Dental Traumatology

Dental Traumatology 2009; **25**: 584–588; doi: 10.1111/j.1600-9657.2009.00821.x

Propolis inhibits osteoclast maturation

Roberta Pileggi[1], Kathryn Antony[2], Kristie Johnson[2], Jian Zuo[3], L. Shannon Holliday[3]

[1]Department of Endodontics, University of Florida, College of Dentistry, FL, USA; [2]University of Florida, College of Dentistry, FL, USA; [3]Department of Orthodontics, University of Florida, College of Dentistry, FL, USA

Correspondence to: Roberta Pileggi, Department of Endodontics, University of Florida, College of Dentistry, Room D-10-37, PO Box 100436, FL, USA
Tel.: +1-352-273-5440
Fax: +1-352-273-5446
e-mail: rpileggi@dental.ufl.edu

Accepted 30 January, 2009

Abstract – Propolis, a natural product produced by the honey bee, has been successfully used in medicine as an anti-inflammatory and antimicrobial agent. Traumatic injuries to the teeth, especially avulsion injuries, present a challenging situation for the clinician because of post-treatment complications, such as inflammatory and/or replacement resorption. Agents that reduce osteoclast numbers and activity may be useful in the treatment of traumatic injuries to the teeth. In this study, we evaluated propolis as an anti-resorptive agent. Calcitriol-stimulated mouse marrow cultures, which contain both osteoclasts and osteoblasts, were exposed to the ethanol extracts of propolis or vehicle control and stained for tartrate-resistant acid phosphatase (TRAP)-activity to identify osteoclasts. A significant, dose-dependent reduction in multinuclear TRAP+ cells was demonstrated, although the propolis treatment accommodated cell growth and survival ($P < 0.05$). Propolis also reduced the formation of actin rings in pure cultures of RAW 264.7 osteoclast-like cells, suggesting that it exerts direct actions on osteoclast maturation. In summary, our data suggest that propolis inhibits late stages of osteoclast maturation including fusion of osteoclasts precursors to form giant cells and formation of actin rings. This supports the hypothesis that it may prove useful as a medicament to reduce resorption associated with traumatic injuries to the teeth.

Traumatic dental injuries in children, adolescents, and adults are a common problem (1). Avulsion injury is characterized by total displacement of the tooth from its alveolar socket. A successful post-replantation is dependent upon immediate treatment (2–5). It is well established that the two major causes of failure after replantation are: (i) inflammatory root resorption and (ii) replacement resorption. Following avulsion and replantation, there is trauma to the attachment apparatus of the root. The neurovascular component of the periodontal ligament (PDL) is damaged, leading to cell necrosis and macrophage activation in the alveolar bone. Osteoclasts precursors are also recruited and activated in the presence of inflammatory mediators which can result in inflammatory resorption.

There is a great deal of controversy relative to the most potent, yet least toxic drug or combinations of drugs to use to promote successful replantation (2).

The medicaments that have been used include camphorated parachlorophenol, cresatin, polyantibiotic mixtures, iodine–zinc iodide solutions, chlorhexidine, formocresol, and sulfonamides. No single antiseptic or antibiotic, or their combination offers outstanding advantages resulting in the exclusion of all others. Inflammatory root resorption in replanted teeth appears to be inhibited by the placement of calcium hydroxide in the root canal (6). The favorable results have been attributed to the ability of calcium hydroxide to elevate the pH of the microenvironment (7). Such alteration theoretically reverses the prevailing phosphatases from acid to alkaline, which presents a more favorable environment for repair (8). However, the alkalinity induced by this agent is transient and resorption has not been inhibited following its application. More recent studies demonstrated the efficacy of calcium hydroxide and chlorexhidine as antimicrobial agents including their effect against *Enterococcus faecalis*, yet the anti-inflammatory property is not evident (9, 10).

Propolis is a natural product produced by honey bees from tree resin. This product contains amino acids, flavonoids, terpenes and cinnamic acid derivatives (11, 12). The precise composition of propolis is dependent upon the types of trees available to the bees, and thus is region dependent. In various *in vitro* experiments, propolis was shown to inhibit the cyclo-oxygenase pathway and eicosanoid synthesis (13–15), suggesting potent anti-inflammatory properties. Propolis from the southeast region presents a high level of artepillin-C (5.0–11.0%) (16), component that had been proven to play an important role against inflammation (17).

