Histological and immunohistochemical evaluation of the effect of local exogenous application of VEGF on bone healing (experimental study in rat)

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ABSTRACT

Background: The repair of bone defects remains a major clinical orthopaedic challenge. Bone is a highly vascularised tissue reliant on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity. Angiogenesis thus plays a pivotal role in skeletal development and bone fracture repair. The role of angiogenic and osteogenic factors in the adaptive response and interaction of osteoblasts and endothelial cells during the multi step process of bone development and repair will be highlighted in this study. This study aimed to identify the role of local exogenous vascular endothelial growth factor in bone healing and to analyze the expression of VEGF by immunohistochemistry in created bone defect after application with different biomaterials in a rat model.

Materials and methods: In this experimental study eighteen male Albino rats, weighing (300-400) gram, aged (6-8) months were used and maintained under control conditions of temperature, drinking and food consumption. The animals were subjected for a surgical operation of medial sides of both tibiae bone (right side was considered as experimental site ,while left be the control one), in control group the bone defect treated with local application of 1µm of normal saline, while experimental treated with local application of 1µm of VEGF .The rats were sacrificed at 3,7,10 days after surgery(six rats for each period). Bone healing was histologically examined with immunohistochemical localization of VEGF

Results: Bone defect treated with local application of VEGF shows an early osteoid tissue deposition with high cell count for osteoblast, osteocyte and osteoclast. Immunohistochemical evaluation for VEGF by stromal cells, reported to be higher with significant difference in VEGF group in comparison to control.

Conclusion: The study illustrated that low application of VEGF could be an effective therapeutic expression for bone injuries; these data are promising for a possible future clinical usage.

Keywords: Vascular endothelial growth factor, bone healing, osteogenic factors. (J Bagh Coll Dentistry 2014; 26(4):108-115).

INTRODUCTION

Skeletal development and fracture repair includes the coordination of multiple events such as migration, differentiation, and activation of multiple cell types and tissues [(1)]. The development of a microvasculature and microcirculation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die [(2)].

The vasculature provides the necessary factors such as growth factors, hormones, cytokines, chemokines and metabolites needed by the surrounding tissue and acts, when needed, as a barrier to limit the movement of molecules and cells. Signals and attractant factors expressed on the bone endothelium help recruit circulating cells, particularly haematopoietic cells to the bone marrow and coordinate with metastatic cells to target them to skeletal regions [(3)].

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 [(4-6)].

Although VEGFs' main effects are on endothelial cells, they also bind to VEGF receptors expressed on monocytes, neurons, chondrocytes and osteoblasts [(7-9)].

Recent studies have shown that the combination of angiogenic and osteogenic factors can stimulate bone healing and regeneration. [(10)(11)]Therefore, the ability to deliver a combined delivery system of growth factors at different rate kinetics locally from biodegradable scaffolds could enhance the reparative mechanism of critical sized bone defects; thus, mimicking the in vivo bone repair conditions.

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 [(12)].Therefore the present study was designed to evaluate the local application of exogenous VEGF on bone healing.

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MATERIALS AND METHODS

All experimental procedures were carried out in accordance with the ethical principles of animal experimentation. In this research, eighteen male Albino rats, weighing (300-400) gram, aged (6-8) months were used and maintained under control conditions of temperature, drinking and food consumption.

Materials

- VEGF165A protein (Rat) (ab51967) lyophilised form from Abcam Company.
- Polyclonal Antibodies of Vascular Endothelial Growth Factor Antibody (VEGF) from Abcam company UK (ab46154).

