

## **EXPRESSION OF VEGF AND BMP7 IN BONE HEALING AFTER TOPICAL, SYSTEMIC FLUORIDE APPLICATION (EXPERIMENTAL STUDY IN RATS)**

**Athraa Y. Alhijazi B.D.S., MSc., Ph.D.<sup>(1)</sup> Ahmed Dheyaa Neamah, B.D.S.<sup>(2)</sup>**

<sup>1</sup>Department of Oral Diagnosis, College of Dentistry, University of Baghdad

<sup>2</sup> Department of Oral Diagnosis, College of Dentistry, University of Babylon

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**ABSTRACT:** *Bone repair is a multistep process involving migration, proliferation, differentiation and activation of several cell types. The effect of topical and systemic application of fluoride on bone healing was studied by histological and immunohistochemical evaluation for expression of VEGF and BMP7. Seventy two Swiss rats were used in the present study. Bone defect was created in rat femur with topical application of fluoridated tooth paste and systemic fluoride through water supplement, while control group left without any application. The animals were sacrificed at periods 3, 7, 14, and 28 days. Six rats for each group and for each period. Histological and immunohistochemical evaluation for VEGF and BMP7 were carried out for all animals. The Histological findings illustrated that all study groups showed deposition of osteoid tissue at 7 days and mature bone illustrated only in paste group at 28 days. The Immunohistochemical findings of VEGF for positive expression by bone marrow stromal cells and bone tissue cells revealed statistically to be the highest in paste group and at 14 days period. While positive expression of BMP7 illustrated positivity in different percentage and the paste group recorded the highest value at 3 days period. It was concluded that fluoridated tooth paste can affect more in bone maturation and had effect on VEGF and BMP7 expression.*

**KEYWORDS:** Fluoride Tooth Paste, Bone Morphogenic Proteins, Vascular Endothelial Growth Factor, Bone Healing.

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## **INTRODUCTION**

Bone is one of the supportive tissues of the teeth, it makes the skeleton of the jaws, also the implants and the prostheses depend on it. So bone healing has a critical importance in dentistry to restore the shape and rigidity of the residual ridge after tooth extraction as well as to enhance the osseointegration with implants (Younis *et al.*, 2009).

Fracture healing can be divided into three phases. The inflammation phase is the first stage of healing. Immediately upon fracture, a blood clot forms, allowing the influx of inflammatory, clean-up cells to the wound area. This is followed by a cytokine cascade that brings the repair cells into the fracture gap. These cells immediately begin to differentiate into specialized cells that build new bone tissue (osteoblasts) and new cartilage (chondroblasts). Over the next few months, these cells begin the repair process, laying down new bone matrix and cartilage. At this initial stage,

osteoclast cells dissolve and recycle bone debris (Fajardo *et al.*, 2009 and Behr *et al.*, 2010). The second, reparative stage begins about two weeks after the fracture occurs. In this stage, proteins produced by the osteoblasts and chondroblasts begin to consolidate into what is known as a soft callus. This soft, new bone substance eventually hardens into a hard callus as the bone weaves together over a 6- to 12-week time period (Peng *et al.*, 2005).

The final step of fracture repair is known as the remodeling phase. At this stage the callus begins to mature and remodel itself. Woven bone is remodeled into stronger lamellar bone by the orchestrated action of both osteoblast bone formation cells and osteoclast bone resorption (Patel *et al.*, 2008).

Fluoride is most concentrated within tissues such as cementum, bone, dentin, and enamel. Fluoride is the ionic form of fluorine, and is instrumental in protecting bone and tooth enamel from loss of constituent minerals.

Fluoride is proposed to enhance the precipitation of hydroxyapatite crystals in solutions of calcium and phosphate (e.g., blood) and therefore tends to prevent the demineralization of bone and teeth (Poureslamiet *al.*, 2011). It has been further proposed that fluoride becomes integrated within the hydroxyapatite crystals, creating enlarge and less soluble crystals. Because these crystals are less soluble and less reactive, dissolution of the tooth structure by acidic by-products of microorganism metabolism cannot occur as readily. Bone is also less likely to demineralize due to the decreased solubility of the fluoride containing crystals (Eberhardet *al.*, 2012).

Most of fluoride supplementation contain it as a sodium fluoride structure that dissolve to free the fluoride ion. Sodium fluoride (NaF) is a colourless to white solid that is moderately soluble in water. It is used in the fluoridation of drinking water and in the manufacture of dental preparations such as toothpaste and fluoridated gel (White *et al.*, 2012).

Many studies based on the use of systemic fluoride in fracture healing to speed the events and strengthen the bone. In this research, dental fluoride tooth paste is used in bone healing of rat femur defect.

## MATERIALS AND METHODS

All experimental procedures were carried out in accordance with the ethical principles of animal experimentation. In this experimental study seventy two male Swiss rats, weighting (0.25 – 0.30 kg), aged 4-5 months were used and maintained under control conditions of temperature, drinking and food consumption. In each rat 2mm bone defects were created in the femur. The study groups include:

- Control group contains (24) rats .
- Paste group contains (24) rats, the bone defect treated with tooth paste containing Fluoride (*LACALUT fluor, 1.476 mg/ml, Germany*).
- Systemic group contains (24) rats, Received systemic supplements of fluoride with water (*Zymafluor (Sodium Fluoride 0.25 mg, Novartis Switzerland)*).

Every single group will composed of 24 rats that will be studied in four periods 3, 7, 14, 28 days (6 rats for each period).

### Application of Topical Fluoride:

In sterilized conditions, the surgical operation was carried on, then drilling the rat femur by 2 mm surgical bur, and application of fluoridated tooth paste. The secondary infection was prevented with antibiotic cover after the operation.

### 2.2 Application of Systemic Fluoride

The fluoride tablets (*Zymafluor (Sodium Fluoride, Novartis Switzerland)*) diluted in the water of the animal in a dose of 0.0625mg daily. That treatment given to the experimental systemic Group from one week before the operation and continues during the period of treatment. The surgical operation was done the same as topical fluoride application but there is no materials added to the defect and left to heal normally.

### Samples preparation:

The samples were collected and delivered to the histological laboratory, and then the histological preparation was done for Hematoxylin and eosin stain as well as for the Immunohistochemical study.

### Immunohistochemical Polyclonal Antibodies

- I. Vascular Endothelial Growth Factor Antibody (VEGF) from Abcam Company UK (ab46154).
- II. Bone Morphogenic Protein7Antibody (BMP7) from Abcam Company UK (ab56023).

### Histological Scoring

The histological examination was done by examining four microscopical fields for each slide for counting the number of the osteocytes, osteoblasts, and osteoclasts. The mean of readings of the 4 microscopical fields was calculated and used in statistical analysis (Xue et al., 2006).