In mid 1950s African bees were introduced in Brazil, which lead to Africanization of Brazilian plants. Park et al. divided Brazilian propolis into 12 groups according to their physiochemical properties (18, 19). The group 3 propolis (G3) was mainly extracted from the popular tree in the region of southern Brazil. The propolis G3 samples that were collected in southern Brazil shows presence of flavonoids and artepillin-C.

Enhancement of pulp healing by propolis has been demonstrated in the reparative process of pulpal tissue with dentinal bridge formation, also from a propolis from the same Brazilian region (20). It has been shown to be biocompatible with the periodontal ligament cells and to be a potential storage media (21–23). In addition, has

demonstrated significant antibacterial activity against oral pathogens, including *E. faecalis* (24, 25).

Because osteoclastogenesis and bone resorption are associated with inflammation, propolis was tested to detect if it had an effect on the formation and activation of osteoclasts-like cells by making use of well characterized *in vitro* culture system (26).

Materials and methods

Propolis extract

Based on our own preliminary studies we found that Brazilian propolis, obtained from the southern region of Brazil in the vicinity of Sao Paulo and extracted with 67% ethanol was biologically active. Similar results were obtained from different batches of Brazilian propolis from this region suggesting the results we obtained are general for propolis obtained from this area of Brazil (21, 23). Extracts consisted of 0.5 g of raw propolis were extracted in 30 ml of 67% ethanol.

In vitro mouse marrow

Osteoclasts were generated from mouse marrow. Four- to six-week-old Swiss-Webster mice (Harlan, Indianapedtolis, IN, USA) were sacrificed by cervical dislocation. Procedure were reviewed and approved by the University of Florida Institutional Animal Care and Usage Committee. Femurs and tibias were dissected free of adherent tissue; the marrow was expelled by cutting both bone ends and flushing the marrow cavity with α-modified minimum essential medium (Sigma, St Louis, MO, USA) plus 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA) (αMEM D10) using a 25-gauge needle. The marrow cells were washed twice with the αMEM medium and plated in 24-well plates at a density of 1 000 000 nucleated cells cm^{-2} in αMEM D10 containing 10 nM 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (26).

Osteoclast-like cells

Pure osteoclast-like cells were generated from the murine cell line RAW 264.7 by stimulation with recombinant receptor activator of nuclear factor kappa B-ligand (RANKL) (27). The cells were plated at 20 000 cells cm^{-2} and cultured for 5–7 days in Dulbecco's modified Eagle's medium (DME, Mediatech, Inc., Manassas, VA, USA) containing 10% fetal bovine serum, penicillin/streptomycin, and 100 mg ml^{-1} GST-RANKL, replacing the medium every 2–3 days.

Cultures were exposed to the ethanol extracts of Propolis (65% ethanol concentration) or vehicle control at the indicated concentrations during the entire culture period.

TRAP and actin ring assays

Cultures were fixed with 2% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP)-activity as described previously (28). Histochemical staining for (TRAP) was performed with a commercial kit (Sigma) and used to identify the osteoclasts. Actin rings were detected by staining, fixed, permeabilized cells with Texas-red-tagged phalloidin (Sigma) as described previously (29). Counters of TRAP+ cells and actin rings were blinded to the treatment groups.

TRAP positive cells were counted under the same magnification by a blinder evaluator under an optical microscope in three categories, mononuclear, multinuclear (2–10 nuclei) and giant (greater than 10 nuclei) as described previously (28–30). The presence of actin rings was statistically analyzed using an ANOVA ($P < 0.05$).

