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Short communication

# Cell death and quantitative reduction of rests of Malassez according to age

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*Background and Objective:* Rests of Malassez are clusters of epithelial cells that remain in the periodontal ligament throughout life. However, it has been reported that the number of these structures decreases with age, and some epithelial cells undergo apoptosis in rests of Malassez of young and adult rats. Therefore, the purpose of the present study was to investigate the incidence of epithelial cell death and the quantitative changes in the rests of Malassez in rat molars of different ages.

*Material and Methods:* Fragments containing the upper molars of rats aged 29, 45 and 120 d were fixed, decalcified and embedded for analysis by light microscopy. In the sections stained by hematoxylin and eosin, the number of rests of Malassez and the number of nuclei of these epithelial structures were obtained. Moreover, the nuclei exhibiting typical features of cell death were also counted in each rest of Malassez. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method for detection of cell death was also carried out.

*Results:* In all groups examined, some rests of Malassez exhibited epithelial cell nuclei with typical features of apoptosis and some of them were also TUNEL positive. From 29 to 120 d of age in rats, the quantitative analysis showed a significant decrease in the total number of rests of Malassez in the cervical, middle and furcation regions of the periodontal ligament. Moreover, a significant decrease of epithelial cell nuclei was concomitant to an increase in the frequency of cell death in the oldest rats.

*Conclusion:* These results suggest that epithelial cell death by apoptosis may be, at least in part, responsible for the reduction in the number of rests of Malassez according to age.

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Rests of Malassez are clusters of epithelial cells that arise from fragmentation of the Hertwig's root sheath and remain in the periodontal ligament throughout life (1,2). Although rests of Malassez exhibit proliferative activity (3,4), it has been reported that the number of these structures decreases with aging (5–8). It is known that cell death by apoptosis plays an important role in the maintenance of the cellular population in the different tissues and organs (9–11). Cell death by apoptosis involves a series of molecular events, including DNA fragmentation, which can be detected *in situ* by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (10,12). Moreover, the complex biochemical cascade that occurs during apoptosis leads to typical morphological changes (9,10).

Recently, the occurrence of apoptosis – detected by the TUNEL method and confirmed by transmission electron microscopy – was verified in some epithelial cells of rests of Malassez in

young and adult rats (13). Therefore, the aim of the present study was to investigate the incidence of epithelial cell death and the quantitative changes in the rests of Malassez in rat molars of different ages.

## Material and methods

Fifteen male Holtzman rats, aged 29, 45 and 120 d, were killed with chloral hydrate (600 mg/kg). The left and right maxillary fragments containing the molars with surrounding periodontal tissues were removed and immediately fixed in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate. After decalcification in 7% EDTA (pH 7.2), the specimens were dehydrated and embedded in paraffin. The sections were stained with hematoxylin and eosin and submitted to the TUNEL method for detection of cell death. Some specimens were also embedded in Historesin (Jung, Heidelberg, Germany) and the sections were stained with hematoxylin and eosin (14).

The TUNEL method, for detection of DNA breaks (12), was used as previously described (15) and according to the Apop-Tag Plus kit (Chemicon Internacional, Chemicula, CA, USA). The reaction was revealed with 0.06% 3,3′-diaminobenzidine (Sigma-Aldrich Co., St Louis, MO, USA) and the sections were counterstained with hematoxylin. Sections of involuting mammary gland were used as positive controls. Negative controls were incubated in a terminal deoxynucleotidyl transferase enzyme-free solution.

In five paraffin sections of the first molar per animal, the number of rests of Malassez and of epithelial cell nuclei was counted in each portion of the periodontal ligament at a magnification of ×500. The nuclei exhibiting typical features of cell death were also counted, at a magnification of ×1000. These typical characteristics included: nuclei with chromatin strongly stained by hematoxylin; nuclei showing a half-moon or a ring shape; and dense bodies strongly stained with hematoxylin. The data were submitted for statistical analysis using the nonparametric Kruskal–Wallis test.

