Anti-Inflammatory and Regenerative Effects of Albanian Propolis in Experimental Vital Amputations

Keywords

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ABSTRACT

This study evaluated the effects of Albanian propolis on the inflamed pulpal tissue after pulpotomy in piglets. In five piglets, two teeth each were infected using special pathogenic microbial flora that was prepared in advance in order to cause inflammation of the pulp. Pulpotomy was performed in the maxillary and mandibular central and lateral incisors. Microbial flora pathogenesis prepared from the section of infected teeth, containing Staphylococcus aureus, Streptococcus viridans, Enterococcus faecalis, Staphylococcus albus and mixed flora were used to cause artificial pulpitis. The first group consisted of piglets with the teeth having pulpal inflammation and regeneration without medication served as the control group. In the second group, the teeth were treated with pure propolis after vital amputation. In the 3rd, 4th and 5th groups, the teeth were treated after vital amputation with the compounds of ZnO+10%, ZnO+20% propolis in ethanol, and with the paste composed of Ca(OH)2+30% oily propolis, respectively. In half of the teeth, propolis based pastes were applied on the pulp and entries of the cavities were isolated with glass ionomer cement while the other half did not receive this treatment and acted as the control. The cavity entries were obturated with chemically polymerized resin composite. Inflammatory response, dominated by polymorphonuclear cells, was observed in the dental pulpal tissue of all the teeth that were not treated with propolis-based paste. Radiography and histopathology analyses were performed to survey the infected pulp tissue treated with propolis up to 3 months. Data were analyzed using Mann-Whitney U test (α=0.05). After vital amputation of the pulp, application of Albanian propolis showed significantly higher anti-inflammatory and regenerative effect creating dentin barrier after 3 months of treatment compared to control group ($p<0.05$). These results were more expressed in the group containing 20% propolis in ethanol+ZnO.

INTRODUCTION

Pulp pathology is one of the major concerns dentistry in that the incidence of complications following pulpitis have been largely treated with pulpectomy only.[3,8,9] In fact, keeping pup vital or regenerate the infected pulp could eliminate tooth extraction and thereby avoid subsequent surgical or reconstructive solutions. Regardless of the methods used for traditional endodontic treatment, the failure rate over time ranges from 30 to 40%.[25,28] In this regard, some attempts have been made to treat pulpal pathology utilizing curative properties of propolis based pastes used in vital amputation one of which is Albanian propolis.

Propolis is a natural medicament with anti-microbial, anti-inflammatory, anti-fungal, immunomodulatory and regenerative properties and its curative features are comparable to those of the antibiotics.[4,5,10-15,17,19] This resinous substance, varying in color from yellow-brown to dark-brown, is collected from trees and shrubs by honey-bees. The main chemicals present in propolis are flavonoids, phenolic and other various aromatic compounds. Flavonoids are well-known plant compounds that have antioxidant, antibacterial, antifungal, antiviral and anti-inflammatory properties.[1,6,16] Propolis as an anti-inflammatory agent has also been shown to inhibit synthesis of prostaglandins. In addition, it contains elements such as iron and zinc that are important for the synthesis of collagen,[2,6,18] and supports the immune system by promoting phagocytic activities, stimulating cellular immunity and augmenting healing effects. Hence, it could be anticipated that Albanian propolis could show regenerative effects in experimental vital amputations.

The objectives of this study therefore were to observe the anti-inflammatory and regenerative action of Albanian propolis on the pulp tissue after vital amputation in vivo in piglets.

MATERIALS AND METHOD

ANIMALS

The experiments were performed at the histopathology laboratories of the University of Medicine Tirana, Albania. The study was performed in accordance with the protocol approved by the Animal Care and Use Committees of University of Medicine, Tirana, Albania (Number: 625). Piglets were obtained from the Institute of Animal Science of the Agricultural University of Tirana, Albania.

Permanent maxillary and mandibular incisors were identified and the cavities were opened in cervical-vestibular direction in matriculated piglets (N=25, 5 per group). In each piglet 2 teeth were included in the study yielding to 10 teeth per group.

