Dexamethasone - loaded polymeric porous sponge as a hard tissue regeneration agent

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Abstract

Background : The aim of this study is to achieve the principles of tissue engineering using biopolymers to be applied in the field of vital endodontic treatment with the aim of stimulating stem cells and engineering and regeneration of dentin tissue. the blend was loaded with the steroidal anti-inflammatory drug, dexamethasone, and the porous drug-loaded bio-sponge was produced by lyophilization. Bio-sponge, as a direct pulp capping agent, was histologically studied compared to calcium hydroxide Ca(OH)2 in an animal experiment.

Results: The results indicated the effectiveness of the bio-sponge as a direct pulp agent, where the dentin bridge was formed faster than Ca(OH)2 treated samples,. There was no inflammatory response in the pulp tissue throughout the follow-up period.

Conclusions : The porous bio-sponge loaded with dexamethasone with a neutral pH resulted in enhancement the odontoblast differentiation from stem cells, resulted in the formation of a renewed dentin bridge without the slightest inflammatory response in the pulp.

1. Introduction

Vital pulp therapy is the treatment of teeth that have a reversible pulp injury in order to help pulp healing and maintain it. The treatment of vital pulp is intended to treat reversible endodontic injuries by capping the pulp and stimulating the formation of the dentin bridge[1, 2]. Direct pulp capping DPC, as known, is one of the vital pulp treatment methods that based on the treatment of exposed vital pulp by applying a capping material onto the exposed pulp in order to form a protective barrier for maintaining the pulp[3, 4]. DPC avoids interference on the pulp and broader therapeutic processes such as endodontics and extractions[5].

Many materials have been used to cover the exposed pulp, and have been extensively histologically and clinically studied and have achieved different success rates, such as zinc oxide eugenol (ZoE)[6], glass ionomer cement (GIC)[7], adhesive systems[8], calcium hydroxide [9], mineral trioxide aggregate (MTA)[10], Biodentine [11] and calcium-enriched mixture cement CEM[12].

It is known that bones and dentin are composed of an extracellular matrix ECM consist of mineral (hydroxyapatite) and organic components which is mainly composed of collagen and glucose aminoglycan GAG[13]. ECM is responsible for cellular metabolism and the forming of new tissues since it gives the mechanical and biochemical properties for the formed tissue[14]. ECM plays a crucial role in regulating the expression of the differentiated phenotype and in supporting both migration and proliferation of fibroblast cells[15].

Chitin, the most abundant polysaccharide in nature after cellulose, is synthesized by an enormous number of living organisms, presents in the exoskeleton of crustaceans, insects and fungal cell walls[16].

Chitin consists of a saccharide backbone with β-1,4-linked glucosamine units and characterized by a high availability of acetyl group[17]. Chitin has a cellulose-like structure with the difference of hydroxyl-group replacement by (-NHCOCH$_3$) at the C$_2$ site[18]. Chitosan is the main derivative of chitin and derived by alkaline deacetylation of chitin[19]. The availability of functional groups allows chemical modifications, making it possible to load and release drugs from chitosan[20].

Chitosan scaffolds with an interconnected porous structure have been shown to be easily manufactured by lyophilization of chitosan solution[21], and have been widely studied in the bone tissue engineering, and it has been shown that it promotes osteoblast cells growth and deposition of the mineralized matrix by cells[22]. The addition of chitosan molecules to the human bone marrow transplantation medium stimulates bone deposition by promoting cell differentiation[23]. It has been suggested that chitosan enhanced bone formation in vivo through indirect mechanisms[23], and it can be used as a standard GAG in tissue regeneration processes[24].

Collagen is the most abundant protein in the body and it is the major component in body tissues, it forms also about 30% of total proteins in mammals[25]. About 28 types of collagen have been identified[26], among which, the type-I collagen is the prevalent type and it is the main component of the ECM[27].

Collagen type-I is the basic protein in animals and it is particularly widespread in the skin, tendons, bones, and dentin, where its function is to absorb and transfer forces[28]. Because of the special properties of collagen that promote the adhesion, proliferation and cellular differentiation, it has been extensively studied in the design of tissue engineering scaffolds; since porous collagen scaffolds have distinctive physical, chemical and biological properties for using in tissue engineering[29, 30].

