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Research Article

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Reparative Calcified Barrier Characterization after Mixing Injectable-Platelet Rich Fibrin with Bioactive Direct Pulp Capping Agents; An Exp. Study.

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Abstract

Aim: *This study investigated the morphology and localization of hard tissue barriers formed after direct pulp capping using i-PRF mixed with bioactive agents (Mineral Trioxide Aggregate (MTA) and Bioactive Bone Graft (BBG)) in dogs' teeth using Scanning Electron Microscope. Materials and methods: A total of 64 teeth were used out of 8 healthy male mongrel dogs. The teeth were randomly assigned into four groups according to capping agents used, they were exposed and capped as follow, Group A; capped with MTA, Group B; capped with MTA+ i-PRF, Group C; capped with BBG, Group D; capped with BBG + i-PRF. Finally the access cavity was restored with Intermediate Restorative Material (IRM). The dogs were euthanized after 1 month; and samples were then prepared for scanning electron microscopic study. SEM was used to assess the morphology, localization and extension of the reparative hard tissue barriers and using an image-processing and analysis software to delimit the perimeters of the root canal area and the hard tissue barrier to determine the percentage of root canal obliteration. Chi-square test was used for intragroup comparisons. Results showed that all groups were statistically different ($P < 0.05$), regarding tissue barrier morphology.*

Keywords: Bioactive Bone Graft, Injectable-Platelet Rich Fibrin, Mineral Trioxide Aggregate, Scanning Electron Microscope.

Introduction

Direct pulp capping is a minimally invasive dental pulp therapy which aims at preserving tooth vitality by allowing pulpal tissue repair and inducing mineralized tissue formation [1]. The success of these treatments depends on the clinical situation as well as the biomaterials used. MTA, hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) are commonly used pulp capping agents [2]. In addition to the initial inflammatory reaction of the pulp, the nature and quality of the newly formed mineralized tissue obtained after direct pulp capping affect the success of the treatment directly. Primary dentin is the tubular dentin that formed by primary odontoblasts during crown formation. However, secondary dentin refers to the physiological dentin that is continuously deposited after the completion of tooth eruption. Tertiary dentin is formed in case of injury as a reactionary dentin that formed due to mild stimuli, produced by the existing odontoblasts. Stronger stimuli lead to the death of odontoblasts. Under favorable conditions, it is found that dental pulp stem cells differentiate into odontoblast-like cells to produce reparative dentin. This reparative dentin is desired during pulp capping procedures [3].

Calcium silicate-based cements have been developed about 20 years ago with the most well MTA formulation which was considered as the gold standard for direct pulp capping procedures However, it has some disadvantages such as prolonged setting time, difficult manipulation, and initial toxicity that are provoked on pulpal tissue due to high PH immediately after its application [1-4]. BBG is resorbable and can be replaced by new bone. It's a silicon-based ceramic made up of two phases: 60% HA and 40% β-TCP. BBG is 70% porous, this increased porosity allows optimal space for vascularization, osteoblast migration and bone deposition, however there are also some disadvantages, such as brittle properties, variable rates of resorption and less revascularization than other pulp capping agents [5].

The creation of an innovative "injectable" formulation of PRF (i-PRF) has been proposed with aiming at providing endodontists with a simple technique utilizing the concentrate of platelets in a liquid form that can be applied alone or in conjunction with bioactive materials providing an adequate perspective for soft tissue as well as hard tissue healing owing to the formation of a three-dimensional fibrin network rich in platelets, type I collagen, leukocytes, growth factors and osteocalcin [6]. It was prepared by the reduction of centrifugation force by the low-speed centrifugation concept which was shown to allow a noticeable increase in regenerative cells, leukocyte, and platelet numbers, as well as growth factors concentration when compared to other formulations of PRF utilizing higher centrifugation speeds [7]. Moreover, i-PRF plays an important role in tissue healing by activating the host defense system because it has the highest number of platelets and defense-fighting leukocytes, allowing for antimicrobial potency against bacterial lipopolysaccharide when compared to other platelet concentrates; also, the leukocytes have a crucial role during wound healing by directing and recruiting various cell types [8,9]. However, i-PRF contains a smaller amount a protein cocktail with different biochemical cues and may work in a synergetic manner, leading to a positive biological impact for tissue regeneration and enhancement of mineralization, and therefore may be the candidate of choice for tissue engineering allows utilizing autologous cues to promote hard tissue regeneration [10]. The advantages of combination of i-PRF with bioactive materials as follow; scaffold material thus helps in forming a favorable hard tissue scaffold with the proper mechanical strength, hydrophilicity and excellent osteoconductivity, wound healing, bone growth and maturation, material stabilization and hemostasis. It also improved the handling properties of bioactive materials [11]. Therefore, this study was conducted to evaluate the effect of i-PRF on bioactive agents used as a direct pulp capping after one month using scanning electron microscopic study as there is no study up to our knowledge was conducted on the effect of i-PRF on localization and morphology of hard tissue barrier.

