Histologic evaluation of the healing potentiality of the human treated dentin matrix scaffold as direct pulp capping material

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Research Article

Keywords: Direct pulp capping, treated dentin matrix scaffold, trans-well migration test, antibacterial activity of treated dentin

Posted Date: July 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1775449/v1

Abstract

Aim

Find a reasonable substitution for current DPC materials.

Methods

The hTDM properties were evaluated by assessing its alkalinity, antibacterial activity, and potentiality to induce cell migration compared to MTA. The acute inflammatory response was evaluated 3 days after iatrogenic pulp exposure by histological examination. However, both chronic inflammatory response and dentin-pulp complex regeneration were evaluated after 3 months.

Results

The results of in vitro assessment revealed high alkalinity of MTA while hTDM exhibited optimum alkalinity. Moreover, the higher antibacterial activity and potentiality of inducing cell migration than MTA. The in vivo assessment ensured the ability of hTDM in optimizing the inflammatory response and dentin-pulp complex regeneration. However, MTA prolonged the inflammatory response and induced the formation of a thick calcific bridge toward the pulp tissue.

Conclusion

hTDM semi-rigid scaffold is a promising novel DPC material.

Introduction

Direct pulp capping (DPC) is a minimally invasive technique aiming to seal the exposure site and preserve pulp vitality. Several materials like calcium hydroxide ($Ca(OH)_2$), tri-calcium silicate-based cement, adhesive resin cement (ARC), and emdogain were used as DPC material [1].

Indeed, these materials developed on empirical bases rather than a deep understanding of the pulp self-healing mechanism [2]. Therefore, they interrupt pulp self-healing mechanism as they exhibit [3] ineffective sealing of the exposure site that increases the susceptibility of bacterial reinfection [4][5][6]. In addition, triggering the formation of a superficial necrotic layer results in a prolonged inflammatory response [7][8]. They induce reparative dentin formation at a supraphysiologic level leads to intra-pulpal calcification. Therefore, intra-pulpal calcification is reported in several studies using different DPC materials [9][10][11].

Nowadays, there are global efforts toward dentin-pulp complex regeneration through tissue engineering strategies using several scaffolds [1]. Collagen, hydroxyapatite (HAP), and hyaluronic acid (HA) scaffolds were used for dentin-pulp complex regeneration.

The low mechanical properties of the collagen scaffolds obstacle using them for DPC [12]. In addition, collagen scaffolds can't induce odontogenic differentiation of DPSCs. Therefore, they need combination with exogenous growth factors like BMP, Nil-like molecule-1 (NELL-1), and FGF-2 [13]. But it makes the preparation technique more complicated and expensive besides the uncontrolled diffusion of the exogenous growth factors [14]. The sub physiologic level is not efficient. While the supraphysiologic level causes inflammation, ectopic mineralization, or toxicity [15]. Kitamura et al. fabricated a composite scaffold of collagen, gelatin, and FGF-2. The incorporation of gelatin into the collagen scaffold controls the release of FGF-2. The results showed regeneration of the dentin-pulp complex and the amputated pulp in rat molars [16]. While the true clinical efficacy of this scaffold is still questionable because of its low mechanical properties and unclear information about FGF-2 dose optimization.

The most important limitation of the HAP scaffold is the high brittleness. Therefore, Okamoto et al developed a composite HAP scaffold combined with a vesico-elastic polymer like polycarbo-lactone (PCL) and BMP-2 to overcome the brittleness and improve its bio-activity. Using this scaffold in the direct pulp capping of rat molars showed a high potentiality to induce calcific bridge formation [17]. However, in vivo degradation of PCL is extended over two years which isn't suitable for the dentin-pulp complex regeneration [18]. Besides, the nature of the formed calcific bridge is osteodentin rather than dentin-pulp complex regeneration [17].

Inuyama et al. established an in vivo study to evaluate the efficacy of the HA spongy scaffold as a DPC material in rat molars. The results showed the formation of reparative dentin toward the residual pulp tissue. The continuous reparative dentin formation leads to intra-pulpal calcification [19]. The low mechanical properties and rapid degradation rate also hinder its clinical application as DPC material [20].

Human-treated dentin matrix (hTDM) revealed high healing and regenerative potentiality of periodontal ligament [21], bone, and dentinal tissues [14]. It was used in direct pulp capping treatment in the forms of human treated dentin matrix paste (TDMP) [22] and injectable treated dentin matrix hydrogel (TDMH) [23]. The low mechanical properties, intra-pulpal calcification, and time-consuming preparation hinder using it in dental clinics [24]. This study presents a novel hTDM semi-rigid scaffold that aims to overcome the limitations of the previous scaffolds and current DPC materials.

Material & Methods

1. Fabrication of hTDM scaffolds

The fabrication procedures of the hTDM scaffolds followed the good manufacturing practice (GMP) and the ethical committee of Faculty of Dentistry, Cairo University and. Forty sound and fully erupted first premolars were extracted from ten patients after obtaining their written informed consent. The extraction procedure was involved in their orthodontic treatment plan. The patients were free from any systemic or local diseases that affect the dental tissues. Their mean age was ranging from 19 to 22 year.

After extraction the periodontal ligament tissue, cementum and a part of root dentin were removed by grinding with high-speed fissure carbide bur under an effective water coolant system. After that, the root surface became flat without curvatures then, the crown was separated from the root by a wheel-shaped disc. Finally, the pulp tissue and pre-dentin were removed using roughly mechanical instrumentation and irrigation with ethylene diamine tetra-acetic acid (EDTA) 10%[22].

The 3D dentin scaffolds were prepared through serial cutting of the prepared root dentin in three directions, two vertical and one horizontal using isomat (precision cutter, PICO 155, USA). After cutting, the 3D dentin scaffolds were in the form of cuboids within 1×1×0.5 mm in diameter.