Dexamethasone, as known, is a synthetic glucocorticoid clinically used as an anti-inflammatory drug[31]. Dexamethasone has been also used to differentiate stem cells into bone cells, as some studies have also reported that BMSCs proliferate and differentiate into bone cells when dexamethasone was added to the culture medium[32].

So we tried to synthesis a bio-polymer sponge consists of collagen and chitosan as a substance to release dexamethasone to use it as a vital pulp therapy agent. We aimed to evaluate the histological response of animal module teeth pulp to this bio-sponge and compare it with Ca(OH)$_2$.

2. Experimental

2. *Experimental*

2.1. Reagents:

Collagen type-I (from bovine flexor tendon; 5162) was purchased from Sigma; Chitosan (deacetylation degree ≥ 75%) was obtained from Sigma. Viscosity-average molecular weight (M_v) of chitosan was determined by viscometry method, the solvent was mixture of 0.3M CH_3COOH/ 0.2M CH_3COONa, intrinsic viscosity was measured using a Ubbelohde viscometer and M_v was calculated using the Mark−Houwink equation $[\eta]= K(M_v)^a$; where K= 0.076 and a = 0.77 and was 332 KD.

Glacial acetic acid (BDH; England). Tris-HCl Buffer (Sigma-Aldrich), dexamethasone (D4902; Sigma-Aldrich), $Ca(OH)_2$ (Dycal). All of the chemical reagents were of analytical grade.

2.2. Preparation of collagen /chitosan sponge:

To dissolve polymers, 1 g of collagen was soaked in 50 mL of precooled water for 24 hours until swilling and then 50 g of 1 M acetic acid was added. The suspension was homogenized with a high-speed mechanical stirrer (RZR 2051). The protein solution was placed in a precooled water ultrasonic bath (3Ltr digital ultrasonic) for 5 minutes until the air bubbles were expelled from the viscous suspension. collagen was kept at a temperature of 4-8°C.

Chitosan solution 1% (w/w) was prepared in 0.5 M acetic acid by magnetic stirring for 48 hours to obtain a viscous clear solution. After complete dissolve, collagen and chitosan solutions were mixed at a 1:1 weight ratio by slowly adding the collagen solution to the chitosan solution with the magnetic stirring of 200 rpm. For homogenizing mixing, a solution was mixed slowly by mechanical stirrer 500 rpm for six hours. air bubbles in solutions were eliminated under vacuum. The mixtures were placed in 24-well cell culture and frozen at -20°C for 24 hours and then lyophilized for 72 hours using (Telstar 2010 freeze dryer).

Dexamethasone loaded-polymer sponge was produced using a bulk method, where dexamethasone solution in ethanol was mixed with chitosan/collagen mixture solution had a concentration 1% w/v (in 0.5 M acetic acid) in order to achieve dexamethasone concentration 1.5mg/10mg polymer, then the solutions were homogenized by magnetic stirrer 200 rpm/min for 6h.

The polymer solution was placed in a 96-well cell culture plate and then frozen and lyophilized.

All mixing processes were carried out in a precooled water bath with a temperature not exceeding 10 °C. Sponges were kept in a temperature not exceeding 10°C until use.

2.3. Animals care:

Twenty adult males of New Zealand white rabbits weighed about 2.5 Kg were used. The animals were individually housed in animals incubator and maintained under clean housing conditions and fed specific standard laboratory chow ad libitum.

2.4. Dental procedures:

Rabbits were divided into four groups, each containing four rabbits. The central teeth were divided into two groups where the dexamethasone-loaded polymer sponge was used to cover the pulp of the upper and lower right incisors and the upper and lower left incisors were used to evaluate the tissue pulp response to $Ca(OH)_2$ capping. In each group, there was one rabbit to use their incisor teeth as a negative control, covered with zinc oxide and eugenol.

After anesthetizing with an intramuscular injection of diazepam (2 ml/kg body weight) and ketamine (1 ml/kg body weight), about a 5 mm tunnel was drilled at the cervical edge of the incisors until the dentin became a sieve. A spherical bur of a 1 mm diameter was used to detect pulp. The bleeding was controlled by a piece of saline wetted cotton. After immersing it in PBS for five minutes until it becomes gelatinous, a polymeric sponge was placed on a pulpal wound. The dental cavity was then closed with reinforced zinc oxide and eugenol. The counterparty was treated in the same way after $Ca(OH)_2$ capping.