Materials and methods

Study design

This study was approved by the Ethical Committee of the Faculty of Dentistry, Suez Canal University.

Sample size determination:

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 0.35 using alpha (α) level of 0.05 and Beta (β) level of 0.05, i.e., power=85%, the approximate minimum sample size (n) was a total of 48 samples for four experimental groups. Eight healthy dogs with complete set of permanent dentition weighing 14-16 Kg, and aged between 10-18 months were used. Four upper and lower incisors were used, for a total of eight teeth in each dog (total sample size = 64) [12-13].

Grouping of teeth

The teeth were divided into four experimental groups (n=12) and two control groups (n=4) based on the pulp capping materials used; Group A: MTA (Angelus, Lodrina, Paraná Brazil) + distilled water, Group B: MTA + i-PRF, Group C: BBG (DM Bone, Meta Biomed, Cheongju-si, Chungbuk, Korea) + sterile saline, Group D: BBG + i-PRF. Finally, IRM was used to restore all of the cavities (Dentsply, Charlote, U.S.A). The observation period was one month. The teeth were randomly divided into four groups. The only one who knew whether A, B, C or D represented which material was the allocator.

Pulp capping procedure

Pre-operative care

Dogs were bathed in Diazinon (Neocidal EC, Ciba-Geigy, Switzerland) at a concentration of 1/1000 ml of water, after that injected subcutaneously with Ivermectin (Ivomec MSD Merk & Co. Inc., USA) at a dosage of 200 mg/kg body weight for parasite control. They were served daily soft food three times a day with pure water. All of the dogs were monitored regularly for any pathological conditions.

Anesthesia

For sedation, 1.0 mL intramuscular diazepam (Chimidarou, Tehran, Iran) was injected half an hour before the experiments, accompanied by intramuscular injections of 10 mg/kg ketamine HCL (Rotex Medica, Germany) and 1 mg/kg Xylazine (Rotex Medica, Germany) by Torad 2000. Every dog was given a subcutaneous injection of atropine sulphate at a dosage of 0.04 mg/kg body weight fifteen minutes before the anaesthetic solution was given [14].

Preparation of i-PRF

After inducing general anaesthesia, 20 mL of blood from each dog was collected in two 10ml glass-coated plastic tubes (Vacutainer; Allschwil, Switzerland) and immediately centrifuged at 400g (700 rpm) and at room temperature for 3 min using a centrifuge (Boca-Raton, FL, EUA). Immediately after centrifugation, the upper yellow fluid (i-PRF) liquid phase was drawn through a plastic syringe as close as possible to the red cells [9]. This yellow fluid was used for mixing with bioactive pulp capping agents in groups B and D.

Cavity Preparation

With 3 percent tincture iodine, the operating area was disinfected. Rubber dam was used for isolation. On the facial surfaces of all teeth, Class V cavities were prepared 1mm coronal to the gingival margin with an inverted cone bur at a high speed of 30,000 rpm. Under copious sterile water spray. The external anatomy of the finished cavities was trapezoid, with proper undercuts at the line angles to keep the capping and temporary filling agents in place. The pulpal floor of each cavity was deepened until the appearance of a pink spot. A sterile sharp probe was used to expose the pulp in the cavity floor's centre. Any bleeding was managed with wetted cotton soaked with sodium hypochloride (NaOCl) 5.25% to estab-

lish haemostasis within one to ten minutes in all cases [14].

