



## IN VIVO STUDY THE EFFECTS OF TOPICAL APPLICATION OF PROPOLIS AND BLACK SEEDS OIL ON PERIODONTIUM HEALING IN CONTROL DIABETIC RABBITS (HISTOLOGICAL AND IMMUNOHISTOLOGICAL STUDY ON VEGF)

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### ABSTRACT

Periodontal diseases are a group of chronic, progressive bacterial infections resulting in inflammation and destruction of tooth supporting tissues. Diabetes is a systemic disease which is a serious oral co-morbidity involving the periodontium. The aim of the present study was to study the effects of topical application of propolis and black seeds oil on periodontium healing of controlled diabetic rabbits. Twelve Newzeland male rabbits were used in this study. All animals were subjected to alloxan injection to induced diabetes which was controlled by insulin. Bilateral defects were induced in periodontium of the distal side of two upper central incisors. Then application of a mixture of propolis and black seeds oil was put in the defect side of right central incisor (Experimental group), whereas distal defect side of left central incisor (Control group) was left to heal normally. The samples were sacrificed at three intervals 3days, 7days, and 10 days (4 rabbits for each interval). Histological and immunohistochemical on VEGF were studied for each interval in both groups. Histological examination showed the acceleration of bone formation and more rapid healing process in experimental side with propolis and black seeds oil than in the control side. Immunohistochemical findings revealed high positive expression for VEGF in experimental side in comparison to control one. Topical application of a mixture of propolis, and black seed was effective in enhancement of periodontium healing in controlled diabetes.

**KEY WORDS:** Periodontium healing ,alloxan, propolis, black seed.

### INTRODUCTION

Periodontium refer to the specialized tissues that both surround and support the teeth, maintaining them in the maxillary and mandibular bones. It provides the support necessary to maintain teeth in function. It consists of four principal components namely: Gingiva, Periodontal ligament (PDL), Cementum and Alveolar bone (Haryanto *et al.*, 2012), (Nanci and Bosshardt, 2006). Propolis is a wax-cum-resin substance that is produced by bees. Mouth environment is rich in bacterial flora which in some conditions may lead to such diseases like caries or diseases of periodontium. The study done in 2013 showed that propolis-based solutions have lower cytotoxic effect on the cells of human gum fibroblasts (Wi ckiewicz *et al.*, 2013).

Wound healing is a complex biological cascade of cellular and biochemical events comprised of three phases: inflammation, proliferation and maturation (Allwayzy, 2013). Wound management still remains an important focus of researches (The use of natural products as an alternative treatment has been on the rise in the last few decades. The study of the effectiveness of topical application of black seed oil on the wound healing in rabbits shows that the black seed oil was enhanced wound healing, and that may be due to its therapeutic and nutritional activities (Al-Mutheffer, 2010). Diabetes has impaired defense mechanisms involving micro- and macro-vasculatures. The increased susceptibility to infection and reduced healing capacity with altered collagen metabolism may explain the increased level of

periodontal destruction (Daniel *et al.*, 2012). There is a clear relationship between degree of hyperglycaemia and severity of periodontitis (Preshaw *et al.*, 2012). Various cytokines and growth factors regulating angiogenesis, the most potent agent acting on vascular endothelium is vascular endothelial growth factor (VEGF) (Padma *et al.*, 2014). VEGF is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. VEGF's normal function is to create new blood vessels during embryonic development and after injury, muscle following exercise, and new vessels (collateral circulation) to bypass blocked vessels (Claesson-Welsh, 2008). Study the effects of topical applications of propolis and black seeds oil on periodontium healing of controlled diabetic rabbits.

### MATERIALS & METHODS

Alloxan (100 mg, England), Insulin (0.1mg/kg B.W). Propolis (10gm), Black seeds oil (10 ml) a mixture was prepared by mixing the two mentioned concentration after propolis had been heated.

Ketamine hydrochloride 50mg and Xylazine 2%

Formalin 10%, ethanol alcohol 96%, xylol, paraffin wax, and Hematoxylin and Eosin (H & E) stain.