2.5. Animal scarification:

Five rabbits in each group were sacrificed with an intraperitoneal overdose of ketamine after 1, 2, 3 and 4 weeks respectively.

2.6. Histological procedures:

Six teeth were assigned in each period of time for each group. The upper and lower jaw were separated and fixed in a 10% formalin for 48-72h. Samples were then washed with continuous water overnight to remove an excess of the fixative. Samples were decalcified by immersion in 10% formic acid for 10 days at room temperature. The samples were then washed with a continuous water stream for 24h, and dried with a series of increasing concentrations of 40, 50, 60, 70, 80, 90 and absolute ethanol and finally with xylene. After complete drying, the samples were embedded in paraffin.

Tissues were cut into sections of 5um thickness, and then stained with Haematoxylin and eosin (H&E) and examined by optical microscopy (Olympus, Tokyo, Japan) at 40x, 100x and 400x magnifications[33].

2.7. Histological evaluation:

Histological evaluation for pulps was carried out according to inflammatory response grades and formation of dentine bridge and its thickness, table (1, 2) shows the standards followed for the histological evaluation[34, 35].

3. Results

3. I. Histopathological evaluation:

1- $Ca(OH)_2$:

In the samples of the first week, there was a necrosis layer under the cover material in all treated samples. There was a congestion layer limited to the coronary pulp tissue under this layer in 25% of the samples, grade (0) of the inflammatory response, which was characterized by some expansion of coronary blood vessels. In addition, a slight inflammatory layer was detected in the coronary section under the cover material in 50% of the samples, grade (1) of the inflammatory response. There was a mild inflammatory layer of grad (2) in the coronary third and slightly extended to the middle third in 25% of the samples.

25% of the second-week samples showed a inflammatory response; grade (1), 25% showed mild inflammation of grade (2), and 50% of samples showed severe inflammation extended to the pulp tissue with partial necrosis; grade (3) inflammatory response.

In the third week, 25% of the samples showed inflammation of grade (1), mild inflammation of grade (2) in 50% of the samples, and there was severe inflammation in 25% of the samples.

In the fourth week, Low degree of inflammation, grade (0), was cleared in 25% of the samples and mild inflammation of grade (2) in 75% of the samples. Fig (7).

1- Bio-Polymer sponge group: (Experimental group):

In samples treated with biopolymer sponges, there were no signs of inflammation or inflammatory cells in all samples during the four weeks, grade (0) of inflammatory response.

2- Control group:

Samples treated with zinc oxide and eugenol showed comprehensive pulp inflammation in 75% of the samples in the first week, grade (3) of the inflammatory response, and of grade (4) in 25% of the samples.

In the second week, all samples showed necrosis of the pulp, grade (4) of an inflammatory response, in all negative controls.Fig (8).

3.2. Evaluation of hard tissue formation:

1- $Ca(OH)_2$:

50% of the first week samples showed a partial formation of the precursor of poorly calcified bridge, grade (1) of forming a hard tissue, deposited directly under the capping material. 50% of the samples showed no formation of hard tissue or formation of precursor dentin, grade (1) of dentine bridge formation.

75 % of the second-week samples showed the development of hard tissue of grade (1), and 25% of the samples exhibited the formation of a calcified hard tissue of grad (2).

In the third week, 50% of the samples showed a grade (2) of a hard tissue formation,

In the fourth week, the hard tissue was heavily deposited and 75% of the specimens owned a grade (3) of a solid tissue formation, and 25% of the specimens exhibited a hard tissue of grade (2). Fig (9).

2- Biopolymers sponge:

In the first week samples, there was dentin vanguard formation of gade (1) in 50% of the samples with dense odontoblast cells in the coronary third of the pulp under capping material, and 25% of the samples showed a hard tissue of grade (2), and 25% of the samples showed no hard tissue formation.

In the second week samples, the dentin bridges of grade (1), (2), (3) in 25%, 50%, 25% of the samples were placed, respectively.

