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Effect of Injectable-Platelet Rich Fibrin on Biocompatibility, Bioactivity and Biomineralization of Bioactive Materials Used as Direct Pulp Capping: An Experimental Animal Study

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Abstract

Vital pulp therapy has been known as one of the treatment options to preserve pulp vitality after being exposed by trauma or caries.

Aim: *This study was conducted to investigate the effect of i-PRF on biocompatibility, bioactivity and bio mineralization of pulp capping agents (Mineral Trioxide Aggregate (MTA) and Bioactive Bone Graft (BBG)).*

Materials and methods: *A total number of 92 teeth of 8 healthy male beagle dogs were used. The teeth were exposed and randomly assigned into four groups, according to the capping agents. Group A; capped with MTA, Group B; capped with MTA+ i-PRF, Group C; capped with BBG, Group D; capped with BBG+ i-PRF. The access cavity was restored with Intermediate Restorative Material (IRM). The dogs were sacrificed at each pre-determined intervals (1 month, and 3 months). Then specimens were prepared for standard histopathological, immunohistochemical examination polyclonal antibodies raised against dentine sialoprotein (DSP). Regarding the histopathological part, Chi square test was used to compare different groups. For the immunohistochemistry, ANOVA and Tukey's post hoc tests were used for intergroup comparisons, while paired t test was used for intragroup comparisons (effect of time within the same group). Kruskal-Wallis and Mann Whitney U tests were used for difference and percent change of sialoprotein marker in each group. Statistical significance was considered at* $P < .05$.

Results: *For the histopathological part, the results of dentine continuity, morphology and thickness after one month showed that there was a significant difference between all groups. Regarding the dentine morphology, there was a significant difference between groups, in one and three months. Results of immunohistochemical part, revealed that after one and three months the best values of immunoexpression of DSP marker, were recorded in groups B and D, followed by group C, with the least value recorded in group A.*

Conclusion: *The findings of the present study suggested that i-PRF promoted a greater and a supportive regenerative ability for the stimulation of odontoblastic differentiation and reparative dentine formation for pulp healing. Furthermore, i- PRF also attenuated the inflammatory condition.*

Keywords: *Bioactive Bone Graft, I-PRF, Mineral Trioxide Aggregate, Sialoprotein Marker.*

Introduction

Direct pulp capping involves the application of a dental material to seal communications between the exposed pulp and the oral cavity (mechanical and carious pulp exposures). It acts as a barrier that protects the dental pulp complex and preserves its vitality. The ultimate goal of pulp capping material is to induce hard tissue formation by the pulp cells (Stanley 1989).

Mineral Trioxide Aggregate (MTA) was introduced to the dental markets in 1993 and its use as a pulp dressing material has been the topic of research since its development, it has demonstrated high success rates in primary and permanent teeth, most probably as a result of its biocompatibility, alkalinity, sealing ability, as well as its unique ability to form a dentine bridge. Several studies reported that MTA induced less pulpal inflammation and more predictable hard tissue barrier formation (Masuda et al., 2010; Al-Hezaimi et al., 2011, Parirokh et al., 2011; Shi et al., 2016). However, it shows long setting time, poor handling characteristics, discoloration and a high cost (Cohen and Combe 1994).

Bioactive bone graft material, BBG ―DM Bone,‖ is resorbable in nature and is able to be replaced by new bone. It is a ceramic constitutes of two-phases of silicon, 60% hydroxyapatite (HA) and 40% beta-tricalcium phosphate (β-TCP), with an optimum balance between HA and β-TCP in the ratio 60:40. DM Bone is 70% porous with interconnected pores of 100~600 microns in diameter. This high porosity allows maximum space for vascularization, osteoblast migration and bone deposition (Sheikh et al., 2017).

Platelet rich fibrin (PRF) is a bioactive construct that stimulates the local environment for differentiation and proliferation of stem and progenitor cells. It acts as an immune regulation node with inflammation control abilities, including a slow continuous release of growth factors over a period of 7–14 days. Such growth factors are rich in fibrin, platelets, leucocytes, monocytes and stem cells (Dohan et al., 2009).

A concept of fabricating growth factors-enriched bone graft matrix (also known as ―sticky bone‖) using autologous fibrin glue has been demonstrated since 2010. Sticky bone provides stabilization of bone graft in the defect, and therefore, it accelerates tissue healing and minimizes bone loss during the healing period. The resultant sticky bone is moldable, preventing micro and macro movement of grafted bone. It also entraps platelets and leukocytes in its fibrin network and it is natural and prevents ingrowth of soft tissues in graft (Al-Azem et al., 2018). The null hypothesis of this study was that there is no significant difference between two pulp capping materials (MTA and BBG) either with or without adding PRF to them. Therefore, this study was conducted to evaluate the effect of platelet rich fibrin

on biocompatibility, bioactivity and bio mineralization of the bioactive materials used as a direct pulp capping after one- and three-months' time intervals.

Materials and Methods

1. Study design:

We worked according to ethical guidelines and regulations of the International Guiding Principles for Biomedical Research Involving Animals (Geneva 2012) (no. 89/2018).

2. Sample size determination

Eight healthy mongrel dogs with complete set of permanent dentition weighing 14-16 Kg, and aged between 10-18 months were used with a total of fourteen teeth in each dog. Six upper and lower incisors were used in each dog for experimental groups (total sample size = 96) and upper and lower canines were used for positive and negative control (total=16) (Faul et al., 2007).

F tests – ANOVA (Yu et al., 2016), were conducted in to determine a sufficient sample size.

Sample size calculation was performed using G*Power version 3.1.9.2, Faul et al., (2007), University Kiel, Germany. Copyright (c) 1992-2014.

The effect size was 0.25 using alpha (α) level of 0.05 and Beta (β) level of 0.05, i.e., power = 95%; the estimated minimum sample size (n) was a total of 48 samples for four experimental groups in one period.

Analysis: A priori: Compute required sample size

Input:	Effect size f α err prob	= 0.35 = 0.05
	Power (1-β err prob)	= 0.95
	Number of groups	= 4
	Number of measurements	= 2
	Corr among rep measures	= 0.5
	Nonsphericity correction ε	= 1
Output:	Noncentrality parameter λ	= 18.0000000
	Critical F	= 2.6937209
	Numerator df	= 3.0000000
	Denominator df	= 102
	Total sample size	= 48 for one period
	Actual power	= 0.9517650

$$f = \frac{\sigma_\mu}{\sigma}$$

$$\sigma_\mu^2 = \frac{\sum_{i=1}^{k} n_j (\mu_i - \mu)^2}{N}$$

Where:

f= is the effect size=0.15; α= 0.05; β=0.1; Power (1- β) = 0.90

3. Grouping of teeth:

The teeth were grouped into four experimental groups (n=24) according to pulp capping materials and two control groups (n=8) as the following; Group 1: MTA (Angelus, Lodrina, Paraná Brazil) + distilled water, group 2: MTA + i-PRF, group 3: bioactive bone graft (BBG) (Cheongju-si, Chungbuk, Korea) + sterile saline, group 4: bioactive bone graft (BBG) + i-PRF. Negative control, teeth were left intact with no pulp exposure and positive control, teeth were exposed and capped with Teflon disc. Finally, all cavities were restored with intermediate restorative material (IRM) (Dentsply, Charlote, U.S.A). Each group were then subdivided into two subgroups (n=12) according to the observation periods. Group T1: 4 dogs were sacrificed after 1 month and group T2: 4 dogs were sacrificed after 3 months for evaluation. The teeth were randomly assessed by the three different observers, without knowing which material was used. The only one who knew whether A, B, C or D represented which material was the allocator.

4. Pulp capping procedure:

4.1. Pre-operative care:

Dogs were bathed in Diazinon (Neocidal EC, Ciba-Geigy, Switzerland) in concentration of 1/1000 ml of water and then were injected subcutaneously with Ivermectin (Ivomec MSD Merk & Co. Inc. U.S.A) at a dose of 200 mg/kg body weight for control of external and internal parasites. They were fed three times a day on regular soft food. Pure water was available all the time. All the dogs were monitored daily for any pathological conditions under supervision of an expert veterinarian.

4.2. Anesthesia:

Half an hour before the procedures, 1.0 mL of intramuscular diazepam (Chimidarou, Tehran, Iran) was injected for sedation, followed by the intramuscular injection of 10 mg/kg of anesthetic agent ketamine HCL (Rotex Medica, Germany) and 1 mg/kg of Xylazine (Rotex Medica, Germany) Torad 2000.

Fifteen minutes before the anaesthetic solution was administered, each dog was premedicated with subcutaneous injection of atropine sulphate at a dose of 0.04mg/kg body weight. After induction of the general anesthesia, 20 mL of blood was drawn from each animal and collected in two 10 mL sterile glass test tubes without any anticoagulant. The sample was centrifuged at 700 rpm for 3 minutes using a laboratory centrifuge (Dohan et al., 2009).

5. Cavity Preparation:

The operating field was disinfected with 3% tincture iodine. Dry field was established with the placement of gauze and cotton roll in the mucobuccal fold (Negm et al., 2016). Class V cavities were prepared on the facial surfaces of all teeth. Approximately, all cavities were prepared 1mm coronal to the gingival margin with inverted cone bur at a high speed 30,000 rpm, under copious sterile water spray. The finished cavities were trapezoid in their external anatomy with proper undercuts at the line angles to retain the capping and temporary filling materials. The pulpal floor of each cavity was deepened until the appearance of a pink spot. A sterile sharp probe was used to create pulp exposure in the center of the cavity floor. The exposed pulp appeared as a red dot. Any bleeding, was controlled with sodium hypochloride (NaOCl) wetted cotton until haemostasis occurred (Abu-Seida, 2012).

