

The Plasma Levels of bFGF and Microbiological Evaluation of Cell Homing Procedure of Incomplete Teeth Apex Maturation in Dogs

Ammar Samir Salim
BDS. (Master student)

Department of Dental Basic Science
College of Dentistry, University of Mosul

Ghada Younis Abdulrahman
MSc, PhD (Assist Prof.)

Department of Dental Basic Science
College of Dentistry, University of Mosul

الخلاصة

الأهداف: تهدف الدراسة لتقييم التغيرات في مستوى bFGF في الدم أثناء إجراء التوجيه الخلوي لللب الأسنان ذات القمم غير المكتملة في الكلاب ، ولتقييم بروتوكول تطهير قناة الجذر) بروتوكول AAE المستخدم في إجراء إعادة وعائية اللب. **المواد والطرائق العمل:** اثنا عشر كلبا من السلالات المحلية تم اختيارها عشوائيا من مناطق مختلفة من مدينة الموصل. تضمنت الدراسة إلى أربع مراحل ، في كل منها ، تم أخذ عينة دم لقياس مستوى bFGF بواسطة kit Canine bFGF ELISA ، المرحلة الأولى (تحفيز العدوى) ، المرحلة الثانية (التطهير) ، المرحلة الثالثة (العلاج) ، والمرحلة الرابعة (بعد ٣ أشهر من المتابعة). في المرحلة الثانية ، تم أخذ عينتين ميكروبيتين قبل وبعد بروتوكول التطهير AAE ، وكذلك المرحلة الثالثة بعد الري النهائي أيضا بواسطة استخدام الإقماغ الوريدية و تم حساب CFU / ml لتقييم فعالية بروتوكول التطهير AAE. **النتائج:** باستخدام تحليل التباين للقياسات المتكرره ، تم تحليل مستويات bFGF في جميع المراحل إحصائيا وتظهر النتيجة معنوية عند مستوى ٥٪ من قيمة $P\text{-value} = 0.04$ تظهر نتيجة العد الميكروبي فرقا ذا دلالة إحصائية عند ١٪ $P\text{-value} = 0.000$. تم تحليل هذه العينات الثلاث إحصائيا عن طريق تحليل التباين أحادي الاتجاه (ANOVA). **الاستنتاجات:** هناك اختلاف في مستويات bFGF في الدم بين المراحل أثناء التوجيه الخلوي لللب ، كما أن بروتوكول AAE للتطهير هو الوسيلة الفعالة لتطهير نظام قناة الجذر المفتوحة.

ABSTRACT

Aims: The study aims to evaluate the blood level changes of basic Fibroblast Growth Factor (bFGF) during pulp cell homing procedure of teeth with incomplete apices in dogs, and to evaluate the root canal disinfection recommendation of American Association of Endodontist protocol used in pulp revascularization procedure. **Materials and Methods:** Twelve local breed dogs randomly selected from different areas of Mosul City. The study was included four stages, the bFGF levels in the blood samples were measured by canine bFGF ELISA kit, stage I control (Induce infection), stage II (Disinfection), stage III (Treatment), and stage IV (3 month follow up). In stage II there are two microbial samples have taken from the root canals before and after AAE disinfection protocol, and also after final irrigation in stage III by the aid of paper point. These microbial samples were diluted and spread on blood agar and after 24hr the colony forming unit per milliliter was calculated CFU/ml to evaluate the effectiveness of AAE disinfection protocol. **Results:** The aid of ANOVA for Repeated Measure Statistical Test, the levels of bFGF in all stages were statistically analyzed. The result show significance at 5% level of significant $P\text{-value}=0.04$. The microbial counting result showed a highly significant difference at 1% $P\text{-value}=0.000$. These three samples have been statistically analyzed by one-way analysis of variance (ANOVA). **Conclusion:** There is a difference in the levels of bFGF in the blood between the stages during pulp cell homing, also AAE protocol for disinfection is an effective method to disinfect the open apex root canal system.

Key words: Cell Homing, Growth factor, bFGF, Incomplete Teeth Apex, Dogs

Salim AS., Abdulrahman Gh Y. The Plasma Levels of bFGF and Microbiological Evaluation of Cell Homing Procedure of Incomplete Teeth Apex Maturation in Dogs. Al-Rafidain Dent J. 2021 ;21 (1):110-123.