Methods

The animals were subjected for a surgical operation. The surgery was performed under a sterilized condition and gentle technique. Every animal was weighted to calculate the dose of general anesthesia that was given to it. The general anesthesia was induced by Intra muscular injection of xylazine 2% (0.4 mg/kg B.W.), plus ketamine HCL 50mg (40 mg/kg- B.W.) also an antibiotic cover with oxytetracycline 20% (0.7 ml/kg) intramuscular injection was given. Both tibiae were shaved and the skin was cleaned with a mixture of ethanol and iodine then a piece of cotton damped with alcohol. Incision was made on the lateral side to expose the medial side of the tibia, the skin and fascia flap was reflected. By instrument drilling, and continuous cooling with irrigated saline, a hole of 1.8mm was made with small round bur at a rotary speed of 1500 rpm. Following the hole preparation, the operation site was washed with saline solution to remove debris from the drilling site. Bone defect was made on both medial sides of right and left tibia bone, as experimental and control sites, respectively.

After operation, drying the area by air, then applied1µm VEGF in experimental site, while normal saline 1 µm was used for control site. Suturing of the muscles was done with absorbable catgut followed by skin suture .The operation site was sprayed with local antibiotic (tetracycline spray). The animals were scarified at intervals 3,7,10 days, using over dose anesthesia. Six animals for each period, in each group. Bone was removed, and the tibia bone was dissected and fixed in 10% buffered formalin. Histological (H&E) and immunohistochemical evaluation was performed under light microscope.

Assessment of Immunohistochemistry results

Positive reading was indicated when the cells display a brown cytoplasmic stain, while negative reading was indicated for absence of immune-reactions depends on positive and negative control.

Immunohistochemical scoring for positive cells expressed VEGF.

Quantification method of Immuno-reactivity was semi-quantitatively estimated the immune-staining score that was calculated as the sum of a proportion score and an intensity score. The proportion score reflects the estimated fraction of positively stained infiltrating cells (score 0, none; score 1, <10%; score 2, 10-50%; score 3, 51-80%; score 4, >80%) [13].

Statistical analysis

For bone cell count and for stromal cells that expressed VEGF was estimated by Mean, S.D, Min., Max., F-test, P-value.

RESULTS

A-Histological findings

1-For control group

At 3 day duration, the group shows bone marrow infiltrated by inflammatory cells with newly formed blood vessels; figure 1. Bone healing site for Control 7 day duration shows fibrous tissue, with progenitor cell and fibroblast cell; figure 2.At 10 day, trabeculated bone formed and coalesce with cutting bone. Osteoblast, osteocyte and reticular cells were showed figure 3.

2- For experimental group; Bone treated with VEGF

Osteoid tissue filled the bone defect with newly formed blood vessels was illustrated at 3 day ; figures(4&5). Trabeculated bone ,coalesce with cutting bone in VEGF group 7 days with highly vascular ostoid tissue;figure6. At 10 days new bone with multiple haverssian canal in nearby cutting bone; figure 7.

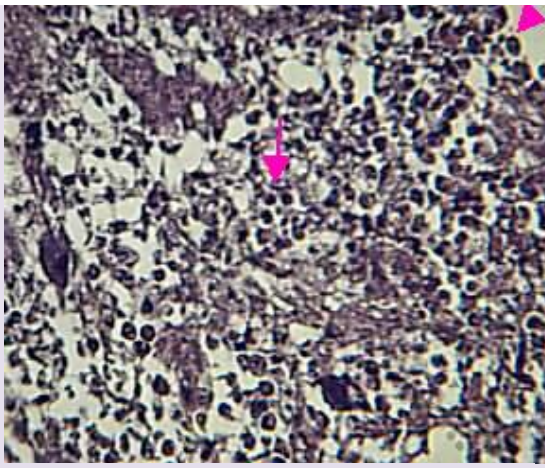

Figure 1: Microphotograph view for control group (3 days) shows progenitor cell (arrow head), inflammatory cell (arrow). H&EX20