The degree of inflammation for each biopsy site was scored as follows: 0, absent; 1, mild; 2, moderate; or 3, severe (Roopali et al., 2007).

### Immunohistochemical scoring of VEGF, BMP7

Quantification method of Immuno-reactivity was semiquantitatively estimated the immune-staining score that was calculated as the sum of a proportion score. The proportion score reflects the estimated fraction of positively stained infiltrating cells. For VEGF and BMP7, it was assessed by identifying and scoring 100 cells in five fields (X40) along bone defect area of different sections according to Suzuki *et al.*, 2010, the scoring is: (Score 0, none; score 1, <10%; score 2, 10-50%; score 3, 51-80%; score 4, >80%).

## RESULTS

### For H &E results for all groups :

- At 3 day period: it shows inflammatory cells infiltration (figure (1)).

- At 7 day period: it shows the deposition of osteoid tissue (figure (2)).
- At 14 day period: it shows the formation of bone trabeculae (figure (3)).
- At 28 day period: it shows immature bone in all groups but the paste group shows the formation of mature bone (figure (4)).

*For Immunohistochemical results of VEGF and BMP7 for all groups:*

- At 3 day period: positive expression of VEGF and BMP7 by inflammatory cells and bone marrow stromal cells in all groups with strong intense identification of VEGF and BMP7 markers in paste group (figure (5,6)).
- At 7,14 and 28 day periods: positivity for expression of VEGF and BMP7 illustrated by osteoblasts, osteoclasts and bone marrow stromal cells ,paste group records a strong expression,while systemic group shows negative expression by stromal cell at 28 day period (figure (7,8,9,10)).

#### Statistic analysis

*Statistic analysis* shows that multiple comparisum by (LSD) for the mean of bone cell count, records to be high in paste group with highly significant value(table 1).Table(2) and figure (11) show that high mean value for inflammatory score 1 is related to paste group and for all periods.

The multiple comparisons by (LSD) method among all pairs of positive VEGF stromal cells and bone cells, show a highly significant difference(table 3;figure 12).

Table (4)andfigure(13)illustrated positive BMP7 by stromal cells and bone cells,that show a highly significant difference with mean value for paste group.

## DISCUSSION

#### *Results of H&E examination:*

The present result illustrates an inflammatory reaction for all groups at 3 day period, as bone defect is an injury, and thus incites an inflammatory response, which peaks 24 h following the injury and it is completed by the first week (Cho *et al.*, 2002). During this time, a complex cascade of proinflammatory signals and growth factors are released in a temporally and spatially controlled manner (Gerstenfeld *et al.*, 2003). Levels of several inflammatory mediators, including interleukin-1 (IL-1), IL-6, IL-11, IL-18, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), are significantly elevated within the first few days (Sfeiret *et al.*, 2005). These signals recruit inflammatory cells and promote angiogenesis (Rundle *et al.*, 2006) Platelets are activated by injury to blood vessels at the fracture site, and release transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and platelet-derived growth factor (Kolare *et al.*, 2010).

Paste group illustrates macrophage and plasma cells, at 3 day duration, this observation is related that monocytes migration and accumulation of macrophages, lymphocytes and plasma cells are established the chronic phase, being the macrophages and their function fundamentals for the transition from inflammatory to the proliferative stage of wound healing (Freitaset *et al.*, 2009).

Persistence of inflammation with poor bone healing in some sample related to systemic group may represent an irritate source to bone tissue and as bone healing commences with an inflammatory reaction which initiates the regenerative healing process leading in the end to reconstitution of bone, but any unbalanced immune reaction during this early bone healing phase may disturb the healing cascade in a way that delays bone healing and jeopardizes the successful healing outcome (Schmidt-Bleek *et al.*, 2012).

At 7 day, all groups show osteoid tissue formation, as the periphery of the bone defect site, includes, stem cells differentiate into osteoblasts. As a result, bone deposits via intramembranous ossification 7–10 days after injury period, primarily as osteoid, a non-mineralized bone.

At 14 day period fluoridated tooth paste group showed bone trabeculae filled a proximately the whole defect in comparison to histologic views for control and systemic groups. This result can be attributed that fluoride stimulates bone formation by osteoblastic stimulation, increases bone formation earlier and to a larger extent in trabecular bone rather than compact bone (Phipps, 1995).

At 28 day period mature bone with osteon formation illustrates in paste group only, while others show immature form. This result could be explained as follows:

1. Fluoride is absorbed in the stomach and small intestine. Once in the blood stream it rapidly enters mineralized tissue (bones and developing teeth). At usual intake levels, fluoride does not accumulate in soft tissue. The predominant mineral elements in bone are crystals of calcium and phosphate, known as hydroxyapatite crystals. Fluoride's high chemical reactivity and small radius allow it to either displace the larger hydroxyl (-OH) ion in the hydroxyapatite crystal, forming fluoroapatite, or to increase crystal density by entering spaces within the hydroxyapatite crystal. Fluoroapatite hardens the tissue and stabilizes bone (Aoba *et al.*, 2003). Chen *et al.*, 2013, demonstrated that fluorine being an electro- negative element and having a negative charge (F-) is attracted by positively charged ions like calcium (Ca<sup>++</sup>). Bones and teeth having highest amount of calcium in the body, attract the maximum amount of fluorine which is deposited as calcium fluoro-apatite.
2. Paste properties include:
  - A. In physics, it is a substance that behaves as a solid until a sufficiently large load or stress is applied, at which point it flows like a fluid, give it a property of staying more time than others form.
  - B. In pharmacology, it consists of fatty base (e.g., petroleum jelly) and at least 25% solid substance (e.g., zinc oxide), (Singh, 2003).

These two properties give the paste an opportunity to stay more time in tissue than others and as the present results of histological demonstrate the presence of matrix within the tissue at 3 days period. Rajan *et al.*, 1988, reported that large amounts of fluoride are ingested from toothpaste and mouthwashes. They found that toothpaste can double the level of fluoride in the blood within five minutes of being used. From

the above mentioned project, it seems that the effects of the fluoride on the bone depends on the type of fluoride used, its rote of application and the affected periods of bone formation, whether in apposition phase of organic or inorganic constituents.

*Results of immunohistochemical findings for VEGF and BMP7*

The present result shows positive expression of VEGF by bone marrow stromal cells, adipocytes, mesenchymal stem cells, endothelial cells, and bone cells include osteoblasts and 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, 2003, 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 and osteoblasts during both bone formation and repair. Two growth factors, BMP-2 and OP-1, with angiogenic and osteogenic activity confirm the importance of these two processes in human skeletal healing. Several growth factors are expressed in distinct temporal and spatial patterns during bone repair. Of these, vascular endothelial growth factor, VEGF, is of particular interest because of its ability to induce neovascularization (angiogenesis), VEGF also acts to recruit and activate osteoclasts as well as stimulate osteoblast chemotaxis, differentiation, and matrix mineralization. Suggesting a functional role for this growth factor in bone formation and remodeling (Mayr-Wohlfart *et al.*, 2002).