In the third week samples, a hard tissue of grade (3) was formed in 75% of the teeth, and grade (2) in 25% of the teeth. The dentin bridge of grade (3) was formed during the fourth week in 100% of the teeth treated with biopolymer sponges. Fig (10).

4. Discussion

Traditional vital pulp treatments practiced in dentistry do not apply tissue engineering principles, that it relies on the formation of the dentin bridge on a tissue reaction to the alkaline substances applied at the exposure site. Since its use in dentistry in 1921, calcium hydroxide has been considered the gold standard for direct pulp capping[36]. calcium hydroxide promotes hard bridge formation slowly, dissolves rapidly after marginal leakage and may dissolve during acid etching before the resin filling, and it does not chemically associate with the tooth or with the restored resin[37].

When calcium hydroxide applied directly to the pulp tissue, necrosis of 2mm depth occurs in the pulp tissue and inflammation in adjacent tissues due to the high pH of the calcium hydroxide[38]. The

formation of hard tissue occurs at the contact area of necrotic tissue and inflamed tissue[39]. Under the necrosis layer, the pulpal stem cells differentiate into the odontoblast like cells, as a tissue reaction, and the dentin bridge matrix is placed[37].

For these reasons, the aim of this study was to try to regenerate dentin by a combination of bioactive and biodegradable polymers. A three-dimensional porous scaffold of a combination of biopolymers analogous to the extracellular matrix for dentin tissue engineering was fabricated. Tissue engineering is a multidisciplinary science that applies chemistry, materials engineering and medicine, aiming to repair and replace damaged or diseased tissues and organs[40]. It requires three-dimensional, porous polymeric scaffolds that create the appropriate mechanical, structural and biological environment for tissue repair and regeneration[41, 42]. Polymers used as a scaffold must be able to mimic the biological structure and functions of the ECM, which is a diverse composition of saccharides, proteins and signaling molecules[43], in terms of chemical and physical structure[44]. Where ECM is responsible for cellular metabolism and forming new tissues[14, 44]. Biopolymers were preferred in tissue engineering applications because of reduced inflammatory reactions, non-cellular toxicity, and biodegradation by blood enzymes in vivo[45].

In our study, we chose to crosslink chitosan and collagen as a result of the excellent properties of both polymers in tissue engineering and drug delivery. Because of its structural similarity to glycosaminoglycans in ECM and the possibility of forming it as porous scaffolds with morphological and mechanical properties similar to those of collagen scaffold, chitosan provides a good choice for tissue engineering applications[46, 47]. Studies have shown that chitosan is a suitable candidate for tissue engineering due to non-toxicity, biocompatibility, and biodegradability[48]. It also has the ability of histological regulation and displays the ability to stimulate cell proliferation[49]. Chitosan has been shown to be highly compatible with osteoblast cells in vitro[50]. This ability appeared in various formulations of chitosan[51]. Good biological compatibility between neurons and chitosan has been reported, and it was found that chitosan is the best membrane for the proliferation of these cells[52]. Chitosan has shown a characteristic enhancement of the vitality of neuronal cells and the results of in vitro cell culture indicate selective adhesion of Schwann cells[53].

Since collagen scaffolds are a distinctive template for renewable cell growth in vitro and in vivo, crosslinked collagen scaffolds have been used in regenerative medicine to promote the regeneration/repair of diseased and damaged tissues[54, 55]. Extensive researches have been done on collagen scaffolds, these scaffolds have been proven to support cellular growth and researches have shown that collagen types-I can form a scaffold that resembles or even fully mimics the structural and biological properties of natural ECM collagen[56].

For our study, we selected high-deacetylated chitosan that was calculated by FT-IR spectroscopy to be 87% (data not shown). This implies that each 1mol of chitosan contains 0.87 mol of free positive amine groups allowing it to crosslink with molecules that have the opposite charge.

It has been found that a high degree of deacetylation enhances the activity of chitosan in each of its antimicrobial properties against a wide range of bacteria and fungi due to a strong positive charge that created by the protonation of free amino groups[57], positive charge adhering negatively bacteria and fungi to chitosan and prevents nutrients from reaching them. It was also found that chitosan with a high degree of deacetylation enhances the activity of fibroblast cells during wound healing processes for the same reason, high positive charge[58].