Pulp treatment

After the incisors pulp exposure, they were capped with one of the four tested capping agents. Group A: MTA was mixed with distilled water (3:1 powder-distilled water ratio) and Group B: MTA was mixed with i-PRF until they had the consistency of wet sand in two groups. Group C: BBG was combined with one to two drops of sterile saline and Group D: BBG was combined with one to two drops of i-PRF, to make a putty-like mixture. The four agents were mixed using metal spatula on a sterile glass slab, then added to the exposure with a fine amalgam carrier and lightly condensed with a moistened cotton pellet. Finally, cavities were restored with IRM [11], the two canines were used as –ve and +ve control in each dog. Teeth were left intact with no pulp exposure in the negative control, and teeth were exposed and capped with Teflon disc in the positive control.

SEM Evaluation

With the quick injection of 20 ml of 5 percent thiopental sodium solution through the cephalic vein, dogs were sacrificed after one month. Surgically, the maxilla and mandible were divided and splitted into two halves at the midline. By sectioning the jaws with a sharp saw, blocks containing a single tooth with its surrounding bone were obtained. The bone blocks containing the teeth were reduced in size to fragments measuring approximately 3 mm of coronal height and 3 mm of root height. The specimens were then stored in 10% formalin at 4°C for 72 h. after that, the pieces were gently ground with water-cooled fine-grained sandpapers until a final thickness of 2 mm on average was reached and root canal entrances were visualized. The specimens were immersed in 2.5% glutaraldehyde (Merck KGaA, Frankfurter Darmstadt, Germany) in a 0.1-mol/L sodium cacodylate buffer at pH 7.4 (Merck KGaA), for 12 h at 4°C. After fixation, the specimens were rinsed with 0.2 mol/L sodium cacodylate buffer at pH 7.4 for 1h with three changes followed by 1min. in distilled water.

Then, they were dehydrated in an ascending ethanol series (25%for 20 min; 50% for 20 min; 75% for 20 min; 90% for 30min; and 100% for 60 min). The specimens were mounted on stubs, sputter-coated with gold and examined with a SEM (XL20, Phillips, Eindhoven, Netherlands). The coronal surface of the specimen was evaluated at X150, X250 and X3000 magnifications. The images were saved as tagged image file format files and were analysed using an image analysis software (Image J software, University of Wisconsin, USA). The perimeter of the root canal area and the perimeter hard tissue barrier were delimited in order to determine the percentage of obliteration and the extension of the barrier [15].

Statistical analysis

Statistical Analysis All data were collected, calculated, tabulated and statistically analyzed using the following statistical tests Qualitative data were presented as frequencies and percentages (%). Chi-square test was used to evaluate qualitative data between the categories. All Statistical analysis were performed using the computer program SPSS software for windows version 26.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) at significant levels 0.05 (P- Value<0.05).

Results

Morphology

The results in table, showed statistically significant difference between MTA, MTA+PRF, BBG and BBG+PRF for Amorphous and Mixed using chi square test at P<0.05, while there was no tubular found. Also, the numbers of amorphous, mixed and tubular was differed from group to other and showed significant difference between them in all groups. For MTA and BBG group, the high number was amorphous with percent 86.67 and 75.0% respectively. While mixed was high in MTA+ i-PRF (85.71%) and BBG + i-PRF (100%) fig.

Table 1: Morphology of the hard tissue barriers formed with each material

Morphology										
	MTA MTA+PRF		**BBG**				**BBG+PRF**			
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**χ2**	**P value**
AMORPHOUS	13	86.67	2	14.29	12	75.00	-	-	19.963	<0.0001**
MIXED	2	13.33	12	85.71	4	25.00	16	100.00	15.412	0.0015**
TUBULAR	0	0.00		0.00		0.00	-	-	0	1
total	15	100.00	14	100.00	16	100.00	16	100.00		
χ^2	19.61	17.732	21.52	32.032						
P value	<0.0001**	<0.0001**	<0.0001**	<0.0001**						