The statistic evaluation of overall periods shows that paste group records the highest value in the mean of positive VEGF; the present study suggests the followings:

1. Paste fluoride initiates formation of a blood clot through biochemical activation.
2. Recruitment and differentiation of mesenchymal cells.
3. Followed by a cellular activation.
4. Finally a cellular response, releases growth factors include VEGF.

As VEGF itself has no osteo-inductive capacity, bone-forming cells were probably recruited from pericytes, circulating cytokines and cells, the blood clot and the fractured bone ends. VEGF potentiates the actions of several cytokines, and it mediates the angiogenic actions of most growth factors. It could therefore complement other cytokines such as basic fibroblast growth factor or bone morphogenetic proteins in enhancing bone healing (Kleinheinz *et al.*, 2002).

Osteoprogenitor cells at the fracture site express bone morphogenetic proteins. These factors, along with inflammatory mediators, recruit mesenchymal stem cells and then guide their differentiation and proliferation. The present result shows strong positive expression of BMP7 by bone marrow stromal cells in paste group, which related to enhancement of infiltration and proliferation of mesenchymal cells that occurs at the bone injury site, and some of these mesenchymal cells differentiate into osteoblasts (Xian *et al.*, 2004). Zhou *et al.*, 2004 study reporting an upregulated expression of some cytokines or growth factors at a various phases of bony repair.

The study has identified the localization and expression of BMP7 in infiltrated mesenchymal cells and osteoblasts during subsequent fibrogenic and osteogenic responses, suggesting its potential role in modulating these two healing responses. At

the bone bridge maturation stage, only BMP-7 expression was seen slightly upregulated and expressed in differentiated osteoblasts and bone marrow cells at the injury site. The present result for systemic group, shows negative expression of BMP7 at 28 day period, Turner, 1995, reported that fluoridated water consumption increases the incidence of fractures

## CONCLUSION

The study concludes that fluoride paste act as an osteostimulation for improving healing of bone injuries or defects through the active stimulation of osteoblast proliferation and differentiation as evidenced by osteoblast marker like bone morphogenic protein 7.