Since free radicals scavenging is one of the most useful properties to be achieved in biomedical compounds that used in regenerative medicine, it prevents the destruction of membrane fats, proteins, and DNA by radical oxygen reactive molecules[59]. Chitosan has been preferred over other biopolymers in a tissue regeneration field due to its ability to dismantle active free radicals through fixation them by free amino and carboxyl groups in chitosan[60].

Dexamethasone, as known, is a synthetic glucocorticoid used clinically as an anti-inflammatory drug. It has been hypothesized also that dexamethasone increases the response of stem cells to materials used for differentiation[61]. Increased vitality and proliferation of stem cells derived from human bone marrow MSCs have been reported as a result of ongoing dexamethasone therapy[32].

Among biopolymers, collagen and polysaccharide, chitosan, are suitable for topical drug delivery systems, providing the advantage of using them as a natural biological material with tissue healing properties[62, 63]. A dug can be linked to polymer matrix by physical methods or by chemical reagents due to the availability of functional groups that is able to interact with the drug in various ways[64]. Whereas glutaraldehyde is one of the most important chemical reagents in the field of drug binding to chitosan and polymers containing amine groups[65-67]. The physical crosslinking between the drug and the collagen matrix plays an important role in loading and releasing the drug from collagen without adding any crosslinking agents that may be cytotoxic when applying this drug-loaded matrix topically[68]. Physical methods of linking a drug to chitosan by electrostatic or hydrogen bonds have been also shown to be effective in controlled drug release[69, 70]. This interaction between the polymer and a drug leads to stable production and loading of the drug with great efficacy and prolonged release of the drug; this has been reported in other research[71].

In the present study, we have used freeze-drying technique to fabricate bio-sponge consisting of chitosan and collagen, so as to benefit from the properties of collagen which promotes cellular adhesion, differentiation as well as its hemostatic properties[18, 72, 73], also to benefit from the adhesive, antibacterial, antifungal and hemostatic properties of chitosan[74-76], that justified our selection of both polymers for crosslinking and histologically study.

Since most studies about biopolymer matrix for medical applications used a different ways to crosslinking polymers to form scaffolds by utilizing functional groups in each component within the mixture, crosslinking treatment is one of the most important issues for bio-scaffolds, consideration

should be given to the intensity of the crosslinking and the preservation of the biological activity and biological properties provided by each component before crosslinking. There are two types of crosslinking methods; Physical and chemical methods. In the chemical methods group, glutaraldehyde is the most comfortable traditional agent used in the treatment of porous collagen scaffolds, and amino biopolymers[77], which achieves a high and undesirable crosslinking degree and potential cytotoxicity of this agents[67, 78].

Physical methods are an attempt to establish a binding without the introduction of cytotoxic chemical reagents and maintain excellent biocompatibility of tissue engineering materials[79].

Here we used the physical linking method by creating ionic bonds between amino groups of chitosan and carboxyl groups of the glutamic and aspartic residues of collagen.

Fig. 1, 2, 3, 4 show FT-IR results of characterization of the polymer blend and dexamethasone-loaded polymers films synthesized by the solvent cast method showed the forming of electrostatic and H-bonding between collagen and chitosan and the forming of H-bonding between dexamethasone and studied homopolymers and polymer blend. Fig. 5 shows SEM images for sponges also showed a porous structure for homopolymer and hybrid sponges, collagen −chitosan sponge at 1:1 weight ratio showed an average pore size of 100 um that is suitable for cell growth[80], dexamethasone was clearly shown immobilized on the surface and embedded in polymer sponges (Fig. 6).

Figure 1. FT-IR for a: dexamethasone, b: collagen, c: chitosan film.

Figure 2. FT-IR for, a: dexamethasone, b dexamethasone-loaded chitosan film, c: chitosan film.

Fig. 3. FT-IR for, a: dexamethasone, b: dexamethasone-loaded collagen, c: collagen film.

Fig. 4. FT-IR for, a: dexamethasone, b: dexamethasone-loaded chitosan:collagen (1:1) film, c: chitosan:collagen (1:1) film.

Fig. 5. SEM for chitosans:collagen (1:1) sponge.

Fig. 6. SEM for dexamethasone loaded chitosan: collagen (1:1) sponge.