The treatment of the dentin was carried out as described by Chen et al., 2017. Dentin cuboids were immersed in the deionized water for 5hrs. The deionized water changed after every 1hr followed by mechanical cleaning for 20min using an ultrasonic cleaner (VGT 1200H, China). After that, the dentin cuboids were exposed to descending concentrations of EDTA (Sigma, USA) started with soaking in 17% EDTA for 5 min then washing with the deionized water for 10 min using the ultrasonic cleaner. They were then sunken in 10% EDTA for 5 min followed by washing with the deionized water for 10 min using the ultrasonic cleaner. Finally, they were sunken in 5% EDTA for 10 min, followed by washing with deionized water using the ultrasonic cleaner. The produced hTDM scaffolds were preserved within a sterile phosphate buffer saline (PBS; Gibco) supplemented with 100 units\ml penicillin (Gibco) and 100 µg\ml streptomycin (Gibco) for 72hr. The last step was washing the hTDM scaffolds with deionized water for 10 min using the ultrasonic cleaner. Finally, they were stored in Dulbecco's modified Engel's media F12 (DMEM-F12; Lonza) at 4^{o}C[24].

2. In vitro assessment of hTDM scaffold

4.1. Alkalinity test

This test was performed at Chemistry laboratory, Faculty of Pharmacy, Delta University for Science and Technology, Egypt. This test included 3 groups; negative control (I), MTA (II) as positive control, and hTDM (III). Group I consisted of 5 tightly closed glass test tubes that contained 5ml DMEM-F12 supplemented with 10% FBS (Gibco), 1% 100 units \ ml penicillin, and 100 µg\ ml streptomycin (Gibco). MTA, and hTDM groups were handled as group I and instead one MTA disc and one hTDM were dropped in each tube containing 5ml complete media. The weight of each disc and scaffold was about 25 mg[25], sterilized using ultraviolet light (UV) for half an hour. All tubes were incubated at 37 ^{o}C and 5% CO_2 for 24 h. The changes in pH were detected by the changes in the complete media color and measuring the pH value by pH meter (HI 2211, USA).

4.2. Antibacterial activity test

This test was performed at Microbiology Department, Faculty of Medicine, Mansoura University, Egypt. To obtain bacterial colonies, infected dentin was excavated from five carious teeth using a sharp spoon excavator after obtaining patients' informed consent. The infected dentin was incubated on a blood agar at 37 ^{o}C and 5% CO_2 for 24h.

After the incubation period, bacterial colonies appeared that were distinguished morphologically into two types. The cloudy white colonies were *streptococcus mutans* and the granular white ones were *staphylococcus aureus*. Colonies of *streptococcus mutans* were cultured in 10, however, *staphylococcus aureus* were cultured in 10 Petri dishes containing.

Antibacterial test against *streptococcus mutans* included 3 groups; negative control (I), MTA (II) as positive control, and hTDM (III). Group I contained *streptococcus mutans* that cultured on blood agar in 5 Petri dishes. A filter paper witted with distilled water was centered in the blood agar. MTA, and hTDM groups were handled as group I and instead one MTA disc and one hTDM scaffold were centered in the blood agar. All Petri dishes were incubated at 37 ^{o}C and 5% CO_2 for 24h. The antibacterial test against *staphylococcus aureus* was designed as *streptococcus mutans* test and instead the *staphylococcus aureus* was cultured on nutrient agar[25].

4.3. Trans-well migration assay

This assay was performed at the National Research Center, Cairo, Egypt. It was performed according to Checklist for Reporting In-vitro Studies (CRIS) guidelines. Trans-wells (upper chamber) with pore size 8.0 µm polycarbonate membrane inserts (Costar, USA), 24 well-plate (lower chambers), and 6th passaged human dental pulp stem cells (hDPSCs) isolated via outgrowth expansion technique from 5 sound molars extracted for surgical purposes were used for this assay.

There were 3 groups; negative control group (I) that was consisted of 3 wells from the 24 well-plate. Each wells (lower chambers) contained 750 µl DMEM-F12 supplemented with 10% FBS (Gibco), 1% 100 unit \ ml penicillin, and 100 µg\ ml streptomycin (Gibco). MTA (II) and hTDM (III) groups were handled as group I and instead MTA discs and hTDM scaffolds were placed with the complete media in the lower chamber.

The trans-wells were inserted into each lower chamber of the 24 well-plate. Each trans-well contained a cell suspension of 2.5 × 10^5 hDPSCs and 200 µl serum-free medium. After that, the cells were incubated at 37 ^{o}C and 5% Co_2 for 24 h. This test had repeated three times under the same conditions. After the

incubation period, the culture medium was removed gently from the trans-well chambers and then washed in PBS (Gibco) twice. The non-migrating cells inside the trans-well were scraped gently using a cotton swab.

According to the protocol of He et al., the cells that migrated to the lower side of the membrane of the trans-well were fixed by immersing in 4% formaldehyde at room temperature for 15 min then washed twice in PBS. The trans-wells were immersed in 100% methanol for 20 min at room temperature to increase the cell membrane permeability. After methanol removal, the trans-wells were washed twice in PBS before staining. The trans-wells were immersed in Giemsa stain for 15 min at room temperature. After that, the Giemsa stain was removed and the trans-wells were washed twice in PBS[26]. After staining migratory cells, they were examined by an inverted light microscope and captured by Olympus photomicroscope at X10 magnification. The cells from three different fields were counted and used for analysis using Image-J (version 1.8.0) software.

3. In vivo assessment of hTDM scaffold

4.1. Study design

The participants were free from any systemic or local diseases that affect the dental tissues. Their upper and lower first premolars were planned to be extracted in their orthodontic treatment plan. Their mean age was ranging from 19 to 22 year.

The sample size was determined using G*Power 3.1.9.2 software (Heinrich- Heine-Universita¨t Düsseldorf, Germany). Means: Difference between two independent means (two groups) and the power analysis was A priori: compute the required sample size. The input parameters were Tails: 2, Effect size d = 0.80, α error probability = 0.05, power = 0.95, and Allocation ratio N2/N1-1. The calculated total sample size required was 64 premolar from 16 patient. According the split-mouth design for each patient, MTA and hTDM scaffolds were assigned randomly to each first premolar (www.randomizer.org). A schematic diagram was drawn for each patient to detect in which premolar the DPC material was assigned. According to follow-up periods, Patients were randomly distributed into two groups using the research randomizer site (www.randomizer.org). In group I, premolars were extracted after 3 days for histologic assessment of the pulp acute inflammatory response. In group II, teeth were extracted after 3 months for histologic assessment of the pulp chronic inflammatory response and dentin-pulp complex regeneration.