5.1. Pulp Treatment:

The exposed pulps of incisors in each quadrant were capped with one of the four tested capping materials, so that each material was represented in each dog. Group A: MTA (Angelus, Lodrina, Paraná Brazil) was mixed with distilled water (3:1 powder-distilled water ratio) until it had the consistency of wet sand. Group B: MTA was mixed with i-PRF until it had the consistency of wet sand. Group C: BBG (Meta Biomed, Cheongju-si, Chungbuk, Korea) was mixed with one to two drops of sterile saline to create a putty-like mixture. Group D: BBG (Meta Biomed) was mixed with one to two drops of i-PRF to create a putty-like mixture. The four materials were mixed on sterile glass slab using metal spatula, which were applied over the exposure by a fine amalgam carrier and condensed lightly with a moistened cotton pellet. Finally, all cavities were restored with intermediate restorative material (IRM), the two canines were used for –ve and +ve control in each dog. Dogs were sacrificed after each observation period by using 20 ml of 5% thiopental sodium solution rapidly injected through the cephalic vein. The maxilla and the mandible were removed surgically and sectioned into two halves at the midline. Blocks containing a single tooth with its surrounding bone were obtained by sectioning the jaws with a sharp saw. The teeth were fixed in 10% neutral buffered formalin for 72

h. The specimens were then further prepared for routine histological analysis, demineralized in 10% formic acid (250 cm + 250 distilled water) for 5 months. The solution was adjusted to pH 7 by addition of Tri-sodium citrate (100 gm + 0.5 Litre distilled water) (Accorinte et al., 2008). A fine needle was used to perforate the specimens to allow acid penetration and the specimens were examined continuously for decalcification. After decalcification, specimens were dehydrated in 70% ethanol then embedded in paraffin blocks. The embedded specimens were serially sectioned into sections of 5µ thickness in a bucco-lingual plane to the tooth main vertical axis, through the capping site and the pulp. Serial sections that showed the deepest part of the cavity and the underlying pulp were selected for histological evaluation. These sections were stained with H&E for evaluation under light microscope (n=8).

Histological evaluation:

For the interpretation of the histopathological changes in the pulp tissue each section was graded according to criteria that were used based on the scoring system developed by Stanley 1988 and modified by Dominquez et al 2003, and an experienced pathologist who was not informed by the tested capping materials investigated the histological samples using a light microscope.

Immunohistochemical examination:

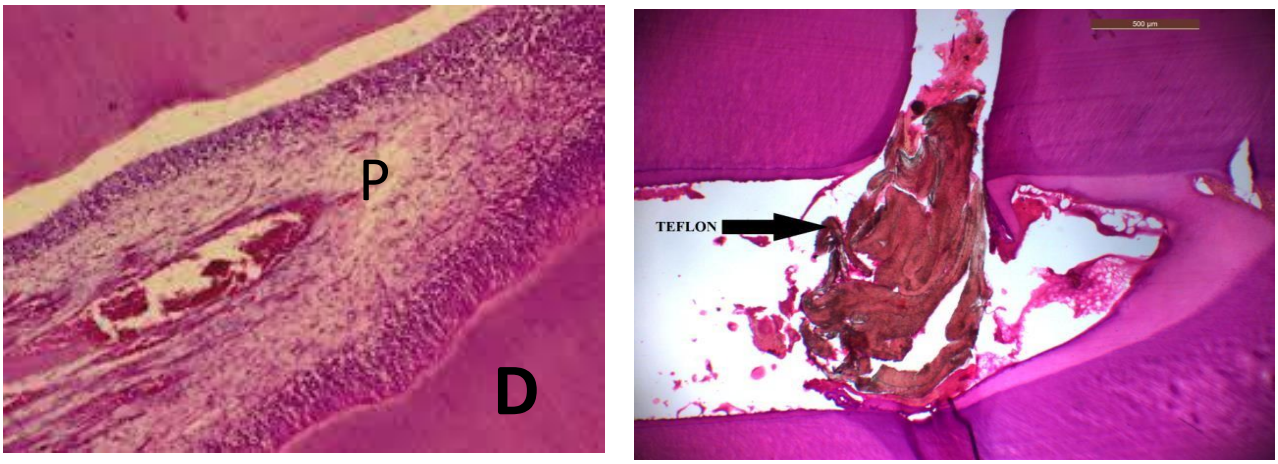
Four specimens (n=4) were de-waxed in xylene, rehydrated in a graded alcohol series, placed in an endogenous peroxide blocker for 10 minutes, and washed with Tris-buffered saline (TBS). The primary anti-dentine sialoprotein (DSP) antibody (Polyclonal Anti-Osteopontin, Biospes, China) was incubated at 4°C for 1 hour, followed by the biotin-streptavidin peroxidase complex. The tissues were counterstained with hematoxylin. A negative control was prepared from samples with no pulp treatment. Positive controls consisted of 1% bovine serum albumin substituted for DSP antibody. The histological sections were examined by the image analyzer computer system using the software Leica Qwin 500 (Leica microsystem, Germany). The amount of staining in the newly formed hard tissue, was measured in the form of an area in a standard measuring frame per 10 fields using a magnification x100 by light microscopy transferred to monitor's screen (Goldberg M, Lasfargues J, 1995).

All data was calculated, tabulated and statistically analyzed using suitable statistical tests. Regarding histopathology, data were presented as frequency (number) and percentages. Chi square test was used to compare different groups, as well as for intragroup comparison between one and 3 months. For

immunohistochemistry ANOVA and Tukey's post hoc tests was used for intergroup comparisons, while paired t test was used for intragroup comparisons (effect of time within the same group). Data related to difference and percent change were non- parametric. Kruskal-Wallis and Mann Whitney U tests were used for intergroup comparison. A p-value of less than 0.05 was considered statistically significant.

Results

Histological examination: Negative control; the teeth presented normal architecture of pulp consisting of delicate, loose connective tissue with numerous blood capillaries. Fibroblasts were numerous and the dentineal wall was outlined with an intact continuous odontoblastic layer of tall columnar cells (figure 1, A). Positive control, the odontoblastic layer were completely destroyed. The loose connective tissue of the pulp were replaced with necrotic tissue, infiltrated with inflammatory cells; extravasation of blood with dilatation of blood capillaries (figure 1, B).



(A) **(B)**

Figure. (9): Representative histological micrographs (H-E Stain, 40x)

(A): Negative control sample: Histological image of the pulp with visible odontoblast cell layer and no inflammatory reaction, D-dentine; O-odontoblasts; P-pulp tissue.

(B): Positive control sample: no hard tissue deposition at exposure area, the pulp was replaced with necrotic tissue, with destroyed odontoblastic layer, D-dentine; P-pulp tissue.

1. Calcific barrier continuity:

At one month, 100% of samples treated with MTA and BBG (group A and C) showed Initial dentine bridge formation extending to not more than one-half of the exposure site (score 3), while those treated with BBG+PRF (group D) showed partial/incomplete dentine bridge formation extending to more than one-half of the exposure site but not completely closing the exposure site (score 2) in 100% of samples. 50% of samples treated with MTA+PRF (group B) recorded score 3 and 50% score 2. Chi square test revealed that the difference between groups was statistically significant (p=0.005), (Table 2, Fig.2,3). At 3 months, groups A, B and C recorded score 2 in 100% of samples, while in group D, 50% of samples recorded complete dentine bridge formation (score 1) and 50% recorded score 2. The difference between groups was not statistically significant (p=0.132), (Table1, Fig.1). Comparison between one and 3 months Groups A and C showed a statistically significant decrease in score from score 3 to score 2 (p=0.00011), while the difference in groups B and D was not statistically significant (p=0.149), (Fig.2-5)

2. Calcific barrier morphology:

At one month, 100% of samples treated with MTA and BBG (group A and C) showed only a thin layer of hard tissue deposition (score 3), while those treated with BBG+PRF (group D) showed only irregular hard tissue deposition (score 2) in 100% of samples. 50% of samples treated with MTA+PRF (group B) recorded score 3 and 50% recorded score 2. The difference between groups was statistically significant (p=0.005), (Table 2, Fig.2,3). At 3 months, groups A, B and C recorded score 2 in 100% of samples, while all samples of group D showed dentine or dentine associated with irregular hard tissue (score 1). The difference between groups was statistically significant (p=0.00019), (Table3, Fig.4,5). Comparison between one and 3 months Groups A, C and D showed a statistically significant decrease in score (p=0.00011), also group B showed decrease in score in 50% of samples, but it was statistically insignificant (p=0.149), (Fig.2-5)

3. Calcific barrier thickness:

At one month, 100% of samples treated with MTA (group A) showed dentine thickness from 0.1–0.25 mm (score 2), all samples treated with BBG (group C) showed <0.1 mm (score 3), also, all samples treated with BBG+PRF (group D) showed >0.25 mm (score 1); while 50% of samples that treated with MTA (group B) recorded score 1 and 50% score 2. The difference between groups was statistically significant (p=0.00), (Table 2, Fig.2,3). At 3 months, groups A, B recorded 50% score 1 and 50% score 2, while groups C and D recorded score 1 in 100% of samples. The difference between groups was not

statistically significant (p=0.299), (Table3, Fig.4,5). Comparison between one and 3 months, Group C showed a statistically significant decrease in score (p=0.00011), while the difference in groups A, B and D was not statistically significant (p=0.149, p=1, p=0.299 respectively), (Fig.2-5)