Results

Mouse marrow cultures contain both osteoclasts and osteoblasts. The osteoblasts support the differentiation of osteoclasts. In the presence of propolis there was a

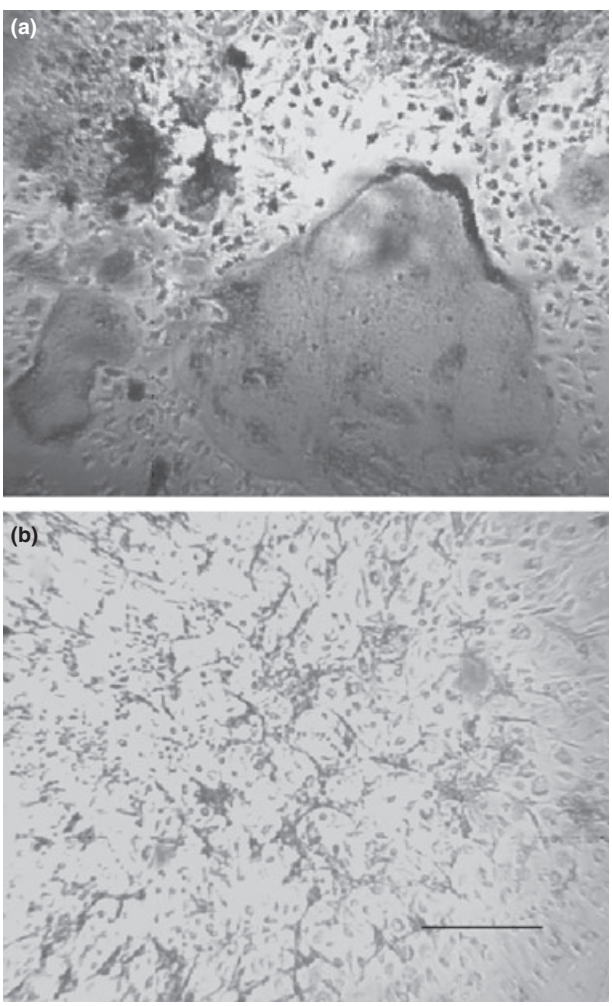

Fig. 1. Propoilis inhibits the formation of multinuclear TRAP+ Cells. Mouse marrow cultures were grown for 7 days in the presence of calcitriol plus vehicle (a) or 10 μl Propolis (b) fixed and stained for TRAP activity to detect osteoclasts. The dark cells are TRAP+. Contrast the numerous giant cells in (a) with the mononuclear cells with a dendritic phenotype in (b). Both images were taken at same magnification and the bar = 25 μm.

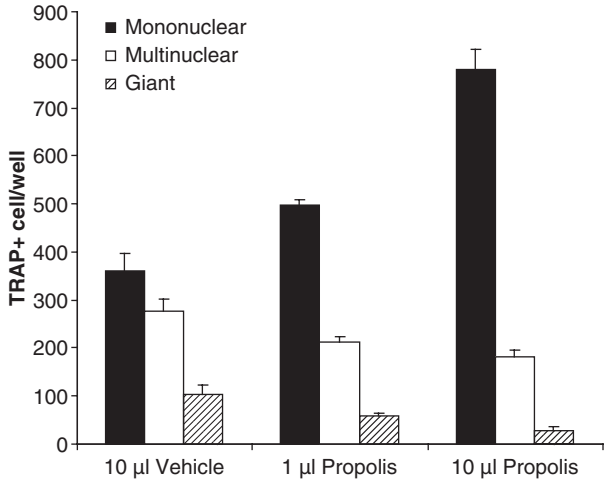

Fig. 2. Propolis enhances formation of TRAP+ mononuclear cells at the expense of TRAP+ multinuclear and giant osteoclasts. Mouse marrow cultures were grown for 7 days in the presence of calcitriol plus vehicle or 1 or 10 μl of the ethanol extract of propolis. Cultures were fixed and stained for TRAP activity, and the numbers of mononuclear, multinuclear (2–10 nuclei) and Giant (>10 nuclei) were tabulated by a counter who was blinded to the treatment conditions. Asterisk denoted significantly different ($n = 4$ per group; $P < 0.05$ compared with the vehicle control).

sharp, dose-dependent reduction in Giant and multinuclear TRAP+ cells and an increase in mononuclear TRAP+ cells (Figs 1 and 2). This suggests that late osteoclast maturation, which is characterized by the fusion of mononuclear precursors, was inhibited by Propolis (Fig. 2). Examination of the cultures indicated that the TRAP+ cells had a dendritic appearance that was quite unlike their normal morphology (Fig. 1b).