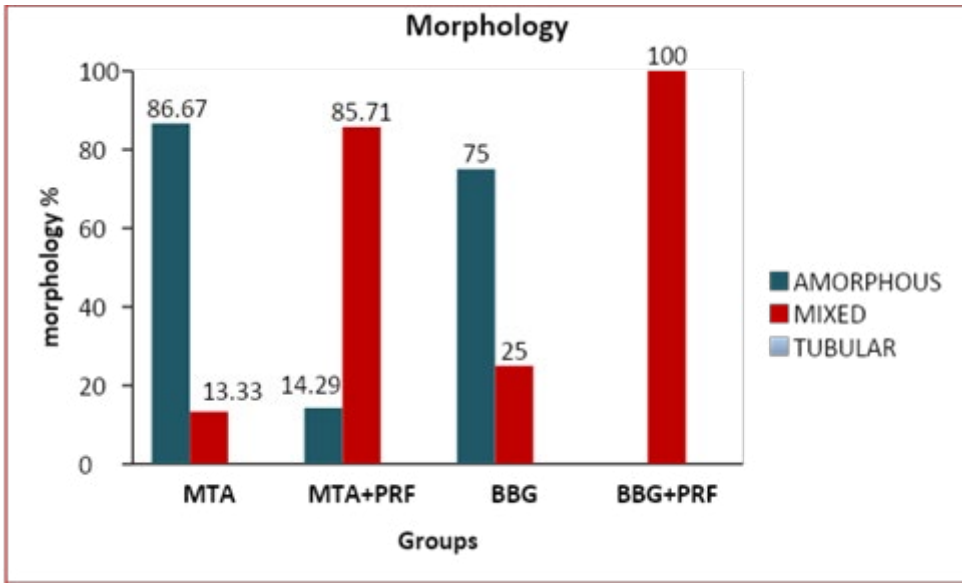

Figure 1: Morphology of the hard tissue barriers formed with each material

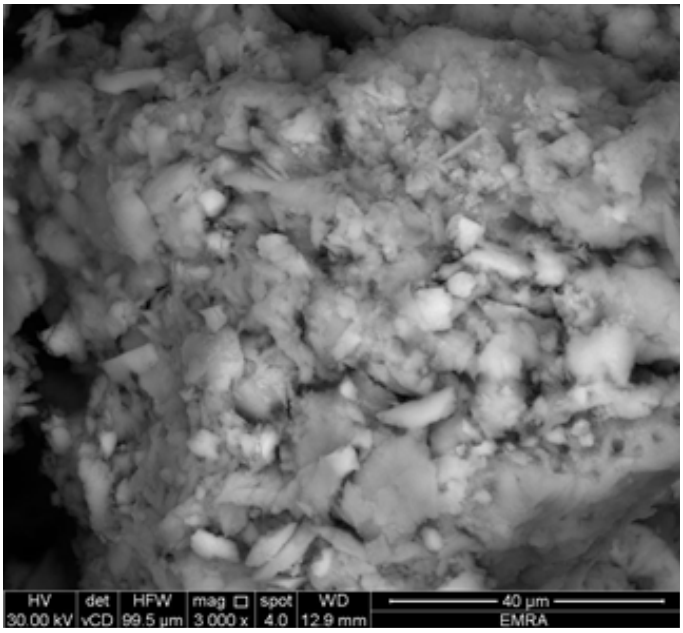

Figure 2: Morphology. Example of an amorphous MTA-induced hard tissue barrier

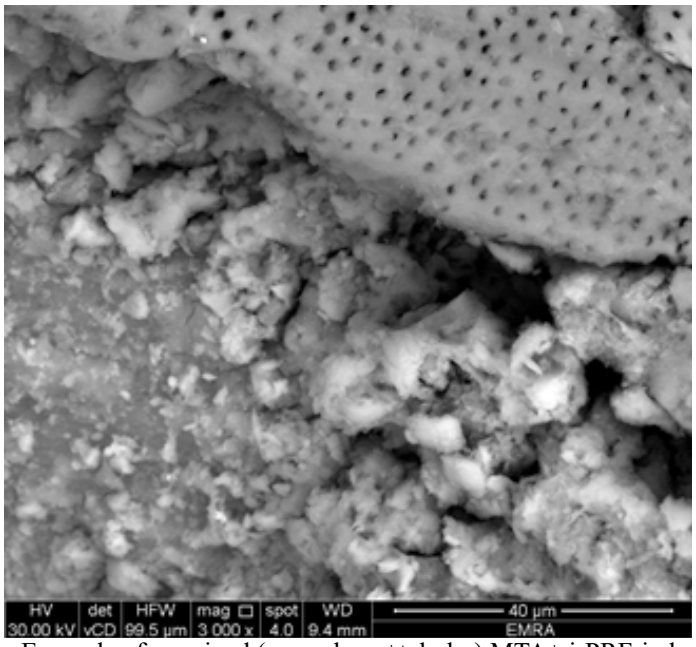

