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Evaluation of the expression of VIII factor and VEGF in the regeneration of non-vital teeth in dogs using propolis

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ABSTRACT

Objective(s): The purpose of the present study was the immunohistochemical evaluation of VEGF and VII factors in dog's teeth pulp revascularized with MTA and propolis.
Materials and Methods: 144 mature and immature two rooted dog's premolar canals were selected. Pulp necrosis and infection were established after 2 weeks and the disinfection of the canals was done with copious NaOCl irrigation and triantibiotic mixture (ciprofloxacin, metronidazole, and minocycline) for 3 weeks. Subsequently, the blood clot was evoked in the canal by periapical tissue irritation with a k-file. The samples were randomly divided into 6 experimental groups: propolis (groups 1, 2), MTA (groups 3, 4), and parafilm (groups 5, 6) in immature and mature teeth. The animals were sacrificed and samples were prepared for immunohistochemical evaluation of VEGF and the VIII factor.
Results: Tissue regeneration was seen in 64.5% of MTA, 38% of propolis, and 0% of parafilm group samples. Expression of VEGF and VIII factor in the propolis group was more than the MTA group and it showed a reduction after 3 months in comparison to 1 month. VEGF and VIII factor were seen in stromal cells in addition to endothelial vessel cells. Overall, expression of angiogenic factors was more in the open apex teeth compared to close apex ones.
Conclusion: According to the results of this study, propolis can induce the expression of VEGF and VIII factor in infected mature and immature dog's teeth and is a suitable biomaterial for the revascularization technique.

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Introduction

Treatment of teeth with open immature apices, especially in cases in which carries or trauma have resulted in pulp necrosis, is a major challenge due to the arrest of tooth root development, thin root canal walls, and lack of normal closure of the apex. In the majority of these root canals, the apical area is wider than the coronal area and there is no barrier to prevent extrusion of root canal filling materials from the root canal into the periapical tissues during root canal treatment due to the large size of the apex. Therefore, the root canal cannot be properly obturated and is susceptible to leakage (1).

The conventional technique for the endodontic treatment of immature permanent teeth with apical periodontitis consists of apexification with calcium hydroxide to create a calcified barrier in the apical area. Some of the disadvantages of this technique include the lengthy nature of the procedure (6–18 months), the need for repeated recall visits to evaluate treatment results, and susceptibility of the tooth to fractures due to the long-term use of calcium hydroxide. Approximately 30% of these teeth fracture during endodontic treatment, especially at the cervical area (2). These disadvantages have resulted in the replacement of apexification with calcium hydroxide with one-session placement of an apical mineral trioxide aggregate (MTA) plug. Despite the high success rate of apexification with MTA plug, the root canal walls remain thin due to a lack of

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root development, and there is a risk for root fracture in case of secondary traumas (3).

The ideal technique for the treatment of a tooth with immature root with a necrotic pulp is revascularization. This technique has some advantages, including the revascularization of the pulp, regeneration of blood vessels and nerves, the potential of root development, and strengthening of dentinal walls by deposition of hard tissues, leading to root resistance against fracture. It is possible to carry out the technique even in immature teeth which are severely infected, with periodontitis and periradicular abscesses (4). One of the first tissue changes in the regeneration of loose connective tissue is the formation of vascular buds, which is associated with the release of angiogenic growth factors such as Factor VIII and VEGF (5). VEGF has the potential to induce differentiation of endothelial cells and their viability and is the most effective and angiogenic factor known to date (6–8). VEGF is expressed in large quantities by odontoblasts and in the sub-odontoblastic layer by endothelial cells. It has been shown that this factor has a key role in the progression of dentinogenesis by induction of the angiogenesis necessary for metabolic needs of odontoblastic cells in the process of matrix secretion (7, 9). Therefore, in the evaluation of the course of regeneration, immunohistochemical studies and assessment of the expression of vascular growth factors are considered valuable and accurate diagnostic techniques. Factor VIII and VEGF are two primary and important factors in this respect (10).