The treatment procedure started in the 2nd month after birth when the piglets weighed 6 to 7 kg. All piglets were sacrificed when they were 6 months old and weighed 35-40 kg.

EXPERIMENTAL PROCEDURES

Solutions of propolis and propolis based curative pastes were prepared in two different ways. Initially, liquid alcohol was extracted from Albanian crude propolis under laboratory conditions which was then evaporated to prepare a soft and dry extract of propolis as described previously.[24,29] In order to achieve this, first propolis were cut it into pieces and partitioned using scissors and fine separators. It was then dissolved in 90% ethanol and filtered by extraction. Extraction was conducted using a device that consisted of a distilling pot having stainless double walls allowing the circulation in water between 70-80°C under vacuum. Evaporation in vacuum and moderate warming ensure the preservation of active ingredients of the propolis. Condensed material was then collected in a glass pot connected to the vacuum extraction device. The amount of the solvent was reduced reaching 20% of 90% ethanol. Starting with the soft and dry extract of propolis, 10% and 20% alcoholic propolis solutions were prepared and 30% propolis was dissolved in vegetable oil. This type of oil was used as it can be mixed with $Ca(OH)_2$, unlike ethanol which immediately causes solidification of the $Ca(OH)_2$.

The teeth selected for the experiments were injured under local anesthesia using an aerator with distilled water with round bur. They were then infected with microbial flora pathogenesis prepared from the section of infected teeth, containing Staphylococcus aureus, Streptococcus viridans, Enterococcus faecalis, Staphylococcus albus and mixed flora to cause pulpitis.[10,11]

The first group consisted of piglets with the teeth that where pulpal inflammation was induced. This group served as the control group. In the second group, the teeth were treated with pure propolis after vital amputation. In the 3rd, 4th and 5th groups, the teeth were treated after vital amputation with the compound of ZnO+10%, ZnO+20% propolis in ethanol, and with the paste composed of Ca(OH)2+30% oily propolis, respectively.

The vital amputation of coronal pulp was performed under anesthesia using mepivacaine hydrochloride 3% (Scandonest 3%, Septodent, Saint-Maur, France) in all cases. After stopping the pulpal hemorrhage using physiological solution and sterile tampons in conditions of full asepsis, a net 3 g amount of propolis and other curative pastes that contained zinc oxide (Biodinamica, Biodinamica Quimica and Farmaceutica Ltd., Ibipora, Brazil) and calcium hydroxide (WP-Dental, Willmann and Pein Gmbh, Bevern, Germany) in sufficient thickness, were placed on the pulp without applying pressure. Treated cavities were isolated with glass-ionomer cement (3M ESPE, St Paul, Minn, USA) and were obturated by chemically-polymerized resin composite (Prime-Dent, Prime Dental Manufacturing, Chicago, USA).

The teeth in all groups were observed for a period of three months after the infection and the treatment. Piglets were sacrificed for radiographic and histologic examinations in the relevant laboratory after three months of observation. Experienced pathologists evaluated the sections dyed with hematoxylin-eosin, Trichrome Vert and Van Gieson.

RADIOGRAPHIC ASSESSMENT OF THE DENTIN THICKNESS

Dentin thickness was measured using a software program (J-F. Madre, Academy of Amiens, Amiens, France) and data were collected from digitized images (i.e. counting elements on the image, measurements of surface light or length). A 1-mm Fixott–Everett metallic grid (Fixott–Everett X-Ray Grid Large Ea,

Miltex Instrument Co, Inc., York, PA, USA) was incorporated in the device holding the X-ray film during exposure for standardized radiological measurements on the digitized images. All the radiographs were subsequently scanned and transferred to the computer for digital analysis. Measurements on the digitized radiographs were performed at baseline (after the pulp-capping procedure) and at 3 months. On each digitized radiograph, the scale for the measurements was determined using the space between two lines of the grid, which was assigned to a value of 1 mm. The dentin thickness on each film was measured with the software accordingly based on the scale in the range of 10/3 mm. One investigator, who was blinded to the objectives of this study and the nature of the pulp-capping material, performed the measurements on the digitized images *(Table 1)*.