4.2. Operative procedures

The operative procedures were performed under local anaesthesia. Class V circular cavities of dimensions 2–3 mm in diameter were prepared on the buccal surface of the included premolars using tungsten carbide round burs ISO #806 314 001534 012 and carbide fissure burs ISO #500 314 107006 008 (Komet, Lemgo, Germany) at ultra-high speed with copious air/water spray. Each bur was used only for 2 cavities to maintain the cutting efficiency. The cavities were prepared at 2–3 mm above the free gingival margin and parallel to the cemento-enamel junction. When the redness of the pulp chamber became visible, a new sterile round tungsten carbide bur ISO #500 314 001001 (0.8 mm in diameter; Komet, Lemgo, Germany) was used under high speed and water cooling to expose the pulp. The size of pulp exposure was 0.8–1.

The cavities were rinsed with sterile saline and dried with sterile cotton pellets. The exposure sites were capped with hTDM scaffold and MTA according to the spilt mouth design. Finally, conventional glass ionomer was used to seal all cavities.

4.3. Histologic evaluation

The extracted premolars were fixed in 10% neutral formalin for 24hr after removal of the apical apexes. After that, washed under gentile running water for 10min, then demineralized using 10% EDTA for 18 month (according to the practical following up for demineralization process). After complete demineralization, teeth were dehydrated and embedded in a melted paraffin wax. The paraffin wax blocks containing teeth were sectioned in the bucco-lingual direction using microtome adjusted at 5μm for the thickness of each section[27].

The sections were stained with Haematoxylin and eosin stains for investigating the pulp inflammatory response. A blinded histologist evaluated each section using a light microscopy (HMF X100). The score of pulp inflammatory response was as in the following; Score (0) means none inflammatory response without or few scattered inflammatory cells. Score (1) means mild inflammatory response with inflammatory cells limited to area of pulp exposure or dentin bridge. Score (2) means moderate Inflammatory response with inflammatory cells infiltration more than one-third, but not all of the coronal pulp. Score (3) means severe inflammatory response and all of the coronal pulp is infiltrated with the inflammatory cells.

The reparative dentin formation and thickness at the exposure site was observed in photomicrographs that were captured with a charge coupled device digital camera (Nikon E8400, Sendai, Japan) (HMF X40). The new dentin thickness was measured at its peripheries and the middle portion then analyzed using Image J Software (Version 1.8.0). The new dentin thickness under both MTA and hTDM was compared with the pre-dentin thickness that representing the physiologic limit of the dentinogenesis process.

4. Statistical analysis

The statistical analyses were performed using SPSS soft-ware (version 20). Comparing between hTDM and MTA in accordance to alkalinity, antibacterial activity, inducing hDPSCs migration potentiality, and dentin bridge thickness were statistically analyzed using one way-ANOVA test. However, the pulp inflammatory response against hTDM and MTA was statistically analyzed using Mann-Whitney test. For all tests, data were expressed as mean ± standard deviation (SD). The statistical significant differences were accepted for p value ≤ 0.05.

Results

1. In vitro assessment of hTDM scaffold

2.1. Alkalinity test

The immediate observation was the change in the color of the culture media from pink to violet after placement of the MTA discs and the color didn't change after adding hTDM. After 24 h, the intensity of the violet color increased with the MTA group. However, the pink color remained stable in hTDM and the control group. The descriptive statistics for the mean and SD of the pH meter readings showed that the highest pH mean was for MTA (9.80 ± 0.44) while the lowest one was for the control (8.20 ± 0.83). The pH means for hTDM was 8.30 ± 0.74. The results of the One way-ANOVA statistical test revealed a significant difference (P = 0.002) among groups. The LSD post-hoc test revealed a significant difference between the control group and MTA (P = 0.001), MTA and hTDM group (P = 0.001) and non-significant difference between control and hTDM (P = 0.798) (Table 1).

Table 1
Results of one way-ANOVA and LSD statistical tests for pH values among different groups.

Groups	Mean ± SD	LSD	*P* value
Control	8.20 ± 0.83	Control x MTA	0.001
MTA	9.80 ± 0.44	Control x hTDM	0.798
hTDM	8.30 ± 0.74	MTA x hTDM	0.001
ANOVA (F ratio, *P* value)	10.955, 0.002		

2.2. Antibacterial activity

The results of culture sensitivity test revealed that there wasn't inhibition zone formed in the negative control group. The MTA formed a small inhibition zone, when cultured with *streptococcus mutans and staphylococcus aureus*. The inhibition zone with *streptococcus mutans* was 0.48 ± 0.02 cm while it was 0.33 ± 0.02 with *staphylococcus aureus*. However, the hTDM scaffold formed an abundant inhibition zone, when cultured with the same types of bacteria. The inhibition zone around the hTDM scaffold was 2.76 ± 0.44 cm with *streptococcus mutans*, while it was 1.58 ± 0.37 cm with *staphylococcus aureus*. This denoting a higher antibacterial activity of the hTDM scaffold when compared to MTA. The results of the Independent samples T-test revealed a significant difference between the groups for *streptococcus mutans* (P = 0.001) and *staphylococcus aureus* (P = 0.001) (Table 2).

Table 2
Results of the independent sample t-test and its statistical significance for streptococcus mutans and staphylococcus aureus culture test between MTA and ChTDM.