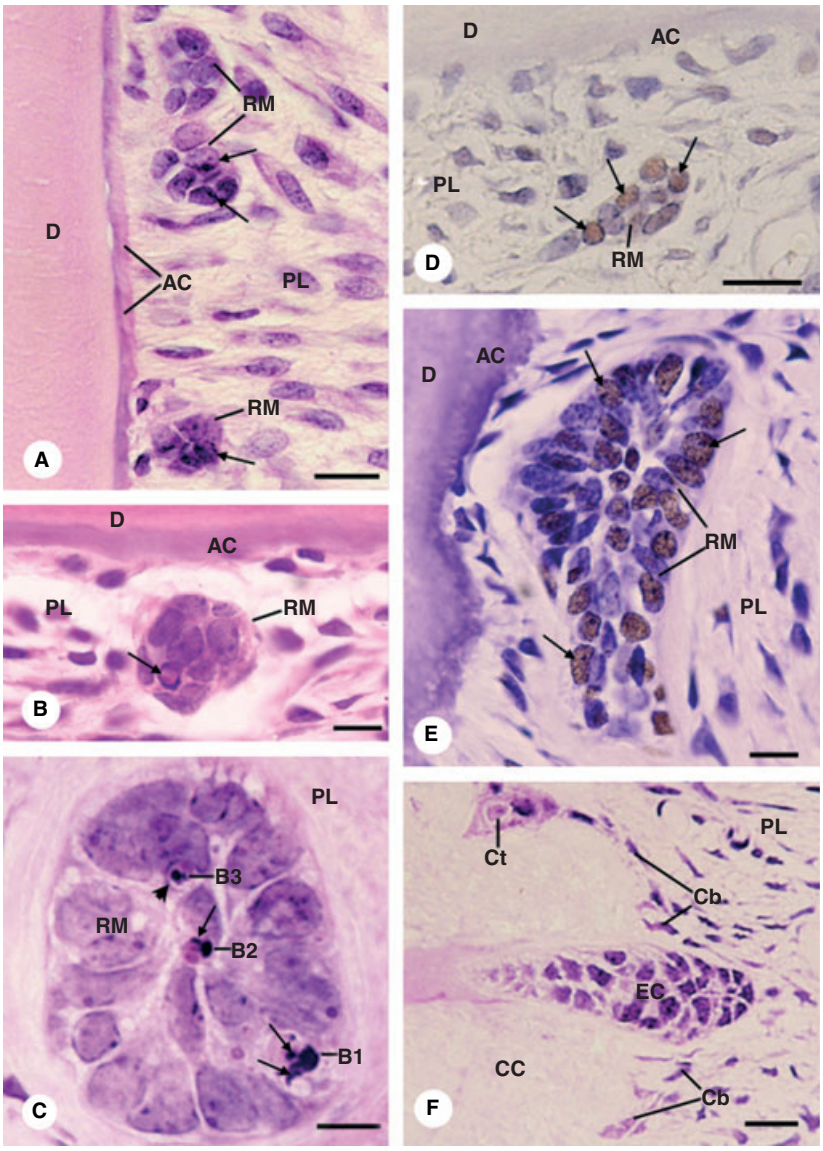


*Fig. 1.* Light micrographs of portions of molar roots stained by hematoxylin and eosin (A, B, C, F) and submitted to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (D, E). (A) Cervical portion of the root from a 29-d-old rat exhibiting small rests of Malassez in the periodontal ligament adjacent to the acellular cementum. Epithelial cells exhibiting nuclei with irregular masses of chromatin strongly stained by hematoxylin (indicated by arrows) are observed. Bar: 20 μm. (B) A furcation portion of a root from a 45-d-old rat shows a rest of Malassez next to the cementum surface (AC), exhibiting an epithelial cell with a ring-shaped nucleus (arrow). Bar: 10 μm. (C) A cervical portion of root from a 120-d-old rat shows a rest of Malassez exhibiting round/ovoid bodies (B1, B2 and B3) strongly stained by hematoxylin. Next to B1 and B2, small dense bodies (indicated by arrows) are also observed. The round body (B3) appears to be inside a vacuolar structure (indicated by an arrowhead) within the rest of Malassez. Bar: 10 μm. (D) A furcation region of root from a 29-d-old rat shows a rest of Malassez exhibiting TUNEL-positive nuclei (indicated by arrows), stained yellow–brown. Bar: 20 μm. (E) A cervical portion of root from a 120-d-old rat exhibits a large rest of Malassez showing several TUNEL-positive nuclei (indicated by arrows) intermingled with TUNEL-negative epithelial cells. Bar: 10 μm. (F) An apical portion of the root from a 45-d-old rat shows a cluster of epithelial cells partially entrapped in the cellular cementum matrix. Bar: 20 μm. AC, acellular cementum; Cb, cementoblasts in the cementum surface; CC, cellular cementum matrix; Ct, cementocyte; D, dentine; EC, epithelial cells; PL, periodontal ligament; RM, rest of Malassez.

## Results

In all groups, rests of Malassez exhibiting 6–10 cells were found in the cervical, middle and furcation regions of the periodontal ligament (Fig. 1A,B,D). However, free rests of Malassez were not observed in the apical portion; in this portion, occasional clusters of epithelial cells were found partially enclosed in the cellular cementum matrix (Fig. 1F). Large rests of Malassez containing numerous epithelial cells were only observed in 120-d-old rats (Fig. 1C,E). Rests of Malassez showing cells with irregular masses of chromatin strongly stained by hematoxylin (Fig. 1A) were found in all groups. In some rests of Malassez, cells showing ring-shaped nuclei (Fig. 1B) and small basophilic round/ovoid bodies were also observed (Fig. 1C). Moreover, TUNEL-positive structures (brown–yellow color) were observed (Fig. 1D,E).