HISTOLOGICAL ASSESSMENT OF PULP RESPONSE AND DENTIN REGENERATION

Three months following pulp capping, the teeth were fixed in 4% formalin and decalcified in 10 to 15% ethylenediaminetetraacetic acid. Sections of 5 to 6 mm thickness from the embedded specimen were stained with hematoxylin-eosin.

Table 1. Scores used for histological examination

Criterion	Scores
Inflammatory cell response	0: None or a few scattered inflammatory cells beneath the site of pulp exposure 1: Mild inflammatory cells as mono- or poly-morphonuclear leukocytes 2: Moderate inflammatory cells infiltration involving the third coronal radicular pulp 3: Severe inflammatory cells infiltration involving the third coronal or more radicular pulp
Tissue disorganization	0: Normal tissue beneath the site of pulp exposure 1: Odontoblast like cells, odontoblasts and pulp tissue pattern disorganization or odontoblasts hyperactivity but normal central pulp tissue pattern 2: General disorganization of the pulp tissue pattern 3: Pulp necrosis
Hard tissue formation	0: No hard tissue formation 1: Slight incomplete hard tissue formation beneath the site of pulp exposure 2: Moderate hard tissue formation beneath the site of pulp exposure 3: Thick hard tissue formation beneath the site of pulp exposure, characterizing a complete dentin bridge

One blinded histologist, analyzed the area of mineralization semi-quantitatively using histomorphometric techniques (Image-pro Express, Bethesda, MD, USA).

Propolis treated specimens were fixed in 10% formalin and decalcified. After they were embedded in paraffin, the specimens were cut by microtome and coloured using Hematoxylin-eosin die. Microscopy examinations were finally performed using a microscope (Richter Optica, Biotech Tissue Microscopa, Carlsbad, California, U.S.A) at x400 magnification.

STATISTICAL ANALYSIS

Statistical analyses were performed using the Prism software (Graph Pad Software, version 3.0, San Diego, CA USA). Mann-Whitney non-parametric test was used to evaluate the histology results in 4 experimental groups and compared with the control group. P values less than 0.05 was considered to be statistically significant.

RESULTS

All the groups of piglets involved in the experiment showed normal development and were in good health until they were sacrificed.

Distribution of scores for each experimental group according to the criteria is presented in Table 2. After vital amputation of the pulp, application of Albanian propolis showed significantly higher anti-inflammatory and regenerative effect creating dentin barrier after 3 months of treatment compared to control group ($p<0.05$) *(Table 3)*.

Radiographic examination of the control group carried out after 2 hours, 5 days and 7 days after the injury and infection of the pulp showed no change in the alveolar bone structure with normal density and continued development of lamina dura *(Figures 1a-b)*.

The histological analysis of the teeth two hours after the injury and infection, presented acute active hyperemia, accompanied by pronounced dilatation of coronary capillaries of the pulp, and polymorphonuclear cells *(Figure 2a-d)*. After 5 and 7 days following injury, coronary purulent inflammation was present in more than 50% of the cases *(Figures 3a-c)*. We extracted the teeth after 2 hours and at the 5th and 7th days after the treatment procedures.

In the second group, where pure propolis was used formation of dentine bridges in the vicinity of the paste were evident. After three months, the radiographic and histologic examinations, showed normal height of the treated tooth and no changes in the periodontal ligament. No necrosis was identified between the paste and the dentin bridges. The length of treated teeth was normal and there were no change in the periodontal ligament. In the fog view, whitening of the luminal canal was noticed right behind the dentin bridge at a thickness of 1-2 mm and the periodontal ligament was uniform.