4. Inflammatory type:

At one month, 100% of samples of all groups recorded score 2 which is chronic inflammation type, with no difference (p=1), (Table 4, Fig.4) At 3 months, groups A, B and C recorded score 2 in all samples, while group D showed no inflammation (score 1) in 100% of samples and the difference between groups was statistically significant (p=0.00019), (Table3, Fig.4,5). Comparison between one and 3 months, Groups A, B and C showed no difference (p=1), while the difference in group D was statistically significant (p=0.00019), (Fig.2-5)

5. Inflammatory intensity:

At one month, 50% of samples treated with MTA (group A), showed moderate inflammation (score 3) and 50% showed sever inflammation (score 4), while 50% of samples treated with MTA+PRF and BBG (groups B and C) recorded score 3 and 50% recorded score 2 which is mild inflammation. Group D recorded score 2 in all cases. The difference between groups was statistically significant (p=0.004), (Table 2, Fig.2,3). At 3 months, groups A, C and D recorded score 1 in 100% of samples, while group B recorded 50% score 1 and 50% score 3 but the difference between groups was statistically insignificant (p=0.132), (Table3, Fig.4,5). Comparison between one and 3 months Groups A, B, C and D showed a statistically significant decrease in score (p=0.00011, p=0.046, p=0.0011, p=0.0011 (Fig.2-5)

6. Inflammatory extensity:

At one month, 50% of MTA treated samples (group A), inflammatory cells were observed in one-third or more of the coronal pulp or in the mid pulp (score 3), while in the other 50% all of the coronal pulp was infiltrated by inflammatory cells (score 4), while groups B, C and D recorded score 3 in 50% of samples and the other 50% of samples were infiltrated with inflammatory cells next to dentine bridge or area of pulp exposure only (score 2). The difference between groups was not statistically significant (p=0.067), (Table 2, Fig.2,3). At 3 months, 100% of samples treated with MTA (group A) recorded score 2, while 50% of group B that treated with MTA+PRF recorded score 1 (absent inflammatory extrensity) and 50% recorded score 2. All samples treated with BBG and BBG+PRF (group C and D)

recorded score1. The difference between groups was not statistically significant (p=0.005), (Table3, Fig.4,5). Comparison between one and 3 months All samples showed decrease in inflammatory extrensity over the time, Groups B and D showed a statistically significant decrease in score (p=0.00011), while the difference in groups A and C was not statistically significant (p=0.149), (Fig.2-5)

7. Odontoblastic layer:

At one month, 50% of samples treated with MTA (group A), showed presence of odontoblast cells and odontoblast-like cells (score 2) and the other 50% showed presence of odontoblast-like cells only (score 3), while 50% of samples treated with MTA+PRF and BBG (group B and C) recorded palisade pattern of cells (score 1) in 50% of samples recorded score 2. All samples treated with BBG+PRF (group D) were score 2. The difference between groups was not statistically significant (p=0.007), (Table 2, Fig.2,3) At 3 months, groups A, C and D recorded score 1 in 100% of cases, while group B recorded 50% score 2 and 50% score 3. The difference between groups was not statistically significant (p=0.0002), (Table3, Fig.4,5). Comparison between one- and 3-months Groups A, B and D showed a statistically significant decrease in score (p=0.00011, p=0.046, p=0.0011 respectively), while the difference in group C was not statistically significant (p=0.149), (Fig.2-5).

	Group A: MTA	Group B: MTA+PRF	Group C: BBG	Group D: BBG+PRF
Histological evaluation	Scores) % (	Scores) % (	Scores) % (	Scores) % (
	1 2 3 50	1 2 3 50	1 2 3 50	1 2 3 50
calcific barrier continuity	100	50 50	100	100
calcific barrier morphology	100	100	100	100
calcific barrier thickness	100	50 50	100	100
Inflammatorytype	100	100	100	100
Inflammatory intensity	50 50	50 50	50 50	100
Inflammatory extensity	50 50	50 50	50 50	50 50
Odontoblastic layer	50 50	50 50	50 50	100

Table 2: Distribution of samples in different groups according to the histologic criteria after one month follow-up

	Group A: MTA				Group B: MTA+PRF				Group C: BBG				Group D: BBG+PRF			
Histological evaluation	Scores)%(				Scores)%(				Scores)%(				Scores)%(			
	1	2	3	50	1	2	3	50	1	2	3	50	1	2	3	50
calcific barrier continuity		100				100				100			50	50		
calcific barrier morphology		100				100				100			100			
calcific barrier thickness	50	50			50	50			100				100			
Inflammatory type		100				100				100			100			
Inflammatory intensity	100				50		50		100				100			
Inflammatory extensity		100			50	50			100				100			
Odontoblastic layer	100					50	50		100				100			

Table 3: Distribution of samples in different groups according to the histologic criteria after three months follow-up

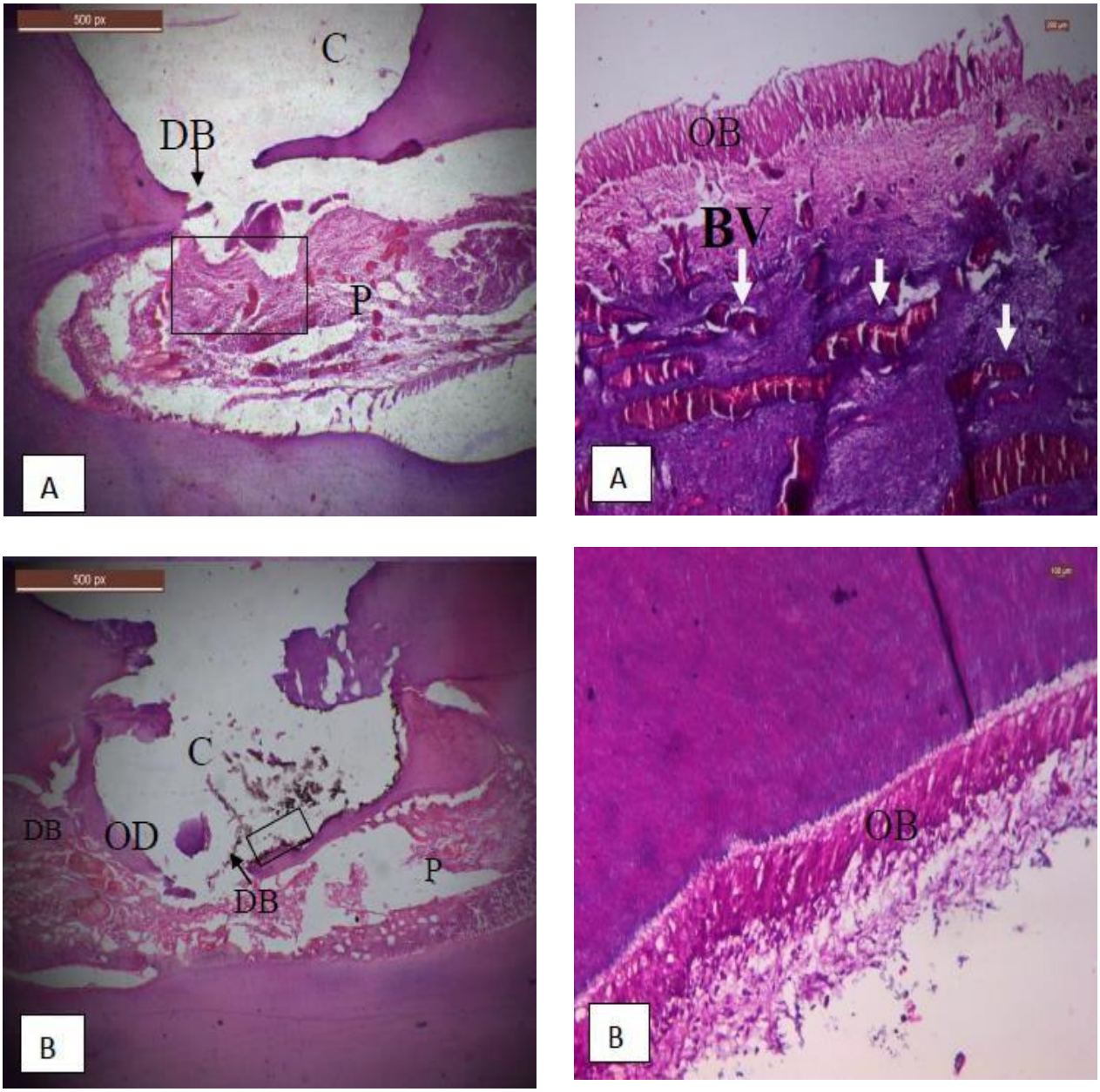


Figure.(2): Histological micrographs after one month of direct pulp capping with group (A): MTA, group (B): MTA+PRF, group (C): BBG, group (D): BBG+PRF, and restoration (H-E Stain, x40, x100), in groups A and C: thin calcified barrier (black arrows), extending not more than half of exposure site with moderate inflammation in the remaining pulp with marked dilation of blood vessels (white arrows) in group A. while in groups B and D: thick irregular hard tissue (black arrows) extend more than half of exposure site and showed mild inflammation. Inflammatory cells were observed in one- third or more of the coronal pulp or in the mid pulp of all groups. The rectangular areas demarcated in micrographs are magnified in the corresponding pictures. C: Cavity, BL: Blood vessels, DB: Dentine bridge, OD: Osteodentine, OB: Odontblast-like cells, T: Tunneling, P: Pulp. Scale bar = 500micro-meter.

