AJMAS
https://journal.utripoli.edu.ly/index.php/Alqalam/index eISSN 2707-7179

Original article

Immunohistochemical Evaluation of Angiogenesis in Diabetes: An *in-Vitro* Experimental Study

Thoraya Elhadi

Department of Oral Biology, Faculty of Dentistry, University of Tripoli, Tripoli, Libya.

ARTICLE INFO

Corresponding Email. *thorayamilad@gmail.com*

***Received**: 06-02-2023*
***Accepted**: 17-03-2023*
***Published**: 21-03-2023*

***Keywords**. Angiogenesis, Diabetes, VEGF, Inflammation.*

ABSTRACT

Background and aims. *Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen, chemotactic agent, and inducer of vascular permeability. It is unique for its effects on multiple components of the wound healing cascade, including angiogenesis and recently shown epithelization and collagen deposition. This study was aimed at evaluating the angiogenesis in diabetes by the expression of VEGF.* ***Methods.*** *A total of 24 adult male rats aged about 6 months and weighing about 250gm was divided into 2 groups. Group I (12 rats) non diabetic. Group II (12 rats) diabetic. For study group, the rats were fasted overnight and diabetes was induced by a single intra peritoneal injection of streptozotocin 60 mg/kg body weight in 0.1 M citrate buffer. All animals, were exposed to surgical wounds (extracted lower right first molar). They were sacrificed as follows 4 rats from each group at intervals of 3day, 7day, 21day after extraction for immune histochemical study.* ***Results.*** *In the present study, immunohistochemically expression of VEGF was detected as brown cytoplasmic reaction. All the examined cases showed positive results for VEGF with different scores.* ***Conclusions.*** *The current results demonstrate expression of VEGF in diabetic rats during healing of extracted socket significantly higher than control group in late period .*

Cite this article. *Elhadi T. Immunohistochemical Evaluation of Angiogenesis in Diabetes: An in-Vitro Experimental Study. App Sci. 2023;6(1):106-112. https://doi.org/10.5281/zenodo.7757752*

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases in which a person has high blood glucose either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced by the pancreas. This resulting high blood sugar produces the classical symptoms of polyuria: frequent urination polydipsia (increased thirst) and polyphagia (increases hunger) [1].

Modelling and establishment of new blood vessels is critical in wound healing and takes place concurrently during all phases of the reparative process. In addition to attracting neutrophils and macrophages, numerous angiogenic factors secreted during the haemostatic phase promote antiogenesis [2].

Resident endothelial cells are responsive to a number of angiogenic factors, including FGF, VEGF, PDGF, angiogenin, TGF-β and TGF-α. A fine balance is kept by the action of inhibitory factors, such as angiostatin and steroids [3] . Inhibitory and stimulatory agents act on proliferating endothelial cells directly as well as indirectly, by activating mitosis, promoting locomotion and by stimulating the host cells to release endothelial growth factors [4].

Initially, there is no vascular supply in the wound center, so viable tissue, which is limited to wound margins, is perfused by uninjured vessels and by diffusion through undamaged interstitium. Capillary sprouts from the surrounding edges invade the wound clot and, within a few days, a microvascular network composed of many new capillaries is formed [5].

Chemotaxis is the abiltiy of cells to move along a chemical gradient. This biochemical mechanism enables cells to reply properly to environmental stimuli that determine proliferation, differentiation and migration. Chemotactic agents act on cell surface receptors to direct the cell migration that is involved in angiogenesis during wound healing [6].

Migration is the consequence of chemotacitc activity and is necessary for angiogenesis. As a complex process that involves coordinated changes in cytoskeletal organization, signal transduction and cell adhesion, migration is dependent on the actin-rich network beneath the plasma membrane and is regulated by physical and chemical factors in the vascular system [7].

Vascular endothelial growth factor (VEGF) is one such candidate. It functions as an endothelial cell mitogen [8], chemotactic agent [9], and inducer of vascular permeability [10]. Other angiogenic growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF-β) have been described, but VEGF is unique for its effects on multiple components of the wound healing cascade, including angiogenesis and recently shown epithelization and collagen deposition [11].