Table 2. Distribution of scores for each experimental group according to the criteria described in **Table 1.**

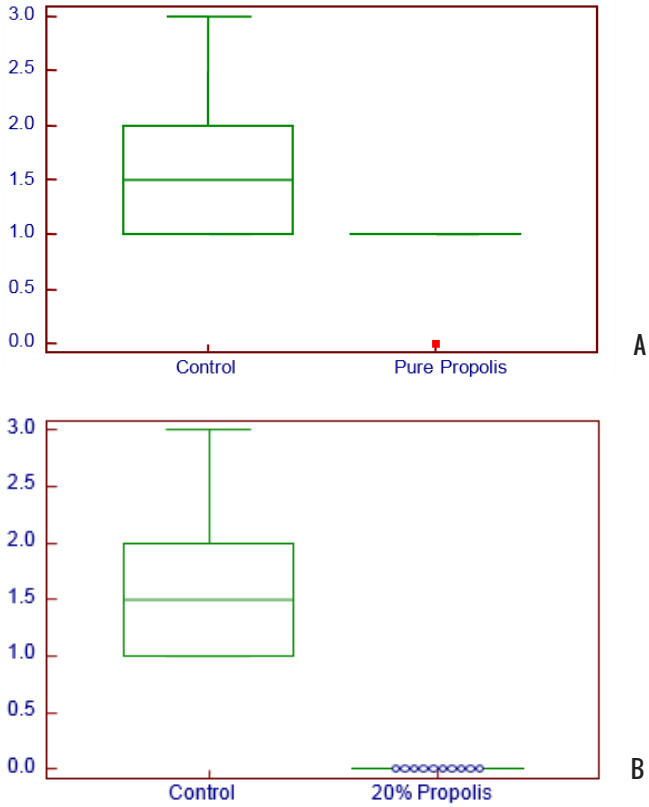
Group number	Treatment	Radiology				Histology				Disintegration			
		0	1	2	3	0	1	2	3	0	1	2	3
1	Control group, no treatment	10	0	0	0	0	5	3	2	0	1	2	3
2	Pure propolis	1	1	2	6	1	9	0	0	10	0	0	0
3	10% propolis in ethanol + ZnO	2	1	3	4	1	6	1	2	3	7	0	0
4	20% propolis in ethanol + ZnO	0	0	2	8	10	0	0	0	10	0	0	0
5	30% oily propolis + Ca(OH)2	1	4	3	2	1	4	3	2	2	8	0	0

Table 3. Mann-Whitney test results based on the independent specimens in experimental groups.

Average rank of first group	13.25
Average rank of second group	7.75
Mann-Whitney U	22.5
Test static Z (corrected for ties)	2.44
Two-tailed probability	P=0.0147

Radiographs indicated the presence of radiopaque bridges in 90% of the cases *(Figure 4a)*. Histological analysis revealed the presence of dentin bridges with no channels of odontoblasts palisades and fibrotic tissue. The pulp in the apical part was normal in all cases.

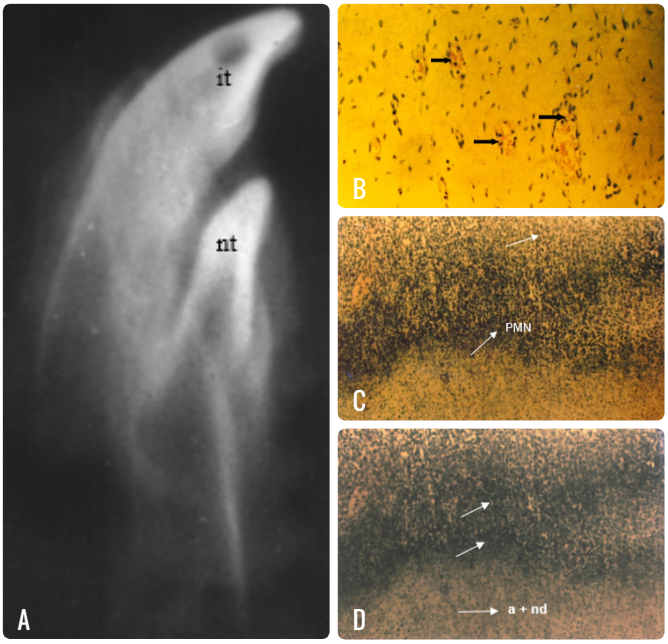
The third group treated with the paste of 10% zinc oxide propolis solution showed presence of radiopaque bridges in 80% of the cases. Apices were closed and no changes were observed in the periodontal ligament *(Figure 4b)*. Histological examinations showed also dentin bridges in this group with no channels and with sound support in the dentin of the radicular channel. Secondary dentin was observed in the upper part where the borders of odontoblasts were slightly destroyed. While slight chronic inflammation in the pulp at the borders was observed, the pulp in the apical region was normal.