Test	Mean ± SD		Lower	Upper	T	df	*P* value
	MTA	hTDM					
streptococcus mutans	0.48 ± 0.02	2.80 ± 0.04	2.33	3.26	113.1	8	0.001
staphylococcus aureus	0.33 ± 0.02	1.60 ± 0.03	1.03	2.16	14	8	0.001

2.3. Cell migration potentiality

The trans-well migration assay results revealed a highest potentiality of the hTDM scaffold in inducing the hDPSCs migration when compared to control and MTA groups. However, MTA exhibited the lowest potentiality in inducing cell migration when compared to control and hTDM groups. The descriptive statistics for the mean and SD of the migrated cells' number showed that the highest mean number was for hTDM (43693.00 ± 50.12) while the lowest one was for MTA (10863.00 ± 40.23) and the mean number of the migrated cells in the control group was 20633.00 ± 60.33. The results of the one way-ANOVA test revealed a significant difference (P = 0.001) among the different groups. The LDS post-hoc test revealed a significant difference between control group and MTA, control group and hTDM group and between MTA and hTDM groups (Table 3).

Table 3
Results of one way-ANOVA and LSD statistical tests for the number of migrated hDPSCs with the different groups.

Groups	Mean ± SD	LSD	*P* value
Control	20633.00 ± 60.33	Control x MTA	0.001
MTA	10683.00 ± 40.23	Control x hTDM	0.001
hTDM	43693.00 ± 50.12	MTA x hTDM	0.001
ANOVA (F ratio, *P* value)	286737700.0, 0.001		

2. In vivo assessment of hTDM scaffold

1.1. Inflammatory response after three days

The histological examination of MTA group after three days revealed score 1 and score (2) acute inflammatory response. Score (1) means mild inflammatory response in which the inflammatory cells are limited to area of pulp exposure. However, score 2 means moderate inflammatory response in which inflammatory cells' infiltration is limited one-third of the coronal pulp. Meanwhile, the histological examination of hTDM group after three days revealed score (0) inflammatory response in which the inflammatory cells are absent. In addition, formation of new blood vessels extended toward the pulp exposure site (Fig. 1).

The results of the Mann-Whitney test of the non-parametric data showed a statistically significant difference ($P \leq 0.001$) between the two groups. The higher mean Rank of the inflammatory response was for MTA group (44.5), while the lower one (25.5) was for hTDM scaffold group (Table 4).

Table 4
Showing the results of the Mann-Whitney test after three days for MTA and hTDM groups

	Groups	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Asymp. Sig. (2-tailed)
Inflammatory Response	hTDM	32	25.50	2144.00	64.000	0.000
	MTA	32	44.50	6112.00		
	Total	64				

1.2. Inflammatory response after three months

The histological examination of the teeth that were treated with MTA after three months revealed Score (1) chronic inflammatory response in which the inflammatory cells are limited to the area of calcific bridge. However, the histological examination of hTDM group after three months revealed Score (0) inflammatory response in which the inflammatory cells are absent.

Regarding the chronic inflammatory response, the results of the Mann-Whitney test of the non-parametric data showed a statistically significant difference ($P \leq 0.001$) between the two groups. The higher mean Rank of the inflammatory response was for MTA group (39.5), while the lower one (25.5) was for hTDM scaffold group (Table 5).

Table 5
Shows the results of the Mann-Whitney test and its statistical significance difference and the mean rank between groups after three months

	Groups	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Asymp. Sig. (2-tailed)
Inflammatory Response	hTDM	32	25.50	816.00	64.000	.000
	MTA	32	39.50	1264.00		
	Total	64				

1.3. Healing process and reparative dentin assessment

The histological examination of the teeth that were treated with MTA after three months revealed that the healing process was accomplished by forming a calcific bridge without any distinct structures. The calcific bridge appeared with an uneven thickness towards the pulp chamber. That reveals to the direction of the healing process towards the pulp chamber rather than the exposure site. There wasn't a distinct odontoblasts layer under that calcific bridge but scattered inflammatory cells instead.

However, the histological examination of hTDM group after three months revealed (Fig. 2) that the healing process was accomplished by formation of new pulp tissue along the hTDM scaffold surface towards the exposure site revealing proper direction of the healing process towards the exposure site. The newly formed pulp tissue appeared with a distinct odontoblast layer. Formation of a homogenous dentin layer with an even thickness sealing the exposure site.

Regarding the dentin thickness, the descriptive statistics for the mean and the standard deviation of the dentin thickness showed that the thickest dentin had observed with teeth treated with MTA (15.06 ± 0.564). However, the thinnest one was the pre-dentin thickness (6.81 ± 0.397), while it was (6.97 ± 0.474) with teeth treated hTDM. The results of one way-ANOVA test for the thickness of the formed dentin revealed a significant difference ($p \leq 0.001$) among the different groups. The LDS post-hoc test results revealed a significant difference between the thickness of dentin formed by MTA and hTDM and pre-dentin thickness ($P \leq 0.199$). However, there was a non-significant difference between the thickness of dentin formed by hTDM and pre-dentin thickness ($P \leq 0.199$) (Table 6).

Table 6

Shows the mean and the standard deviation of the dentin thickness

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
hTDM	32	6.97	0.474	.084	6.80	7.14
MTA	32	15.06	0.564	.100	14.86	15.27
Pre-dentin	32	6.81	0.397	.070	6.67	6.96
Total	96	9.61	3.902	.398	8.82	10.41

Discussion

The alkaline pH (7.3–8) is crucial for the odontogenic differentiation of DPSCs [7]. The alkalinity test results reveal a non-significant difference between hTDM and the control group (pH = 8.2). That ensures the ability of the hTDM to maintain the micro-environment pH. However, MTA raised the pH value to 9.8. After adding the MTA disc to the complete medium, its pink color changed into violet immediately. That indicates the high alkalinity of MTA. These results were consistent with studies of Tsai et al. and Luczaj-Cepowicz et al. that detected the immediate increase of MTA pH value from 10.2 to 12.5 and then decreased to 10.2 again after 3hrs [8][28].

The results of the antibacterial test reveal bacteriostatic activity of the hTDM. The PDGF & FGF released from the hTDM exhibit bacteriostatic activity against Gram-positive and Gram-negative bacteria [29] [30]. Contrary to MTA, the results reveal a low antibacterial activity of the MTA against the cariogenic bacteria. These results were consistent with the study established by Huang et al. that investigated the antibacterial activity of MTA. It revealed the inferiority of the MTA when compared to Biodentine and Ca $(OH)_2$ [31].