VEGF is produced by many cell types that participate in wound healing: endothelial cells, fibroblasts, smooth muscle cells, platelets, neutrophils, and macrophages [12]. The objective of this study to explain different between normal angiogenesis and pathological angiogenesis in diabetes.

METHODS

Experimental animals

Twenty-four adult male rats weighing about 200-250 gms, and aged about 6 months were used in this study. These animals were obtained from the Institute of Medical Research Alexandria University. Animal were housed in specially designed wire mesh bottom cages, three animals per cage. All the animals were supplied a regular and the same diet adlibitum throughout the whole experimental period. The study included 24 rates grouped into 12 rats each (Diabetic and control group).

Data collection procedure

For study group, the rats were fasted overnight and diabetes was induced by a single intraperitoneal injection of streptozotocin 60 mg/kg body weight in 0.1 M citrate buffer [13]. Streptozotocin (STZ) was freshly prepared immediately before injection, and it was kept in cold store and refrigerator temperature (2-8c°) away from light. If it is not used fresh, streptozotocin solution can exhibit reduced ability to induce diabetes [14]. At the time the animals were entered the study, their body weight and blood glucose level were recorded then they were measured 48 hours after the administration of streptozotocin, then every two weeks throughout the time of the experiment.

Each animal received general anaesthetic solutions of 10% (40 ml/k.gm body weight) and zylazine 2% (5 mg/k.gm body weight)

The surgical site was disinfected using iodine swab, extraction of mandibular right 1st molar of all animals was carried out. One fourth of the animals in each group were sacrificed at 3, 7, and 21day following tooth extraction. Jaws were dissected, and specimens were fixed in 10% formalin saline for 7 days to be prepared for immunohistochemical study.

Immunostaining for VEGF using anti-rabbit polyclonal Antibody, clone Po-A (ready to use). Was done according to kit manual. Antigen retrieval using citrate buffer. (PH 0.6) was done for 1 hour at 40 c°[15].

Immunohistochemical technique of VEGF was done according to Shi et al [15,16].

RESULTS

In the present study, immunohistochemical expression of VEGF was detected as brown cytoplasmic reaction. All the examined cases showed positive results for VEGF but in different scores.

In the first 3 days of studied cases, the VEGF immune staining was detected in inflammatory cells, endothelial cells, fibroblast, osteoblast cells. In group I, score for the VEGF expression was ranged from 2 – 3 Fig. 1(A), in the median (2.0- 3.0) (Table 1). In group II, score for the VEGF expression was ranged from 2 – 3 Fig. 2 (A), in the median (2.0-3.0).

After 7 days of tooth extraction, the VEGF immune staining was detected in endothelial cells, fibroblast, osteoblast cells and residual inflammatory cells. In group I, score for the VEGF expression was ranged from 2 – 3 Fig.1 (B), in the median (2.0-3.0). In group II, score for the VEGF expression was ranged from 2 – 3 Fig. 2 (B), in the median (2.0-3.0).

In the 21 days, the VEGF immune staining was detected in inflammatory cells, endothelial cells, fibroblast, osteoblast cells. In group I, score for the VEGF expression was ranged from 1 – 2 Fig. 1 (C), in the median (1.0-2.0). Using Mann Whitney test. To compare between the different periods Wilcoxon signed ranks test was applied, in group I, no significant changes between 3 days and 7 days (p=1.000), whereas statistically significant changes were observed between 3 days and 21 days (p=0.038) and statistically significant change between 7 days and 21 days (p=0.059). In group II, no significant changes between 3 days and 7 days (p=1.000), and no significant changes between 3 days and 21 days p2 (0.083) and no significant changes between 7 days and 21 days (p=0.083).

Table 1.