The effects of Propolis on the mixed cultures could have been direct or indirect through osteoblasts in the cultures. We next tested whether propolis affects the differentiation of pure cultures of RAW 264.7 cells to differentiate into osteoclast-like cells in the presence of recombinant RANKL. Here, the number of actin rings, characteristic cytoskeletal markers formed by mature osteoclasts (30), were counted. We found that propolis completely blocked the formation of actin rings at relatively low doses (Fig. 3). There was a statistical difference between propolis (groups: 1 μl (SD = 0) and 10 μl (SD = 0) and ethanol's 1 μl (SD = 79.9) and 10 μl (SD = 37.5) ($P < 0.01$).

Discussion

In this study the effects of Brazilian propolis on osteoclast formation and activity were examined *in vitro*. Propolis for the first time was shown to inhibit both osteoclast formation (numbers of TRAP+-multinuclear cells) and maturation (production of actin rings). This inhibition occurred at concentrations of propolis that did not preclude cell growth and survival. This inhibition was first detected in mouse marrow cultures. These cultures contain both osteoclasts and osteoblasts and stimulation of osteoblasts with calcitriol increases their production of RANKL, which in turn stimulates osteoclast progenitors to mature into osteoclasts (30). Thus while propolis inhibited osteoclast formation, we could not discern whether the effects were directly on osteoclasts, or indirectly on osteoblasts. Consequently, propolis was tested on osteoclast-like cells generated by stimulation of RAW 264.7 cells with recombinant RANKL. In this experiment, actin ring formation was blocked, indicating that propolis osteoclasts.

Our data suggest that propolis inhibits some aspect of the pathway leading to mature, active osteoclasts. Previous studies have indicated that propolis has anti-inflammatory effects by inhibiting the cyclo-oxygenase pathway and eicosanoid synthesis after corneal injury (15). Osteoclastogenesis requires activation of nuclear factor kappa B, which is a hallmark of inflammation (31). A reasonable hypothesis is that this pathway may be a primary target of propolis, but future studies will be required to test that idea, and to identify the active components of propolis.

Root resorption is the main challenge to clinicians when dealing with traumatized teeth (32). Numerous studies have attempted to find a medicament that would ameliorate this problem. To date, calcium hydroxide is the treatment of choice, but the data related to its effect on resorption and bacterial growth are still controversial. Andreasen and Kristerson (33) demonstrated that placement

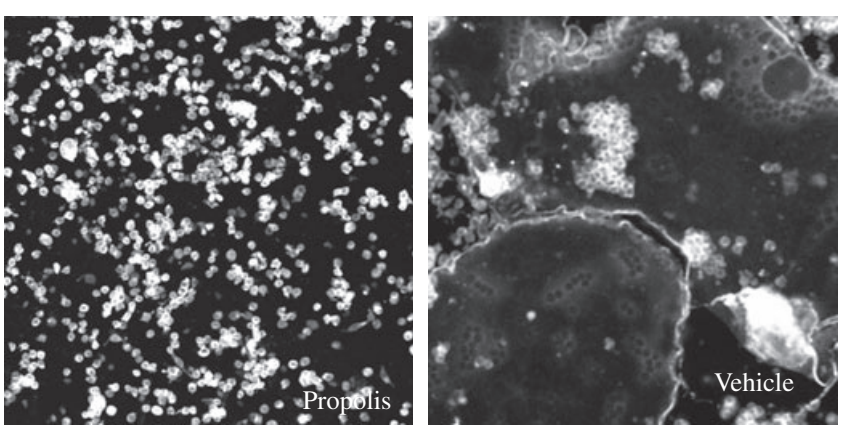

Fig. 3. Propolis blocks formation of actin rings. Raw 264.7 cell were treated with RANKL plus vehicle or 10 μl propolis. Cells were fixed after 5 days of culture and stained with Texas-red-tagged phalloidin to detect filamentous actin. Note that in control cultures actin rings formed, indicated by arrows. No actin rings were detected in cultures treated with Propolis. Bar equals 25 μm. Pictures taken at same magnification.

of calcium hydroxide in the canals following replantation resulted in noticeably more replacement resorption than in teeth that were filled with gutta-percha or those in which the pulps were extirpated and received no root canal fillings. Furthermore, Dumsha and Hovland in a primate model demonstrated that calcium hydroxide was ineffective in preventing resorption following avulsion injuries (34).