The results of the Trans-well migration test revealed a higher ability of hTDM to induce DPSCs migration than MTA. That is due to the release of TGF-β1 from the hTDM [32]. However, the high alkalinity of MTA may inhibit the DPSCs migration. These results were consistent with the study established by Avery et al. that detected a high bio-activity of hTDM via testing its ability to induce BMSCs proliferation and migration [15].

In the in vivo assessment, the teeth treated with MTA showed scores of 1 and 2 pulp inflammatory response after 3days. That reveals a prolonged inflammatory phase that delays the proliferative and regenerative phases. However, MTA inhibits the expression of Bcl-2 and Bcl-xL and upregulates Bax expression[8]. Therefore, a superficial necrotic layer formed at the MTA-pulp tissue interface. During this time the pulp-self healing mechanism tries to tolerate the pulp injury and compensate for the necrotic layer that prolongs the inflammatory phase more than the physiologic level.

While in the second follow-up period (after three months), the teeth treated with MTA expressed a score (1) pulp inflammatory response even after calcific bridge formation. The calcific bridge thickness formed by MTA was thicker than the normal pre-dentin. Moreover, it was formed towards the pulp tissue obliterating the pulp chamber. These results are in agreement with the studies of Liu et al. and Oliveira et al. that detected uncontrolled mineralization and intra-pulpal calcification after using MTA as a DPC material [9],[10].

However, the teeth treated with the hTDM scaffolds exhibited a non-inflammatory response score (0) besides the formation of new blood vessels at the exposure site after three days. The novel hTDM proves the extreme advantages of being a natural body product having high biocompatibility with the pulp tissue. Furthermore, it didn't disturb the pH in the intra-pulpal environment.

Furthermore, the histologic sections' examination of the teeth treated with hTDM after three months revealed the formation of an organized new pulp tissue with a distinct odontoblast layer along the hTDM surface. In addition, sealing the exposure site with newly formed dentin that adhered to the scaffold surface and the normal existing dentin makes the scaffold a part of the tooth structure. That explains the retention of the hTDM scaffold in histologic sections even after 18 months of the decalcification process.

The comparison between the new dentin and the pre-dentin thickness revealed new dentin formation at the physiologic rate. That is due to the presence of DGP in the hTDM scaffold (as a natural component) that inhibits bio-mineralization by preventing additional minerals' deposition [33]. In addition, the ability of the hTDM scaffold to release the endogenous cytokines and growth factors at the physiologic level [15]. Optimizing the mineralization process is mandatory to avoid intra-pulpal calcification. Therefore using the hTDM scaffold as a direct pulp capping material didn't alter the physiologic pulp-self healing mechanism.

The suggested healing mechanism induced by the hTDM scaffold involves four phases. The first one is hemostasis which starts a few seconds after the pulp exposure. The uncontrolled bleeding from the exposure site was intended before scaffold placement to allow wetting of the scaffold surface with blood. As a result of the high surface wettability of the scaffold, a resilient union formed between the blood clot and the scaffold surface [21]. This union provides a sealing barrier at the exposure site [34]. The growth factors released from the blood clot and hTDM co-ordinate to startup the inflammatory phase in the healing mechanism.

The inflammatory phase involves early and late inflammatory responses. The early inflammatory response started 24hr after the pulp exposure under the influence of PDGF and IL-6 released from the platelets in the blood clot and hTDM scaffold. IL-6 attracts neutrophils to the injury site, while PDGF activates the resident M2 macrophages and T-cells to release IL-1, IL-6, and IL-ß1 for attracting more neutrophils. The attracted neutrophils and activated M2-macrophages

phagocytize the micro-organism and foreign bodies at the exposure site. At the end of the early inflammatory response, M2 macrophages release TNF-α inducing neutrophils apoptosis [35].

The late inflammatory response started after 48hr after the pulp exposure. The monocytes were attracted to the exposure site and activated into M1 macrophages under PDGF and IL-6 influence. The M1 macrophages completed the phagocytosis process and released collagenase for cleaning the injury site. After 3days, the inflammatory phase ended, and M1 macrophages underwent apoptosis [36]. Therefore, after three days, the histologic sections of the teeth treated with hTDM scaffold showed a non-inflammatory reaction.

The third phase is the proliferative phase which starts immediately after the inflammatory phase. The released VEGF and FGF-2 from the hTDM scaffold[37] [30] and macrophages [36] orchestrate that phase. In response to VEGF, the pericytes detached from the outer wall of the blood vessels and then differentiated into new endothelial cells. After that, they migrated to the low oxygen tension area where they formed new blood vessels through angiogenesis. The formation of new blood vessels is a pre-requisite for the last phase of the healing mechanism “Regenerative phase” [34]. After three days, the histologic sections of the teeth treated with the hTDM scaffold showed the formation of new blood vessels at the exposure site. This observation ensured the power of the hTDM in optimizing the healing process at the physiologic level without any deviation. However, FGF-2 induced proliferation and migration of the fibroblasts and DPSCs.

During the regenerative phase, after fibroblasts’ crawled from the cell rich-zone to the exposure site, they drilled tunnels in the provisional matrix of the fibrin clot, then produced collagen, fibronectin, and proteoglycan. These products of the fibroblasts were the components of the new extracellular matrix of the regenerated pulp tissue towards the hTDM surface. Under the influence of TGF-β1 released from the hTDM, the DPSCs differentiated into pre-odontoblasts [38]. The hTDM scaffold released dentin sialophospho-protein (DSPP) that induced the pre-odontoblasts differentiation into mature odontoblasts [39]. But, DSPP wasn’t the only factor that controlled the odontogenic differentiation of the pre-odontoblasts. Many previous studies ensured the nano-tubular pattern and high surface wettability of hTDM. These surface properties enhance DPSCs spreading, attachment, adhesion, and odontogenic differentiation. That alters their morphology by forming long cytoplasmic processes (odontoblastic processes) toward the opened dentinal tubules at the scaffold surface [40][41][42]. Cellular migration to the hTDM scaffold explain its ability to direct the dentinogenesis towards the exposure site rather than the pulp tissue.