The drugs can be loaded into the polymer matrix or condensed on the surface of the matrix, the maximum loading in the drug formulation can be achieved by incorporating the drug during the formation of pharmaceutical molecules[81]. Here we used the physical method of linking the drug to the polymer sponge by hydrogen bonding between dexamethasone and the polymer mixture before lyophilized.

Drug release study from the sponge shown that the release of drug from the hybrid sponge was faster than the chitosan sponge, which ended in 16h, and was slower than the collagen sponge which completed within 5h. The formulation based on collagen-chitosan (1:1 wt %) blend was able to control drug release within 10h[82] . Thus, the dexamethasone loaded -blend of high molecular weight chitosan and collagen, to form a bio-sponge hydrogel for DPC, will have advantages of both collagen and chitosan properties, in addition to the properties of prolonging released dexamethasone, which is about 30 times more effective than cortisone[83].

Our results revealed that the combination of bio-polymers that act as a natural extracellular matrix loaded with a steroidal anti-inflammatory drug, that acts as an osteoconductivity agent, can be considered as an effective alternative to high-alkaline mineral oxides in order to avoid its side effects occurring in a teeth pulp, such as pulp calcifications, tissue burns, and pulp stones. Bio-polymer sponge with a neutral PH activates stem cells to differentiate into odontoplast cells and form the dentin bridge.

The results revealed that the dentin bridge was formed without any inflammatory response in the pulp tissues, maybe due to the release of dexamethasone, and faster than that in the $Ca(OH)_2$ group, with a shorter duration. The dentin bridge was regular and thick under the bio-scaffold, and The odontoblasts layer appeared to exist under the formed dentin bridge, which indicates that the formation of the hard tissue was as a renewed dentin layer and not as a tissue reaction towards $Ca(OH)_2$ alkalinity.

Conclusion

In this study, a porous bio-sponge consisting of high deacetylated chitosan and collagen type-I loaded with dexamethasone was fabricated by the freeze-drying method. The results indicated that the porous bio-sponge with a neutral pH proved its superiority over $Ca(OH)_2$ and resulted in the formation of a renewed dentin bridge with a dense odontoblast layer beneath it within one week without any burns in the dental pulp and without the slightest inflammatory response in the pulp. That means; The presence of bio-sponges stimulated the stem cells of the dental pulp to differentiate into odontoblast-like cells.

Declarations

List of abbreviations:

Calcium htdroxide: $Ca(OH)_2$

Declarations:

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

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Tables

Table 1: Scoring system of inflammatory response.

Grade	Characterization
0	Absence of inflammatory response or low degree of inflammation limited to the capping site.
1	The slight inflammatory response in the coronary third of the pulp
2	Mild inflammation extends over the coronary third and includes the middle one-third of the pulp
3	Severe inflammation involving the whall pulp
4	Necrosis of whole pulp

Table 2: Scoring system of dentine bridge formation

Characterization	Grade
No deposition of hard tissue	0
Partially formed hard tissue or mild hard tissue formed at the interface of the grafting material	1
Moderate hard tissue deposition distant from the exposure area	2
Heavy hard tissue deposition distant from the exposure area	3