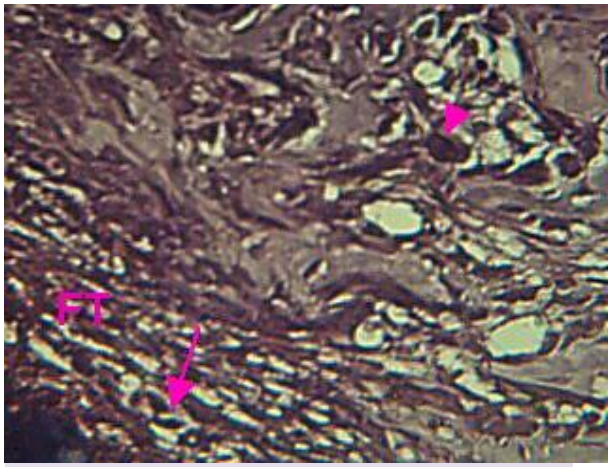

Figure 2: View for bone healing site (control7 days) shows fibrous tissue (FT) with progenitor cell (arrow head), fibroblast (arrow). H&EX20

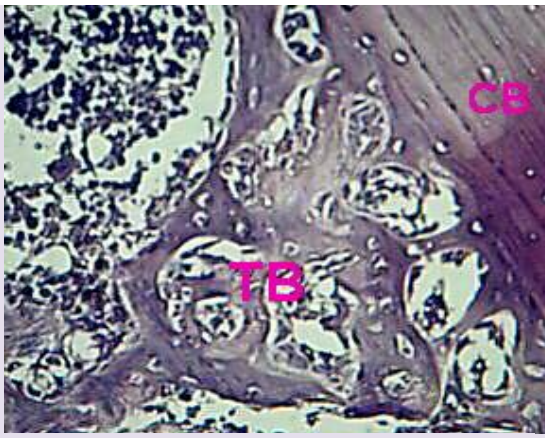

Figure 3: View for trabeculated bone (TB) coalese with cutting bone (CB) in control group 10 days. H&EX20

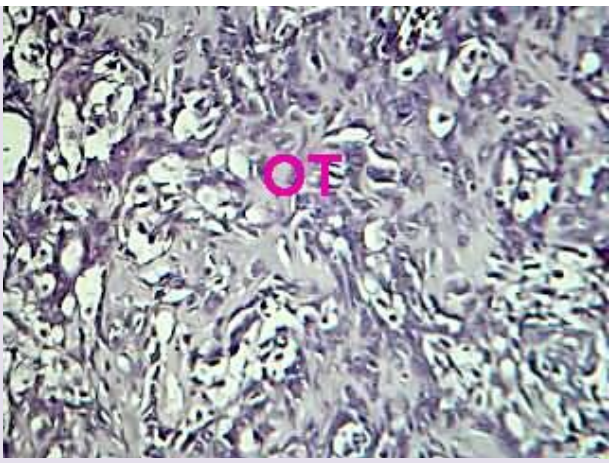

Figure 4: View for osteoid tissue (OT) filled the bone defect in VEGF group at 3 days. H&EX20