Monoclonal antibodies: Vascular Endothelial Growth Factor Antibody (VEGF) from Abcam Company UK (ab1316)

Detection Kits System, Abcam Company England (ab80436) Experimental design

### Experimental design

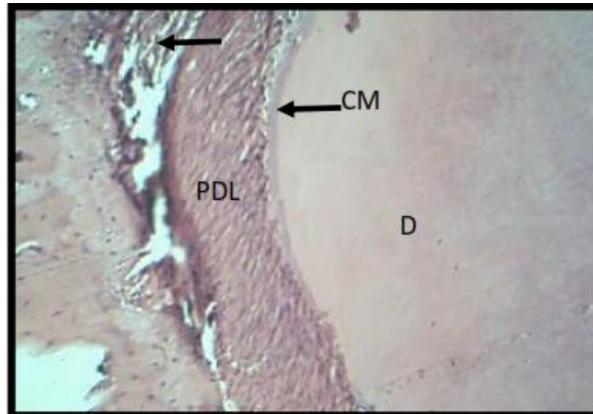
Twelve Newzeland male rabbits were used in this study. All animals were injected by 150 mg/kg B.W.) of alloxan intravenously in the external marginal vein in rabbit's ear. After elevation of blood glucose level, they received daily insulin treatment subcutaneously as a treatment in a dose of 0.1mg/kg B.W. to control the hyperglycemia (Wang *et al.*, 2010). Bilateral defects were induced in periodontium of the distal side of two upper central incisors. Then application of a mixture of propolis and black seeds oil was put in the defect side of right central incisor (Experimental group), whereas distal defect side of left

central incisor (Control group) was left to heal normally. The samples were sacrificed at three intervals 3days, 7days, and 10 days (4 rabbits for each interval). Histological and immunohistochemical on VEGF were studied for each interval in both groups.

### RESULTS

#### Histological findings

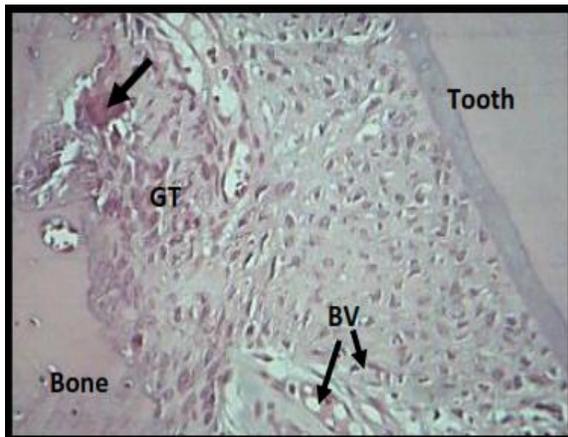
Histological examination of 3 days duration shows defect area with some bundles of regenerating collagen fibers of granulation tissue infiltrating defect area (Figure-1).



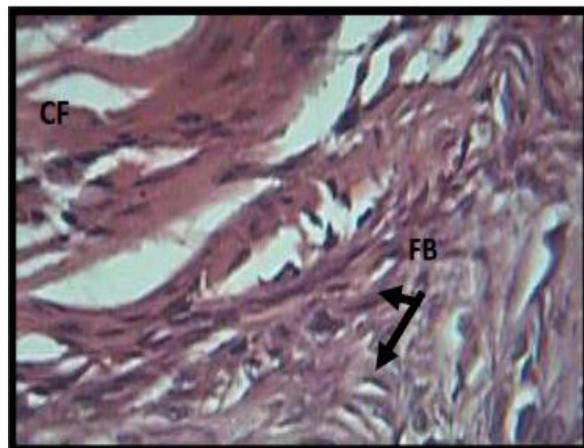
**FIGURE 1:** View after 3days shows granulation tissue at defect area (arrow), root dentin (D), cementum (CM) and periodontal ligament (PDL). H&EX10.

### Experimental group

Histological findings of experimental group of 3days duration show remodeling granulation tissue infiltrated by inflammatory cells with large numbers of fibroblasts at defect side and numerous blood vessels (Figure 2, 3).



**FIGURE2:** View at defect site of control group after 3days shows granulation tissue (GT) infiltration by inflammatory cells, blood clot (arrow) and blood vessels (BV). H&EX20.