Figure 3: Morphology. Example of an mixed (amorphous+tubular) MTA+ i-PRF-induced hard tissue barrier.

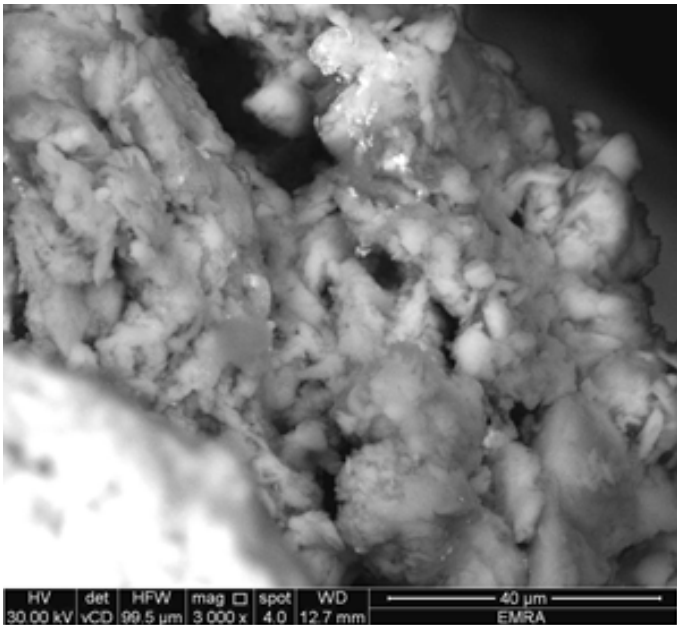

Figure 4: Morphology. Example of an amorphous BBG-induced hard tissue barrier.

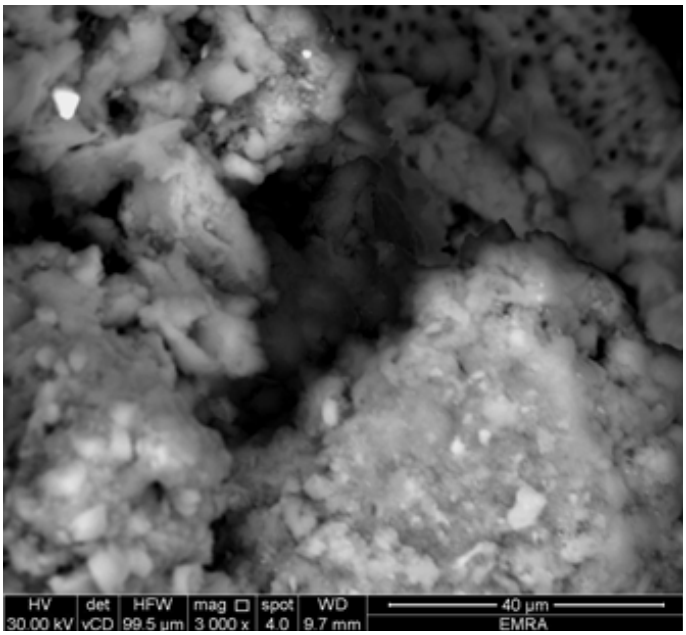

Figure 5: Morphology. Example of a mixed (amorphous+tubular) BBG+ i-PRF-induced hard tissue barrier.