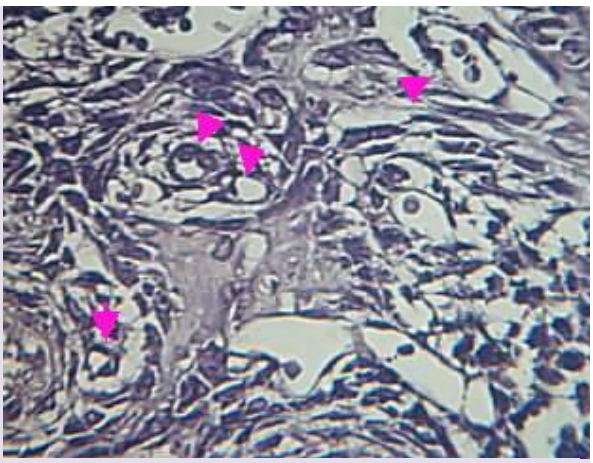

Figure 5: New blood vessels (arrow heads) in developing osteoid tissue. H&EX40

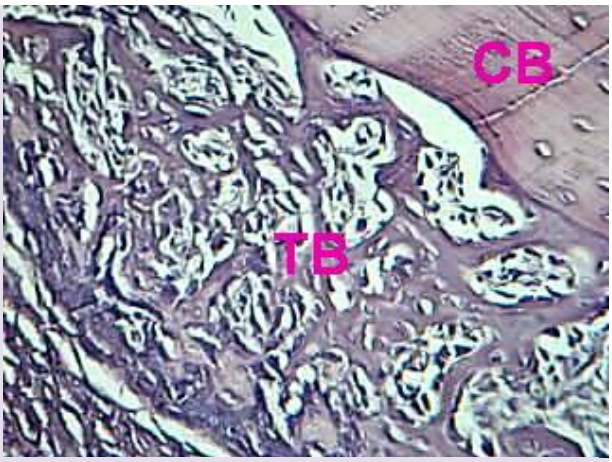

Figure 6: Trabeculated bone (TB), Coalesce with cutting bone (CB) in VEGF group 7 days. H&EX20

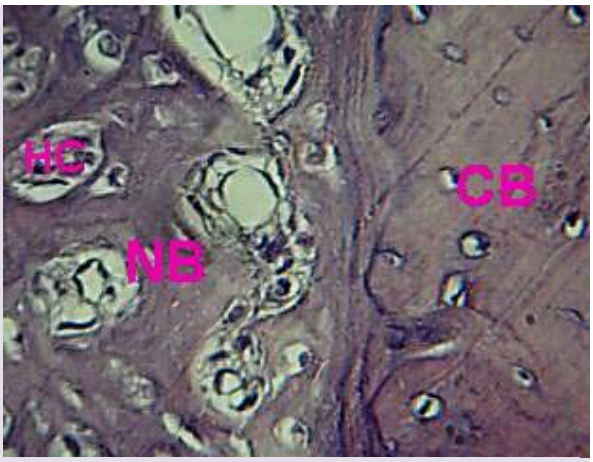

Figure 7: New bone (NB) with multiple haversian canal (HC), in nearly cutting bone (CB) in VEGF group 10 days. H&EX20

B-Immunohistochemical findings

1-Expression of VEGF in control group

Immunohistochemical view for control group at 3 and 7 days period a positive expression of VEGF by progenitor cell and endothelial cell were detected in figures 8 & 9.At 10 day duration, the fibroblast cell and active osteocyte cell show positive VEGF expression, figure10.

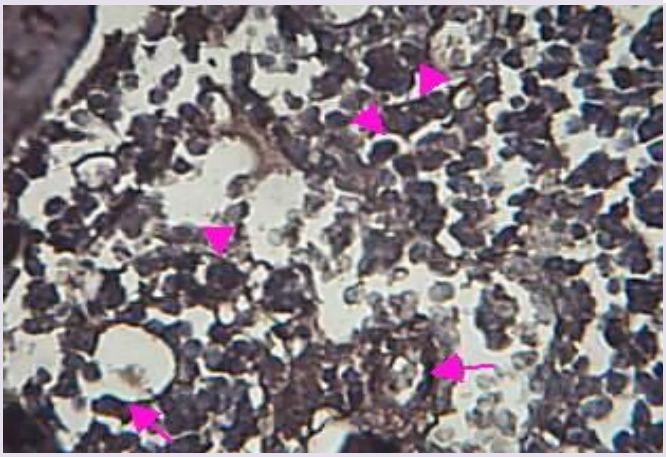

Figure 8: Immunohistochemical view for control group 3 days shows positive expression of VEGF by progenitor cell (arrow heads), endothelial cell (arrows). DAB stainX40