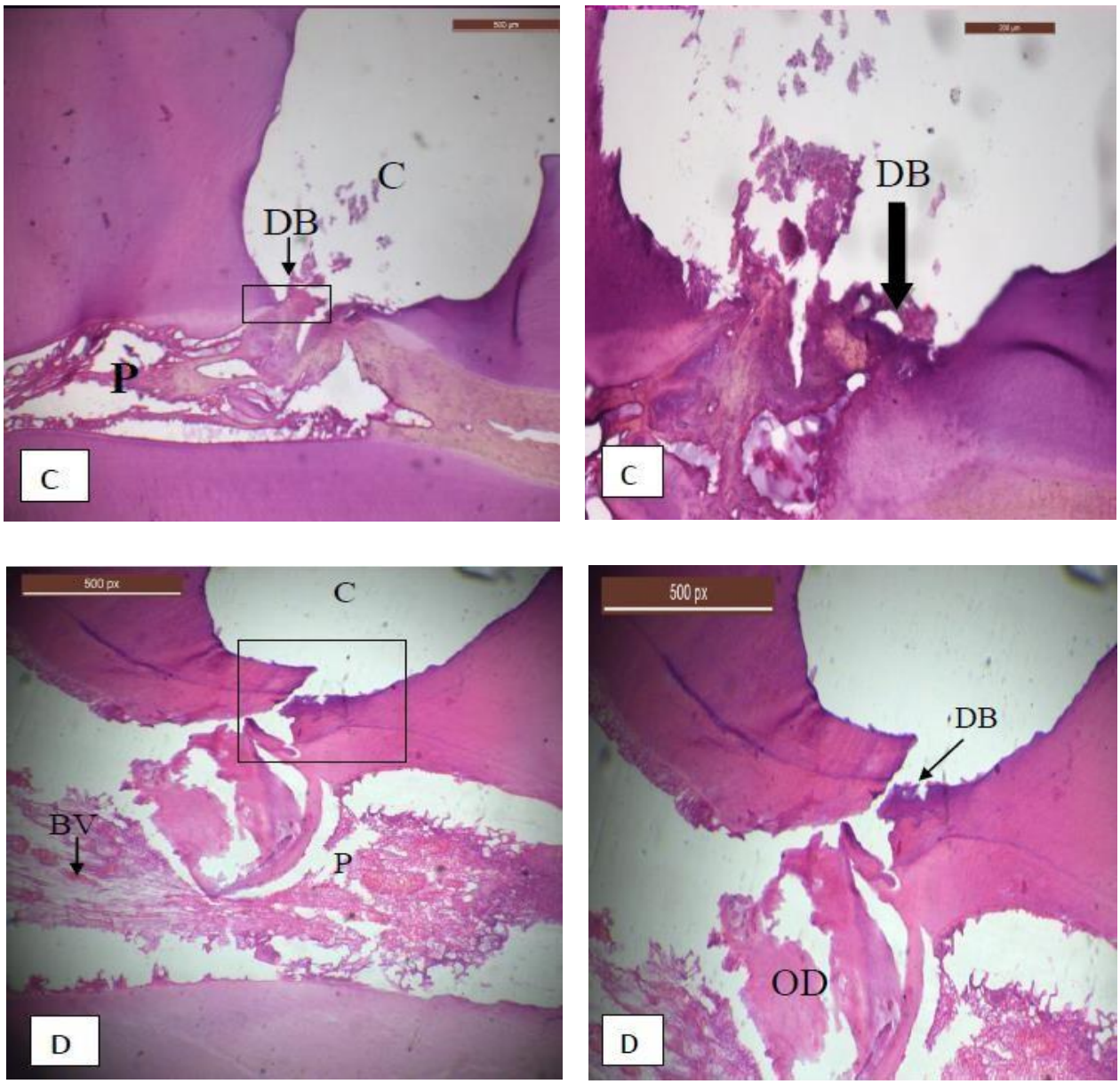


Figure. (3): Histological micrographs after one month of direct pulp capping with group (A): MTA, group (B): MTA+PRF, group (C): BBG, group (D): BBG+PRF, and restoration (H-E Stain, x40, x100), in groups A and C: thin calcified barrier (black arrows), extending not more than half of exposure site with moderate inflammation in the remaining pulp with marked dilation of blood vessels (white arrows) in group A. while in groups B and D: thick irregular hard tissue (black arrows) extend more than half of exposure site and showed mild inflammation. Inflammatory cells were observed in one-third or more of the coronal pulp or in the mid pulp of all groups. The rectangular areas demarcated in micrographs are magnified in the corresponding pictures. C: Cavity, BL: Blood vessels, DB: Dentine bridge, OD: Osteodentine, OB: Odontblast-like cells, T: Tunneling, P: Pulp. Scale bar = 500micro-meter.

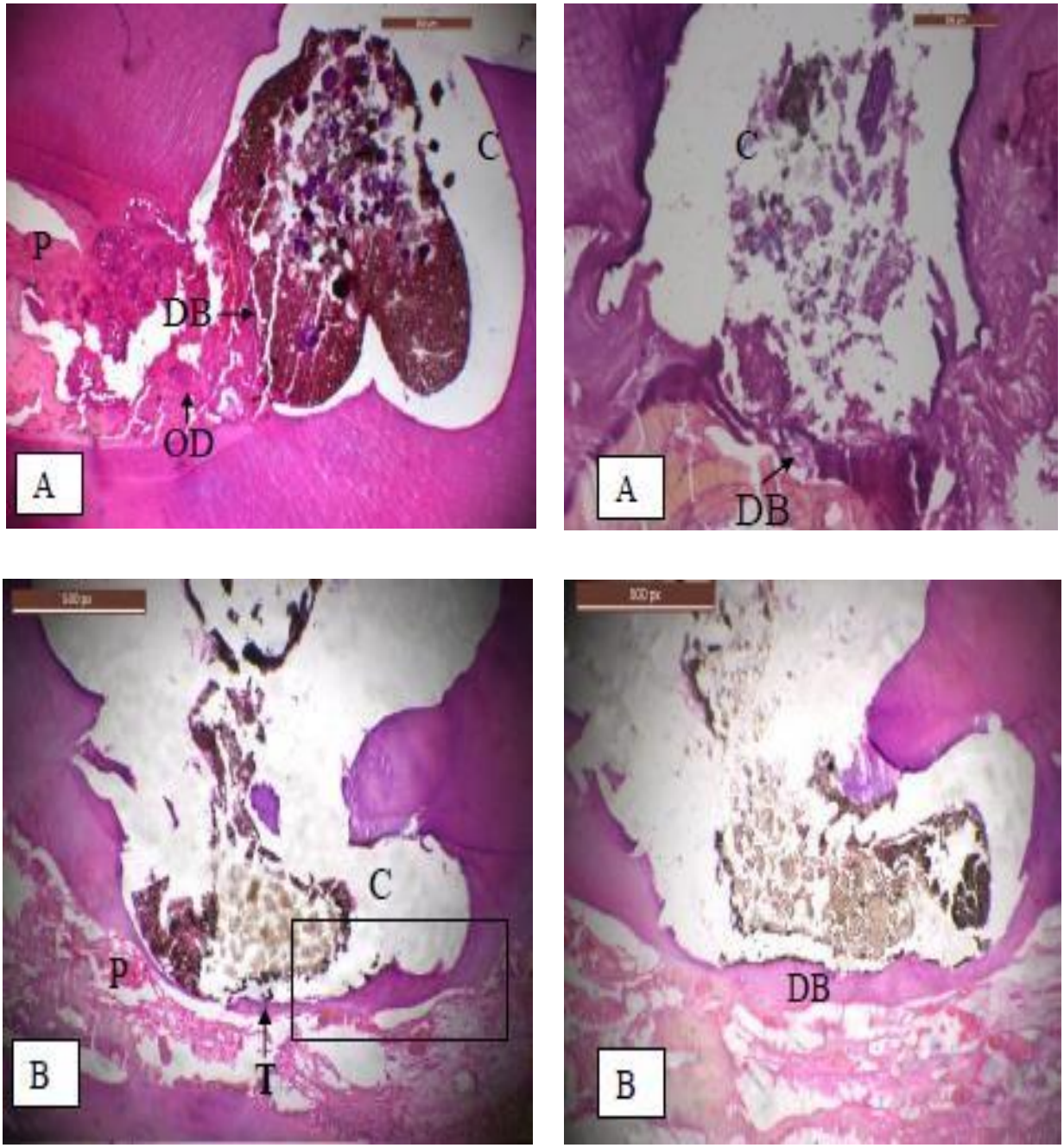


Figure. (4): Histological micrographs after three months of pulp capping with group (A): MTA, group (B): MTA+PRF, group (C): BBG, group (D): BBG+PRF, and restoration (H-E Stain, x40, x100), A, B, C showed closure more than half of exposure site (black arrow), with odontoblast-like cells in group C (white arrow). The dentine bridge with tunnel defect is observed in group B. group D showed complete irregular hard tissue (black arrow) with no inflammation. The rectangular areas demarcated in micrographs were magnified in the corresponding pictures. C: Cavity, BL: Blood vessels (arrow head), DB: Dentine bridge, OD: Osteodentine, OB: Odontblast-like cells, T: Tunneling, P: Pulp. Scale bar = 500micro-meter.