DOI: [10.33899/rden.2020.128138.1049](https://doi.org/10.33899/rden.2020.128138.1049) ©2021, College of Dentistry, University of Mosul

Received: 25/8/2020

Sent to Referees: 5/9/2020

Accepted for Publication: 30/9 /2020

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INTRODUCTION

The pulp tissue will be injured and inflamed, after trauma to the tooth or progression of dental caries till reach it, it starts the sequence of pulp necrosis, which leads to loss of pulp vitality, also atrophy of tooth development. One of the most critical cases that will make endodontics and pediatric dentists confuse about the best treatment plan are those cases with periapical periodontitis and open apex tooth.^(1,2,3) Pulp necrosis can be managed by the root canal treatment (RCT) in teeth with mature apex; however, if the formation of root is not completed, a combination of dentin thin walls and open apices, make the accomplishment of RCT challenging⁽⁴⁾. There are different treatment strategies to treat apical periodontitis with immature apexes, by apexification, which require to induce a calcified barrier in an open apex root. ⁽⁵⁾ Either by calcium hydroxide paste, or mineral trioxide aggregate plug (MTA plug) .

Although the high success rate of apexification which has been reported to be -between 74% and 100%, but may result in abnormal morphology of the root such as the calcified tissue formation inside the root canal, also calcium hydroxide placing for long-term may weaken the dentin and may induce root fracture ⁽⁶⁾ . So, the other strategies used to treat these types of cases are biological strategies in order to replace vital tissue in the space of root canal which has recently attracted great attention under the term of “Regenerative Endodontics”⁽²⁾.

Dental pulp regeneration which termed cell homing or “revitalization” technique, which is defined as the attraction and recruitment of endogenous cells, which can be include stem/progenitor cells, into a region to be regenerated or into an anatomic compartment.⁽⁷⁾ Stem cells homing is defined as the recruitment of endogenous stem cells from bone marrow and other niches by signaling “mobilization” factors to the site of injury to induce repair ⁽⁸⁾.

Growth factors (GFs) are naturally occurring substances which are polypeptides, that have the ability to stimulate cell growth, proliferation, cellular differentiation, healing and are major growth-regulatory molecules from cells in culture and in vivo. ⁽²⁾The growth factors utilized should have three functions: (1st) promote the root canal angiogenesis; (2nd) enhance the endogenous stem cell migration and (3rd) induce mineralization. ⁽⁸⁾

Basic fibroblast growth factor (bFGF or FGF-2) is a type of protein which firstly isolated from the pituitary glands of bovine. One member of the FGF family (minimally composed of 23 polypeptides) is FGF-2. FGF-2 stimulates mesenchymal cell proliferation during the bud and cap stage during the development of tooth germ, therefore has an important effect in dentin repair and odontogenesis.⁽⁹⁾

The aims of this study are the evaluation of blood level changes of bFGF in the dogs during pulp cell homing

procedure of teeth with incomplete apices. Also, to evaluate the root canal disinfection protocol used in pulp revascularization procedure (cell homing) that recommended by of American Association of Endodontist (AAE) through microbial counting of the root canals microbial samples

MATERIALS AND METHODS

The study was approved by Research Ethics Committee board (University of Mosul, College of Dentistry, REC reference No. D.B.S./4/2432019-2)

Local breed dogs (n=12) female, aged approximately 5 months and the dogs have been randomly selected from different areas in Mosul City. The animals have been kept and cared for standardized separated cages with good air ventilation and the good entrance of sun light. All the dogs were vaccinated with (BIOCAN DHPPI+L, LYOPHILISATE, England), The study has been taken lower permanent second premolar, and X-ray radiograph has been taken to each dog with self-developing film, and by using the film positioning system, so that to take reproductive radiograph which was used during the comparison between the stages and ensuring the state of the incomplete apex formation. The anesthesia protocol which has been used in this research, atropine (0.04mg/kg) intramuscularly (IM), then immediately followed by the mixture of ketamine (0.1mg/kg) with xylazine (1mg/kg), in the same syringe, the

administer intramuscularly (IM). The maintenance dose every 30 minutes the same mixture of ketamine-xylazine in order to maintain adequate anesthesia during procedure.^(10,11)

This study has divided into four stages each stage has two divisions the clinical work and laboratory work (serological work). Stage I (induce infection), Stage II (disinfection), Stage III (treatment) and Stage IV (three months follow up).