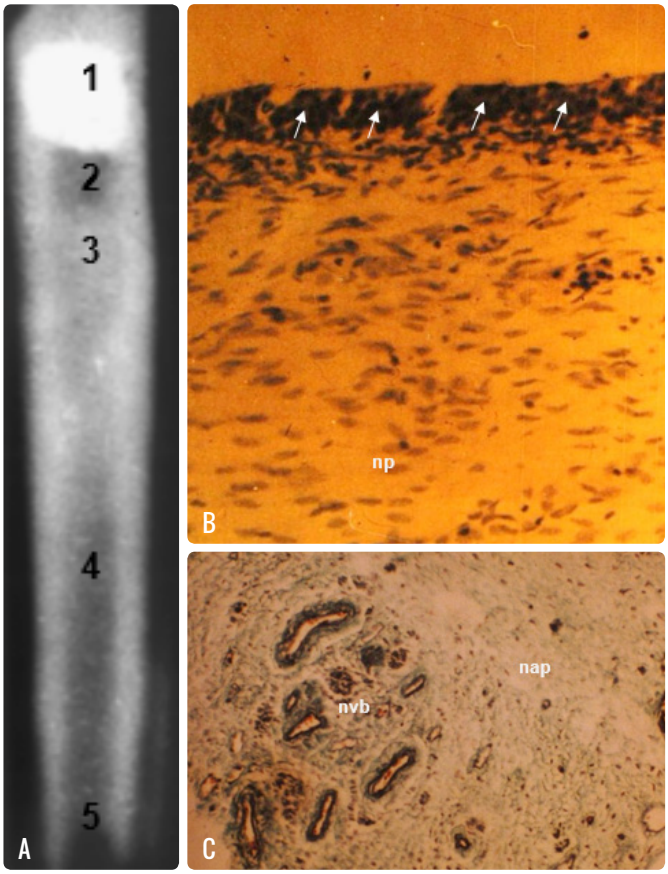
Figures 1a-b: Comparison of a) pure propolis, b) 20% Propolis treated group compared to the control group.

In the fourth group treated with the paste consisting 20% propolis and zinc oxide, radiographic and histologic examinations of the fragments of bones in all treated cases showed formation of dentin bridges with a thickness of 2-3 mm immediately after the application of the paste. Length of the teeth was normal and the process of apexification was continuing *(Figure 5a)*. Histological examinations of solutions showed normal apical pulp and the existence of blood capillaries in the pulp. The borders of odontoblasts showed regular palisade positioning. Finally, new dentin with no channels was noticed in the borders *(Figure 5b)*. Dentin bridges were very well supported by the walls of the radicular channel in all the cases of the fourth group *(Figure 5c)*.

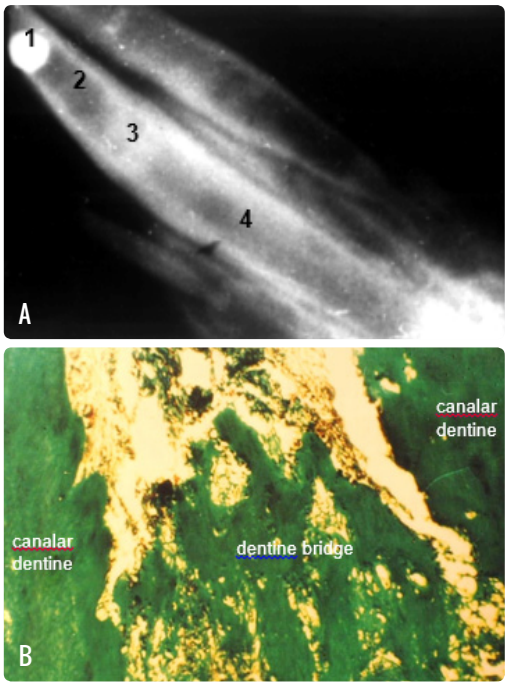
In the fifth group treated with calcium hydroxide and 30% oily propolis, immediately after application of the paste, necrotic spots, inflammation and regeneration of the pulp of the root were observed *(Figure 6a)*. The histological analysis showed dystrophic processes in the pulp, fibrosis of the pulp, partial dentin bridge formation with amorphous structure with no support in the dentin walls of the channel. The dentin bridges were porous with no distinct border between the bridge and the pulpal tissue. Pathological resorption of the pulp was also evident *(Figure 6b)*.