Localization

The results in table, showed no statistically significant difference between MTA, MTA+PRF, BBG and BBG+PRF for central, Peripheral, And Centro-Peripheral using chi square test at P<0.05. For MTA and BBG group, the high number was central with percent 80.0 and 66.67% respectively. While Peripheral was high in MTA+ i-PRF (71.43%) and BBG+ i-PRF (68.75%) fig...

Table 2: Localisation of the hard tissue barrier formed with each material

Localization										
	MTA MTA+PRF		**BBG**				**BBG+PRF**			
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**χ^2**	**P value**
CENTRAL	12	80.00	4	28.57	10	66.67	5	31.25	5.77	0.123
PERIPHERAL	3	20.00	10	71.43	6	40.00	11	68.75	5.467	0.141
CENTRO-PERIPHERAL		0.00		0.00		0.00		0.00	0	1
total	15	100.00	14	100.00	15	100.00	16	100.00		
χ^2	15.615	10.868	9.509	11.38						
P value	<0.0001**	0.004**	0.0086**	0.0034**						

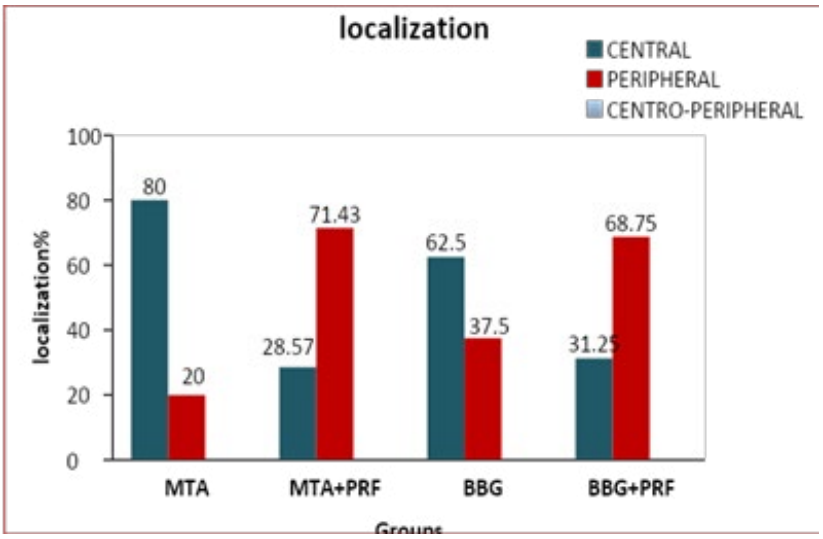

Figure 6: Localisation of the hard tissue barrier formed with each material

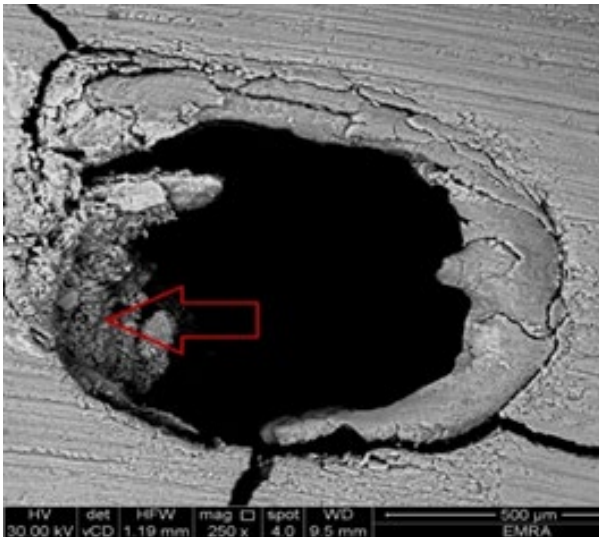

Figure 7: Localisation. Example of a Central MTA, induced hard tissue barrier.