After extraction	3days	7 days	21 days
Group I **Sig. bet. periods** **p1=1.000, p2=0.038*,** **p3 = 0.059**	3.0 (2.0-3.0)	3.0 (2.0-3.0)	1.0 (1.0-2.0)
Group II **Sig. bet. periods** **p1 = 1.000, p2 = 0.083,p3= 0.083**	3.0(2.0-3.0)	3.0 (2.0-3.0)	2.0 (2.0-3.0)
P	1.000	1.000	0.042*

Comparison between the two studied groups according to expression of VEGF during the healing of extracted socket in diabetic rats. p1: p value for comparing between 3 days and 7 days, p2: p value for comparing between 3 days and 21 days, p3: p value for comparing between 7 days and 21 days. *: Statistically significant at p ≤ 0.05

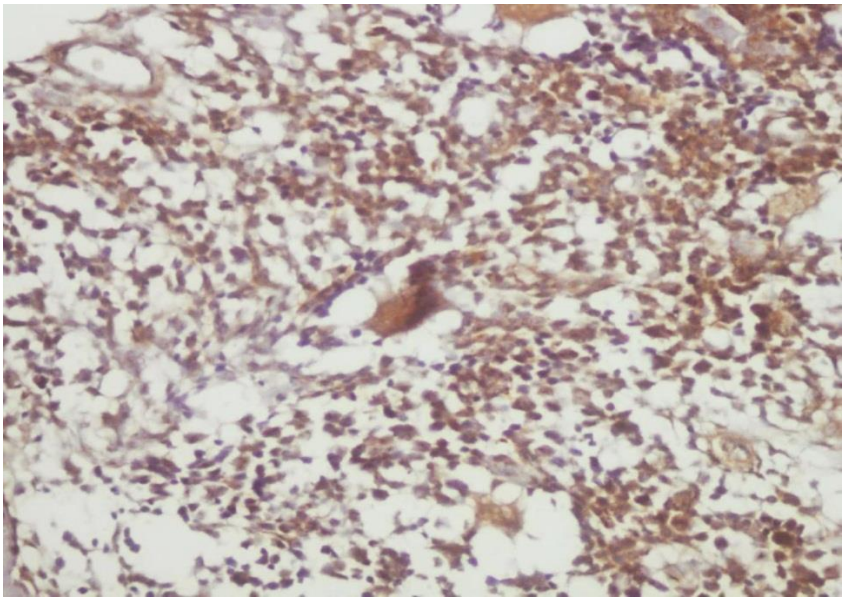

FIGURE 1 (A). Group I after 3 days showing strong cytoplasmic brown colouration in ≥ 76% of inflammatory cells and endothelial cells (VEGF, Avidin biotin, DAB x 400).

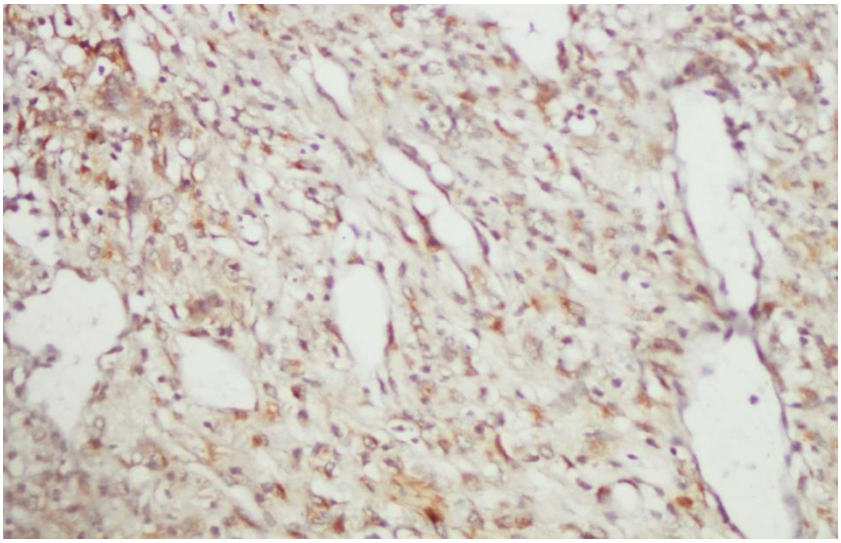

FIGURE 1 (B). Group I after 7 days showing moderate cytoplasmic brown colouration in ≥26 ≤75% of endothelial cells, fibroblast, and residual inflammatory cells (VEGF, Avidin biotin, DAB x 400).