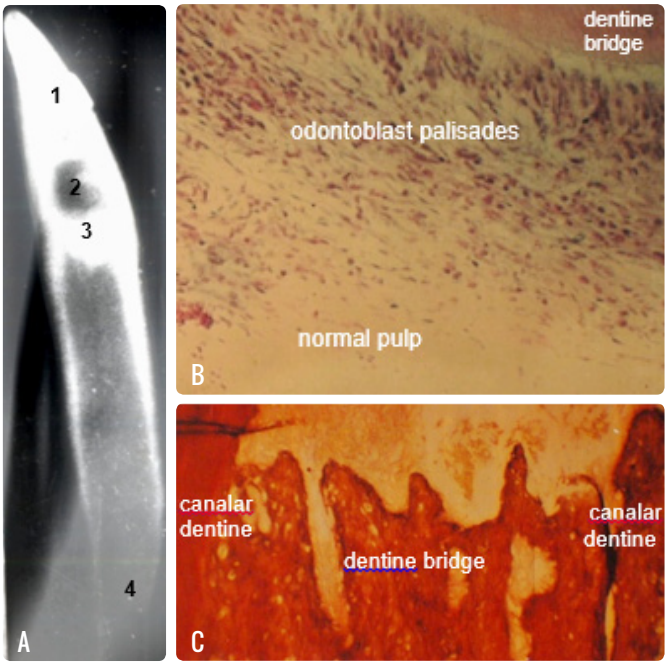
Figures 2a-d: a) Bone fragment with infected teeth from first group (control) showing the accident in the pulp with inoculated infection. Note the central infected incisor and normal lateral incisor without infection (infected tooth-it), (normal tooth-nt), b) Two hours after injury and infection, died with hematoxylin-eosin showing acute active hyperemia (a), c) After 5 days of infection. Note the polymorphonuclear cells (PMN) and acute purulent inflammation (a), d) After 7 days of infection. Note the dense infiltrate with inflammatory cells (abscesses-a and necrotic detritus-nd) restricted in granulation tissue.



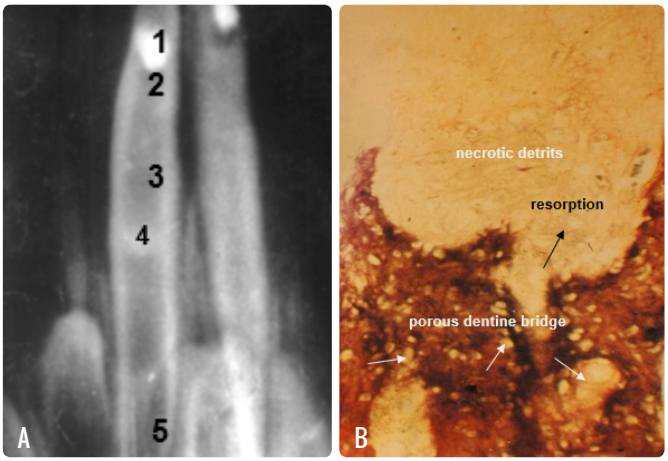
Figures 3a-c: a) Definitive filling (1), propolis paste (2), dentin bridge (3), lumener canal (4) and in complete apex formation in group 2 treated with pure propolis, b) 3 months after treatment (normal pulp-np) (a), odontoblasts palisades (dyed with hematoxylin-eosin), c) 3 months of treatment (normal apical pulp-nap), neuro vascular bundles (nvb) died with Trichrome Vert.