This study started at 19 August 2019 and was completed at 5 March 2020 in the animal house of the College of Dentistry at Mosul University.

Stage I (induce infection) started by given adequate general anesthetic solutions, 3ml of the blood has been taken from the cephalic vein and rapidly transferring it to ethylenediaminetetraacetic acid (EDTA) vacutainer tube then with gentle movement to ensure complete mixing of the blood with EDTA. This sample is the control sample which is before beginning the procedure.

The dog was repositioned on ventral recumbence and cover it with surgical towels just allowing the mouth to be unwrapped, an access cavity has been opened by the aid of a round bur with high-speed hand-piece, a cotton pellet with supragingival plaque has been inserted inside the pulp chamber, then the access cavity has been closed with glass ionomer filling material as a temporary filling . This stage has been continued for about (2-

4) weeks to ensure the periapical periodontitis which occurred as radiolucency around the apical area by the aid of periapical radiograph. Figure (1A)

In the laboratory, the plasma has been separated by centrifugation at 3500 rpm (round per minute) for 15 minute, then the plasma has been drained from the tube and transferred into eppendorf tube, stored in deep freeze -24°C .

The level of basic fibroblast growth factor (bFGF) in the plasma specimens of these dogs have been measured by using a special enzyme linked immunosorbent assay (ELISA) kit, which called Canine basic fibroblast growth factor (cbFGF) ELISA kit (MyBioSource.com, USA), in order to measure the level of BFGF in the blood.

After 2-4 weeks from stage I, a radiograph has been taken to ensuring that chronic periapical periodontitis has been established and stage II now has established .

According to the American Association of Endodontics (AAE) “ clinical considerations for a regenerative procedure revised 4/1/2018”, ⁽¹³⁾ and according to the European Society of Endodontology ⁽¹⁴⁾, these cases have been treated.

After administration of general anesthetic solution as described before, the blood sample was collected as in stage I, after repositioning of the dog on ventral recumbence, the tooth has been isolated with a rubber dam, disinfected with

chlorhexidine 2% and with Povidone-iodine 3% till dry , the temporary restoration has been removed by using round bur and cotton pallet, then the microbial sample has been taken from the canals with paper point size 20, transferred to the laboratory inside microbiological vial that contains brain heart infusion broth .

Before the disinfection of root canals system has been taken place, the working length of the two canals of the tooth have been established by using size 20-file inside the canals and take a radiograph. As in Figure(1B).

According to AAE and European Society of Endodontology 20ml of sodium hypochlorite 1.5% ^(13,14) has been used to irrigate the canal system by aid of 20ml disposable syringe and 27 gauge double side vent needle in order to reduce or prevent extrusion of sodium hypochlorite to periapical area. Also in the way to reduce the cytotoxic effect on periapical cell (stem cell of periapical papillae), the needle has been 1mm shorter than the working length. The irrigation with sodium hypochlorite 1.5% last long for about 10 minutes, then irrigation with normal saline and another irrigation with 20ml of EDTA 17% for about 5 minutes; lastly final irrigation with normal saline⁽¹³⁻¹⁴⁾. The canals then have been dried with size 80 paper point, these paper point taken as intra-canal microbial sample and transferred in to sterile microbial vial contain BHI broth as microbial sample after irrigation filled with

Incomplete Teeth Apex Maturation in Dogs

calcium hydroxide paste^(13,14) (calasept@plus, Sweden) then a cotton pellet put over it and glass ionomer filling material close the access cavity. This stage last long for 4-5 weeks. The blood samples of stage II have been used to measure the bFGF in this stage same as laboratory work of stage I.

The microbiological samples have been diluted in order to calculate the bacterial number (CFU) per milliliter, 1ml added on three blood agar plates then spread with L-shape glass rod, those blood agar plates have been incubated for about 24 hr, colonies have been counted and calculated by the following equation:

$$\text{Bacteria/ml} = \text{Number of colony (CFU)} * \frac{1}{1\text{ml Diluent}}$$

After about 4-5 weeks of stageII step of disinfection, the treatment step (stage III) has to be started. After administration of a good anesthesia protocol, then blood sample has been taken, also after proper rubber dam isolation and disinfection with chlorhexidine 2% and then with Povidone-iodine 3% till dry, the temporary filling material has been removed by the aid of round bur and exposed the cotton pallet, the cotton pallet has been removed by using a proper probe, 20ml of 17% EDTA solution with in 20ml disposable syringe with double side vent irrigation needle used to irrigated (agitated) the calcium hydroxide that present inside the root canal in order to remove it from the tooth, also in order to the opening of

dental tubules that contain signaling molecules and factors that will stimulate the cell migration.⁽¹⁵⁾ The irrigation procedure prolonged about 10 minutes.