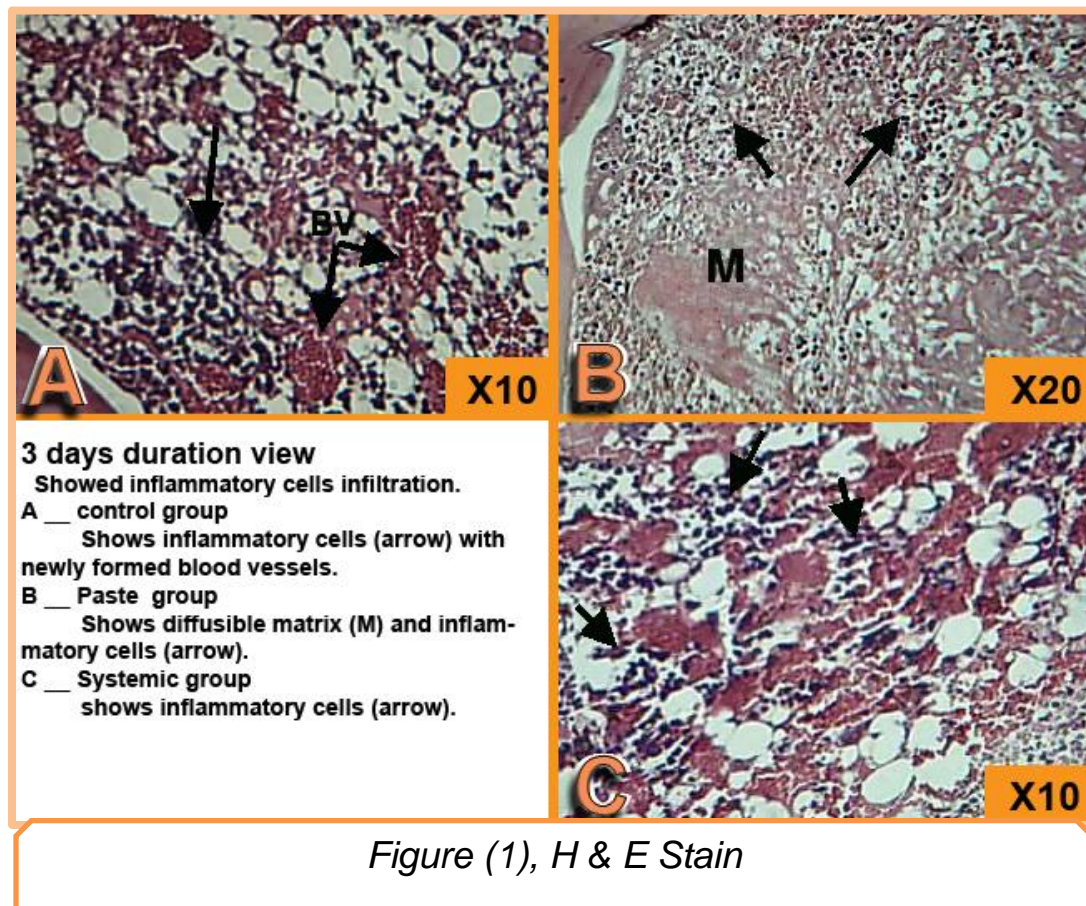
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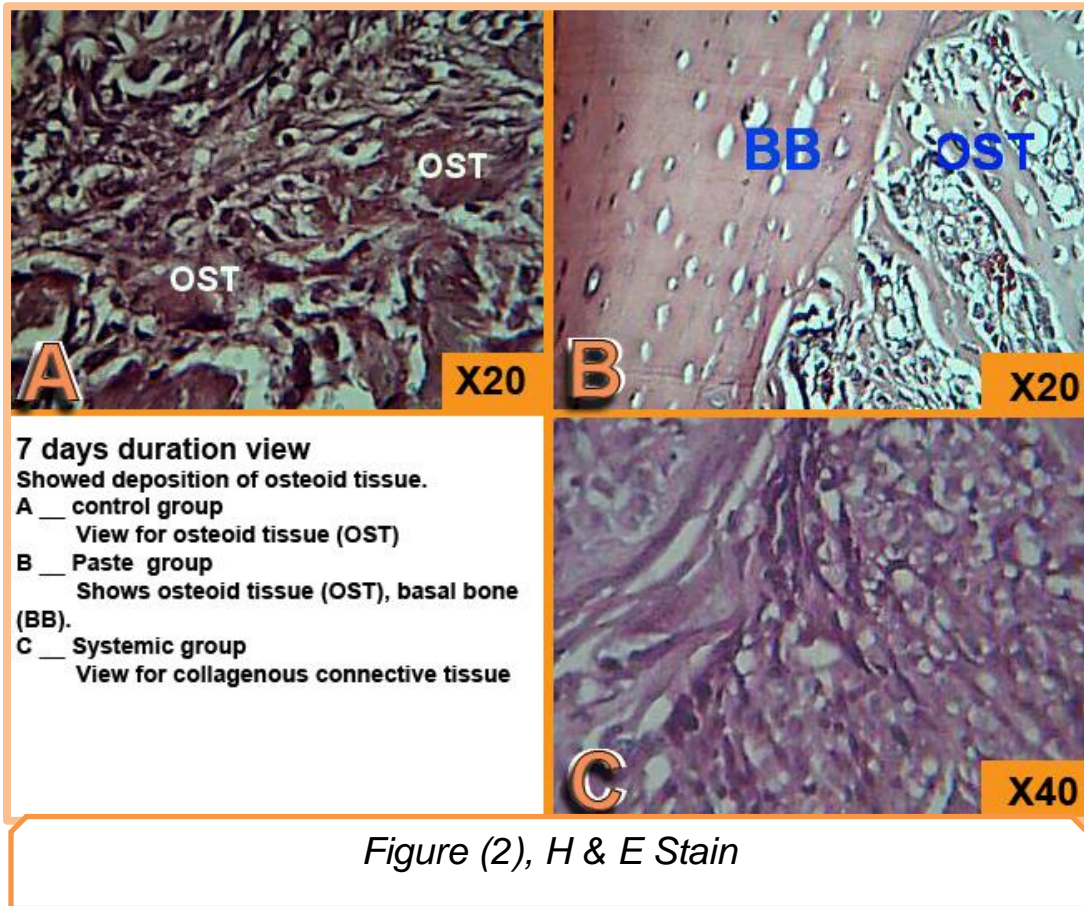
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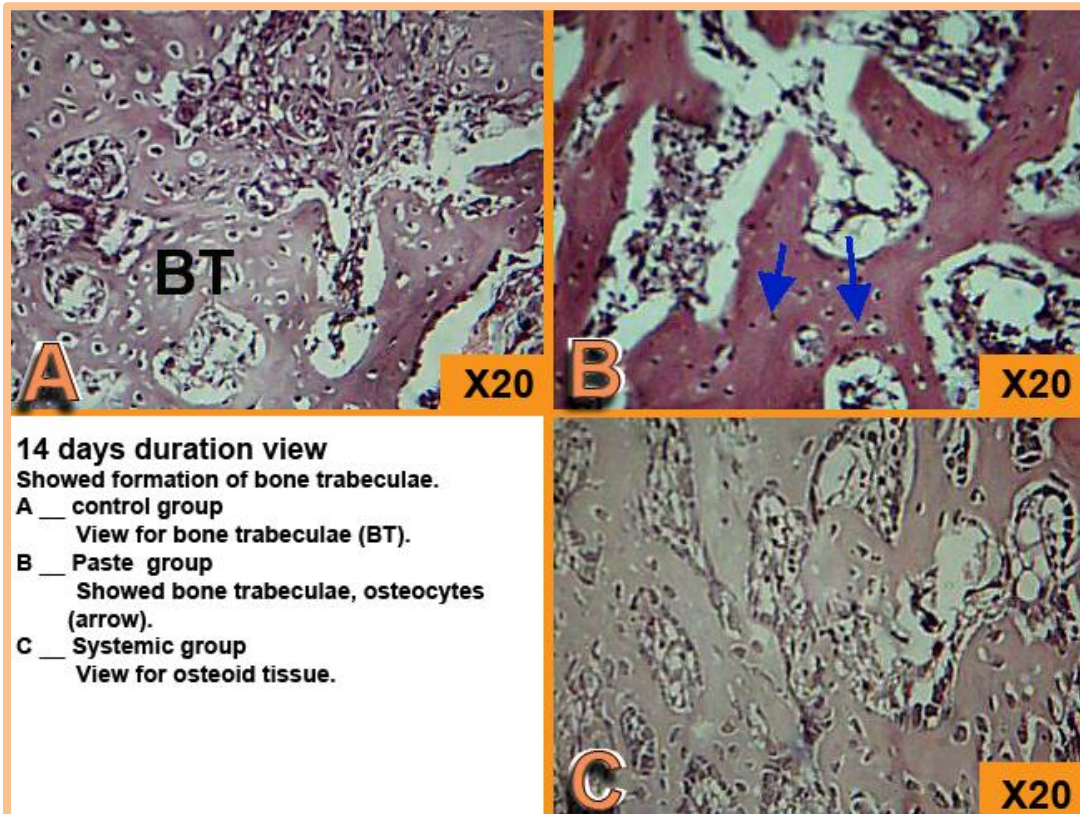