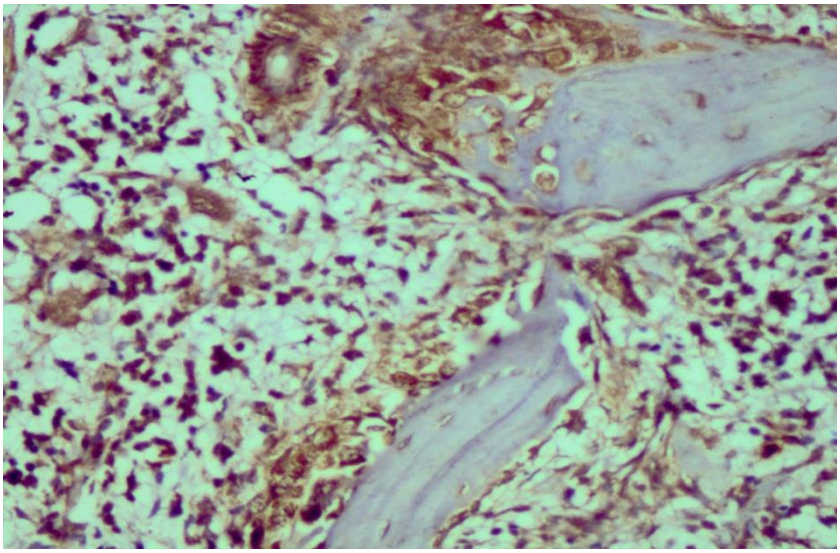

FIGURE 2 (A). Group II (diabetic)after 3 days showing strong cytoplasmic brown colouration in ≥ 76% of inflammatory cells, osteoblast, fibroblast cells and endothelial cells (VEGF, Avidin biotin, DAB x 400).

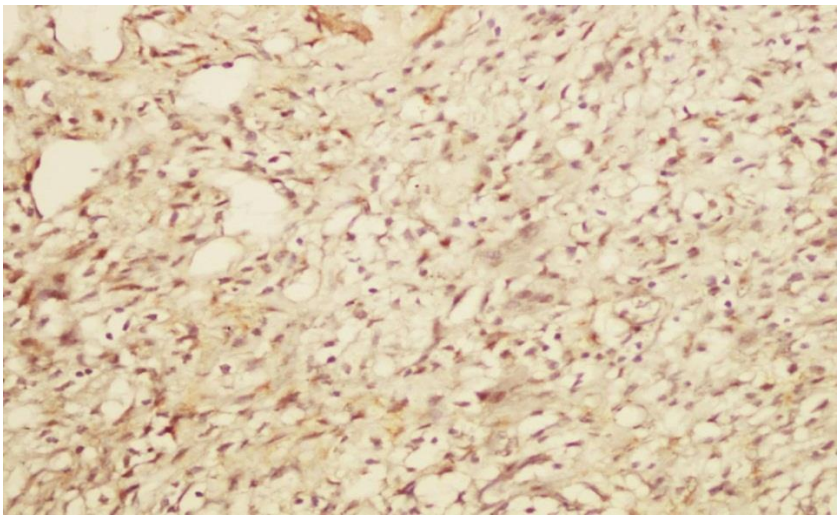

FIGURE 2 (B). Group II(diabetic) after 7 days showing strong cytoplasmic brown colouration in ≥ 76% of inflammatory cells, endothelial cells and fibroblast cells. (VEGF, Avidin biotin, DAB x 400).