After completion of irrigation with 17% EDTA, then 5ml of 0.9% normal saline has been used to irrigate the canal in order to reduce the toxicity of EDTA, then the canals have been dried with size 80 paper points. These paper points transferred inside the microbiological vial that contain BHI-broth, as a microbiological sample after completion of final irrigation.

After complete dryness of the canals, the next step which was the most important step, which was the induction of bleeding inside the canals, the induction of bleeding or entrance of blood inside the canals has been done by the aid of size 25 head-storm file through entering the file about 2mm over the working length of the canals this step make an injury in the periapical area that allows the blood to flow inside the canals, the level of blood inside the canal has been monitored to ensure that not exceed the level cemento-enamel junction (CEJ), then the blood has been allowed to clot for about 15 minute. The next step was the application of white mineral trioxide aggregate (MTA) over the blood clot, mixing of MTA powder with sterile distilled water in a ratio of 1:1, with the aid of suitable sterile MTA applicator the mixed MTA has been applied over the blood clot, by using the wet head of size 80 paper point, the mixed MTA has been condensed and spread over the blood clot

After application of MTA a suitable wet cotton pellet has been applied over the MTA in order complete of setting, then glass ionomer filling material close the access cavity Figure (2) , at this point the clinical work has been finished and the follow up after 3 months which was the last stage (stage IV) to see the result of the whole procedure.

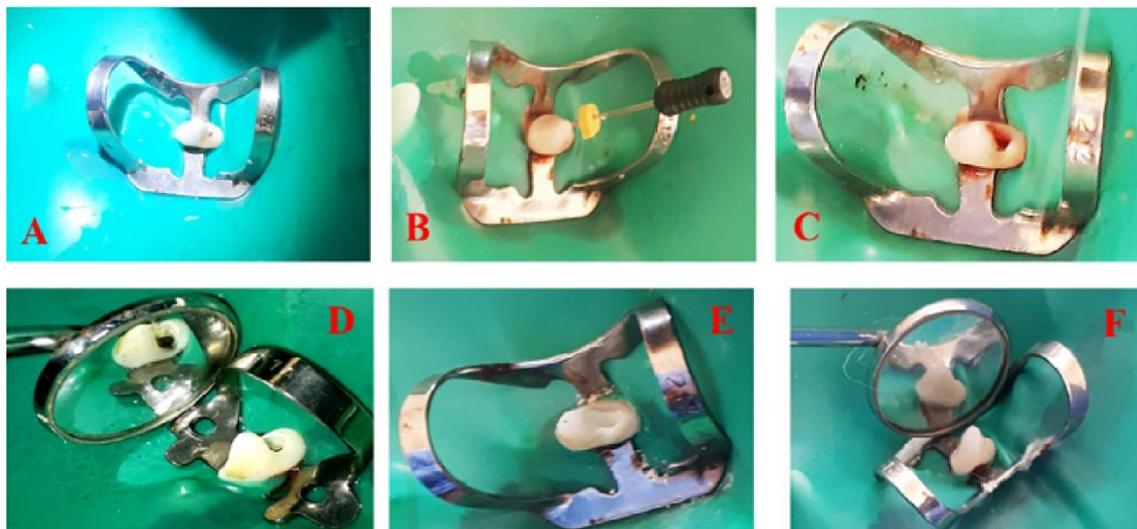
The blood samples of stage III have been used to measure the bFGF in this stage same as laboratory work of stage I,

the microbial sample that has been taken after final irrigation with normal saline has been diluted, cultured and counted. The last stage of this experiment which has been begun after 12 weeks (3 months) of stage III (treatment stage). In this stage, the animal has given anesthesia protocol and the last blood sample has been taken, also the final follow up periapical radiograph Figure(1C). The blood samples of stage IV have been used to measure the bFGF in this stage same as laboratory work of stage I.