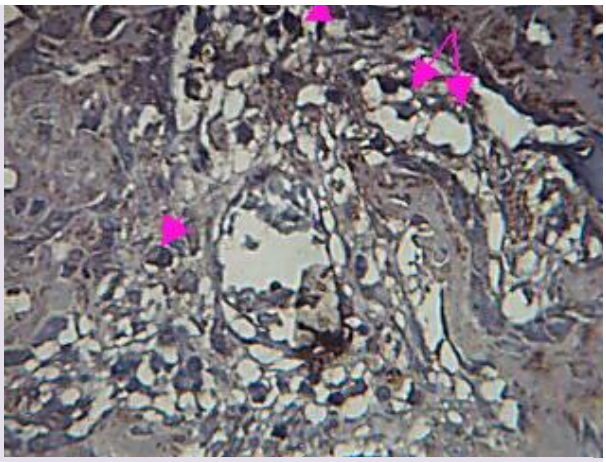

Figure 9: Progenitor cells (arrow heads) and endothelial cell (arrows) show positive VEGF expression in control group 7 days. DAB stainX20

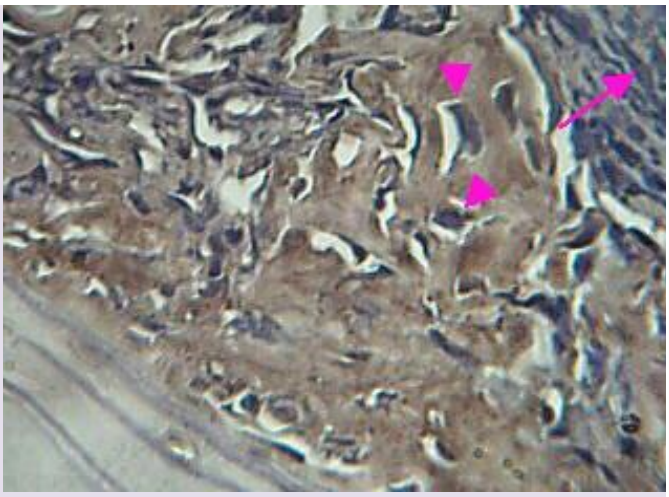

Figure 10: Fibroblast (arrow), active osteocyte (arrow heads) show positive VEGF expression in control group 10 days. DAB stain X40

2- Expression of VEGF in VEGF treated group

View for VEGF group 3 days shows positive VEGF expression by progenitor cells and fibroblast ,figure11.Osteoblast ,active osteocyte and osteoclast show positive VEGF expression at 7 and 10 day periods, figures 12,13,14.

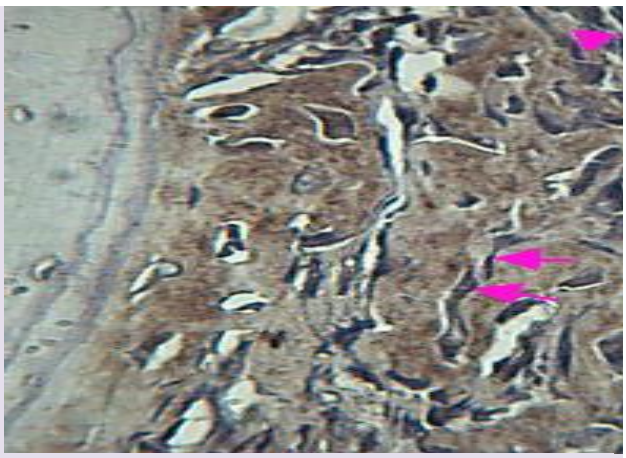

Figure 11: Immunohistochemical view for VEGF group 3 days shows positive VEGF expression by progenitor cells (arrow heads) and fibroblast (arrows). DAB stainX40