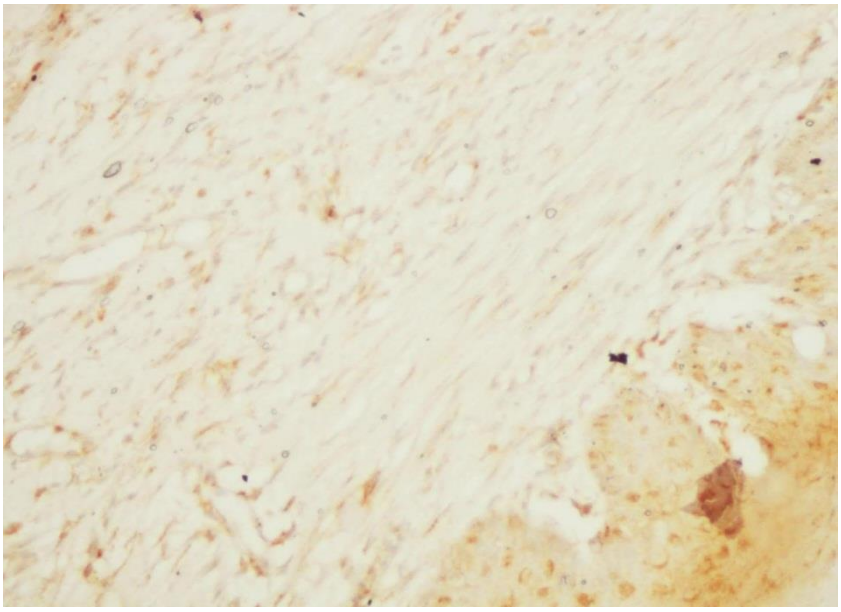

FIGURE 1 (C). Group I after 21 days showing weak cytoplasmic brown colouration in ≥11 ≤ 25 % of keratinocytes and fibroblast cells (VEGF, Avidin biotin, DAB x 400).

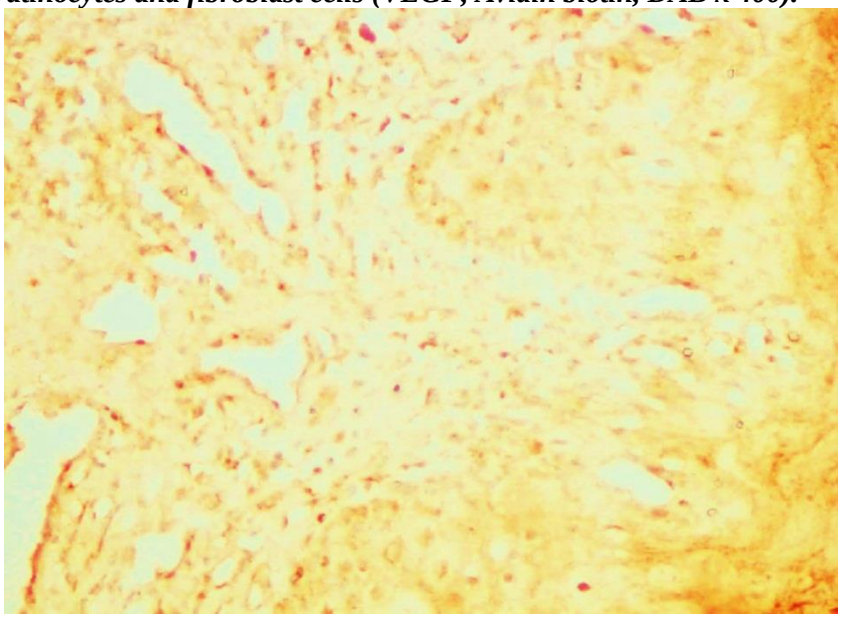

FIGURE 2 (C). Group II after 21 days showing strong cytoplasmic brown colouration in ≥76% of keratinocytes, endothelial cells and fibroblast cells (VEGF, Avidin biotin, DAB x 400).

DISCUSSION

VEGF is a growth factor discovered in 1989 by ferrara etal. That specifically act on vascular endothelial cells and characteristically vascularization and vascular hyper permeability [17]. The VEGFs and their corresponding receptors are key regulators in a cascade of molecular and cellular events that ultimately lead to the development of the vascular system, either by vasculogenesis, angiogenesis or in the formation of the lymphatic vascular system [17]. Although VEGFs' main effects are on endothelial cells, they also bind to VEGF receptors expressed on monocytes, neurons, chondrocytes and osteoblasts [18].