At present, there is a lot of evidence on the use of propolis in the treatment of ulcers resistant to treatment, and it has been shown that honey decreases inflammation severity, derides necrotic tissues, decreases edema, and induces angiogenesis and formation of granulation tissue (11). Considering the properties mentioned above in relation to propolis, the present study was designed to evaluate the inductive effect of propolis on the expression of angiogenesis factors and pulpal regeneration of non-vital teeth with mature and immature apices.

Since there is no comprehensive study on revascularization in necrotic teeth despite different case reports and serial reports in relation to revascularization, and since the expression of VEGF and factor VIII, which are important angiogenesis factors, has not been evaluated, the present study was undertaken to assess the expression of VEGF and factor VIII in regeneration of the pulp of necrotic teeth with mature and immature teeth in dogs using propolis at two one- and three-month intervals.

Materials and Methods

In the present study, 8 dogs with good systemic health, which were 6 months old and were from a mixed Iranian breed, were used. The dogs had two-rooted premolars with open and closed apices. A total of 144 canals were selected from healthy two-rooted maxillary and mandibular premolars, with 112 open and 32 closed apices. A total of 72 root canals were assigned to each one-month and 3-month interval. The protocol of the study was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran (code: 900124).

The first treatment session

Half an hour before the study, the animals received intramuscular injections of diazepam (Chemie Darou, Tehran, Iran), ketamine (Rotex MEICA, Germany), and xylazine (Rotex MEICA, Germany), administered by a veterinarian. The animals' vital signs were monitored during the whole procedure. The development of the root apex was assessed by radiographic examinations and teeth with coronal widths of ≥1 mm and <1 mm were assigned to open apex and closed apex groups, respectively. Then, since evaluation of VEGA and factor VIII were to be carried out at 1-month and 3-month intervals, the teeth on the right side of jaws in each dog were assigned to group A and those on the left side were assigned to group B. First, the teeth in group A were treated and after two months the same treatment procedures were carried out on teeth in group B. Therefore, the factors in groups A and B were evaluated at 3-month and 1-month postoperative intervals, respectively. The teeth in group A (left side) underwent anesthesia with 2% lidocaine containing epinephrine at a concentration of 1:100,000 (Darou Pakhsh, Iran) and an access cavity was prepared with a #2 round diamond bur (Dia, South Korea) in a high-speed handpiece. The tooth lengths were determined by radiography using the bisecting angle technique. Then the pulpal tissue of the root canal was removed with a #35 broach (Dentsply/Maillefer, Switzerland). In order to create necrotic and infected root canals, which was the aim of the first treatment session, the teeth were left exposed to the oral cavity for two weeks without the use of temporary restoration (12). At the end of this treatment session, the dogs received a dexamethasone injection and were monitored by the veterinarian until they gained consciousness.

The second treatment session

After two weeks the dogs once again underwent general anesthesia. The aim of this treatment session was to disinfect the root canals. The teeth were isolated by rubber dam (SUPA, Iran), using the split dam technique. The external tooth surfaces and the rubber dam were disinfected by 0.2% chlorhexidine mouthwash (Iran Najo, Tehran, Iran). After cleaning the access cavity, the root canals of teeth with open apices were disinfected with 5.25% NaOCl and copious amounts of normal saline (Razi Serum Company, Iran) without any mechanical instrumentation. However, teeth with closed apices were prepared using the step-back technique. After final irrigation of the root canals with normal saline, the canals were dried with sterile

paper points and triantibiotic paste was placed in the root canals by an amalgam carrier, with equal portions of minocycline, ciprofloxacin, and metronidazole, similar to the protocol introduced by Hoshino (13). Then the teeth were dressed with Cavit (Aria Dent, Tehran, Iran), leaving the triantibiotic paste in place for 3 weeks.

The third treatment session

Water-based propolis was prepared in Mashhad Razi Research Institute. Each ml of propolis contained 50 mg of the polyphenol effective material. The material was prepared in paste form for easy placement in the root canal in groups 1 and 2.