Several techniques have been suggested to retard or prevent resorption by maintaining the highest possible numbers of viable periodontal ligament cells. These methods include: storage of the teeth in saliva or milk prior to replantation (35). The use of dexamethasone to decrease root resorption has been advocated, but while this decreased progressive root resorption, it increased ankylosis (36). Moreover, the results of applying these techniques were inconsistent and unpredictable in their capacity to inhibit root resorption.

Andreasen, had associated inflammatory resorption with five factors: injury to PDL, initial external resorption exposing dentinal tubules, presence of necrotic and infected pulp communicating with resorbed area via dentinal tubules, possible presence of bacteria on PDL, and maturation of tooth (37).

Recently, a study using propolis had demonstrated no differences on resorption when using fluoride or propolis on the root surface of replanted rat teeth (38). However the concentration of propolis used was different than our study and the resorption was analyzed only 2 months following replantation.

As stated in the introduction, we utilized the propolis from southern of Brazil due to its anti-inflammatory and antimicrobial properties (16–25) which are fundamental against resorption.

In summary, this report suggests that propolis inhibits osteoclastogenesis and osteoclast activation in tissue culture. At least some of these effects are direct actions of propolis on osteoclasts.

Currently, we are examining whether propolis is effective at reducing root resorption with a pitting assay and the different chemical components of propolis.

References

1. Gassner R, Bosch R, Tuli T, Emshoff R. Prevalence of dental trauma in 6000 patients with facial injuries: implications for prevention. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1999;87:27–33.
2. Andreasen JO, Kristerson L. The effect of limited drying or removal of the periodontal ligament. Periodontal healing after replantation of mature permanent incisors in monkeys. Acta Odontol Scand 1981;39:1–13.
3. Dumsha TC. Luxation injuries. Dent Clin North Am 1995;39:79–91.
4. Dumsha TC. Management of avulsions. Dent Clin North Am 1992;36:425–37.
5. Pileggi R, Dumsha TC. The management of traumatic dental injuries. Tex Dent J 2003;120:270–5.
6. Trope M, Yeşilsoy C, Koren L, Moshonov J, Friedman S. Effect of different endodontic treatment protocols on periodontal repair and root resorption of replanted dog teeth. J Endodon 1992;18:492–6.
7. Trope M. Clinical management of the avulsed tooth: present strategies and future directions. Dent Traumatol 2002;18:1–11.
8. Tronstrad J, Andreasen JO, Hasselgren G, Kristerson L, Riis I. pH changes in dental tissues after root canal filling with calcium hydroxide. J Endodon 1981;7:17–21.
9. Evanov C, Liewehr F, Buxton TB, Joyce AP. Antibacterial efficacy of calcium hydroxide and chlorhexidine gluconate irrigants at 37 degrees C and 46 degrees C. J Endod 2004;30:653–7.
10. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. J Endod 2006;32:93–8.
11. Khalil ML. Biological activity of bee propolis in health and disease. Asian Pac J Cancer Prev 2006;7:22–31.
12. Sforcin JM. Propolis and the immune system: a review. J Ethnopharmacol 2007;113:1–14.
13. Paulino N, Teixeira C, Martins R, Scremin A, Dirsch VM, Vollmar AM, et al. Evaluation of the analgesic and anti-inflammatory effects of a Brazilian green propolis. Planta Med 2006;72:899–906.
14. Ribeiro LR, Mantovani MS, Ribeiro DA, Salvadori DM. Brazilian natural dietary components (annatto, propolis and mushrooms) protecting against mutation and cancer. Hum Exp Toxicol 2006;25:267–72.
15. Ozturk F, Kurt E, Inan UU, Emiroglu L, Ilker SS. The effects of acetylcholine and propolis extract on corneal epithelial wound healing in rats. Cornea 1999;18:466–71.
16. Matsuda AH, de Almeida-Muradian LB. Validated method for the quantification of artepillin-C in Brazilian propolis. Phytochem Anal 2008;19:179–83.
17. Kano Y, Horie N, Doi S et al. Artepillin C derived from propolis induces neurite outgrowth in PC12m3 cells via ERK and p38 MAPK pathways. Neurochem Res 2008;33:1795–803.
18. Park YK, Ikegaki M, Alencar SM, Moura FF. Evaluation of Brazilian propolis by both physicochemical methods and biological activity. Honeybee Sci 2000;21:85–90.
19. Park YK, Ikegaki M, Alencar SM. Classification of Brazilian propolis by physicochemical method and biological activity. Mensagem Doce 2000;58:2–7.
20. Bretz WA, Chiego DJ Jr, Marcucci MC, Cunha I, Custodio A, Schneider LG. Preliminary report on the effects of propolis on wound healing in the dental pulp. Z Naturforsch [C] 1998;53:1045–8.
21. Martin MP, Pileggi R. A quantitative analysis of Propolis: a promising new storage media following avulsion. Dent Traumatol 2004;20:85–9.
22. Ozan F, Polat ZA, Er K, Ozan U, Deger O. Effect of propolis on survival of periodontal ligament cells: new storage media for avulsed teeth. J Endod 2007;33:570–3.
23. Gjerstsen A, Storthz KA, Pileggi R, Liu Z. Cell proliferation and apoptosis of periodontal ligament cells following storage in various media. J Endod 2007;33:340.
24. Koo H, Rosalen PL, Cury JA, Park YK, Bowen WH. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. Antimicrob Agents Chemother 2002;46:1302–9.
25. Koo H, Gomes BP, Rosalen PL, Ambrosano GM, Park YK, Cury JA. In vitro antimicrobial activity of propolis and Arnica montana against oral pathogens. Arch Oral Biol 2000;45:141–8.
26. Holliday LS, Dean AD, Greenwald JE, Glucks SL. C-type natriuretic peptide increases bone resorption in 1,25-dihydroxyvitamin D3-stimulated mouse bone marrow cultures. J Biol Chem 1995;270:18983–9.
27. Krits I, Wysolmerski RB, Holliday LS, Lee BS. Differential Localization of Myosin II Isoforms in Resting and Activated Osteoclasts. Calcif Tissue Int 2002;71:530–8.
28. Holliday LS, Dean AD, Lin RH, Greenwald JE, Gluck SL. Low NO concentrations inhibit osteoclast formation in mouse marrow cultures by cGMP-dependent mechanism. Am J Physiol 1997;272:F283–91.