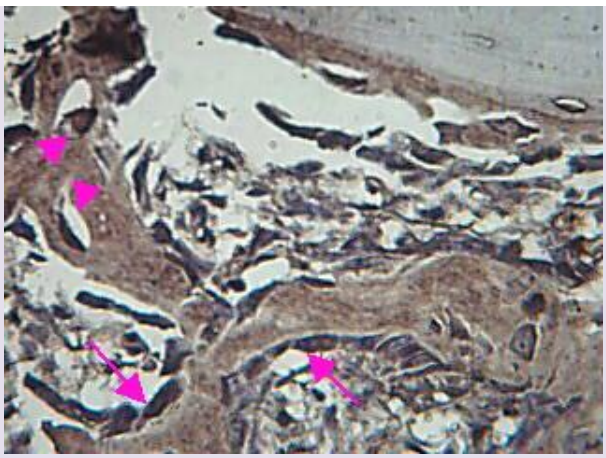

Figure 12: Osteoblast (arrows) and osteocyte (arrow heads) show positive VEGF expression in VEGF group 7 days. DAB stainX40

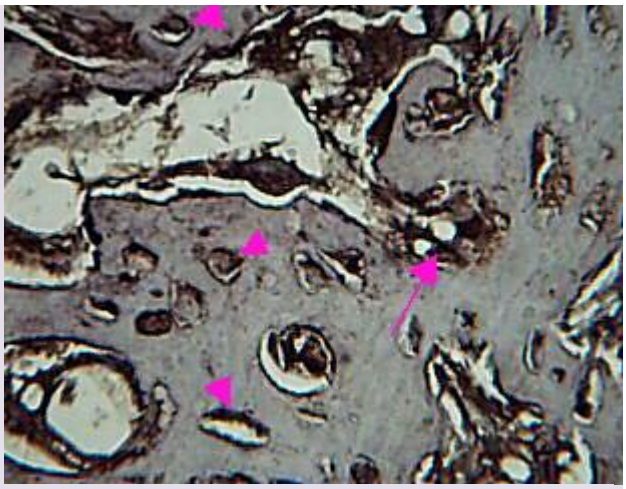

Figure 13: Osteoblast (arrow), osteocyte (arrow heads) show positive VEGF expression in VEGF group at 10 days. DAB stainX40

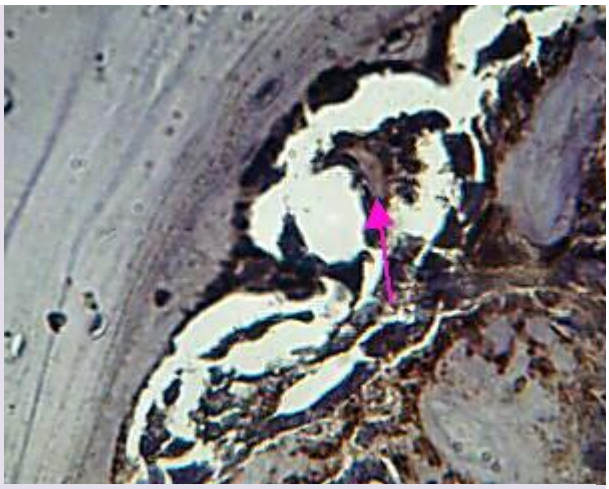

Figure 14: Osteoclast cell (arrow) show positive VEGF expression. DAB stainX40

C. Statistical analysis

Statistic analysis for the mean of count of bone cells includes osteoblast, osteocyte and osteoclast in the periods 7 and 10 days, shows that VEGF group illustrates a high mean value with highly significant differences in comparison with control group, table (1).

For the positive expression of VEGF by stromal cells in study groups, statistic analysis demonstrated a high significant value for VEGF group in comparison to control and in periods (3, 7 and 10 days) as shown in table 2.