After 3 weeks the temporary restoration was removed after a rubber dam was placed under general anesthesia. The triantibiotic paste was removed using 10 ml of sterile saline solution with the use of a hand file. Then a #30 hand K-file (Dentsply/Maillefer, Switzerland) was used to induce hemorrhage in teeth with open apices and in teeth with closed apices; #15 to #30 files (Dentsply/Maillefer, Switzerland) were used 2 mm beyond the apex. After hemorrhage was observed in all of the root canals, it was stopped by a large sized paper cone at the cervical third of the root canal. Then a computer was used to randomly assign teeth into 6 groups for each one-month and 3-month interval and the pulp chamber space was filled as follows:

Group 1: Propolis in teeth with open apices (n=24)
Group 2: Propolis in teeth with closed apices (n=7)
Group 3: MTA in teeth with open apices (n=20)
Group 4: MTA in teeth with closed apices (n=6)
Group 5: Parafilm in teeth with open apices (n=12)
Group 6: Parafilm in teeth with closed apices (n=3)

In all groups, the coronal double seal was provided by MTA and glass-ionomer (Gold Label, GC Corporation, Tokyo, Japan) after treatment.

As mentioned previously, in order to assess angiogenic factors at 1-month and 3-month intervals, the teeth on dog's left side (group B) were treated 2 months after the teeth on the right side (group A). One month after treating the teeth on the left side of the dogs and taking radiographs and observation of complete development of the roots, the animals were sacrificed using vital perfusion.

Immunohistochemical evaluation

The upper and lower jaws of the animals were separated at appropriate places and the teeth were separated from the jaws by block section and decalcified. After decalcification and embedding the teeth in paraffin, a microtome was used to prepare 4-µ-thick cross-sections from the paraffin blocks longitudinally. Then the samples were sent to the laboratory for immunohistochemical tests. The histological cross-sections were evaluated under a binocular light microscope (Nikon, Japan) in a blind manner by a pathologist. After histological evaluation of the samples and confirmation of formation of blood vessels, two cross-sections from each sample underwent immunohistochemical staining. All the laboratory procedures were carried out using the protocol and instructions of the Dako Company available in each immunohistochemistry kit. An IHC study was performed for the cases which showed regeneration as follows: after deparaffinization and hydration of slides, antigenic retrieval was done by incubation with molar citrate buffer 1% (pH = 6) in a microwave oven for 12 min. For IHC, the streptavidin-biotin immunoperoxidase procedure was performed by incubation at room temperature for 10 min with 3% hydrogen peroxide; 60 min with an antibody against VEGF and factor 8 (DakoCytomation), then streptavidin peroxides (DakoLsab 25 system, peroxidase kit; Dako, Glostrup, Denmark) and finally with chromogen (diaminobenzidine hydrochloride). Counterstaining was done with Mayer's hematoxylin and VEGF, and factor 8 expression was evaluated as the quantitative score. The quantitative score was classified depending on the percentage of positive cells. VEGF code number was M7273 and factor 8 (M0616) was diluted 1/150 in R Tris buffer. Hemangiomas tissue was considered as positive control. Negative control was done after elimination of primary antibody from samples (14).

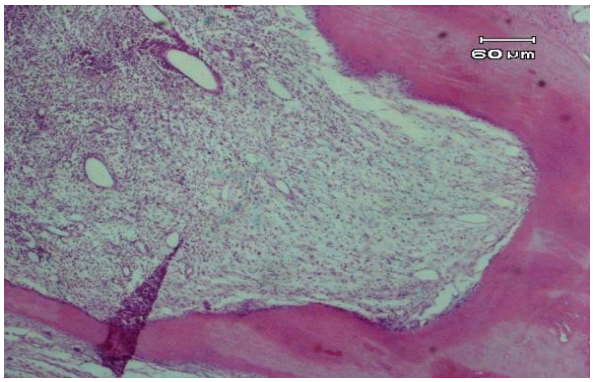

Figure 1. Regenerated pulp-like tissue in the root canal. (H&E staining ×400)