Conclusions

The in vitro assessment of hTDM scaffold exhibited that it is an excellent nano-environment for DPSCs migration. The hTDM scaffold provides optimum pH that favorable for dentinogenesis process as compared to MTA. In addition, superior antibacterial activity of hTDM scaffold as compared to MTA.

The in vivo assessment revealed the high biocompatibility of hTDM scaffold with the pulp tissue. Besides, its superiority in directing the healing process towards the exposure site rather than the residual pulp tissue avoiding pulp chamber obliteration. The ability of hTDM scaffold to optimize the mineralization process at the physiologic level makes it a reasonable natural substitute for the current DPC material.

Recommendations

- For a precise understanding of the healing mechanism by hTDM scaffold, further researches recommended at earlier and later endpoints.
- Cryopreservation of the hTDM scaffold is recommended to overcome time-consuming preparation technique.
- Although the results of the present study favor the use of hTDM scaffold as a novel DPC material, it should be considered that the present research was conducted under optimal circumstances where all teeth used were intact Therefore, further studies using larger sample size and including carious teeth complicated with reversible pulpitis are recommended to confirm the current results.
- Further studies are recommended for acquiring the hTDM scaffold a bactericidal effect without decreasing its cellular biocompatibility.

Declarations

Funding

No funding

Informed consent

Written informed consent

Consent of publication

Not applicable

Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Acknowledgements

Not applicable

References

1. D. G. Moussa and C. Aparicio, “Present and future of tissue engineering scaffolds for dentin-pulp complex regeneration,” Journal of Tissue Engineering and Regenerative Medicine. 2019, doi: 10.1002/term.2769.
2. M. Okamoto, Y. Takahashi, S. Komichi, P. R. Cooper, and M. Hayashi, “Dentinogenic effects of extracted dentin matrix components digested with matrix metalloproteinases,” Sci. Rep., vol. 8, no. 1, pp. 1–17, 2018, doi: 10.1038/s41598-018-29112-3.
3. J. C. Farges *et al.*, “Dental Pulp Defence and Repair Mechanisms in Dental Caries,” *Mediators of Inflammation*, vol. 2015. 2015, doi: 10.1155/2015/230251.
4. P. Trongkij, S. Sutimuntanakul, P. Lapthanasupkul, C. Chaimanakarn, R. H. Wong, and D. Banomyong, “Pulpal responses after direct pulp capping with two calcium-silicate cements in a rat model,” Dent. Mater. J., vol. 38, no. 4, pp. 584–590, 2019, doi: 10.4012/dmj.2018-225.
5. L. E. Monfoulet *et al.*, “The pH in the microenvironment of human mesenchymal stem cells is a critical factor for optimal osteogenesis in tissue-engineered constructs,” Tissue Eng. - Part A, 2014, doi: 10.1089/ten.tea.2013.0500.
6. G. M.G. *et al.*, “Calcium silicate and calcium hydroxide materials for pulp capping: Biointeractivity, porosity, solubility and bioactivity of current formulations,” J. Appl. Biomater. Funct. Mater., 2015, doi: 10.5301/jabfm.5000201 LK - http://xv9lx6cm3j.search.serialssolutions.com/?sid=EMBASE&issn=22808000&id=doi:10.5301%2Fjabfm.5000201&atitle=Calcium+silicate+and+calcium+hydroxide+materials+for+pulp+capping%3A+Bi
7. N. Akhlaghi and A. Khademi, “Outcomes of vital pulp therapy in permanent teeth with different medicaments based on review of the literature,” Dental Research Journal. 2015, doi: 10.4103/1735-3327.166187.
8. C. L. Tsai *et al.*, “Mineral trioxide aggregate affects cell viability and induces apoptosis of stem cells from human exfoliated deciduous teeth,” BMC Pharmacol. Toxicol., vol. 19, no. 1, pp. 1–10, 2018, doi: 10.1186/s40360-018-0214-5.
9. Q. Liu, Y. Ma, J. Wang, X. Zhu, Y. Yang, and Y. Mei, “Demineralized bone matrix used for direct pulp capping in rats,” PLoS One, vol. 12, no. 3, pp. 1–13, 2017, doi: 10.1371/journal.pone.0172693.
10. T. M. Oliveira *et al.*, “Clinical, radiographic and histologic analysis of the effects of pulp capping materials used in pulpotomies of human primary teeth,” Eur. Arch. Paediatr. Dent., 2013, doi: 10.1007/s40368-013-0015-x.
11. A. B. Paula *et al.*, “Evaluation of dentinogenesis inducer biomaterials: An in vivo study,” J. Appl. Oral Sci., vol. 28, pp. 1–13, 2020, doi: 10.1590/1678-7757-2019-0023.
12. M. M. Villa, L. Wang, J. Huang, D. W. Rowe, and M. Wei, “Bone tissue engineering with a collagen-hydroxyapatite scaffold and culture expanded bone marrow stromal cells,” J. Biomed. Mater. Res. - Part B Appl. Biomater., 2015, doi: 10.1002/jbm.b.33225.
13. J. Wu *et al.*, “Effects of Nel-like molecule-1 and bone morphogenetic protein 2 combination on rat pulp repair,” J. Mol. Histol., vol. 50, no. 3, pp. 253–261, 2019, doi: 10.1007/s10735-019-09822-2.
14. X. Gao *et al.*, “Nano-structured demineralized human dentin matrix to enhance bone and dental repair and regeneration,” Appl. Sci., vol. 9, no. 5, 2019, doi: 10.3390/app9051013.
15. S. J. Avery, L. Sadaghiani, A. J. Sloan, and R. J. Waddington, “Analysing the bioactive makeup of demineralised dentine matrix on bone marrow mesenchymal stem cells for enhanced bone repair,” Eur. Cells Mater., 2017, doi: 10.22203/eCM.v034a01.
16. C. Kitamura, T. Nishihara, M. Terashita, Y. Tabata, and A. Washio, “Local regeneration of dentin-pulp complex using controlled release of FGF-2 and naturally derived sponge-like scaffolds,” International Journal of Dentistry. 2012, doi: 10.1155/2012/190561.
17. M. Okamoto *et al.*, “Performance of a biodegradable composite with hydroxyapatite as a scaffold in pulp tissue repair,” Polymers (Basel)., 2020, doi: 10.3390/POLYM12040937.
18. S. Sánchez-González, N. Diban, and A. Urtiaga, “Hydrolytic degradation and mechanical stability of poly(ε-Caprolactone)/reduced graphene oxide membranes as scaffolds for in vitro neural tissue regeneration,” Membranes (Basel)., vol. 8, no. 1, 2018, doi: 10.3390/membranes8010012.
19. Y. Inuyama *et al.*, “Effects of hyaluronic acid sponge as a scaffold on odontoblastic cell line and amputated dental pulp,” J. Biomed. Mater. Res. - Part B Appl. Biomater., 2010, doi: 10.1002/jbm.b.31497.
20. C. Chung, M. Beecham, R. L. Mauck, and J. A. Burdick, “The influence of degradation characteristics of hyaluronic acid hydrogels on in vitro neocartilage formation by mesenchymal stem cells,” Biomaterials, 2009, doi: 10.1016/j.biomaterials.2009.04.040.
21. D. L. Baker, S. A. Stanley Pavlow, and U. M. E. Wikesjö, “Fibrin clot adhesion to dentin conditioned with protein constructs: An in vitro proof-of-principle study,” J. Clin. Periodontol., 2005, doi: 10.1111/j.1600-051X.2005.00714.x.
22. P. Mehrvarzfar, P. V. Abbott, F. Mashhadiabbas, M. Vatanpour, and S. Tour Savadkouhi, “Clinical and histological responses of human dental pulp to MTA and combined MTA/treated dentin matrix in partial pulpotomy,” Aust. Endod. J., vol. 44, no. 1, pp. 46–53, 2018, doi: 10.1111/aej.12217.
23. A. A. Holiel, E. M. Mahmoud, W. M. Abdel-Fattah, and K. Y. Kawana, “Histological evaluation of the regenerative potential of a novel treated dentin matrix hydrogel in direct pulp capping,” Clin. Oral Investig., pp. 2101–2112, 2020, doi: 10.1007/s00784-020-03521-z.
24. J. Chen *et al.*, “Treated dentin matrix paste as a novel pulp capping agent for dentin regeneration,” J. Tissue Eng. Regen. Med., 2017, doi: 10.1002/term.2256.
25. A. A. Elreash, H. Hamama, W. Eldars, G. Lingwei, A. M. Zaen El-Din, and X. Xiaoli, “Antimicrobial activity and pH measurement of calcium silicate cements versus new bioactive resin composite restorative material,” BMC Oral Health, vol. 19, no. 1, pp. 1–11, 2019, doi: 10.1186/s12903-019-0933-z.
26. X. He *et al.*, “IFN-l 3 regulates human dental pulp stem cells behavior via NF-l B and MAPK signaling,” Sci. Rep., 2017, doi: 10.1038/srep40681.