In all groups, the cervical region showed the highest incidence of rests of Malassez (Fig. 2). From 29 to 120 d of age, the number of rests of Malassez decreased significantly (by 80–95%) in cervical, middle and furcation regions (Fig. 2). Moreover, the total number of epithelial cell nuclei also decreased significantly (by 70%); this reduction was concomitant to the increase (50%) observed in the frequency of cell death (Table 1).

## Discussion

Our quantitative results showed that in 120-d-old rats, the number of rests of Malassez in all the regions of the periodontium decreased significantly in comparison to the number of rests of Malassez in 29- and 45-d-old rats. Some authors have suggested that reduction in the number of rests of Malassez with age could be explained by degeneration of these structures followed by calcification (5,6); moreover, a possible epithelial–mesenchymal transformation into cementoblasts has been also suggested (1,3,16). On the other hand, Cerri & Katchburian (13) demonstrated that some epithelial cells undergo apoptosis in the rests of Malassez. Considering the morphological features combined with the TUNEL positivity, our results confirm the occurrence of cell death in the rests of Malassez. The TUNEL method reveals DNA fragmentation that occurs during cell death by apoptosis (9,10,12). Moreover, apoptosis in the cells of the rests of Malassez has been confirmed by typical ultrastructural characteristics, as shown by Cerri & Katchburian (13). Therefore, the high incidence of apoptosis in the rests of Malassez of 45- and 120-d-old rats, associated with the decrease (70%) in the total number of epithelial cell nuclei, strongly suggest that cell death may be, at least in part, responsible for the reduction in the number of rests of Malassez in the oldest rats.

As also described by other authors (6,17,18), our results revealed a high incidence of rests of Malassez in the cervical region in all groups. The incidence of rests of Malassez in this region may be explained by the presence of a constant inflammatory reaction in the gingiva; thus, the production of cytokines (tumor necrosis factor, transforming growth factor-α, interleukin-1 and interleukin-6) could stimulate the proliferation of epithelial cells of the rests of Malassez (2,6,19). This suggestion is reinforced by the presence of large rests of Malassez containing numerous epithelial cells in this portion of periodontium of 120-d-old rats.