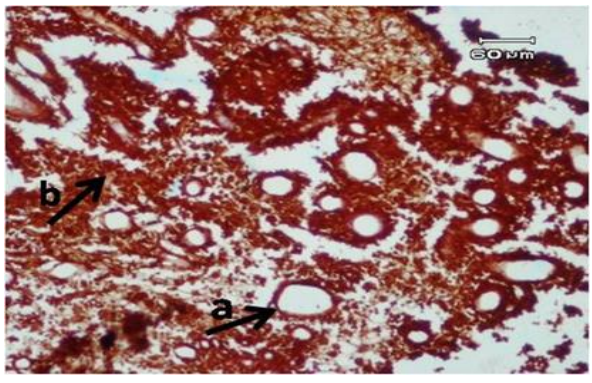

Figure 2. Immunohistochemical staining of VIII factor: severe staining in vessel walls and stromal cells (×400)

Table 1. Expression rates of VEGF and factor VIII at 1- and 3-month intervals

	One month Follow-up		Three month Follow-up	
Material	VEGF	VIII	VEGF	VIII
MTA	%20	%60	%40	%37
Propolis	%75	%72	%59	%54

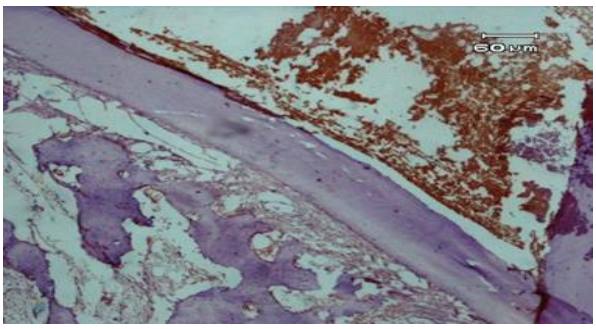

Figure 3. Immunohistochemical staining of VEGF with grade 4+ intensity in 88% of cells: (×400)

Results

Based on histological observations, the regenerated tissues consisted of pulp-like soft tissue and vessels and hard tissues, including cementum-like tissue and inorganic tissue (Figure1). On the whole, in 54.3% of cases, regenerated tissue was observed within the root canal. Regardless of the status of the apex before treatment, no regeneration was observed in the parafilm group (0%). In the propolis and MTA groups, regeneration was observed in 64.5% and 38% of cases. Table 1 presents the expression rates of VEGF and factor VIII at 1- and 3-month intervals.

Based on the results of the immunohistochemical evaluation, the expression rates of VEGF and factor VIII were higher at the 1-month interval compared to the 3-month interval and higher in the propolis group compared to the MTA group (Table 2).

The expression rates of VEGF and factor VIII in the propolis group with open apices were higher than those with closed apices, and in the MTA group, they were higher in the closed apices compared to open apices. Immunohistochemical evaluation showed that in a group of regenerated samples, VEGF and factor VIII were observed in stromal cells with high intensity, in addition to the endothelial cells of blood vessel walls (Figures 2, 3).

Table 2. Expression rates of VEGF and factor VIII in open and closed apex teeth

	Closed Apex				Open Apex			
	One-month		Three-month		One-month		Three-month	
Material	VEGF	VIII	VEGF	VIII	VEGF	VIII	VEGF	VIII
MTA	68%	60%	59%	45%	41%	47%	39%	40%
Propolis	37%	42%	30%	38%	75%	66%	67%	56%

Discussion

Proper endodontic treatment is associated with a large number of problems in immature necrotic teeth. Several techniques are used to treat such teeth, including one-session and multi-session apexification and use of an MTA plug. Despite the high success rate of apexification techniques, this treatment modality does not result in the continuation of root development and the tooth becomes susceptible to fracture (1–3). On the other hand, revascularization and induction of a blood clot in the root canal lead to thickening of the root canal wall and intracanal growth of the vital tissue. The blood clot can play the role of a scaffold for the migration of periapical stem cells into the root canal and formation of a new pulpal tissue (12). In the present study, similar to studies by Nagy *et al* (1), Becerra *et al* (2) and Nagata *et al* (4), revascularization protocol was implemented, which consisted of minimum instrumentation or non-instrumentation in conjunction with the use of 5.25% NaOCl and triantibiotic paste to disinfect the root canal and induce hemorrhage, followed by application of MTA in order to seal and induce regeneration.