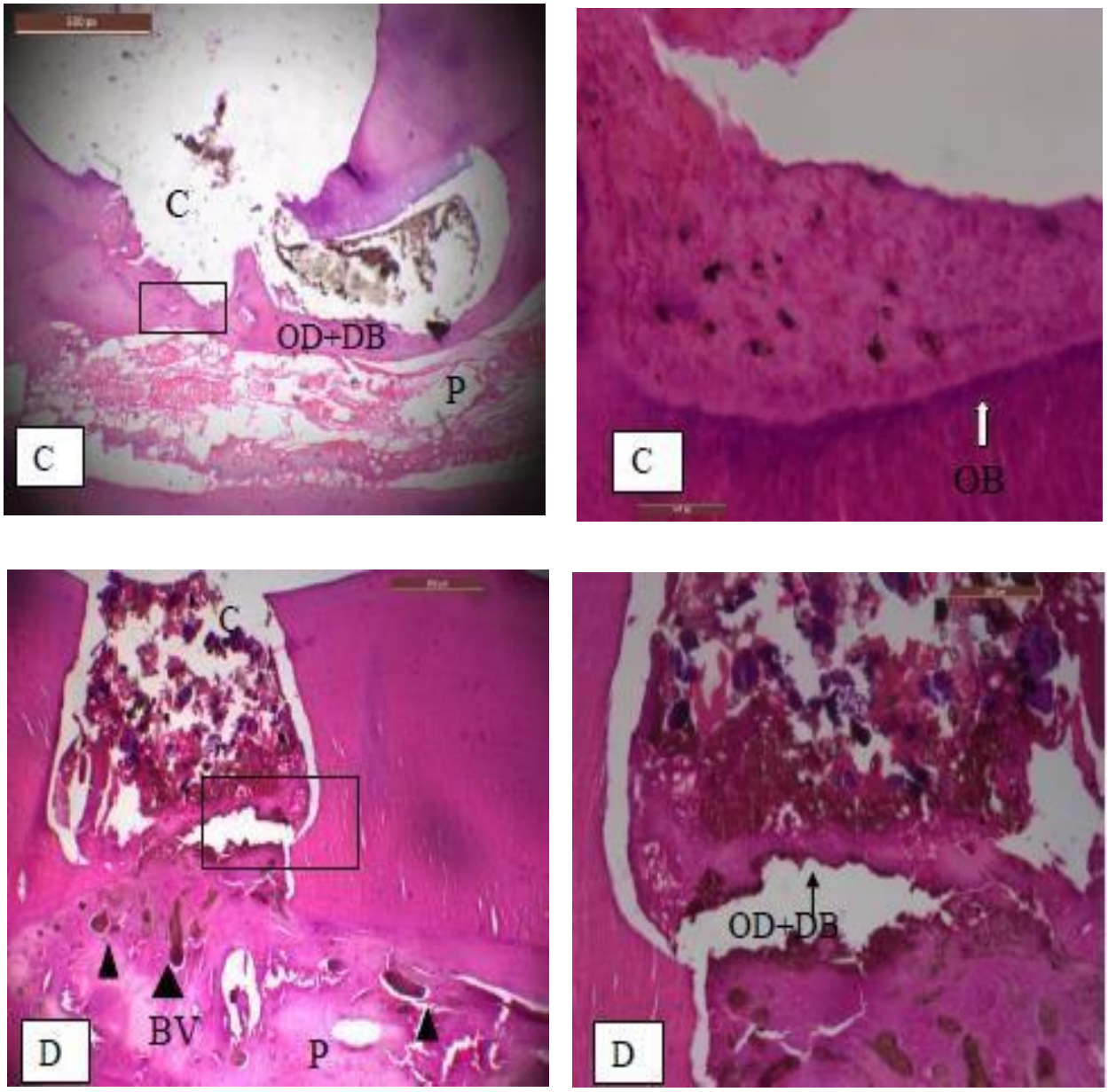


Figure. (5): Histological micrographs after three months of pulp capping with group (A): MTA, group (B): MTA+PRF, group (C): BBG, group (D): BBG+PRF, and restoration (H-E Stain, x40, x100), A, B, C showed closure more than half of exposure site (black arrow), with odontoblast-like cells in group C (white arrow). The dentine bridge with tunnel defect is observed in group B. group D showed complete irregular hard tissue (black arrow) with no inflammation. The rectangular areas demarcated in micrographs were magnified in the corresponding pictures. C: Cavity, BL: Blood vessels (arrow head), DB: Dentine bridge, OD: Osteodentine, OB: Odontblast-like cells, T: Tunneling, P: Pulp. Scale bar = 500micro-meter.

Immunohistochemical Examination:

A- Comparison between groups, at one and three months, the highest mean values of area percent of immunoexpression marker (sialoprotein) were recorded in groups B and D, followed by group C, with the least value recorded in group A. ANOVA test revealed that the difference between groups was statistically significant (p=0.00, 0.002). Tukey's post hoc test revealed no significant difference between groups B and D.

B -Intragroup comparison (Effect of time within the same group), in all groups A, B, C, D, the area percent of immunoexpression increased by time and a higher mean value was recorded after 3 months. This difference was statistically significant (p=0.025, 0.043, 0.005, 0.018) respectively, (Table 4, Fig.6, 7).

Time	Group A (MTA)		Group B (MTA+PRF)		Group C (BBG)		Group D (BBG+PRF)		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
1 month	.32^{c}	.26	3.624^{a}	1.11	.973^{b}	.51	3.616^{a}	1.28	0.00*
3 months	1.26^{c}	.65	4.96^{a}	1.36	3.70^{b}	1.48	5.94^{a}	2.57	0.0002*
P-value	.025*		.034*		.005*		.018*		

C.I.= confidence interval, Significance level P<0.05, *significant

Tukey's post hoc test: Within, the same observation, means with different superscript letters are significantly different

Table (4): Descriptive statistics and comparison between groups for area percent of immunoexpression.

C-Intergroup comparison of difference by time:

The greatest mean difference (increase) by time was noted in group D, followed by group C, Then group B, with the least value recorded in group A. Kruskall Wallis test revealed that the difference was statistically significant (p=0.032). Wilcoxon signed Rank test revealed no statistically significant difference between groups C and D (Table 5).

Area percent		Mean	Std. Dev	Std. Error	Sig.
Difference	Group A(MTA)	.94[c]	.60	.27	0.032*
	Group B MTA+PRF	1.34[b]	1.02	.46	
	Group C(BBG)	2.72[a]	1.08	.48	
	Group D BBG+PRF	3.62[a]	2.37	1.19	

C.I.= confidence interval, Significance level P<0.05, *significant, NS=non-significant

Wilcoxon signed Rank test: median with different superscript letters are significantly different

Table (5) Descriptive statistics and comparison between groups for difference by time of area percent of immunoexpression (Kruskall Wallis test)

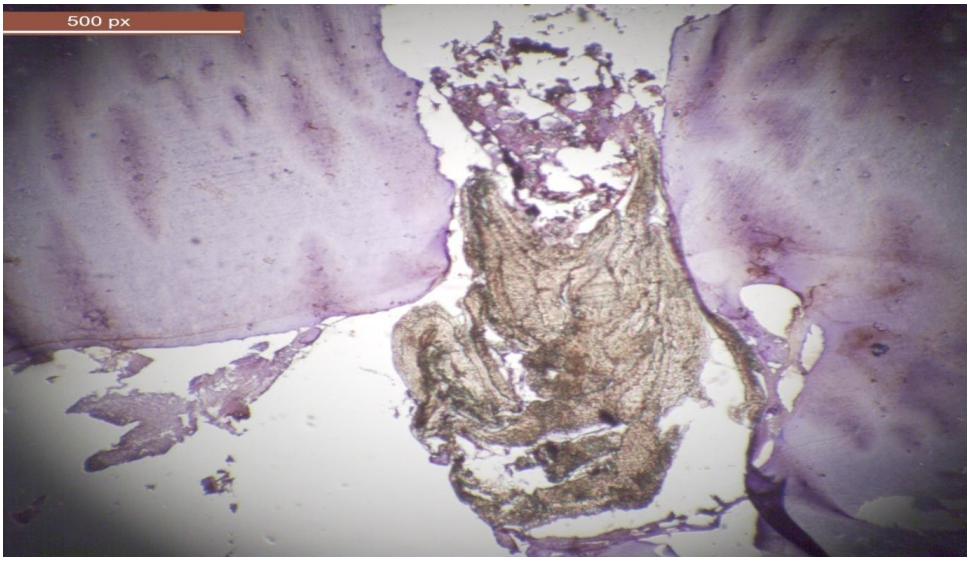


Figure. (8a): Positive control section. No immunoreactivity was observed in the dentine or predentinee. The tooth was included after placement of Teflon disc above the exposure then IRM.