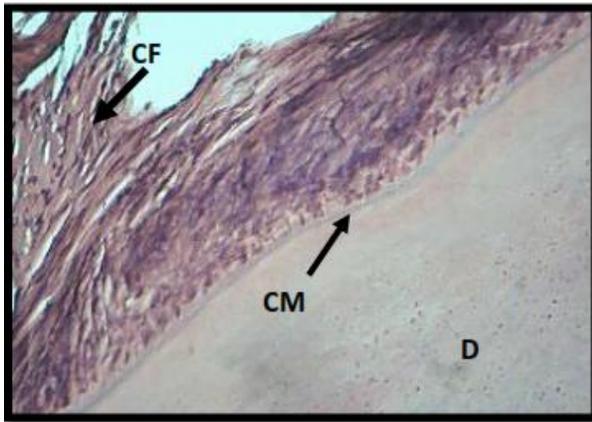


**FIGURE 3:** Magnified view after 3 days shows collagen fibers (CF) and fibroblasts (FB). H&EX40.

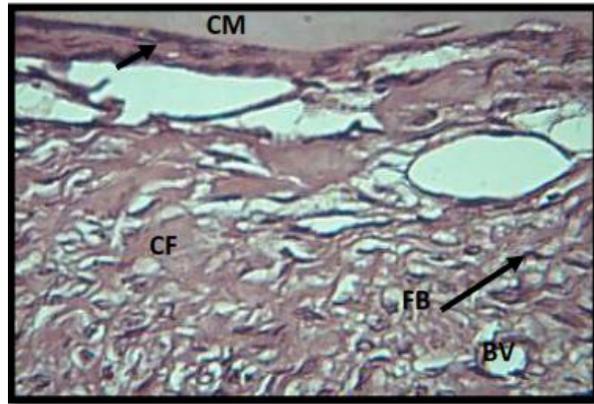
### Seven days duration

#### Control group

Microphotograph view shows new collagen fibers almost filling the defect area fibroblasts, blood vessel, cementoblasts at cementum surface (Figures 4, 5).



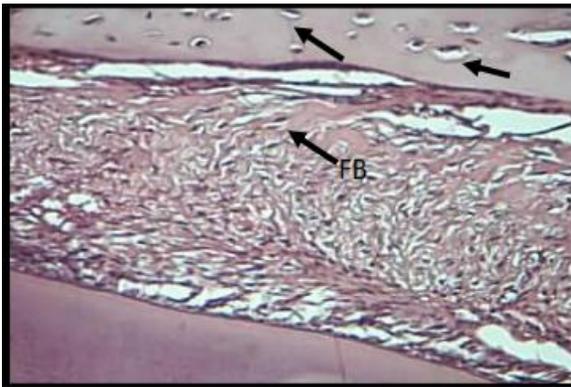
**FIGURE 4:** View of 7days duration shows regenerating collagen fibers(CF). H & E X 20.



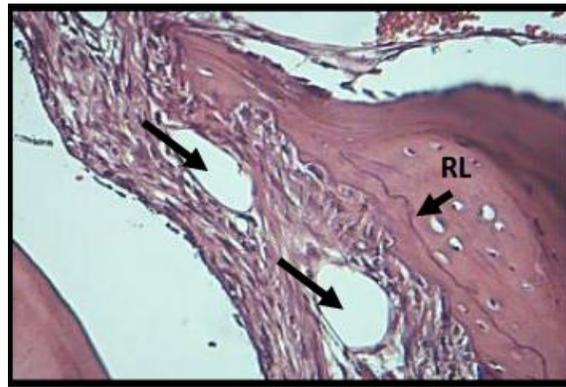
**FIGURE5:** Magnified view after7days shows collagen fibers (CF) and fibroblasts (FB), blood vessel (BV), cementoblasts (arrow) at cementum surface. H& EX40.

**Experimental group**

View of defect area after 7days of material application shows remodeling of collagen fibers with formative fibroblasts, bone matrix entrapping osteocytes, cementum and dentin (Figure 6, 7).



**FIGURE 6:** View of 7days duration shows organized collagen fibers (CF) and fibroblasts (FB) osteocytes in bone matrix (arrows). H&EX20



**FIGURE 7:** View of 7days duration shows new bone formation with reversal line (RL), and numerous interstitial spaces(arrows ). H&EX40

**Ten days duration**

**Control group**

Histological view of 10 days duration shows the remodeling collagen fibers occupying the operating site associated with fibroblasts (Figures 8).



