Values of the different parameters expressed as mean \pm SD

	1 month	3 months	5 months
Number of ERMs/mm	4.34 \pm 1.51 a	1.48 \pm 0.89 b	0.27 \pm 0.50 c
Size/Area of ERMs (μm^2)	213.61 \pm 61.84 a	431.91 \pm 180.21 b	203.43 \pm 82.23 a
Height of periodontal ligament (PL.h) (μm)	141.42 \pm 29.25 a b	162.06 \pm 28.21 b	124.72 \pm 18.67 a
Area of cementum at furcation (C.Ar) (μm^2)	3418.96 \pm 905.88 a	19365.76 \pm 5500.52 b	32182.76 \pm 7114.51 c
Bone area (B.Ar/T.Ar) (%)	25.26 \pm 2.37 a	44.70 \pm 3.95 b	46.81 \pm 7.80 b

Means with same letter do not differ significantly ($p \geq 0.05$).

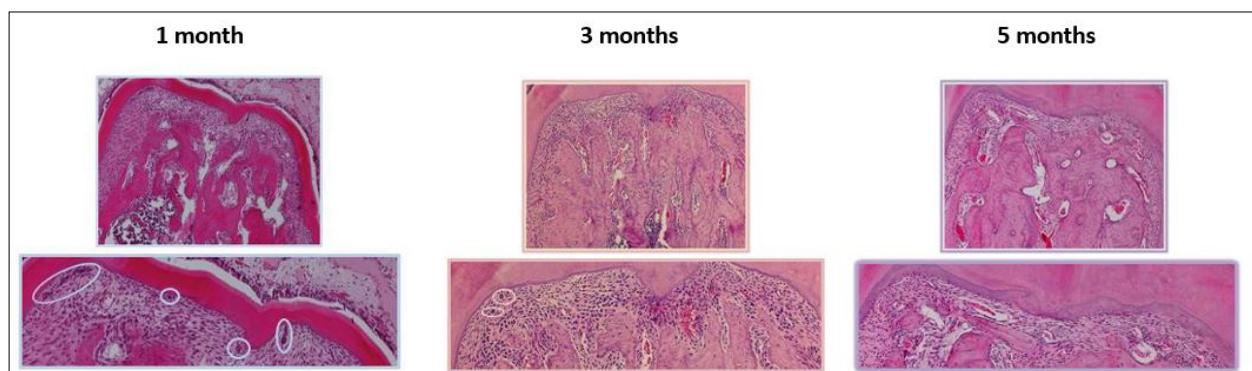


Fig. 4: Microphotographs of mesiodistal sections of lower molar of Wistar rats aged 1, 3 and 5 months, showing the decline in number of ERMs, the increase in cementum area at the level of the furcation and the reduction of the periodontal space as animal chronological age increases. H&E. 40 and 100X.

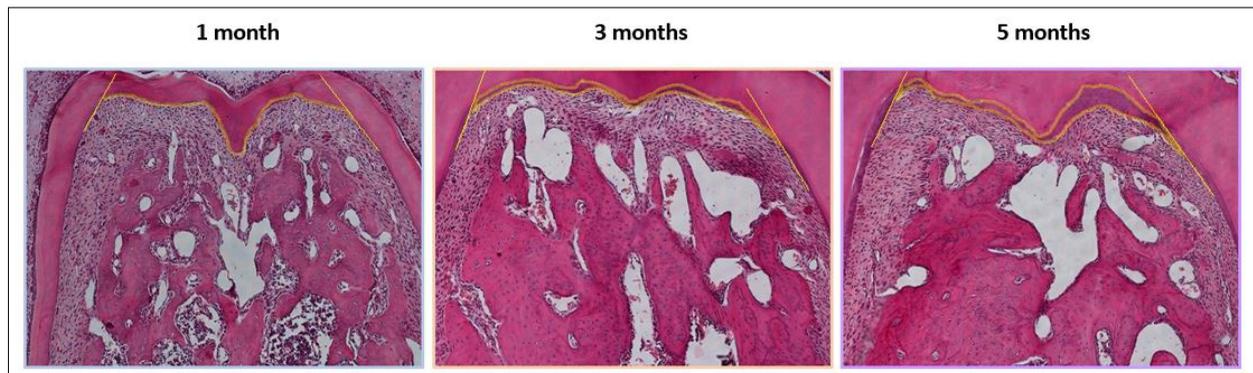


Fig. 5: Microphotographs of mesiodistal sections of lower molar in Wistar rats aged 1, 3 and 5 months, showing delimitation established for evaluating the cementum area in the furcation zone in the 3 age groups. H&E.100X.