Recent studies have shown that the combination of angiogenic and osteogenic factors can stimulate bone healing and regeneration [19]. Both osteogenesis and angiogenesis are integrated parts of bone growth and regeneration. Combined delivery of osteogenic and angiogenic factors is a novel approach in bone regenerative engineering. Exogenous addition of vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs) together with an osteoconductive scaffold is a very promising method to enhance bone repair [20]. Therefore, the present study was designed to evaluate the expression of VEGF on healing of extracted socket in diabetic rats. Impaired wound healing is a well-documented phenomenon both in experimental and clinical diabetes [21]. Several mechanisms for diabetes-

impaired wound healing are proposed that are mostly related to impairment of macrophage function. angiogenic response, and extracellular matrix (ECM) deposition [22].
The present result shows positive expression of VEGF by inflammatory cells, precursor endothelial cells, keratinocytes, and bone cell include osteoblasts and active osteocytes in different periods in all groups but in different score. And that was in agreement with E. Fadhil et al, were found positive expression of VEGF by bone marrow stromal cells, adipocytes, mesenchymal stem cells, precursor endothelial cells, and bone cell include osteoblasts and active osteocytes in different periods in all groups but in different score Therefore, his data provide evidence that VEGF activity is essential for appropriate bone formation and mineralization in response to injury [23].
VEGF is produced by many cell types that participate in wound healing: endothelial cells, fibroblast, smooth muscle cells, Platelets, neutrophils, and macrophages [12].
The platelet is the first vascular component to appear in the wound site, followed by neutrophils, and then maerophages. Activated platelets release VEGF, particularly after thrombin stimulation [24].
On present study, Figure 1 (A, B) & Figure 2 (A, B) show positive cytoplasm immune staining in endothelial cell of newly formed capillaries. One of VEGF's roles in wound healing is in stimulation of angiogenesis. Wound healing angiogenesis involves multiple steps including vasodilation, basement membrane degradation, endothelial cell migration, and endothelial cell proliferation [25].
VEGF induces endothelial cell migration in wound healing through two primary mechanisms, chemotaxis and vasodilatation. In the initial phase of angiogenesis, endothelial cells migrate before mitotic division [26].
On present study fig.1(C) & fig. 2 (C) showing granulation tissue formation established healing responses. An essential feature of normal wound repair is the formation of granulation tissne, i.e. fibro vascular tissue containing fibroblasts, collagen and blood vessels, which is the hallmark of an established healing response [27].
In addition, VEGF is not only contributing to vasculogenesis in the emberyonic period and angiogenesis of normal tissues such as smooth muscle, cardiac muscle, and liver but is also secreted at solid cancer and inflammatory tissues and is closely involved in pathological angiogenesis in the many diseases including cancer, chronic rheumatoid arthritis and diabetic retinopathy [28].
Presently, VEGF is used clinically as a blood test item. The circulating VEGF concentration has been reported to be significantly higher in patients with various diseases in which VEGF is associated with pathological angiogenesis than in healthy individual [29].
Kakizawa et al, Compared the serum VEGF concentration between diabetic patents and healthy controls and reported that it was significantly higher in diabetic patients [30].
The expression of VEGF different between the groups were not significantly different at 3 and 7 days, but it was significantly different at 21 days.

CONCLUSION

Expression of VEGF in diabetic rats during healing of extracted socket was higher than control group in late period. Diabetes is considered an important risk factor for severe infection of the wound and impaired healing of extraction wound in comparison with control group. Expression of VEGF with periodontal disease is recommended.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

REFERENCES

1. Mc Cance DR,Hanson RL, Peititt DJ, Bern-en PH, Hadden DR, Knowler WC. Diagnosing diabetes mslktiis-do we need new criteria Diabetologia .1997; 4: 247-55.
2. Servold SA. Growth Factor impact on wound healing.Clin Podiatr Med Surg .1991; 8: 937-53.
3. Ribatti D,Vacca A, Roncali L, Dammacco F. Angiogenesis under normal and pathological conditions.Haematologica.1991; 76: 311-20.
4. Ferrara N.Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog Horm Res.2000; 55:15-35.
5. Robson MC,Steed DL,Franz MG.Wound healing:biologic features and approaches to maximize healing trajectories. CurrProbl Surg .2001; 38: 72-140.
6. Hsu S, Thakar R, Li S.Haptotaxis of endothelial cell migration under flow Methods. Mol Med.2007; 139: 237-50.
7. Li S,Huang NF, Hsu S. Mechanotransduction in endothelial cell migration.J Cell Biochem.2005;96: 1110-26.