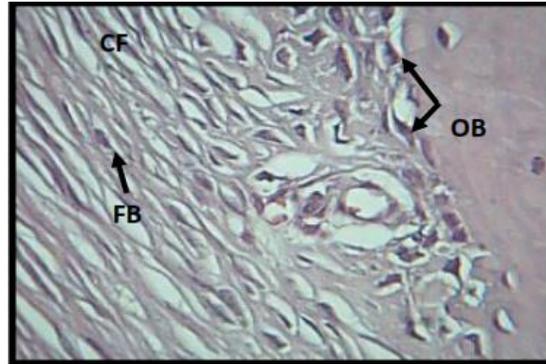
**FIGURE 8:** View of 10days duration shows organized collagen fibers, fibroblasts (FB), cementoblasts (arrows), cementum (CM) and dentin (D). H & E X 40.

**Experimental group**

View of 10days duration shows the new regenerated principal fibers that are reattached to cementum of root ,and bone(Figures 9,10)



**FIGURE 9:** View of 10days duration shows reattachment of collagen fibers (CF) to cementum (C) and cementoblasts (CB). H&EX20.

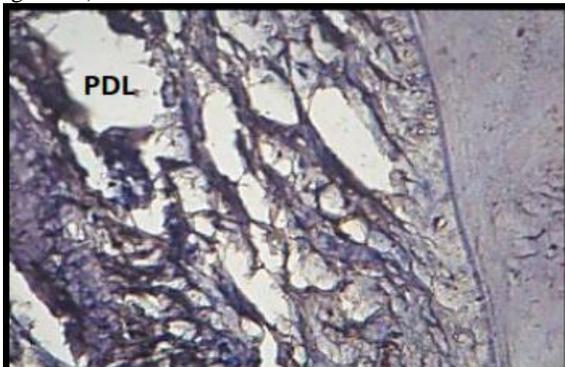


**FIGURE10:** Other view of 10days duration shows collagen fibers (CF), fibroblasts (FB), bone and osteoblasts (OB). H&EX40.

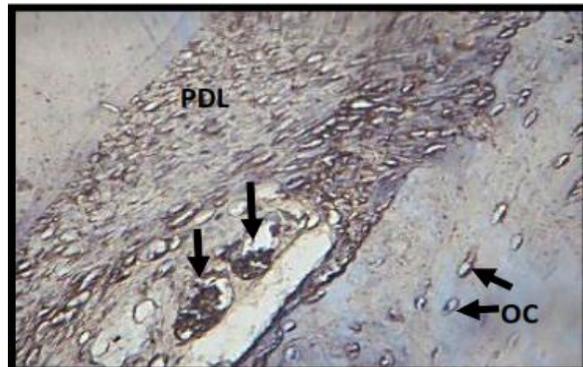
**Immonohistochemical results**

**Three days duration**

View of operated site of control group shows positive expression of VEGF by periodontal ligament fibers, (Figure11). The experimental group showed detection of positive expression of VEGF by collagen fibers, interstitial tissue and osteocytes (Figure 12).



**FIGURE 11:** View of 3days duration of control group shows positive expression of VEGF by periodontal ligament fibers (PDL). DAB stain with counter stain hematoxylinX40.



**FIGURE12:** View of operated site in experimental group shows positive expression of VEGF by periodontal ligament fibers, interstitial tissue (arrows)and osteocytes of bone(OC). DAB stain with counter stain hematoxylinX40.

**Seven days duration**

Immunohistochemical localization of VEGF was detected by collagen fibers and fibroblasts in control group (Figure13)and positively stained gingival epithelial cell layers and fibrous connective tissue 795gingival in experimental group(Figure14).



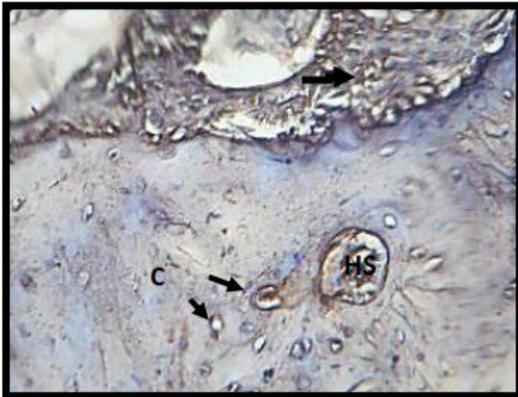
**FIGURE 13:** View of positive localization of VEGF expressed by periodontal collagen fibers and interstitial tissue(arrows). DAB stain with counter stain hematoxylinX20.