(A) Chronic periapical periodontitis (red cycle) (B) Working length estimation (C) Application of calcium hydroxide and glass ionomer filling material

Figure (1): Radiograph of Stage II (Disinfection)



(A) Tooth after agitation with 17% EDTA (B) Over instrumentation by 40k-file 2mm beyond the apex (C) Bleeding fill the canal (D) Blood clot formation after 15 minute (E) MTA application (F) Final restoration by glass ionomer filling material

Figure (2): Clinical Procedure During Stage III

RESULTS

The concentration levels of bFGF in the blood have been measured by the aid of Canine bFGF- ELISA kit in order to compare among the stages of this procedure.

According to the manufacture manual, the samples and reagents have been added and then entered the ELISA reader in order to get the absorbance of the standards and samples, and use the

standard absorbance and the concentrations to draw the standard curve as in then use this standard curve to estimate the concentration of the samples. By the aid of ANOVA for Repeated Measure Test using SPSS from IBM version 25, the result showed significant difference ($P=0.04$) at 5% level of significance as in Table (1)⁽¹⁶⁾ Figure (3) shows the mean differences among the groups.

Table (1): ANOVA for Repeated Measure Test of bFGF Concentration in Blood

Source2	Type III Sum of Squares	df**	Mean Square	F	Sig.
Stages Sphericity Assumed	.009	3	.003	3.105	.040

* The level of significance $\alpha=0.05$, $p=0.05$
 ** Degree of freedom

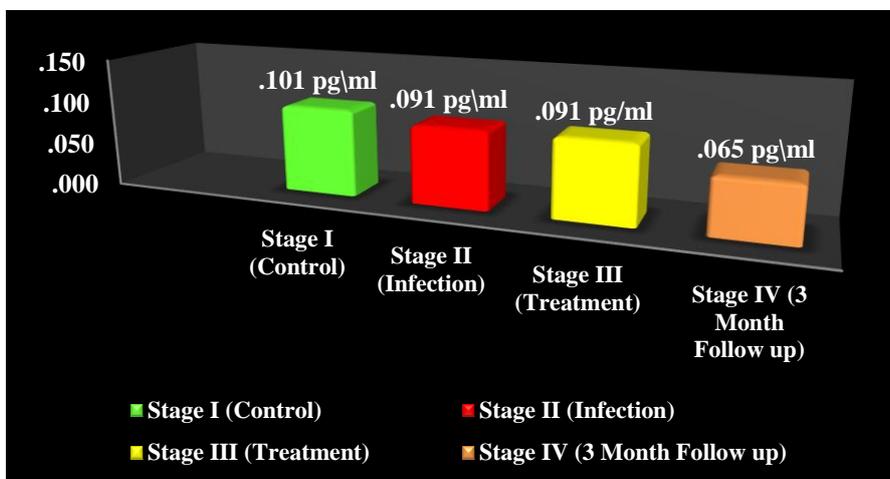
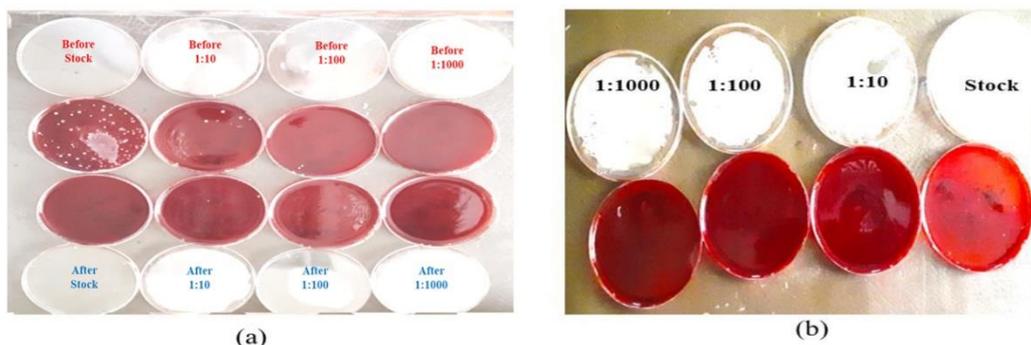


Figure (3): Mean bFGF Levels Differences Among The Stages

The bacterial samples have taken from the root canal area stage II (before irrigation protocol, and after irrigation

protocol) and stage III (after final agitation of calcium hydroxide with 17% EDTA and normal saline) Figure (4).