29. Zuo J, Jiang J, Chen SH, Vergara S, Gong Y, Xue J et al. Actin Binding Activity of Subunit B of Vacuolar H(+)-ATPase Is Involved in Its Targeting to Ruffled Membranes of Osteoclasts. J Bone Miner Res 2006;21:714–21.
30. Holliday LS, Welgus HG, Hanna J, Lee BS, Lu M, Jeffrey JJ et al. Interstitial collagenase activity stimulates the formation of actin rings and ruffled membranes in mouse marrow osteoclasts. Calcif Tissue Int 2003;72:206–14.
31. Walsh NC, Crotti TN, Goldring SR, Gravallese EM. Rheumatic diseases: the effects of inflammation on bone. Immunol Rev 2005;208:228–51.
32. Tronstad L. Root resorption–etiology, terminology and clinical manifestations. Endod Dent Traumatol 1988;4:241–52.
33. Andreasen JO, Kristerson L. The effect of extra-alveolar root filling with calcium hydroxide on periodontal healing after replantation of permanent incisors in monkeys. J Endod 1981;7:349–54.
34. Dumsha T, Hovland EJ. Evaluation of long-term calcium hydroxide treatment in avulsed teeth – an in vivo study. Int Endod J 1995;28:7–11.
35. Trope M. Clinical management of the avulsed tooth. Dent Clin North Am 1995;39:93–112.
36. Keum KY, Kwon OT, Spangberg LS, Kim CK, Kim J, Cho MI et al. Effect of dexamethasone on root resorption after delayed replantation of rat tooth. J Endod 2003;29:810–3.
37. Andreasen JO, Andreasen FM. Textbook and color atlas of traumatic injuries to the teeth, 3rd edn. Copenhagen/St Louis: Munksgaard/CV Mosby; 1994.
38. Gulinelli JL, Panzarini SR, Fattah CM, Poi WR, Sonoda CK, Negri MR et al. Effect of root surface treatment with propolis and fluoride in delayed tooth replantation in rats. Dent Traumatol 2008;24:651–7.