Table 1: Descriptive statistics of the bone cells count (H&E) and groups' difference in each duration

Bone cells	Duration	Groups	Descriptive Statistics				Groups' difference	
			Mean	S.D.	Min.	Max.	F-test	p-value
Osteoblast	7 days	Control	8.75	0.13	8.6	8.9	3140.64	0.000 (HS)
		VEGF	17.23	0.17	17	17.4		
	10 days	Control	6.83	0.10	6.7	6.9	25.25	0.000 (HS)
		VEGF	6.85	0.06	6.8	6.9		
Osteocyte	7 days	Control	10.80	0.08	10.7	10.9	3108.89	0.000 (HS)
		VEGF	14.25	0.13	14.1	14.4		
	10 days	Control	8.68	0.10	8.6	8.8	6762.42	0.000 (HS)
		VEGF	13.13	0.10	13	13.2		
Osteoclast	7 days	Control	1.75	0.96	1	3	9.02	0.002 (HS)
		VEGF	1.88	0.10	1.8	2		
	10 days	Control	0.70	0.36	0.3	1	8.44	0.003 (HS)
		VEGF	0.19	0.08	0.1	0.3		

Table 2: Descriptive statistics and duration difference of the positive stromal cells expressed by VEGF marker

Groups	Duration	Descriptive Statistics				Duration difference	
		Mean	S.D.	Min.	Max.	F-test	p-value
Control	3 days	20.43	0.10	20.3	20.5	69771.89	0.000 (HS)
	7 days	51.63	0.10	51.5	51.7		
	10 days	80.45	0.37	80.1	80.9		
VEGF	3 days	64.43	0.38	64.1	64.8	8046.75	0.000 (HS)
	7 days	64.08	0.10	64	64.2		
	10 days	85.33	0.26	85.1	85.7		

DISCUSSION

The present study used exogenous VEGF in defect bone related to its ability to couple angiogenesis with bone formation and remodeling. In addition, VEGF may act as a central mediator for other factors in promoting bone healing

The present study shows an early osteoid deposition in VEGF group at 3 day period is related to osteoid tissue formation in bone defect site that includes, stem cells differentiate into osteoblasts that enhanced by vascular endothelial growth factor (VEGF) which has been implicated in angiogenesis . As a result, bone deposits via intramembranous ossification 3–7 days after injury period, primarily as osteoid, a non-mineralized bone. Goad et al. [14] Illustrated that angiogenesis is essential to both normal and pathological bone physiology.

At 7 day period VEGF group shows bone trabeculae filled a proximately the whole defect in comparison to histologic view for control. This result can be attributed that VEGF directly promotes the differentiation of primary osteoblast. [15,16] The present statistics analysis for the mean value of osteoblast, active osteocyte and osteoclast in VEGF group were recorded to be a highly significant differences in comparisum to other groups .These findings may be discuss as followings:

1. Mechanism of effect of exogenous VEGF in bone healing
   During bone formation and fracture healing there is a cross-talk between endothelial cells and osteoblasts. Previous study showed that vascular endothelial growth factor A (VEGF-A) might be an important factor in this cross-talk, as osteoblast-like cells produce this angiogenic factor in a differentiation-dependent manner. Moreover, exogenously added VEGF-A enhances osteoblast differentiation. [17]
2. VEGF also acts to recruit and activate osteoclasts as well as stimulate osteoblast chemotaxis, differentiation, and matrix mineralization. Our results implicate VEGF in intramembranous ossification.
3. It has suggests that exogenous VEGF play an important role in the regulation of bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation.
4. Formation of new capillaries, a critical component of tissue growth and repair, is a recognized process in the development, formation, and remodeling of bone. Vascular endothelial growth factor (VEGF), a potent angiogenic factor with specific mitogenic actions on endothelial cells, is produced in a regulated manner by many cell types, including osteoblasts [18]. The present result shows positive expression of VEGF by bone marrow stromal cells, adipocytes, mesenchymal stem cells, precursor endothelial cells, and bone cells include osteoblasts and active osteocytes in different periods in all groups but in different score. Therefore, our primarily data provide evidence that VEGF activity is essential for appropriate bone formation and mineralization in response to injury.