Research showed that MTA promotes the growth of stem cells by inducing signaling molecules (15). In addition, MTA might induce formation of hard tissues with mild inflammation in a span of two weeks, which might be attributed to the release of large amounts of calcium ions or induction of secretion of BMP-2 and TGF-β1 from periodontal fibroblasts (16). Researchers reported that direct contact of MTA with cells might increase the expression of genes similar to dentin osteocalcin and sialoproteins. Therefore, direct contact is necessary for the differentiation of cells into odontoblast-like cells. In addition, it was reported that MTA increases the secretion of VEGF if it directly contacts the pulpal cells (17).

In general, at present, there is a lot of evidence available on the use of propolis for the treatment of ulcers resistant to treatment and it has been shown that it is a good dressing material, reduces inflammation, derides necrotic tissues, decreases edema, and induces angiogenesis and granulation tissue formation (11,18). Study showed that direct pulp capping with the flavonoid type of propolis in rats can delay inflammation of dental pulp and induce reparative dentin (19). Researchers histologically evaluated the dental pulp with direct pulp capping with propolis and compared it with MTA and Dycal and reported more severe inflammation in human premolars treated with Dycal in comparison with propolis and MTA at 15- and 45-day intervals. Propolis and MTA resulted in dentin bridge formation in more teeth compared to Dycal; the dentin bridge was close to the pulp cap material and the pulpal response to direct pulp capping with propolis was comparable to that with MTA (20). Despite the availability of studies on the effects of propolis on wound healing and decreasing the severity of inflammation, the present study was undertaken to

assess the effect of propolis on the induction of the principal vascular growth factors of the pulp in non-vital teeth with mature and immature apices. Parafilm was selected in the present study as a separating material in control groups due to its neutrality and lack of the property to irritate vital tissues so that the effects of MTA and propolis on the induction of regeneration could further be explained.

VEGF is an important regulator of physiologic and pathologic angiogenesis. This factor is expressed in the healthy pulp and dentin matrix (7). Studies have shown that odontoblast-like cells and undifferentiated pulp cells express high amounts of VEGF *in vitro* (9). On the other hand, the release of VEGF present in the dentin matrix can initiate the revascularization and anastomosis processes like an active molecule (21). The presence of VEGF is on the rise from the first day of an inflammatory process after formation of granulation tissue but then it decreases gradually (5, 6). On the other hand, formation of tooth calcified tissues is visible on the 14th day of regeneration processes on histological views and approximately 3 months later on radiographic views (22). Therefore, in the present study 1- and 3-month intervals were selected to evaluate the relationship between VEGF and continuation of root development during regeneration processes.

Dissanayaka reported that due to the presence of VEGF in endothelial cells, concomitant culturing of these cells with pulpal stem cells results in the appearance of angiogenic phenotype (23). On the other hand, based on the results of a study by Li in 2010 the number of endothelial cells with positive factor VIII in cellular groups exposed to VEGF was higher so these factors are in relation with each other (24). Although some studies have evaluated the effect of MTA on the expression of VEGF during pulp capping and formation of a dent in the bridge (17), there is no study available on the amount of this factor during revascularization in the root canal. Therefore, the main reason for selecting these two factors in the present study was their key and primary role in the process of angiogenesis and tissue regeneration (21). Selection of immunohistochemical techniques for the evaluation of tissue regeneration increased the importance and value of the study due to the sensitivity and accuracy of these studies; on the other hand, this study is a unique study due to the absence of similar studies.

The importance of establishing a coronal seal after endodontic treatment has been shown and accepted in various studies (25). Various techniques have been proposed to establish a maximum seal and decrease microleakage. In the present study, similar to studies by Thibodeau (26), Petino (27) and Chueh (28), the double technique with the use of MTA and glass-ionomer was used to achieve a maximum seal.