27. A. Kabiraj, J. Gupta, T. Khaitan, and P. T. Bhattacharya, “Principle and Techniques of Immunohistochemistry – a Review,” Int J Biol Med Res, 2015.
28. E. Luczaj-Cepowicz, G. Marczuk-Kolada, M. Pawinska, M. Obidzinska, and A. Holownia, “Evaluation of cytotoxicity and pH changes generated by various dental pulp capping materials — an in vitro study,” Folia Histochem. Cytobiol., vol. 55, no. 2, pp. 86–93, 2017, doi: 10.5603/FHC.a2017.0008.
29. G. R. Jiménez-Gastélum, E. M. Aguilar-Medina, E. Soto-Sainz, R. Ramos-Payán, and E. L. Silva-Benítez, “Antimicrobial Properties of Extracellular Matrix Scaffolds for Tissue Engineering,” *Biomed Res. Int.*, vol. 2019, 2019, doi: 10.1155/2019/9641456.
30. J. G. Smith, A. J. Smith, R. M. Shelton, and P. R. Cooper, “Antibacterial activity of dentine and pulp extracellular matrix extracts,” Int. Endod. J., vol. 45, no. 8, pp. 749–755, 2012, doi: 10.1111/j.1365-2591.2012.02031.x.
31. M. H. Huang, Y. F. Shen, T. T. Hsu, T. H. Huang, and M. Y. Shie, “Physical characteristics, antimicrobial and odontogenesis potentials of calcium silicate cement containing hinokitiol,” Mater. Sci. Eng. C, 2016, doi: 10.1016/j.msec.2016.04.016.
32. *et al.*, “Effect of EDTA on TGF-β1 released from the dentin matrix and its influence on dental pulp stem cell migration,”*Braz. Oral Res.*, 2016, doi: 10.1590/1807-3107BOR-2016.vol30.0131.
33. M. Widbiller *et al.*, “Cell Homing for Pulp Tissue Engineering with Endogenous Dentin Matrix Proteins,” J. Endod., 2018, doi: 10.1016/j.joen.2018.02.011.
34. C. Theoret, “Physiology of Wound Healing,” in Equine Wound Management: Third Edition, 2016.
35. S. A. Eming, P. Martin, and M. Tomic-Canic, “Wound repair and regeneration: Mechanisms, signaling, and translation,” Science Translational Medicine. 2014, doi: 10.1126/scitranslmed.3009337.
36. S. Singh, A. Young, and C. E. McNaught, “The physiology of wound healing,” Surgery (United Kingdom). 2017, doi: 10.1016/j.mpsur.2017.06.004.
37. R. Li *et al.*, “Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration Biomaterials Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration,” no. March 2019, 2011, doi: 10.1016/j.biomaterials.2011.03.008.
38. K. M. Fawzy El-Sayed *et al.*, “The Dental Pulp Stem/Progenitor Cells-Mediated Inflammatory-Regenerative Axis,” Tissue Engineering - Part B: Reviews. 2019, doi: 10.1089/ten.teb.2019.0106.
39. M. Goldberg, A. B. Kulkarni, M. Young, and A. Boskey, “Dentin: Structure, composition and mineralization,” Front. Biosci. - Elit., vol. 3 E, no. 2, pp. 711–735, 2011, doi: 10.2741/e281.
40. M. Y. Shao *et al.*, “The presence of open dentinal tubules affects the biological properties of dental pulp cells Ex Vivo,” Mol. Cells, 2011, doi: 10.1007/s10059-011-0010-1.
41. L. Sadaghiani, H. B. Gleeson, S. Youde, R. J. Waddington, C. D. Lynch, and A. J. Sloan, “Growth factor liberation and DPSC response following dentine conditioning,” J. Dent. Res., vol. 95, no. 11, pp. 1298–1307, 2016, doi: 10.1177/0022034516653568.
42. K. M. Galler *et al.*, “EDTA conditioning of dentine promotes adhesion, migration and differentiation of dental pulp stem cells,” Int. Endod. J., 2016, doi: 10.1111/iej.12492.