(a) Before and After Irrigation Protocol (b) After final irrigation in stage III

Figure (4): Blood Agar Plate After 24hour

These three samples have been statistically analyzed by one-way analysis of variance (ANOVA) by the aid of SPSS from IBM version 25 and the result as in Table (2). Figure (5) has compared the means of stageII (before irrigation protocol,

and after irrigation protocol) and stage III (after final agitation of calcium hydroxide with EDTA). The statistical analysis shows high significant difference between these three groups.

Table (2): (a) Descriptive Statistics (b) ANOVA (c) Duncan Multiple Range Test for Total Bacterial Count During the Procedure

(A) Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Stage II before	27	8744.44	12946.081	2491.47	3623.14	13865.7	400.00	43000.0
Stage II after	27	47.4444	72.87520	14.0248	18.6160	76.2729	.00	220.00
Stage III	32	1.0313	1.51305	.26747	.4857	1.5768	.00	5.00
Total	86	2760.62	8236.9163	888.209	994.629	4526.62	.00	43000.0

(B) ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1409212429.791	2	704606214.895	13.420	.000
Within Groups	4357764818.302	83	52503190.582		
Total	5766977248.093	85			

(C) Duncan Multiple Range Tests

Treatment	Samples NO.	Subset for alpha = 0.05		
		1	2	3
Stage III	32	1.0313		
Stage II before	27		47.4444	
Stage II after	27			8744.4444

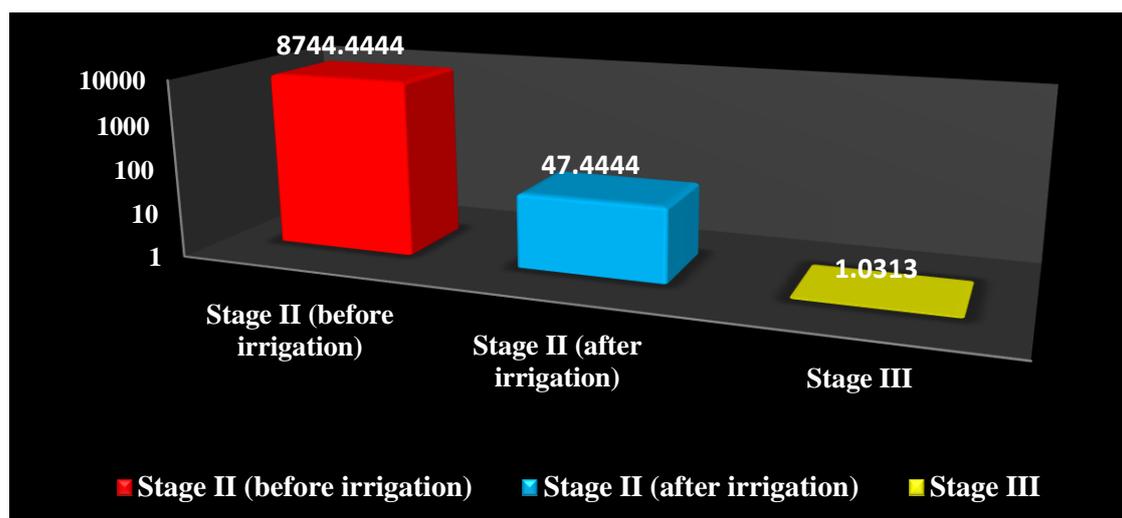


Figure (5): Mean of CFU/ml in Stage II Before Irrigation, Stage II After Irrigation, and Stage III

DISCUSSION

This study has been evaluated the blood levels change of bFGF during the normal state or control (stage I) and during the infection and disinfection (stage II, stage III) and lastly after 3month follow up of revascularization procedure (pulp cell homing procedure), Figure (3) show the difference in the mean of blood concentration in each stage during the procedure, and when taking a look on this figure you will note that the central columns have same levels, these levels occur during infection and disinfection, the period from infection to complete disinfection about (7-8) weeks these levels

slightly higher than the last column (3 months follow up) in the same figure that means the bFGF may increase during infection. This is due to the response of the body to infection (inflammation) and increasing bFGF release from fibroblast, endothelial cells, and macrophage, in order to the increasing angiogenesis, as its part of tissue healing processes in that injured area.⁽¹⁷⁾

In the Figure (3), the last column showed decreasing the mean concentration of bFGF after 3months follow up that means that the levels of this factor return back to the normal level in the blood.