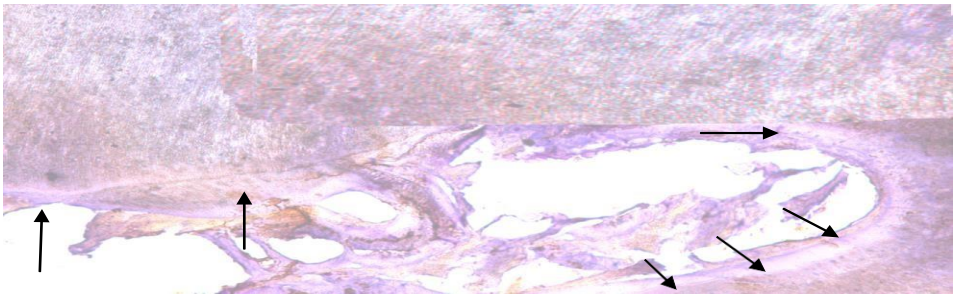


Figure. (8b): Negative control section Immunolocalization of DSP.

DSP expression is predominantly observed in odontoblasts and well-formed dentine (arrows). Tissue sections stained negative (brown) for DSP which is a marker for terminally differentiated odontoblast cells.

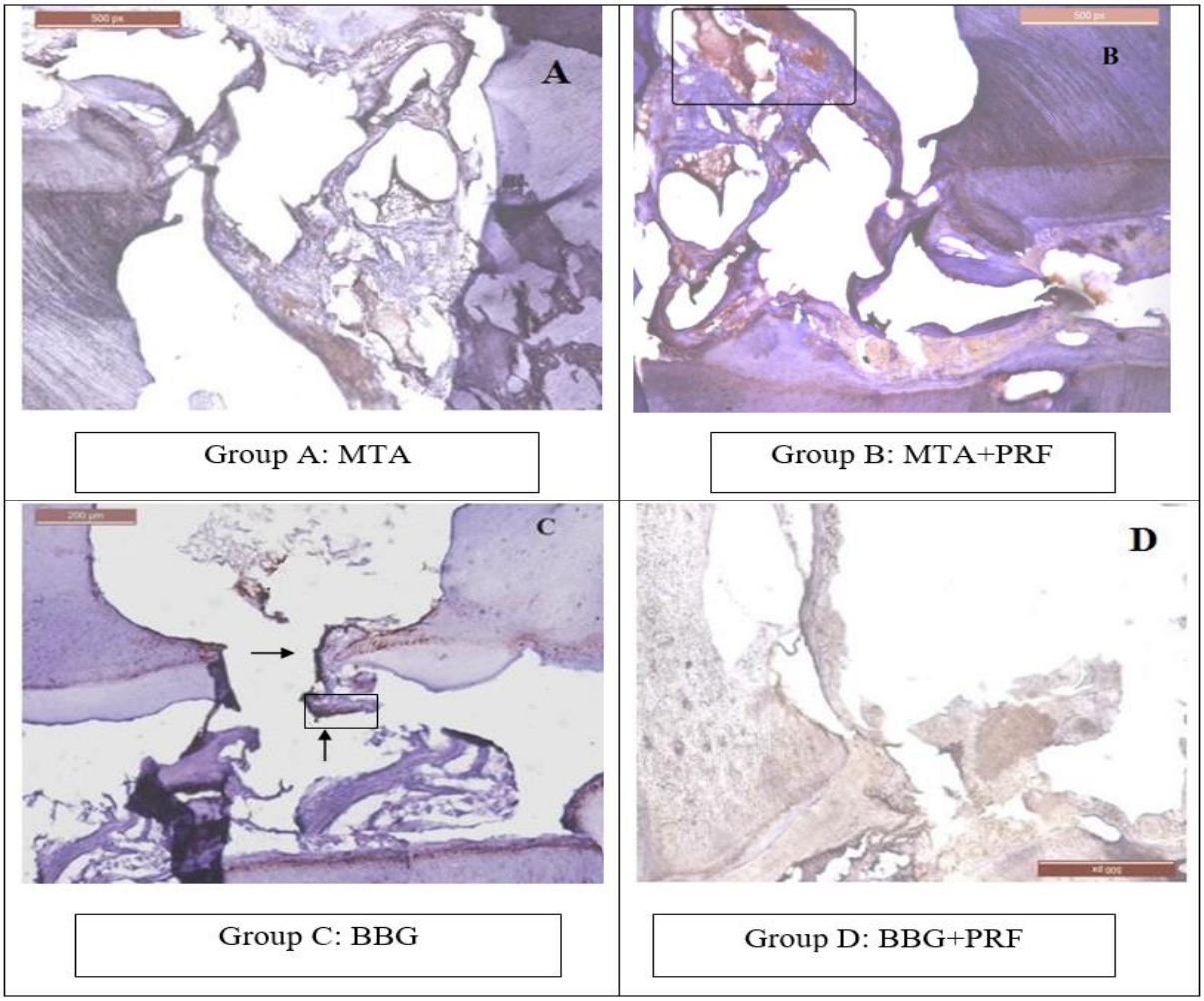


Figure. (6): Immunohistochemical photomicrographs of the regenerated dentine-pulp complex after one month. (A) Black arrow representing Dentine Sialoprotein marker, it was either undetectable or very faint in MTA treated group (original magnification X100). (B), (C) and (D). Dog's pulp capped with MTA+PRF, BBG, BBG+PRF; respectively, demonstrating immunostaining for DSP (original magnification X100), the hard tissue and odontoblasts are moderately stained.

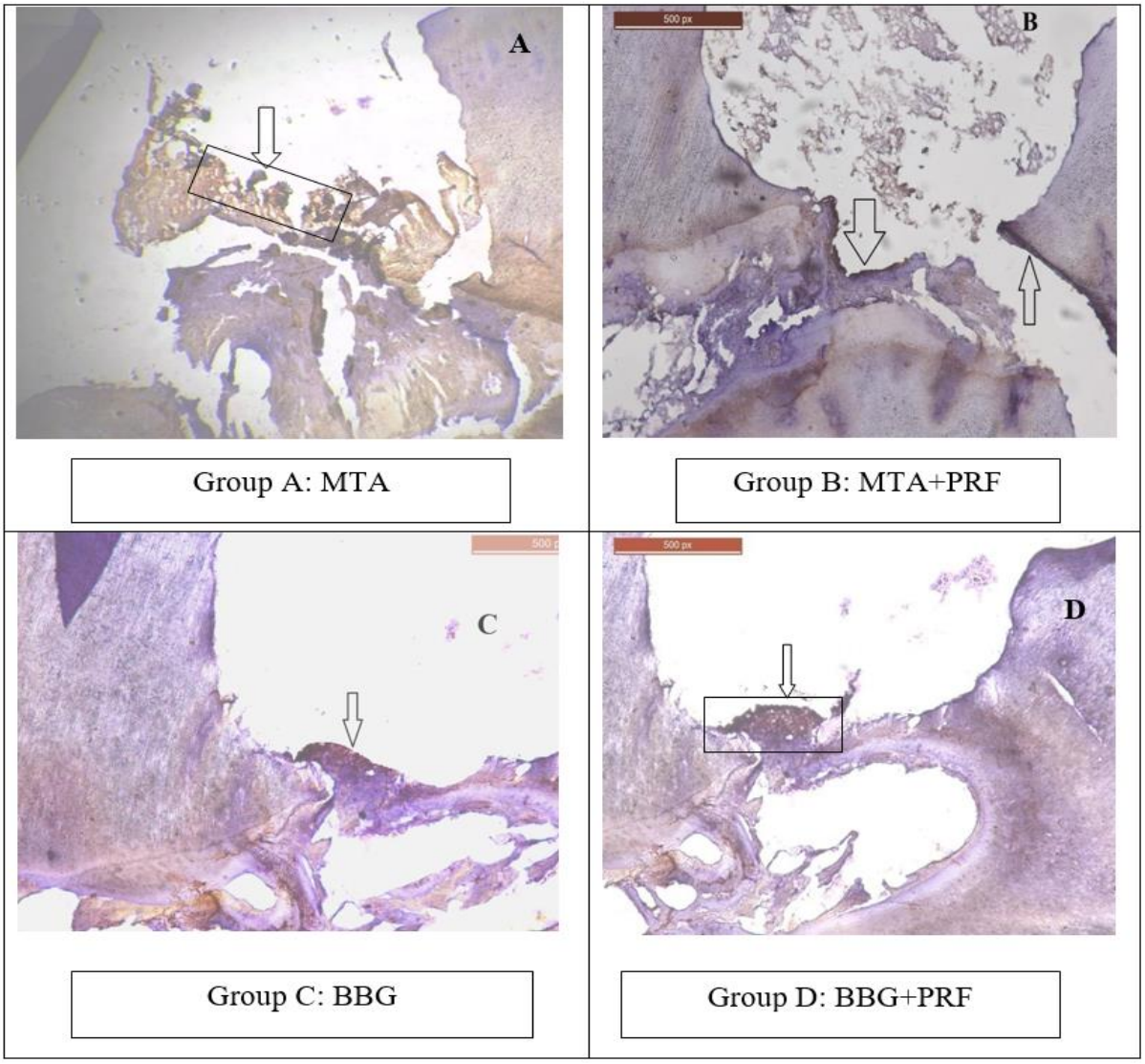


Figure. (7): Immunohistochemical photomicrographs of the regenerated dentine-pulp complex after three months. (A), (B), (C) and (D), DSP staining (brown) can be seen in the dentine (D) and predentine (PD) (white arrows) and rectangular areas showed the newly formed hard tissue (Positive reaction at the active formation site of reparative dentine can be observed). The white arrows representing the marked stain (strongly immune-satining) in the newly formed hard tissue lining the exposed dentine. (Original magnification X100). In groups B, C and D more intense DSP staining in the layer of cells lining the calcific bridge which most likely corresponds to hard tissue producing cells.