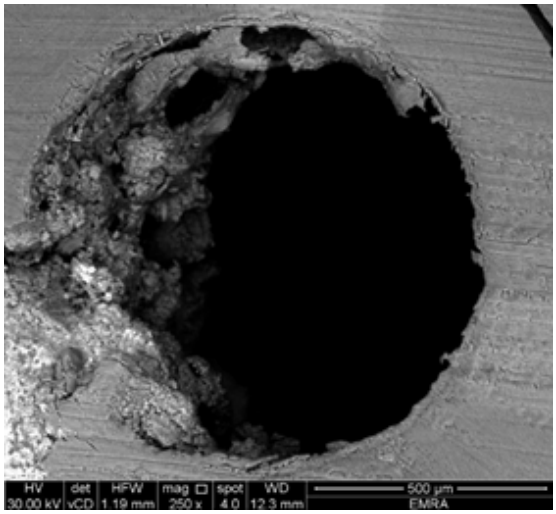

Figure 8: Localisation. Example of a peripheral MTA+ i-PRF, induced hard tissue barrier.

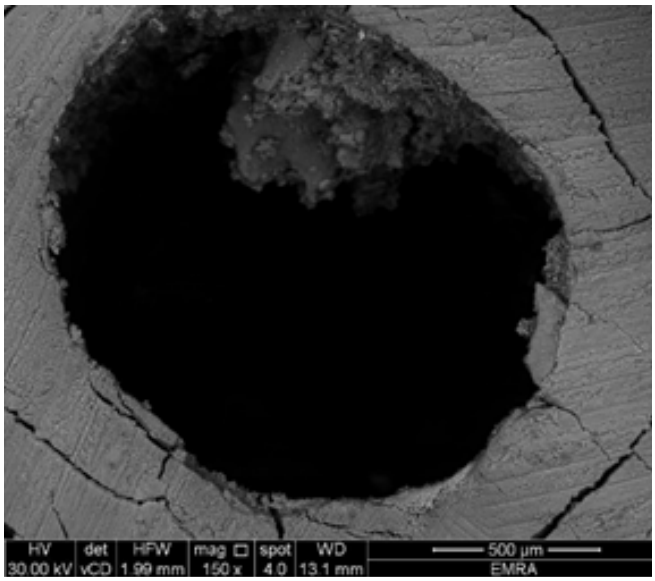

Figure 9: Localisation. Example of a Central BBG-induced hard tissue barrier.

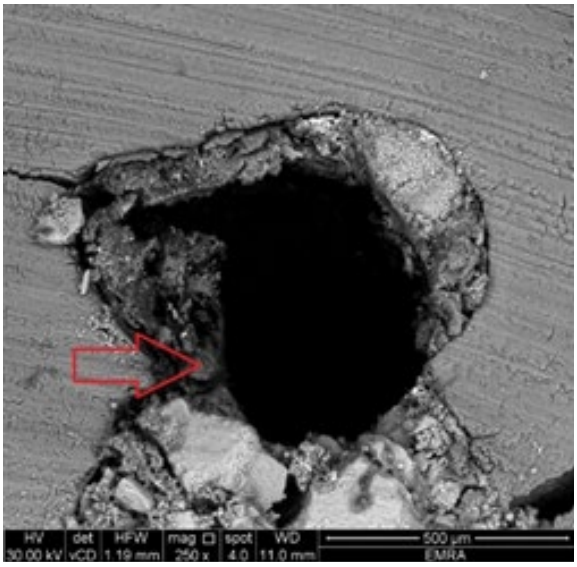

Figure 10: Localisation. Example of a peripheral BBG+ i-PRF, induced hard tissue barrier.

Discussion

Direct pulp capping treatment is intended to preserve pulp vitality in selected cases. It has been shown that one of the most important properties of a pulp-capping material is its ability to induce the hard tissue formation with high-quality [16]. Though many studies have identified the tertiary dentin, most of them have only made a histological characterization this dentin. Some of them evaluated the volume of the formed mineralized dentin by cone beam computerized tomography [17]. In this study, for the first time to the best of our knowledge, we evaluated the nature and character of the newly formed hard tissue after direct pulp capping using i-PRF mixed with bioactive materials using electron microscopy. Based on the present study that showing the good quality of the dentinal bridge obtained using these agents with i-PRF, our hypothesis in this current study was that the chemical nature of the reparative tissue is similar to that of normal dentin.