Figure (3), H & E Stain

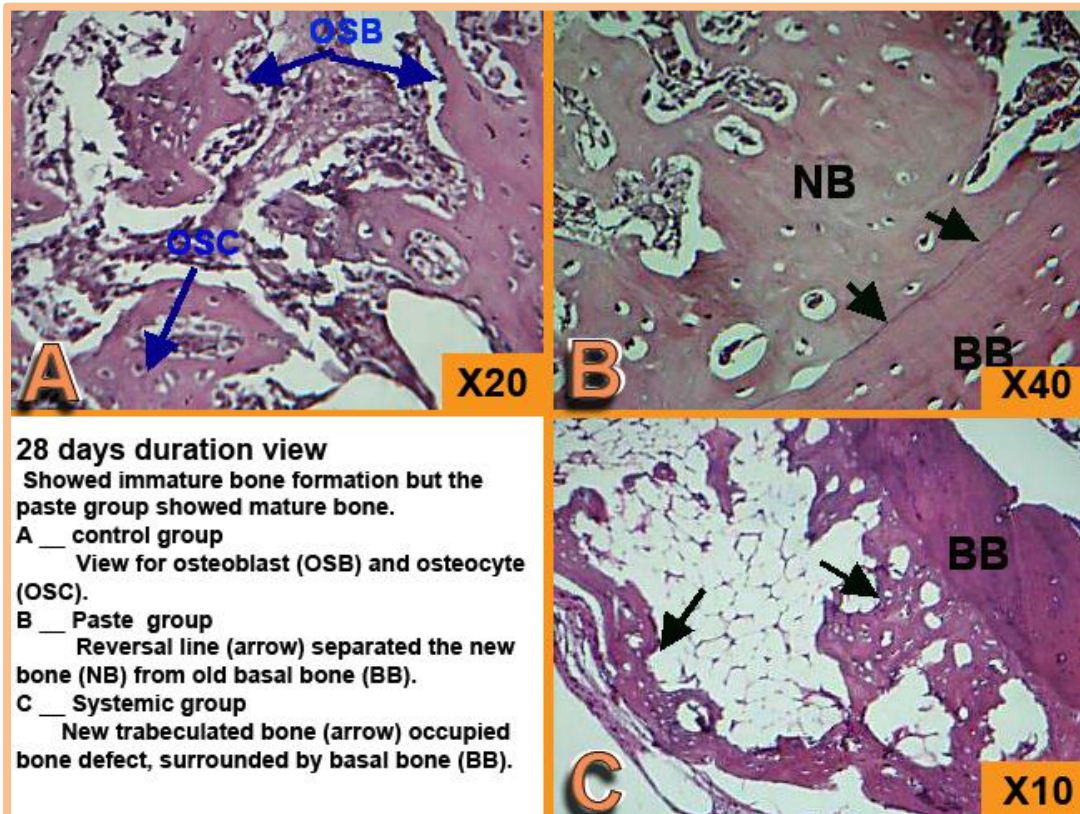


Figure (4), H & E Stain



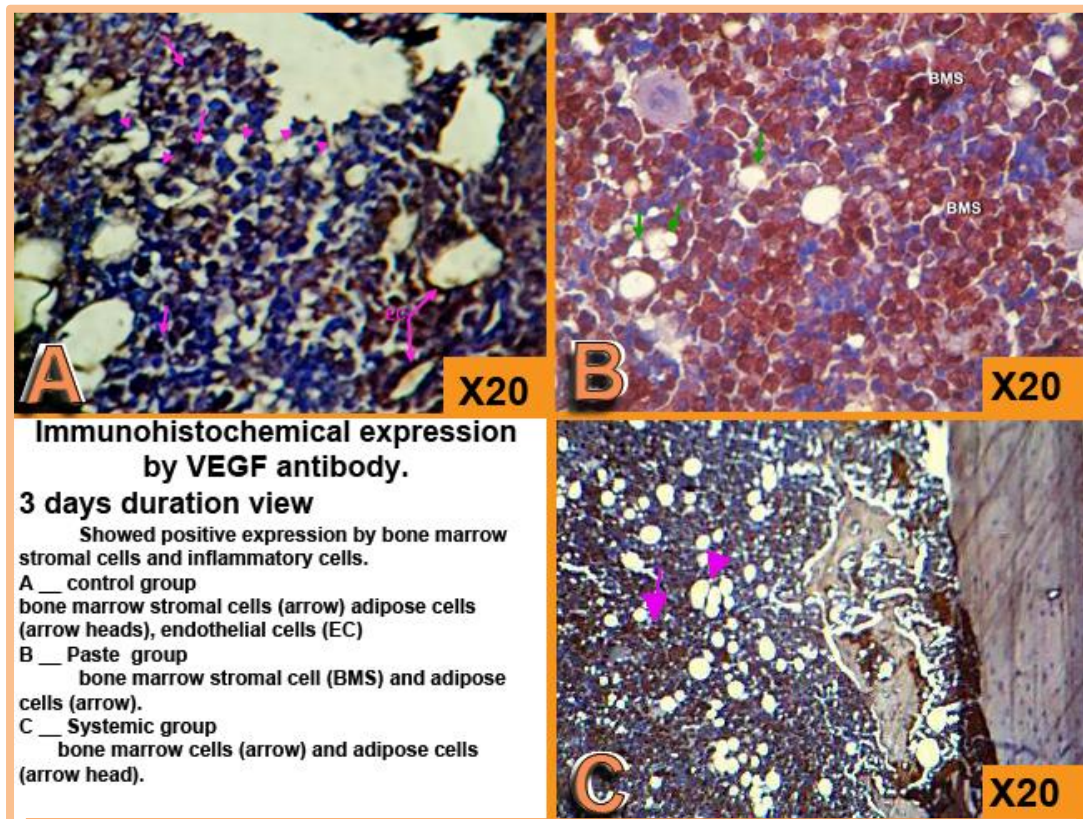


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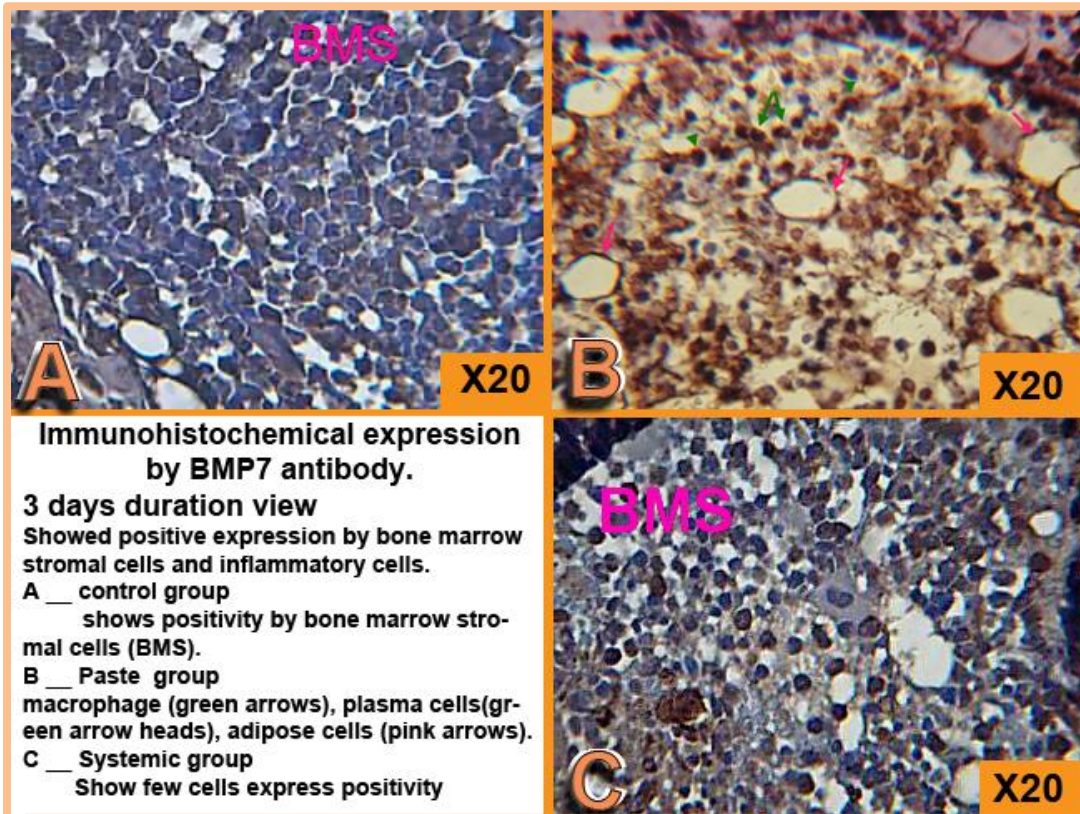