Discussion

Direct pulp capping is the use of a biocompatible material on pulp that has been exposed unintentionally during removal of caries or by traumatic injuries. Studies have shown that both bacteria and toxicity of materials damage the pulp. The effective pulp capping material should be biocompatible, provide a biological seal, prevent bacterial leakage and stimulate dentine bridge formation (Schuurs et al., 2000). In 1995, MTA was introduced by Torabinejad for sealing all the existing pathways between the root canal system and the outer surface of the tooth. This material became known as an appropriate material for pulp capping, because of its several good features such as high sealing effect, high pH, biocompatibility, long-term stability, prevention of bacterial leakage, and stimulation of cementum, bone and dentine formation (Ford et al., 1996). However, MTA does not have good handling properties when prepared according to the manufacturers' instructions, and the setting time is relatively long after mixing (Choi et al., 2013).

Calcium phosphate ceramics and hydroxy-apatite have been advocated as alternative pulp capping agents. When a mixture of biodegradable hydroxyapatite/beta-tricalcium phosphate ceramics is used, the material shows an ability to dissolve, break down, allowing new bone formation and remodelling to take place (Sheikh et al., 2017).

Among the newly introduced approaches to tissue regeneration, platelet rich fibrin (PRF) are shown to have significant effect in healing in different surgical fields such as orthopedic surgery, cardiovascular surgery, and maxillofacial surgery (Carlson and Roach, 2002). PRF was first described by Choukroun et al. 2001, and it is called the second generation platelet concentrate. It has several advantages over traditionally prepared PRP (Kalaskar and Damle 2004). Preparation of PRF does not require thrombin and calcium chloride for activation and the process is even easier and less time consuming than preparation of PRP gel. In this study, a liquid formulation of platelet rich fibrin (PRF) termed injectable-PRF (i-PRF) without the use of anti-coagulants was investigated (Dohan 2009).

The tissue response was evaluated histopathologically; as histological assessment is considered the most reliable method to evaluate the condition of dental pulp (Hashem et al., 2018). Sections were obtained from the capped teeth and examined for presence and distribution of reparative dentine, presence of inflammation and odontoblastic layer according to scoring system of Dominquez et al 2003, which is more precise than other scoring systems (Min et al. 2008).

Moreover, an immunohistochemical technique was applied for detection the amount or area percentage of sialoprotien marker, as dentine sialoprotein (DSP) is mainly expressed in odontoblasts and is

considered a marker of the differentiation of dental pulp cells into odontoblasts (Parirokh et al., 2005). The DSP gene product, phosphophoryn, is a hyperphosphorylated protein which comprises nearly 50% of the noncollagenous protein in dentine (Saito et al., 2000). Also, DSP is considered a positive regulator of hard-tissue mineralization acting on dentine, and its stimulation might lead to enhanced mineralization.

The experimental animals selected to carry out this study were dogs. Because the use of animals in in-vivo studies is more advantageous than in human beings. As the experimental conditions can be rigidly controlled. Furthermore, the mechanism of induction and synthesis of dentine are the same in human beings and a number of laboratory animals, even though the rate of dentineogenesis may differ (El Ashry et al., 2016). Also, the pulp size provides a suitable sample for histopathological evaluation. Beside that dogs' dentition provides a good number of teeth in each animal; that allows the comparison of more than one material or technique in the same dog.

In the present study, two evaluation periods were selected, the first period (one month) in order to show the primary response of tissues and second period (3months) for the final judgment of the procedure (Parirokh et al., 2011), as showed that not less than two weeks are needed for new odontoblasts to start to differentiate and form a barrier of new dentine (Parirokh et al., 2005).

According to histopathological study, the results of dentine continuity, morphology and thickness after one month showed that there was a significant difference between groups figure (2, 3). All samples of groups A (MTA) and C (BBG), showed initial thin layer of irregular calcified tissue. However, group D (BBG+PRF) and most of group B (MTA+PRF) showed better results as the dentine bridge extended to more than one-half of the exposure site with irregular pattern. These data indicates that all tested materials represent a biocompatible substrate to which hard tissue productive cells could attach and produce fibrous connective tissue for further mineralisation and neodentineal bridge formation. The ability of MTA to induce neodentineal bridge formation may be due to its sealing ability, biocompatibility, cytokine release induction or osteoconductivity (Asgary et al., 2006). Similar studies also observed the ability of MTA to form reparative dentine in the form of partial or incomplete dentine bridge (Nowicka et al., 2013., Bansal et al., 2014, Akhavan et al., 2017, Daniele 2017, Okamoto et al., 2018, Ricucci et al., 2018, Dammaschke et al., 2019, Suhag et al., 2019). However, other studies found complete reparative dentine bridge formed after capping with MTA. This confliction may be due to different examination test used in some studies as micro-CT cone beam (Nowicka et al., 2015), different methodology and materials used in comparison with MTA, different time intervals of follow up which was 7, 14, 21 and 30 days (Junior and Holland, 2004, Parirokh et al., 2005, Queiroz et al.,

2005, Chacko and Kurikose 2006, Parolia et al., 2010, Khalil et al., 2013, Nowicka et al., 2013, Liu et al., 2015, Nowicka et al., 2015, Bollu et al., 2016, Shi et al., 2016). BBG is an osteoconductive material that provide scaffold or template which would allow vascular ingress, cellular infiltration and attachment with calcified tissue deposition (Kiliç and Güngormüs, 2016). Osteoconductivity of BBG results from partial dissolution of HA and Beta-TCP crystals increasing the supersaturation of calcium and phosphate ions in the microenvironment causing the precipitation of apatite-like crystals which incorporate the carbonates in the biological fluid (LeGeros et al., 2003). Moreover, both MTA and TCP had alkaline pH values that remained consistently high for 14 days, although the pH values of MTA were significantly higher than those of TCP, alkaline environment might promote reparative dentine formation (Tagaya et al., 2005, Ferracane et al., 2010, Lee et al., 2014). On the other hand, the current results were in disagreement with Frank et al., (1991), who observed a continuous, well-calcified bridge, after capping pulp of premolars in young patient with HA. Similarly, Yildirim et al., 2007, Honda et al., 2009 and Gala-Garcia et al., 2012 observed the histo- pathological pulp response following direct pulp capping with a composite of beta-tricalcium phosphate-hydroxyapatite bioceramic (BC), and showed that a complete dentine bridge is formed one month, but here the study was on rat.

Concerning morphology of dentine bridge formed with MTA and BBG, Al- Hezaimi et al., 2011, Ricucci et al., 2014 and Dammaschke et al., 2019 stated that no tubular dentine could be identified from any of the specimens capped with MTA. In contradiction to our results, Hua et al., 2001, showed that the teeth capped with Hydroxyapatite compound preparation showed both osteo-like dentine and tubular like dentine formation without necrotic layer after capping with HA material. Also, Zarrabi et al., 2010 and Tran et al., 2012 showed that more tubular pattern of the dentineal bridge is formed after capping with MTA. The limited number of samples might be the reason for results discrepancies.

The better result of groups B (MTA+PRF) and D (BBG+PRF) was attributed to the addition of PRF. PRF is a concentrated suspension of the growth factors found in platelets. These growth factors are involved in wound healing and are postulated as promoters of tissue regeneration (Raja and Naidu 2008). PRF offers several advantages including promoting wound healing, graft stabilization, wound sealing, and hemostasis and improving the handling properties of graft materials (Woo et al., 2016). The present results were in consouauos with Zhang et al., 2012, they reported that the repair and regenerative potential of PRF enhanced cellular metabolism in combination with the sealing ability of MTA, enhanced the clinical success outcomes in pulpotomy and apexification procedures. Moreover,

the present results were in full accordance with Mazor et al., 2009, Zhang et al., 2012, Bölükbası et al., 2013, Shivashankar et al., 2013, Woo et al., 2016, Azzaldeen et al., 2019.

Regarding the time factor (one and three months) groups A (MTA) and C (BBG) showed extension of initially formed thin dentine bridge to partial/incomplete dentine bridge with irregular hard tissue deposition, this might be due to that when MTA undergoes gradual dissolution in tissue fluid, releasing calcium ions slowly that is responsible for the formation of the adherent HA layer on the MTA surface. Because of the porous nature of MTA, the precipitation continues internally within MTA and thus leads to a change in the overall composition of MTA. Histologically, this layer has been described as dentineal bridge, osteotypic matrix, osteodentine, and reparative dentine in various studies (Holland et al., 2001, Tziafas et al., 2002). Several materials also, namely calcium phosphate cements and hydroxyapatite cement, elicit biologic responses essentially similar to that of MTA (Holland et al., 1999, Saidon et al., 2003) with similarity in their mode of biologic action to gradual release of Ca and ability to form HA layer, that may attributed to significant decrease in score in group C (BBG). The previously reviewed results were in agreement with Jabber et al., 1991, Hayashi et al., 1999, Kato et al., 2011, Danesh et al. 2012, Nowicka et al., 2013., Bansal et al., 2014, AlShwaimi et al., 2016, Akhavan et al., 2017, Daniele 2017, Okamoto et al., 2018, Ricucci et al., 2018, Dammaschke et al., 2019, Suhag et al., 2019.