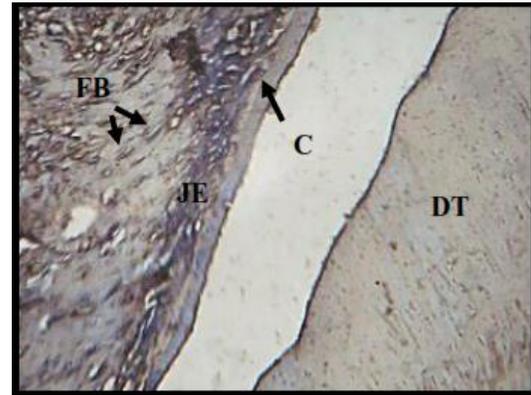
**FIGURE 14:** View of positive localization of VEGF expressed by new epithelium (NE)and dermal connective tissue (CT). DAB stain with counter stain hematoxylinX40.

**Ten days duration:**

Immunohistochemical localization of VEGF was detected by collagen fibers and fibroblasts in control group (Figure 15) and positively stained new junctional epithelium and fibrous connective tissue of gingiva dentinal tubule of dentin was noticed in experimental group (Figure 16).



**FIGURE 15:** View of control group shows positive localization of VEGF expressed by fibroblasts (arrow), cementocytes (C) and (HS). DAB stain with counter stain hematoxylin X 40.



**FIGURE 16:** View of positive localization of VEGF expressed by new junctional epithelium cells (JE), fibroblasts (FB) cementocytes (C) and dentinal tubules (DT) in experimental group. DAB stain with counter stain hematoxylin X 40.

**DISCUSSION**

An extensive body of literature reports that diabetes is a risk factor for gingivitis and periodontitis, and the degree of glycemic control is a determining factor in the vulnerability to oral health complications that are three to four times higher as compared to systemically healthy individuals (Preshaw *et al.*, 2012). Treatment of periodontal disease and reduction of oral inflammation may have a positive effect on the diabetic condition (Mealey, 2006). As revealed by histological examination the response of the injured periodontium to combined use of propolis and black seeds oil was detected at 3 days which showed that defect site was characterized by inflammatory cells infiltration, there was beginning of the proliferation of fibroblast, immature cellular fibrous connective tissue. At the 7 days, more regular principles fibrous connective tissue seen, with reattachment of collagen fibers to bone and root was noticed and it seemed to be more obvious with time progression during healing process at 10 days mature fibrous connective tissue was present in the operated area and gingiva appeared covered by complete thickened layer of epidermis in agreement with the findings of Al-Mutheffer, 2010 who used black seed oil. Fusen *et al.*, 2003 investigated the association between VEGF, diabetes mellitus and periodontitis and concluded that VEGF is increased in gingival tissues of diabetic patients especially those with periodontal diseases. The reported increase in the release of IL-1 & TNF- from peripheral blood mono-nuclear cells of periodontitis patients compared with healthy subject might explain the higher concentration of VEGF since these cytokines can induce the expression of VEGF. The immunohistochemical findings of this study revealed that the expression of VEGF was more evident in experimental groups by various cellular and fibrillar elements of the periodontium throughout healing intervals, in accordance with a study conducted in 2014 which demonstrated that VEGF levels are prominent even in the healthy gingival

samples of controlled diabetic patients (Ramya and Kumar, 2014). A study by Keles *et al.*, 2010 on VEGF expression levels of gingiva in gingivitis and periodontitis patients with/without diabetes mellitus it was stated that VEGF expression is probably related to both maintenance of periodontal health and periodontal tissue destruction. Cetinkaya *et al.*, 2007 investigated the association between VEGF expression and vascularisation with regard to the number and diameter of blood vessels and concluded that VEGF may be related more to the healing stage of periodontal disease than to the destruction stage. Therefore, it can be stated that studies regarding the role of VEGF in the pathogenesis of periodontal diseases have had conflicting results.

**CONCLUSION**

It can be concluded that combined topical application of propolis and black seeds oil shown efficacy in the treatment of periodontium minor injury in controlled diabetics. The use of this mixture is of particular importance because of more pronounced response of periodontium healing.

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