8. Ferrara N,Henzel WJ.Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells.Biochem Biophys Res Commun.1989;16:851–8.
9. Yoshida A, Anand-Apte B, Zetter BR. Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. Growth Factors. 1996;13:57–64.
10. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF.Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. 1983;219:983–5.
11. Stojadinovic OKA, Golinko M, Tomic-Canic M, Brem H. A novel, non-angiogenic mechanism of VEGF: stimulation of keratinocyte and fibroblast migration. Wound Repair and Regeneration. 2007:A30.
12. Uchida K, Uchida S, Nitta K, Yumura W, Marumo F, Nihei H. Glomerular endothelial cells in culture express and secrete vascular endothelial growth factor. Am J Physiol .1994;266:F81–8.
13. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev. 2004; 25: 612-28.
14. Martinez G, Al-Dalian SM, Menedez S, Guilian A, Leon OS.Ozone treatment reduces blood oxidative stress and pancrease damage in a streptozotocin –induced diabetes model in rats.Acta Farm Bonaerense. 2005; 24: 49-7.
15. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed paraffin-embedded tissue, an enhancement method for immunohistochemical staining based on microwave oven heating of tissue section. J Histochem Cytochem. 1991; 39: 741-8.
16. Ruiz PA , DE Toledo OA , Nonaka CFW , Pinto LP, DE Souza LB . Oral Sci . 2010;18:12-9 .
17. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc Res.2005; 65: 550-63.
18. Ferrara N, Gerber HP, Le Couter J. The biology of VEGF and its receptors. Nat Med. 2003; 9: 669-76.
19. Geiger F, Bertram H, Berger I,Lorenz H,Wall O, Eckhardt C, Simank HG, Richter W .Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects . J Bone Miner Res .2005; 20: 2028-35.
20. Cui Q, Dighe AS, Irvine JN JR. Combined angiogenic and osteogenic factor delivery for bone regenerative engineering. Curr Pharm Des. 2013; 19: 3374-83.
21. Lioupis C. Effects of diabetes mellitus on wound healing. J Wound Care. 2005;14:84–86 .
22. Maruyama K, Asai J, Ii M, Thorne T, Losordo DW, D'Amore PA. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing .Am J Pathol . 2007;170:1178–1191.
23. Fadhil E, Athraa Y, J Bagh Coll Dentistry. 2014; 26 (4): 108 -115.
24. Brem H, Folkman J. Angiogenesis and basic fibroblast growth factor during wound healing.American Academy of Orthopedic Surgeons . 1994. p. 213-22.
25. Folkman J,Brem H.Angiogenesis and inflammation. Basic Principles and Clinical Correlates.1992; 821-39.
26. Yoshida A, Anand-Apte B, Zetter BR. Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. Growth Factors.1996;13:57–64.
27. Brem H, Folkman J. Angiogenesis and basic fibroblast growth factor during wound healing.Bone Formation and Repair . 1994; 213-22.
28. Toi M,Matsumoto T,Bandoh H.Vascular endothelial growth factor : its prognostic implications . Lancet oncol.2001;2:667 – 673.
29. Ferrari G,Scagolitti GV . Serum and urinary vascular endothelial growth factor levels in non – small cell lung cancer patients.Eur J cancer. 1996; 32: 2368 – 2369.
30. Kakizawa H, Ito Y, Fujiwara K, Kato T, Makino M, Vchimura K, et al. Change of VEGF and ET-1 concentration in the blood of the patients with the diabetes mellitus.Bulletin of the fujita Medical society. 2000; 24: 189 – 195.