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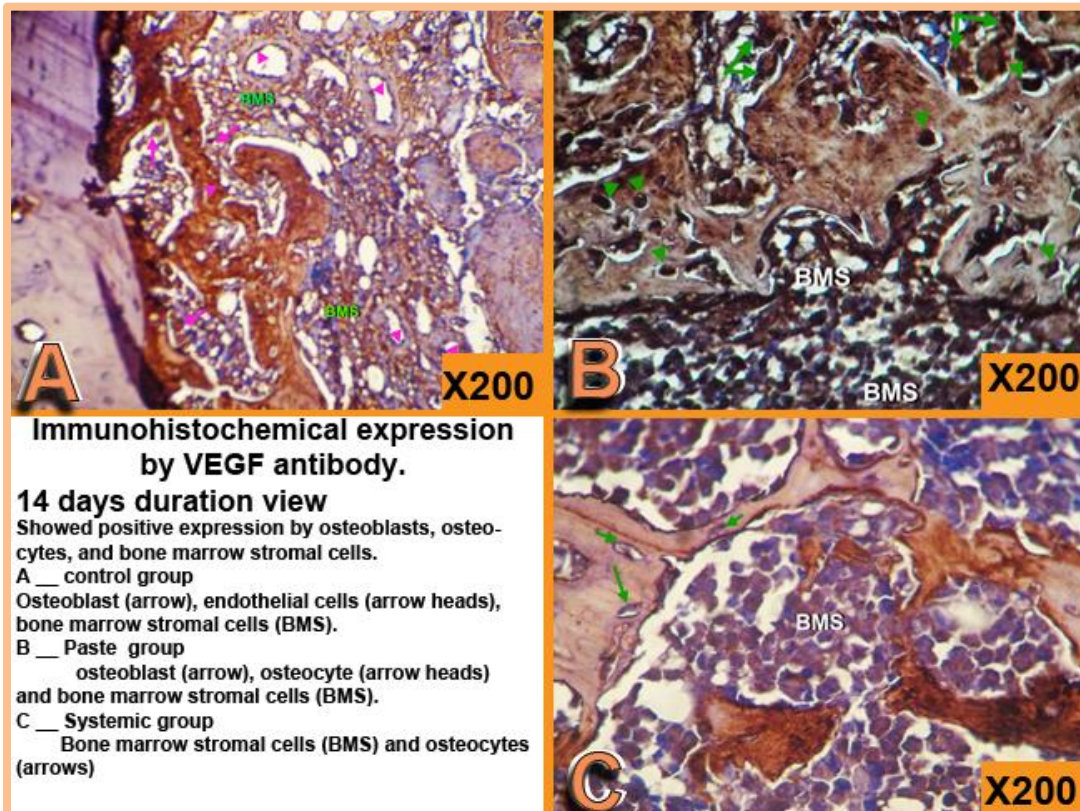


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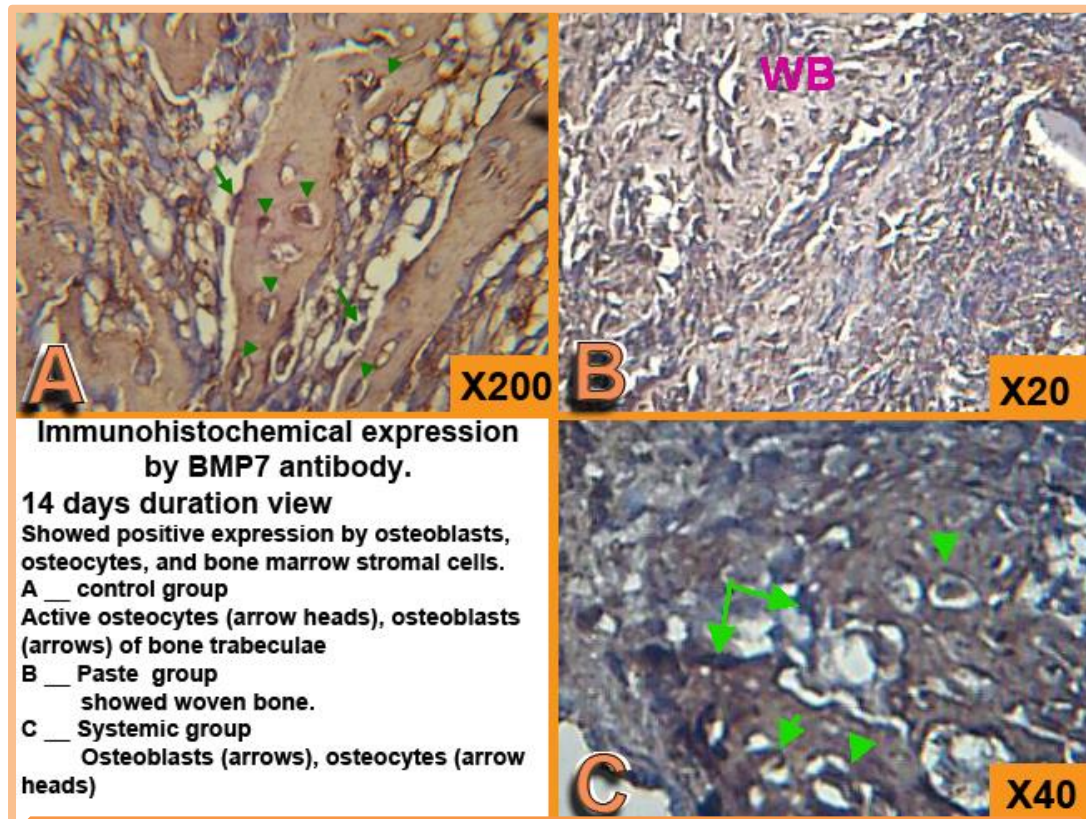