Carano and Filvaroff [19] reported that the intimate connection, both physical and biochemical, between blood vessels and bone cells has long been recognized. Genetic, biochemical, and pharmacological studies have identified and characterized factors involved in the conversation between endothelial cells (EC) and osteoblasts (OB) during both bone formation and repair.

REFERENCES

1. Colnot C, Romero DM, Huang S, Helms JA. Mechanisms of action of demineralized bone matrix in the repair of cortical bone defects. Clin Orthop Relat Res 2005; 435: 69-78.
2. Schmid J, Wallkamm B, Hammerle CH, Gogolewski S, Lang NP. The significance of angiogenesis in guided bone regeneration. A case report of a rabbit experiment. Clin Oral Implants Res 1997; 8: 244-8.
3. Brandi ML, Collin-Osdoby P. Vascular biology and the skeleton. J Bone Miner Res 2006; 21: 183-92.
4. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc Res 2005; 65: 550-63.
5. Ferrara N, Gerber HP. The role of vascular endothelial growth factor in angiogenesis. Acta Haematol 2001; 106: 148-56.
6. Rossant J, Howard L Signaling pathways in vascular development. Annu Rev Cell Dev Biol 2002; 18: 541-73.
7. 7-Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003; 9: 669-76.
8. Storkebaum E, Carmeliet P VEGF: a critical player in neurodegeneration. J Clin Invest 2004; 113: 14-18.
9. Bluteau G, Julien M, Magne D, Mallein-Gerin F, Weiss P, Daculsi G, Guicheux J VEGF and VEGF receptors are differentially expressed in chondrocytes.Bone. 2007; 40: 568-76.
10. Geiger F, Bertram H, Berger I, Lorenz H, Wall O, Eckhardt C, Simank HG, Richter W Vascular ndothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. J Bone Miner Res 2005; 20: 2028-35.
11. Peng H, Usas A, Olshanski A, Ho AM, Gearhart B, Cooper GM, Huard J VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone

healing through modulation of angiogenesis. J Bone Miner Res. 2005; 20: 2017-27.
12. Cui Q, Dighe AS, Irvine JN Jr.Combined angiogenic and osteogenic factor delivery for bone regenerative engineering. Curr Pharm Des 2013; 19(19): 3374-83.
13. Suzuki S, Dobashi Y, Hatakeyama Y, Tajiri R, Fujimura T, Heldin CH, Ooi A. Clinicopathological significance of platelet-derived growth factor (PDGF)-B and vascular endothelial growth factor-A expression, PDGF receptor-β phosphorylation, and microvessel density in gastric cancer. BMC Cancer. 2010; 30(10): 659.
14. Goad DL, Rubin J, Wang H, Tashjian AH Jr, Patterson C.Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinol 1996;137(6): 2262-8.
15. Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE, Löwik CW.Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinol 2002; 143(4): 1545-53.
16. 16-Saadeh PB, Mehrara BJ, Steinbrech DS, Dudziak ME, Greenwald JA, Luchs JS, Spector JA, Ueno H, Gittes GK, Longaker MT.Transforming growth factor-beta1 modulates the expression of vascular endothelial growth factor by osteoblasts. Am J Physiol 1999; 277(4 Pt1): C628-37.
17. 17-Mandracchia VJ, Nelson SC, Barp EA. Current concepts of bone healing. Clin Podiatr Med Surg 2001; 18(1): 55–77.
18. Athanasopoulos AN, Schneider D, Keiper T, Alt V, Pendurthi UR, Liegibel UM, Sommer U, et al. Vascular Endothelial Growth Factor (VEGF)-induced Up-regulation of CCN1 in Osteoblasts Mediates Proangiogenic Activities in Endothelial Cells and Promotes Fracture Healing. J Biol Chem 2007; 282(37): 26746–53.
19. Carano RA, Filvaroff EH. Angiogenesis and bone repair. Drug Discov Today 2003: 8: 980-980.