Figures 4a-b: a) Definitive filling (1), propolis paste (2), dentin bridge (3), and lumener canal (4) in group 3 treated with ZnO+10% propolis in ethanol, b) 3 months after treatment dyed with Trichrome Vert.



Figures 5a-c: a) Definitive filling (1), propolis paste (2), dentin bridge (3), and closed apex (4) in group 4 treated with ZnO+20% propolis in ethanol, b) 3 months after treatment (normal pulp), odontoblast palisades and dentin bridge formation (dyed with hematoxylin-eosin), c) 3 months after treatment showing canal dentin, dentin bridge died with Van Gieson.



Figures 6a-b: a) Definitive filling (1), propolis paste (2), necrosis (3), dentin bridge (4), and apex (5) in group 5 treated with $Ca(OH)_2$+30% oily propolis, b) 3 months after treatment showing porous dentin bridge, resorption and necrotic debris died with Van Gieson.

DISCUSSION

In this study, vital amputation of coronary pulp of the teeth was performed until the entrance of the radicular channel in piglets after which artificial pulpitis was created and treated with different preparations of Albanian propolis. We assumed that the radicular pulp obtained was vital and not inflamed. The propolis was expected to eliminate the traumatic infection caused by the dental bur. Based on the results of this study, since the application of propolis in 3 groups (groups 2, 3 and 4th) showed both radiological and histological differentiation significantly compared to the control group, the hypothesis could be partially accepted.

Several previous studies were performed only in complete sterile conditions. In fact, it is impossible to diagnose inflammatory complications in the teeth that were covered. Therefore, the critical evaluation of the healing in amputated pulp after covering could only be performed histologically. In this study, three months after application, the pulp in the teeth treated with pure propolis, dentin bridges were formed, with odontoblastic profile with no inflammatory response. This might be attributed to the anti-inflammatory properties of flavonoids and caffeic acid present in the composition of the propolis that are well-known to decrease inflammation. Increasing of propolis concentration to 20% in ethanol mixed with zinc oxide even signified more promising results. The dentin barrier quality observed in radiological and histological examinations, confirmed no inflammation accompanied with high quality dentin barrier formation.

Although antibacterial properties of the pastes could be verified and effective results of the use of propolis could be observed, in this study, the healing of the teeth could not be excluded based on immune capabilities of the vital pulp. Sterile inflammatory phases are typically observed during the first months after vital amputation but the results in this study already after 3 months verified the regenerative effects of the propolis pastes used.

Numerous previous studies reported necrosis with the use of calcium hydroxide.[3,8,9,13,14,17,20,22] The results obtained from the fifth group where $Ca(OH)_2$+30% oily propolis was used in this study have highlighted resorption that could be due to the presence of calcium hydroxide in the composition of the paste.

According to the methodology employed in this study, the inflammation was identified already two hours after the application of the pastes. It can be thus stated that the method used to injure and inoculate the microbial flora caused active acute hyperemia. The existence of experimental pulpal inflammation was recognized by not only erythrocytes but also with the presence of polymorphonuclear leukocytes.

In this in vivo study piglets were used due to the closest structural resemblance of their teeth to the human teeth, while many previous studies were conducted on mice, dogs, monkeys, and humans.[7,8,17,20,21,27]

Propolis was mixed with zinc oxide in this study that is a neutral powder commonly used in dentistry. The absorbance capabilities of ZnO allowed to removing the ethanol from the composition. The anti-inflammatory regenerating capabilities of propolis were not evident in group 2 where pure propolis was used and not mixed with ZnO. Future studies should elaborate clinical use of the compounds tested in this study.

CONCLUSIONS

After vital amputation of the pulp, application of Albanian propolis showed anti-inflammatory and regenerative effects creating dentin barrier after 3 months of treatment in vivo in piglets. These results were more expressed in the group containing 20% propolis in ethanol+ZnO. The potential advantages of the use of propolis should be followed in endodontically treated teeth clinically.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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