The first column (control) in Figure(3) showed the highest concentration than other stages, this can be explained that the experimental dogs are a local breed and before the experiment start, the medical health of these dogs have been checked and given a total vaccine as mentioned in above, but, due to different medical problems, that mean any type of infection or inflammation during the first stage this may affect the level of this factor in the blood. This is approved by the lower level in the last stage follow up after return to the normal state and when the medical state has been controlled.

All mean concentrations of each stage are statistically analyzed by the aid of ANOVA for Repeated Measure Test, as in Table (1). This statistical test takes the mean concentration values of each stage and compare it with other value and depending on that the first value is the control, in relation to time (change depending on time), this test shows that there is a statistical difference among these group depending on the time at 5% level of significance as in Table (1).

All these data indicate that there is a relation between the cell homing and the bFGF level in the blood.

Different studies have been evaluated the effects of basic fibroblast growth factor (bFGF) during pulp revascularization (pulp cell homing) by different ways.

One In-vitro isolate to investigate the effect of bFGF on cell migration and cell

differentiation, on human dental pulp stem cell, the study concluded that the bFGF has a possible role in the regeneration of dentin-pulp complex, but also presented that bFGF has an inhibitory effect on alkaline phosphatase (ALKase) that will possibly inhibit calcification in regenerated area.⁽¹⁸⁾

Another in-vitro study found that the use of bFGF was acting primarily on cell proliferation and when combined with transforming growth factor, beta 1(TGFβ1) was seen to have stimulatory effect on odontoblastic differentiation HDPC.⁽¹⁹⁾ Other study found that control release of FGF-2 suggested to stimulated the dentinal bridge-like osteodentin formation on the regenerated pulp surface⁽²⁰⁾.

There are other different in-vivo studies use bFGF controlled released gelatin hydrogels as a scaffold, according to Kikuchi et al.⁽²¹⁾ has found that there were angiogenesis and formation of dentin like particles; also, Ishimatsu et al.⁽²²⁾ found that bFGF controlled release gelatin hydrogels could start the angiogenesis and able to induce cell differentiation.

All previous studies (In-vitro and In-vivo) have been demonstrated that the bFGF has an effective role in cell migration, cell differentiation and angiogenesis. These effects in some studies were dose-dependent and the most suitable dose that used ranged from 1 to 50 ng/ml of bFGF.^(23,24,25) All previous studies are in agreement with this study in which there was an effect of bFGF on pulp cell homing.

In this study, there are significant changes in the bFGF level in blood.

On another hand, one study was performed on dog teeth, found no effect of bFGF on cell differentiation to odontoblast-like cell.⁽²⁶⁾

This study has evaluated the effectiveness of irrigation protocol recommended by American Association of Endodontist (AAE). The evaluation depends on microbial counting (CFU/ml) of intra-canal samples before and after the irrigation protocol (stage II), and after final irrigation (stage III), as a method to evaluate the effectiveness of AAE irrigation protocol of such cases (open apex cases). Figure (4) represented the means of the bacterial count in three different samples. The mean of stage II before irrigation (red column) is higher than stage II after the irrigation (blue column), this occurs due to the higher numbers of bacteria in the canals due to the induction of infection in stage I (introduce supragingival plaque inside the canals), and after disinfection protocol, the numbers of bacteria are noticeably decreased to a very low number in comparison with their number before using irrigation AAE irrigation protocol, then in stage III (after 4-5 week of calcium hydroxide paste application and then agitated by 20ml of 17% EDTA for 5 minute), the number of bacteria decreased or became near to zero.

As mentioned before the numbers of bacteria were statistically analyzed by one-

way analysis of variance (ANOVA) as in Table (2). The result shows high significant difference between those groups, this means that the recommendation of AAE for canal disinfection has excellent effects on the bacteria present in the root canals. Sasanakul, et al. found that irrigation with 1.5% of NaOCl may be an effective way to disinfect large canals.⁽²⁷⁾

CONCLUSION

There are impotent rules of basic Fibroblast Growth Factor (bFGF) in pulp cell homing procedure (pulp revascularization), and the change in the blood level mean that there is body response to this type of treatment

The disinfection protocol American Association of Endodontics (AAE) is an effective method for disinfection of wide root canal with open apex and also may allow to release growth factor presence in dentinal tubules in order to enhance the homing of the cells present in the periapical area.

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