Observation of tissue regeneration in the root canals of more than 50% of samples, irrespective of the type of the inductive material and the status of the pulp, is consistent with the majority of studies carried out to date on revascularization techniques (26–28). The results of the present study showed tissue infiltration in 38% and 64% of MTA and propolis samples, respectively, with no vital tissue regeneration in the parafilm samples. Therefore, the inductive effect of MTA and propolis on regeneration and angiogenesis was shown. Since the direct contact of MTA with cells is necessary for its inductive effect and release of growth factors (17), it is possible that the presence of parafilm has prevented MTA from bringing about its effect. Another reason for regeneration not occurring in the parafilm group might be related to questionable setting of MTA in the vicinity of parafilm due to the absence of moisture. The expression rate of VEGF and VIII factors at the one-month interval was higher than that at the 3-month interval, which might be attributed to the conversion of granulation tissue to pulp-like tissue during the first month and a decrease in the intensity of inflammatory processes. The pulpal stem cells differentiate into endothelial cells over time.

In the present study, higher amounts of VEGF and factor VIII were found in the regenerated tissues in the perivascular endothelial and stromal cells. The expression of these factors by stromal cells is an indication of the acquisition of the capacity to secrete these factors by these cells and the capacity of stem cells to differentiate into endothelial cells. According to the present study, propolis can induce angiogenesis and expression of VEGF and factor VIII. Achieving similar results in relation to the density of blood vessels in the propolis and MTA groups might reflect the obvious effect of this material on inducing upregulation of genes related to angiogenesis. The antibacterial property of propolis and the slow rate of disintegration of its constituents are other advantages of this material for its long-term effect on tissue healing and induction of regeneration. In other words, better results can be expected in this field.

Based on the results of a study by Otsby on teeth with closed apices (29), the hypothesis of the possibility of the success of this technique in teeth with closed apices was introduced. A large number of case reports are about teeth with immature apices and it has been shown that revascularization of replanted teeth with wide apices of almost 1.1 mm is more successful (1, 2, 4). This finding suggested that in revascularization of teeth with closed apices and necrotic pulps it is necessary to instrument the tooth apex up to 1–2 mm and destroy the isthmus so that sufficient space can be created for the influx of hemorrhage into the root canal system. In the present study, this technique was used for the regeneration of teeth with closed apices and in a number of samples with closed apices, too, tissue regeneration and induction of growth factors were observed. This tissue consisted of pulp-like soft tissue and blood vessels and the hard tissue consisted of cementum-like and mineral tissue; this result is unique and paves the way for further studies on regenerative treatments is such teeth.

Conclusion

Based on the results of this study, more VEGF and factor VIII were induced in the propolis group compared to the MTA group. No regeneration was observed in the parafilm group samples. More factors were induced in teeth with open apices compared to teeth with closed apices. More factors were induced at one-month interval compared to the 3-month interval, irrespective of the type of the inductive material.