In rat molars, the formation of cellular cementum occurs rapidly in the apical portion (1). Thereby, epithelial cells of the Hertwig's root sheath are generally trapped inside the dentino–cementum junction (15,20,21) and some undergo cell death (15). In the present study, epithelial structures were also partially entrapped in the cellular cementum, as also described in pig molars (22). These findings could explain the absence of free rests of Malassez in the apical portion of the

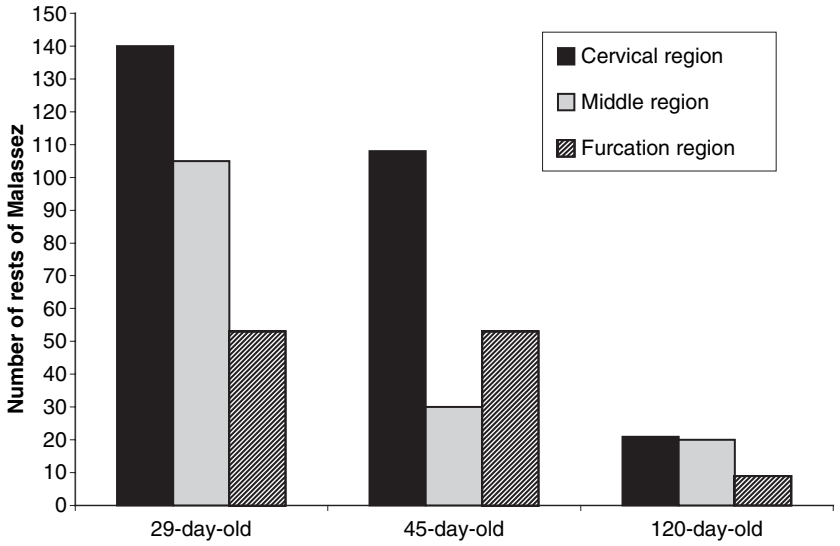


*Fig. 2.* Total number of rests of Malassez in the cervival, middle and furcation regions of the periodontium in 29-, 45- and 120-d-old rats.

*Table 1.* Number of rests of Malassez (RM), total number of nuclei of epithelial cells and frequency of cell death in the rests of Malassez of 29-, 45- and 120-d-old rats

| Groups | Number of RM | Number of nuclei | Frequency (%) of cell death |
|---|---|---|---|
| 29-d-old rats | 59.6 ± 13.24 | 227.4 ± 64.52 | 5.18 ± 2.27 |
| 45-d-old rats | 38.2 ± 7.59 | 171.2 ± 33.99 | 11.33 ± 7.48 |
| 120-d-old rats | 10 ± 7.34 | 61.6 ± 38.08 | 11.36 ± 7.48 |

periodontium in all ages of rats analyzed.

Although the rests of Malassez remain in the periodontal ligament throughout life, their exact function is unknown. Epithelial cells of rests of Malassez exhibit high levels of osteopontin – a glycoprotein that participates in the early tooth root development (23) and in cementum repair in adult rats (3). Thus, the participation of these structures in the formation and maintenance of the cementum could explain the high frequency of rests of Malassez in the young rats and their permanence in the adult rats. Epithelial rests also secrete prostaglandins (24) and interleukin-1α (25), which stimulates bone resorption, maintaining the periodontal ligament space. The significant reduction in the total number of epithelial cells in rats from 29 to 120 d of age, verified in the present study, could explain the reduction of the periodontal ligament space that usually occurs with age (26).

In conclusion, the number of rests of Malassez decreases significantly in all regions of the periodontal ligament according to age. The inverse relationship between the incidence of apoptosis and the number of epithelial cells in rats from 29 to 120 d of age indicates that cell death may be responsible for the quantitative reduction in the number of rests of Malassez.

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