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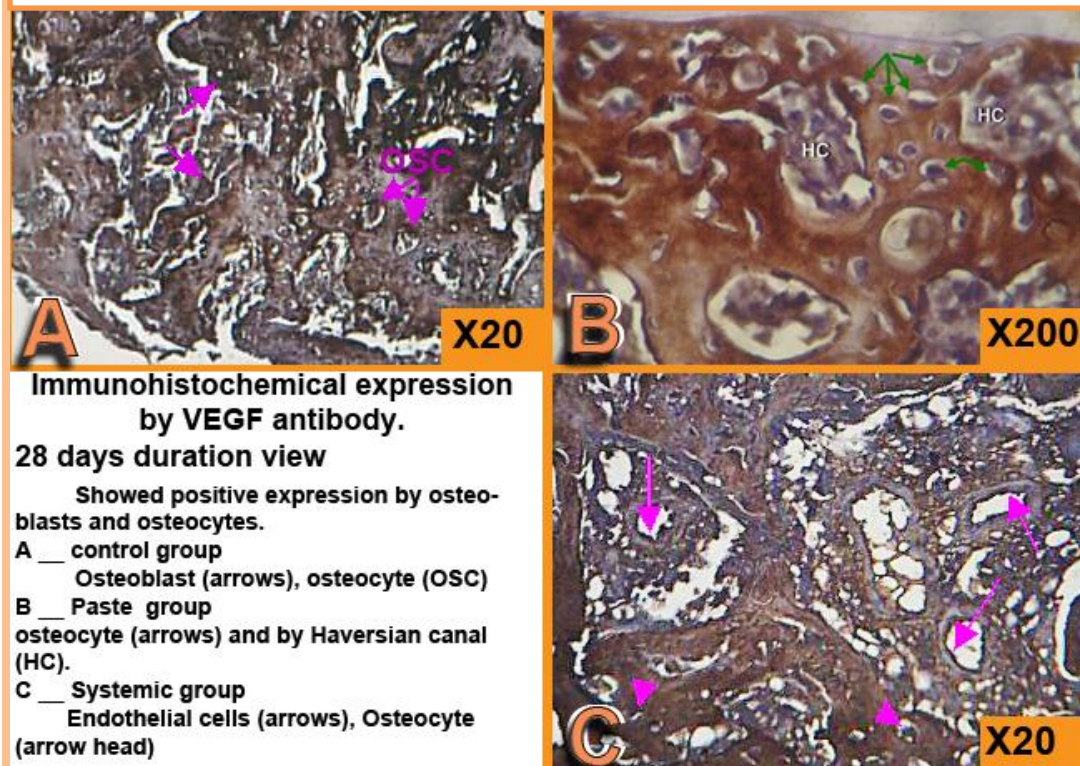


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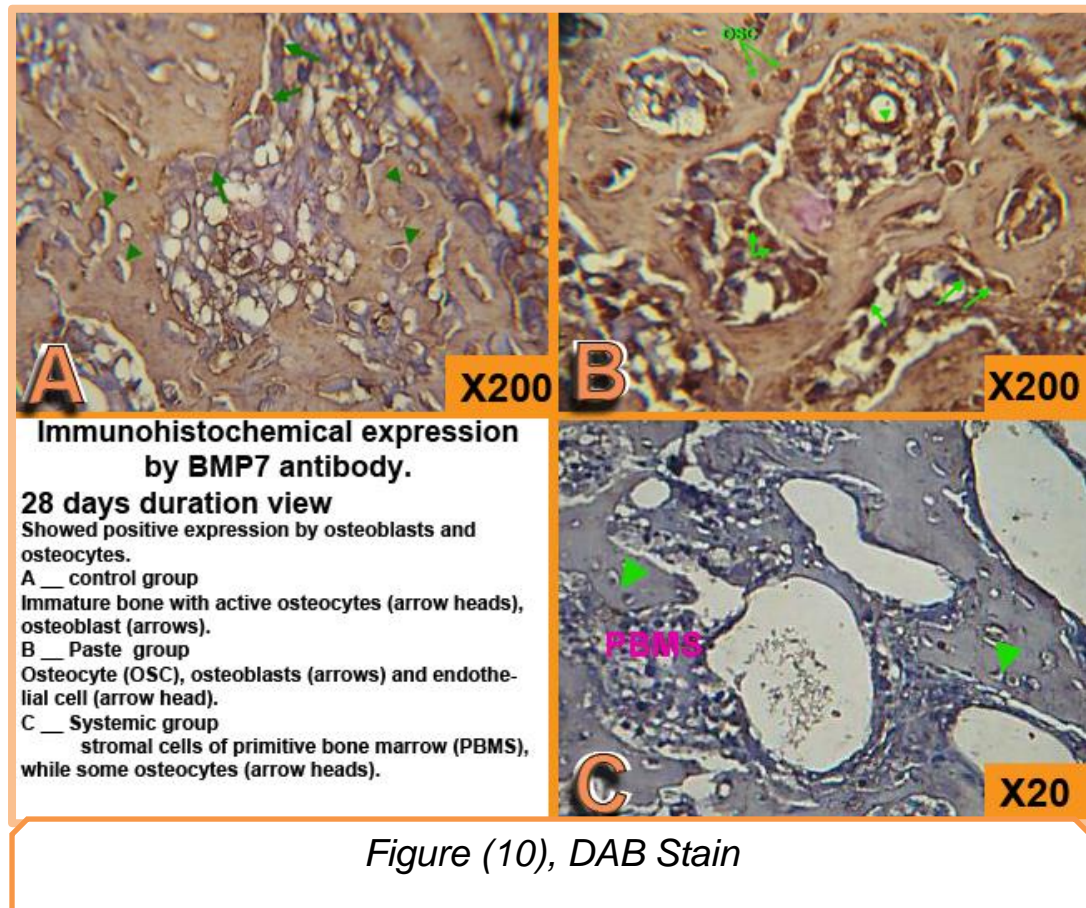


Table (1): Multiple Comparisons by (LSD Method) among all pairs of H&E Bone cells count Parameter According to different Groups and types of bone cells in compact form

Factor	LSD test				
	(I) group	(J) group	Mean Difference (I-J)	Sig. <sup>(*)</sup>	C.S.
Groups	Control	Paste	-2.97	0.000	HS
		Systemic	2.08	0.000	HS
	Paste	Systemic	5.06	0.000	HS

(\*) HS: Highly Sig. at  $P < 0.01$ ; Non Sig. at  $P > 0.05$

Table (2): Statistics for of the studied groups distributed due to (Scoring of inflammatory cells) Parameter

Score	Mean	Std. Error	95% C. I.	
			L. B.	U. B.
1	3.2	0.199	2.804	3.596
2	1.5	0.199	1.104	1.896
3	1.0	0.199	0.604	1.396