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References

1. Nagy MM, Tawfik HE, Hashem AA, Abu-Seida AM. Regenerative potential of immature permanent teeth with necrotic pulps after different regenerative protocols. J Endod 2014; 40: 192-198.
2. Becerra P, Ricucci D, Loghin S, Gibbs JL, Lin LM. Histologic study of a human immature permanent premolar with chronic apical abscess after revascularization/revitalization. J Endod 2014; 40:133-139.
3. Mente J, Leo M, Panagidis D, Ohle M, Schneider S, Lorenzo Bermejo J, *et al.* Treatment outcome of mineral trioxide aggregate in open apex teeth. J Endod 2013; 39:20-26.
4. Nagata JY, Gomes BP, Rocha Lima TF, Murakami LS, de Faria DE, Campos GR, *et al.* Traumatized immature teeth treated with 2 protocols of pulp revascularization. J Endod 2014; 40:606-612.
5. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 2003;18:696-704.
6. Nakanishi Y, Izumi K, Yoshizawa M, Saito C, Kawano Y, Maeda T. The expression and production of vascular endothelial growth factor in oral mucosa equivalents. Int J Oral Maxillofac Surg 2007; 36:928-933.
7. Virtej A, Løes S, Iden O, Bletsa A, Berggreen E. Vascular endothelial growth factors signalling in normal human dental pulp: a study of gene and protein expression. Eur J Oral Sci 2013; 121:92-100.
8. Ferrara N, Gerber HP, LeCouter J .The biology of VEGF and its receptors. Nat Med 2003; 9: 669-676.
9.Botero TM, Son JS, Vodopyanov D, Hasegawa M, Shelburne CE, Nor JE. MAPK signaling is required for LPS-induced VEGF in pulp stem cells. J Dent Res 2010; 89:264-269.
10. Li WW ,Talcott KE, Zhai AW, Kruger EA, Li VW. The role of therapeutic angiogenesis in tissue repair and regeneration. Adv Skin Wound Care 2005; 18:491-500; quiz 1-2.
11. Jastrzębska-Stojko Z, Stojko R, Rzepecka-Stojko A, Kabała-Dzik A, Stojko J. Biological activity of propolis-honey balm in the treatment of experimentally-evoked burn wounds. Molecules 2013; 18:14397-1413.
12. Yamauchi N, Yamauchi S, Nagaoka H, Duggan D, Zhong S, Lee SM, *et al*. Tissue engineering strategies for immature teeth with apical periodontitis. J Endod 2011;37:390-397.
13. Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, *et al. In-vitro* antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J 1996; 29:125-130.
14. Artese L, Rubini C, Ferrero G, Fioroni M, Santinelli A, Piattelli A. Vascular endothelial growth factor (VEGF) expression in healthy and inflamed human dental pulps. J Endod 2002; 28:20-23.
15. Torabinejad M, Parirokh M. Mineral trioxide aggregate: a comprehensive literature review--part II: leakage and biocompatibility investigations. J Endod 2010; 36:190-202.
16. Guven G, Cehreli ZC, Ural A, Serdar MA, Basak F. Effect of mineral trioxide aggregate cements on transforming growth factor beta1 and bone morphogenetic protein production by human fibroblasts in vitro. J Endod 2007; 33:447-450.
17. Paranjpe A, Smoot T, Zhang H, Johnson JD. Direct contact with mineral trioxide aggregate activates and differentiates human dental pulp cells. J Endod 2011; 37:1691-165.
18. de Groot AC. Propolis: a review of properties, applications, chemical composition, contact allergy, and other adverse effects. Dermatitis 2013; 24:263-282.
19. Sabir A, Tabbu CR, Agustiono P, Sosroseno W. Histological analysis of rat dental pulp tissue capped with propolis. J Oral Sci 2005; 47:35-38.
20. Parolia A, Kundabala M, Rao NN, Acharya SR, Agrawal P, Mohan M, *et al*. A comparative histological analysis of human pulp following direct pulp capping with Propolis, mineral trioxide aggregate and Dycal. Aust Dent J 2010; 55:59-64.
21. Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. Arch Oral Biol 2000; 45:1013-1016.
22. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res 2010; 89:219-229.
23. Dissanayaka WL, Zhan X, Zhang C, Hargreaves KM, Jin L, Tong EH. Coculture of dental pulp stem cells with endothelial cells enhances osteo-/odontogenic and angiogenic potential *in vitro*. J Endod 2012; 38:454-463.
24. Li YL, Li H, Hu MJ, Tang JM, Wang JN. Effect of mesenchymal stem cells transfected with human vegf-165 gene carried by adenovirus on revascularization for hind limb ischemic necrosis in rat model. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2010; 18:1568-1573.
25. Kenneth M. Hargreaves Sc. Pathway of the Pulp. 10th ed. Mosby; 2011.
26. Thibodeau B, Teixeira F, Yamauchi M, Caplan DJ, Trope M. Pulp revascularization of immature dog teeth with apical periodontitis. J Endod 2007; 33:680-689.
27. Petrino JA, Boda KK, Shambarger S, Bowles WR, McClanahan SB. Challenges in regenerative endodontics: a case series. J Endod 2010; 36:536-341.
28. Chueh LH, Huang GT. Immature teeth with periradicular periodontitis or abscess undergoing apexogenesis: a paradigm shift. J Endod 2006; 32:1205-1213.
29. Nygaard-Ostby B, Hjortdal O. Tissue formation in the root canal following pulp removal. Scand J Dent Res 1971;79:333-349.