Figures

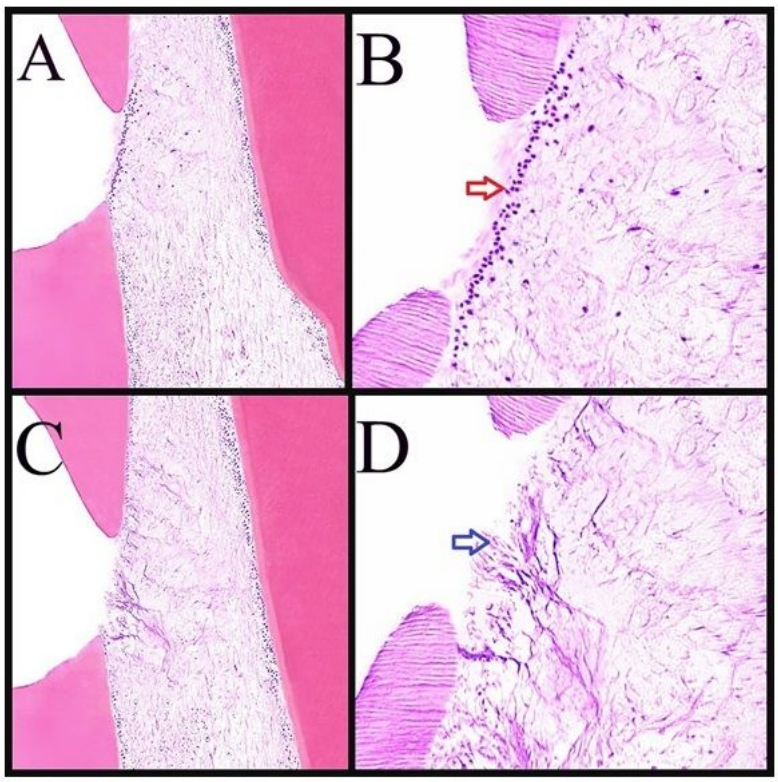

Figure 1

Photomicrographs show pulp inflammatory response after three days. (A) Teeth treated with MTA *(40X)*. (B) A higher magnification of (A) shows inflammatory cells (red arrow) at the exposure site and others scattered in one third of the coronal pulp (*100X*). (C) Teeth treated with hTDM scaffold (*40X*). (D) A higher magnification of (C) shows score 0 inflammatory response and formation of new blood vessels (blue arrows) toward the exposure site (*100X*).

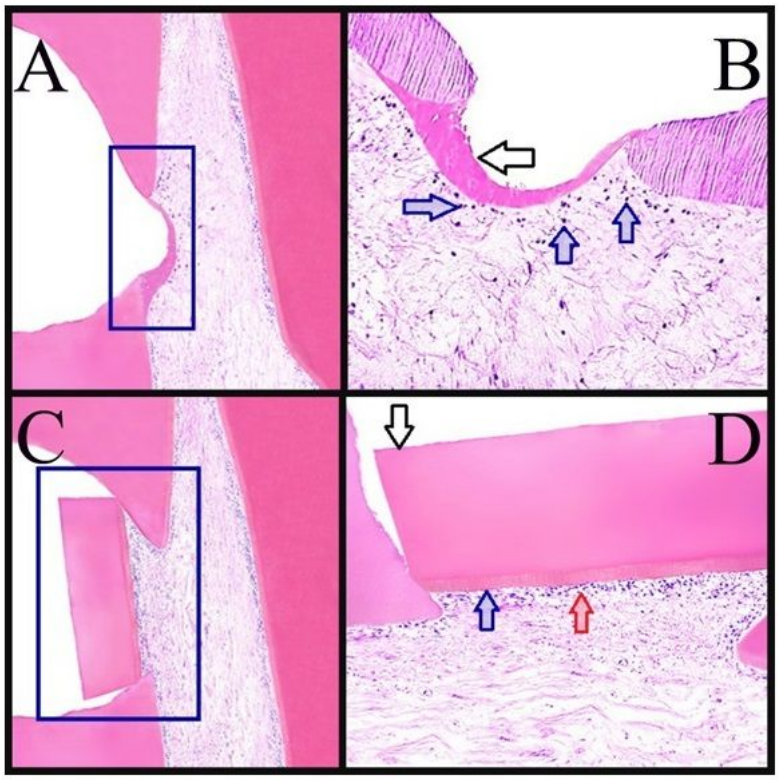

Figure 2

Photomicrographs showing pulp inflammatory response and dentin-pulp complex regeneration after three months. (A) Teeth treated with MTA *(40X)*. A higher magnification of the squared area of (A) shows score 1 (mild) inflammatory response (blue arrows) and formation of calcific bridge (black arrow) with un even thickness towards the pulp chamber (*100X*). (C) Teeth treated with hTDM scaffold (*40X*). (D) A higher magnification of (C) shows score 0 inflammatory response, formation of homogenous dentin (blue arrow) and odontoblastic layer (red arrow) along the hTDM scaffold (black arrow) (*100X*).