4	0.3	0.199	-	0.696
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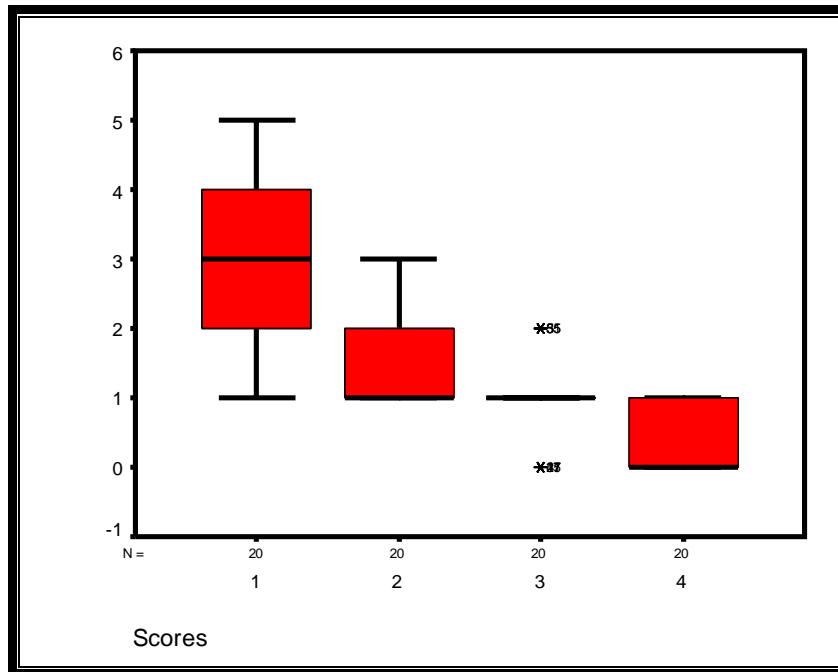


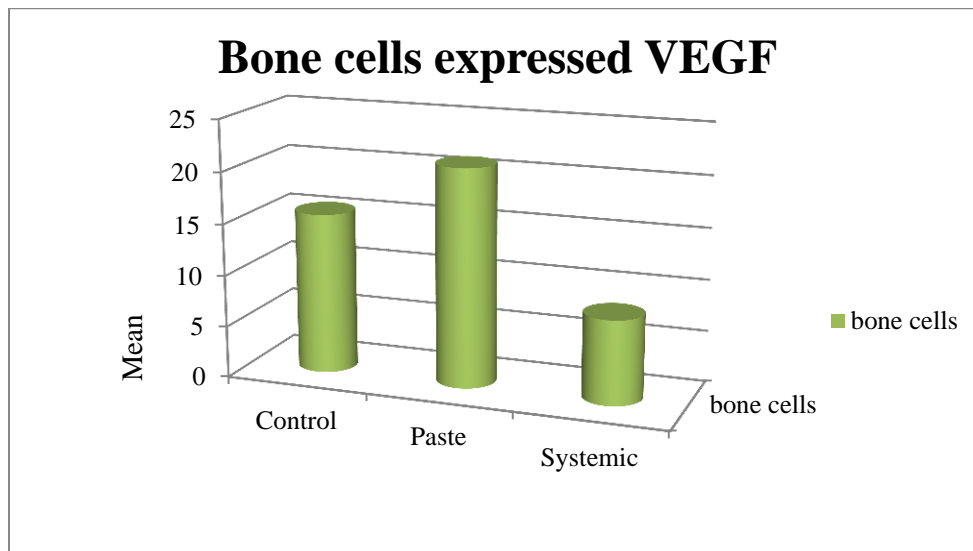
Figure (11): Stem-Leaf (Explorer) Plots for the (Scoring of inflammatory cells) Parameter in studied trials

Table (3): Multiple Comparisons by (LSD Method) among all pairs of Positive Stromal Cell expressed VEGF According to different Groups and periods of treatment in compact form

Factor	LSD test				
	(I) group	(J) group	Mean Difference (I-J)	Sig. <sup>(*)</sup>	C.S.
Group <sub>s</sub>	Control	Paste	-29.75	0.000	HS
		Systemic	1.00	0.527	NS
	Paste	Systemic	30.75	0.000	HS

<sup>(\*)</sup> HS: Highly Sig. at  $P < 0.01$ ; Sig. at  $P < 0.05$ ; Non Sig. at  $P > 0.05$

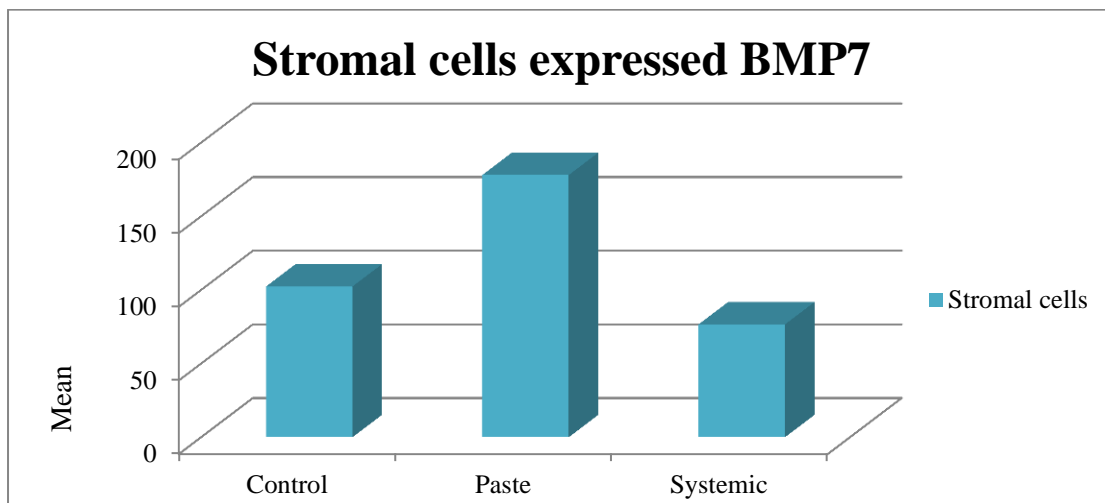




Figure(12) Mean of positive bone cell expressed VEGF in different groups

Table (4): Multiple Comparisons by (LSD Method) among all pairs of Positive Bone cells count for BMP7 Parameter According to different Groups and types of bone cells in compact form

Factor	LSD test				
	(I) group	(J) group	Mean Difference (I-J)	Sig. <sup>(*)</sup>	C.S.
Groups	Control	Paste	0.22	1.000	NS
		Systemic	2.72	0.029	HS
	Paste	Systemic	2.94	0.008	HS

(\*) HS: Highly Sig. at  $P < 0.01$ ; Non Sig. at  $P > 0.05$ 

Figure(13) Mean of positive stromal cell expressed BMP7 in different groups