The inflammation that observed in this study, in all groups was chronic in the first month. But after 3 months period, only group D (BBG+PRF) showed no inflammation with absent or very few inflammatory cells figure (5). This might be related to, the presence of leukocytes and cytokines along with small amounts of lymphocytes in PRF can play a significant role in the self-regulation of inflammatory and infectious phenomenon (Toffler et al., 2009). PRF includes dense fibrin network with leukocytes, cytokines, glycoproteins and growth factors. Basically it is also known that PRF (often referred to as leukocyte) contains a supra-physiological concentration of leukocytes, which are one of the primary cells in the body responsible for fighting against bacterial invasions. Therefore, we hypothesized that their incorporation into LPS-induced environments may facilitate a pro–wound healing environment. LPS is the major component of the bacterial membrane of gram-negative bacteria that contributes to pulpitis (Rupf et al., 2010). It was shown that the expression levels of inflammatory-related genes was significantly reduced after treatment with liquid PRF, including TNF-a and IL-1b (genes expressed during pulpitis) (Hirsch et al., 2017). It was further found that liquid PRF partially inhibited the transport of nuclear factor kappa B (p65) to the nucleus and therefore may reduce the activation of the downstream signaling pathways responsible for creating an inflammatory response

with hDPCs. This explains, the relative existence of mild inflammation of the pulpal tissue following pulp capping using PRF. This also clarifies the statistically significant decrease in score in groups B (MTA+PRF) and D (BBG+PRF) in inflammatory extensity and intensity to absent and mild. Also, chronic and moderate inflammation in groups A (MTA) and C (BBG) might be referred to high alkalinity of both materials that causes denaturation of adjacent cells, tissue proteins and a few bacteria that might be present in the exposed area. As the materials set, the pH changes and the cell injuries subside with an improvement of inflammatory extensity and intensity (Amini et al., 2009). These current results are compatible with Parirokh et al., 2011, Shahravan et al., 2011, Tabatabayi et al., 2017, who concluded that mild to moderate inflammation and dentine bridge formation occurred in the pulpal tissue of the dog's incisor teeth capped with PRF, which shows promising effects in healing of the wounded pulp. Similarly, Chetna et al., 2018, indicated that PRF showed promising results in case of pulpal inflammation with irreversible signs and may be a good treatment option in comparison to endodontic treatment in pulpotomy case with deciduous molar. Also, Chai et al., 2019, concluded that, liquid PRF also attenuated the inflammatory condition and maintained a supportive regenerative ability for the stimulation of odontoblastic differentiation and reparative dentine in hDPCs. Likewise, Gala-Garcia et al., 2012, observed that after direct pulp capping of mechanically exposed teeth in rats with a composite of beta-tricalcium phosphate-hydroxyapatite bioceramic (BC), were associated with moderate inflammation. On the other hand, Frank et al., 1991, found that HA as a pulp capping agent, caused no inflammation in the pulp. Many studies have conflicting results, as they did not find any sign of inflammation after capping with MTA. This might be due to different samples number and different methodology (Zhu and Xia, 2003, Queiroz et al., 2005, Yun et al., 2007 and Gomes-Filho et al., 2011, Bansal et al., 2014, Da-Fonseca et al., 2016, Shi et al., 2016). Also, Karanth et al., (2013), Simsek et al., (2015), Benetti et al., (2018) , Emerenciano et al., (2018), recognized that MTA Angelus induced the mildest reaction after 7 and 15 days after subcutaneous polyethylene tube implantation containing MTA in rats.

During the process of dentineogenesis, a highly controlled extracellular events occur. This process is tightly controlled by odontoblasts, which secrete extracellular matrix (ECM) proteins and regulate dentine mineralization. ECM comprises collagenous and non-collagenous proteins (NCPs) (Butler 1995). Among NCPs, dentine sialophosphoprotein (DSPP) is the most abundant ECM protein in dentine and is processed into three major forms: dentine sialoprotein (DSP), dentine glycoprotein (DGP) and dentine phosphoprotein (DPP) (Yamakoshi, 2006). Among them, DSP and DPP are chiefly expressed in odontoblasts and dentine (Discher et al., 2009). DSP and peptides derived from DSP are able to induce dental primary/stem cell differentiation and biomineralization (Lee et al., 2012). This

explained the presence of palisade appearance of elongated and polarized odontoblast like cell layer in groups B (MTA+PRF) and D (BBG+PRF). The intended role of PRF in the defects was to deliver the growth factors in the early phase of healing. The viable platelets in PRF releases growth factors like Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor (TGF), Epithelial Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) in about the same concentration for a duration from 7 to 28 days. Achieving the peak level on day 14, that is coincided with cell ingrowth. Therefore, it directs stem cell migration, proliferation, and differentiation and supplements angiogenesis (Dohan et al., 2010, Strauss et al., 2020). Also, group A (MTA) and group C (BBG) induced differentiation and maturation of odontoblastic cells and this may be due to the influx of calcium ion released from MTA and CP cements that stimulate odontoblastic differentiation and mineralization (Woo et al., 2016). Several studies were in agreement with the present results, they found that MTA, PC cements and PRF induced the proliferation and differentiation of HDPCs into odontoblasts or odontoblast-like cells (Tsukamoto et al., 1992, Higashi and Okamoto 1996, Yun et al., 2007, Kuratate et al., 2008, Masuda et al., 2010, Zarrabi et al., 2010, Asgary et al., 2014, Jaberiansari et al., 2014, Liu et al., 2015, Torun et al., 2015, Chen et al., 2016, Woo et al., 2016, and Duo et al., 2020). Also, Ogata et al. 2005, concluded that mixing of HA with B-TCP, showed greater ability in osteogenesis than HAp by increasing collagen synthesis and calcification of the extracellular matrix. The expression of DSPP mRNA concomitantly increased with maturation of odontoblasts with the start of dentine mineralization, which is considered a terminal phenotypic marker of mature odontoblasts (Bleicher et al., 1999). As the HA forms the main inorganic portion of dentine, whereas type I collagen constitutes most of the organic portion (Butler 1995), DPP interact and covalently crosslinked to type I collagen fibrils through their core proteins forming a needle- like structure to capture Ca2+ ions and bond to hydroxyapatite. This leads to nucleation of hydroxyapatite crystals around and inside the collagen fibrils and so, leading to conversion of the predentine to dentine at the mineralization process (Feng et al., 1998, Saito et al., 2000, Dechichi et al., 2007).

Results of immunohistochemical study, revealed that after one and three months highest mean values were recorded in groups B (MTA+PRF) and D (BBG+PRF), and followed by group C (BBG), with the least value recorded in group A (MTA), figure (6, 7). The difference was significant between these groups, the presence of PRF, significantly increased the gene expression of DSPP during early phases and facilitated the expression of bone sialoprotien at later stages. All of these genes are expressed in both bones and teeth and in both osteoblasts and odontoblasts (Gu et al. 2000; Gundberg 2000; Qin et al. 2002; Shiba et al. 2003; Sumita et al. 2006). Several studies were in-consouauos with current results

after studying the effect of PRF on DSP expression (Chen et al., 2015, Woo et al., 2016, Kim et al., 2017, Hong et al., 2018, Cao et al. 2020). Although hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Gronthos et al. 2000, Torabinejad M, Hong C, Lee S, et al. Investigation of mineral trioxide aggregate for root-end filling in dogs. J Endod. 1995; 21: 603– 608.

Miura et al. 2003) have often been used as scaffolds for pulp regeneration, they are exogenous and have certain limitations in terms of their degradation rates, but Ogata et al. 2005, showed the great effect of Hydroxyapatite/soluble calcium phosphate composites in osteogenesis than HAp by increasing collagen synthesis and calcification of the extracellular matrix. However, MTA does not have good handling properties when prepared according to the manufacturers' instructions, and the setting time is relatively long after mixing (Torabinjad et al. 1995 and Choi et al., 2013), which also explain the marked increase in percent of marker expression by time, as it takes a lot of time to set plus the high PH around 11-12.5 initially (Lee et al., 2014). However, these studies have also demonstrated that the initial toxicity of MTA decreased in a time-dependent manner, the current results indicate that these materials are biologically acceptable materials for pulp tissue. These results in agreement with Tecles et al., 2008, Kim et al., 2016, who showed that MTA when used as a pulp capping material, it causes increase in DSP by time with reparative hard formation. Abdul-Qader et al., 2015, expressed high expression level of bone sialoprotein (BSP) and dentine sialophosphoprotein (DSPP) genes, after using B-TCP as a scaffold and studied their effect on HDPCs viability. Despite positive results, the slow degradation property of the B-TCP has been also suggested (Zhang et al., 2015, Karayürek et al., 2019) to jeopardize the actual bone-regeneration capacity of the PRF owing to the slow resorption property which may retard the replacement of new bone formation, these conflicted results may be due to humans model was used, as well as different methods of examination (clinically and radio-graphically).

We rejected the null hypothesis, as we found significant difference between the tested materials.

In conclusion, adding i-PRF to either MTA or BBG produced favourable outcomes.

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