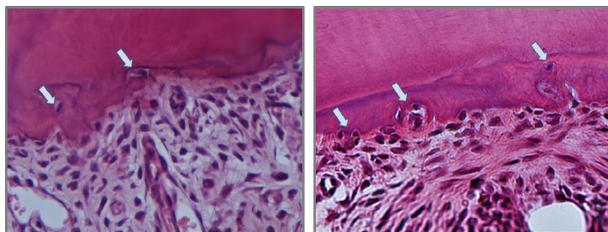


Fig. 6: Microphotographs of mesiodistal sections of lower molar in Wistar rat aged 5 months, showing cell nuclei compatible with ERMs embedded in the cementum (arrows). H&E. 40X.

DISCUSSION

The results of this study show that the number of ERMs of lower molars in Wistar rats declines significantly as animal chronological age increases, and that there is major variability in size. The decline in number of ERMs was accompanied by a significant increase in interradicular cementum area at the level of the furcation and the interradicular bone area, entailing a reduction in height of the periodontal ligament when the animals were 5 months old.

The decline observed in number of ERMs with increase in age agrees with different papers published since 1950. Wentz et al.³³ suggests that the decline in number of ERMs with age may be because they degenerate and then become calcified. Studies on human ERMs have also reported that they decline as age increases, and studies on older people report the onset of ERM calcification, which persist as cementum-like structures/cementicles³⁴. A study by Gonçalves et al.³⁵ suggests that such decline may be partly due to cell apoptosis. In our study, the decline with age was accompanied by, and probably related to, an increase in the cementum area and interradicular bone volume and the consequent reduction in the height of the periodontal ligament.

Sims et al.³⁶ also reports a reduction in the thickness of the periodontal space as age increased in mice.

It is important to note that although our study did not find isolated partial calcifications in the periodontal ligament, it did find nuclei of cells compatible with possible ERMs embedded in the cementum associated to the interradicular space. The hypothesis that the nuclei observed in the radicular cementum matrix may have originated from nearby ERMs is also supported by the well-known fact that the topographic zone of the furcation and radicular cervical third is not characterized by presenting cellular cementum.

Hasegawa et al.³⁷ showed that during cementum repair, ERMs express osteopontin and ameloblastin, suggesting that they may be involved in cementum repair. More recent findings suggest that within the ERMs, there is a cell population with properties similar to those of undifferentiated mesenchymal cells, which, under the right stimulus, can differentiate into cells capable of synthesizing mineralized matrices in a particular microenvironment³⁸.

It has been more than twenty years since enamel matrix derivatives were introduced in clinical periodontics for use in regenerative therapies. Nevertheless, further research is still needed for better characterization of their effects on the behavior of different tissues and on the different cells³⁹. In this context, it is worth noting that ERMs can express proteins typical of the enamel matrix, such as amelogenin and ameloblastin¹⁶. These proteins may have a strong effect on cell activity of the insertion periodontum^{18,19}. Considering that ERMs may be a natural source of these proteins within the periodontal ligament itself, it is essential to continue learning about the behavior of ERMs in different contexts and under different stimuli.

The current study also found an increase in interradicular bone volume as chronological age increases, in agreement with other studies conducted by our group⁴⁰. This finding poses a question that is yet unanswered, regarding whether there is any association between the increase in bone volume found and the behavior and/or presence of ERMs in the periodontal ligament. In further studies we will endeavor to ascertain whether there are mechanisms that operate in said association and how they work.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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CONCLUSIONS

Further studies are needed to learn more about the role of ERMs and their association with the cementum, periodontal ligament and bone tissue, both in health and in situations requiring periodontal repair and/or regeneration mechanisms. In addition to providing insight regarding the biology of ERMs, this study contributes to a better characterization of an animal model that is extensively used in research on different aspects of oral pathology.

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