But only the inner layer of reparative dentin bridge formed in groups B and D, that mixed with i-PRF was similar to primary dentin. The quality of a calcified bridge that formed at the exposure site has been recognized as an important factor for the clinical success of direct pulp capping [18]. The main objective of an ideal capping material in exposed pulp might be complete reconstitution of the pulp periphery with a dentin-like matrix. SEM results in our study confirmed a protective dentin bridge at the site of pulp exposure and at higher magnification, dentinal tubules were seen in the newly formed mineralized tissue after capping with i-PRF with MTA and BBG, evoking a calcified tissue resembling tubular dentin specially in inner layers.

In regards to morphology, there was a statistically significant difference between MTA, MTA+ i-PRF, BBG and BBG+ i-PRF, while there was no tubular newly formed bridge found. For MTA and BBG group, the high number of samples was amorphous with percent 86.67 and 75.0% respectively, the authors reported that the amorphous areas in calcified bridge were characterized as precipitations of coarse calcium granulations, forming a superficial granular zone, which is associated with the initial synthesis and deposition of disorganised dentin matrix, one of the characteristics of a bioactive material is its ability to form an apatite-like layer on its surface when it comes in contact with physiologic fluids in vivo or with simulated body fluids Indeed, the inadequacy for true regeneration in mammals has been attributed partially to the absence of blastema formation at the site of injury (reprogramming of terminally differentiated stem cells) and partially to the rapid fibro-proliferative response that accompanies chronic inflammation and wound healing [15,19-22].

While mixed (tubular and amorphous) was high in MTA+ i-PRF (85.71%) and BBG + i-PRF (100%), despite of the external layer is irregular and atubular, but the internal one exhibits the dentinal characteristics with irregular and randomly distributed tubules. The concentration of growth factors in i-PRF plays a significant role in hard tissue formation, for example, the production of transforming growth factor-b1 has a positive effect on bioactive material in a cell culture environment encouraging for cell viability and proliferation [23,24]. It has been also noticed that, the inflammatory pulp response to MTA and BBG with i-PRF seems to be less intense [11].

This might be attributed to the fact that, immediately after preparation for use, MTA has a significantly lower pH (approximately 10.2) and during the setting period, which takes about 4 h, the pH of MTA can increase to 12.5, this initial pH of MTA, when it has been placed in contact with the exposed pulp tissue, justify the occurrence of a more severe pulp injury [25]. Therefore, there is a relationship between the influence of agents' pH and the pulp response pattern, Torabinejad et al. (1995). This process more likely represents a repair response that produces calcified scar tissues by pulpal fibroblasts [15]. In regards to localization, no statistically significant difference between MTA, MTA+ i-PRF, BBG and BBG+ i-PRF. For MTA and BBG group, the high number of samples was central with percent 80.0 and 66.67% respectively. While Peripheral was high in MTA+ i-PRF (71.43%) and BBG+ i-PRF (68.75%), the tested MTA and BBG cements presented a limited diffusion of their components from the pulp-capping site to the interior of the pulp tissue, maybe, it might have been an important cause to the presence of a central hard tissue barrier in several specimens capped with MTA and BBG.

However, Platelet concentrates can increase dental pulpal cells (DPC) proliferation and differentiation, suggesting potential applications of these as a biological molecule to promote the regeneration of lost or injured dental pulp tissues and stimulate reparative dentinogenesis Moreover, i- PRF induced great alkaline phosphatase activity, dentin sialophosphoprotein, dentin matrix protein, and mRNA expression of TGF-β, osteocalcin, PDGF, and type I collagen that stimulates the differentiation of osteoblasts and the deposition of the mineral matrix [26-28]. I-PRF was selected to be used in this study owing to its various advantages; it is in a liquid form which can be easily mixed with bioactive pulp capping materials and It acts as a source of growth factors and a scaffold at the same time. Our conclusion; most hard tissue barriers formed in the root pulps capped with i-PRF + DPC materials exhibited dentinal tubules with predominance of peripheral hard tissue barriers in the specimens.

Ethical Approval

This paper approved by Research Ethics committee (REC) of Faculty of Dentistry, Suez Canal University, Established according to WHO-2011, standards. Serial no. 89/ 2018.

Competing interests

The authors declare no conflict of interest.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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