Figures
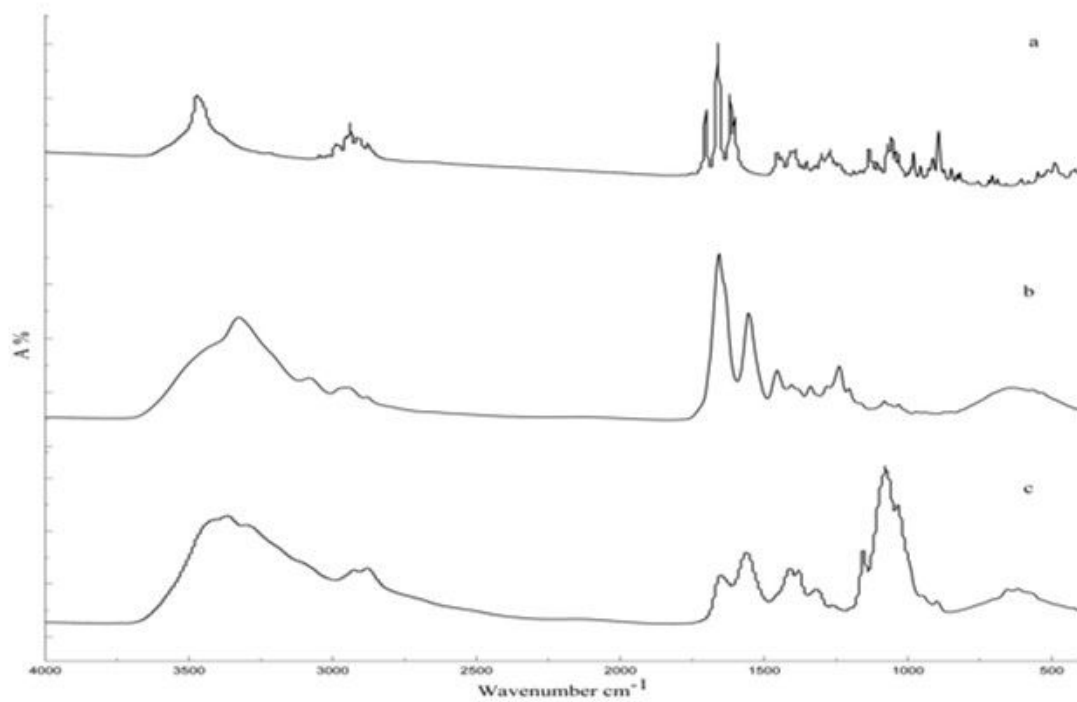
Figure 1

FT-IR for a: dexamethasone, b: collagen, c: chitosan film.
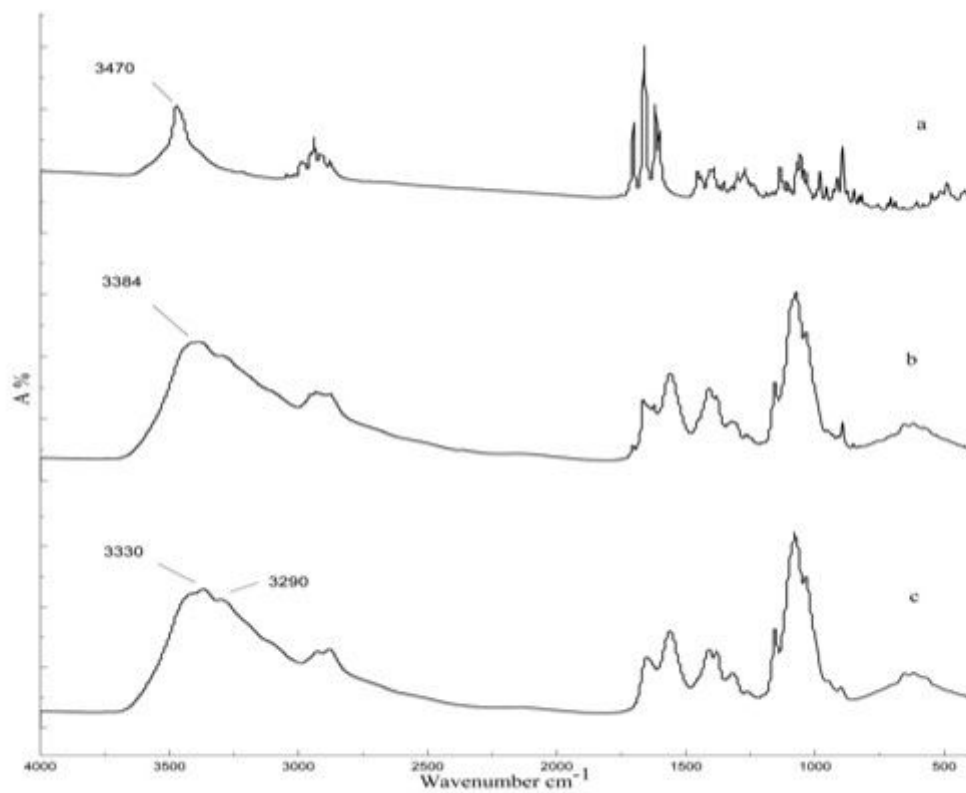
Figure 2

FT-IR for, a: dexamethasone, b dexamethasone-loaded chitosan film, c: chitosan film.
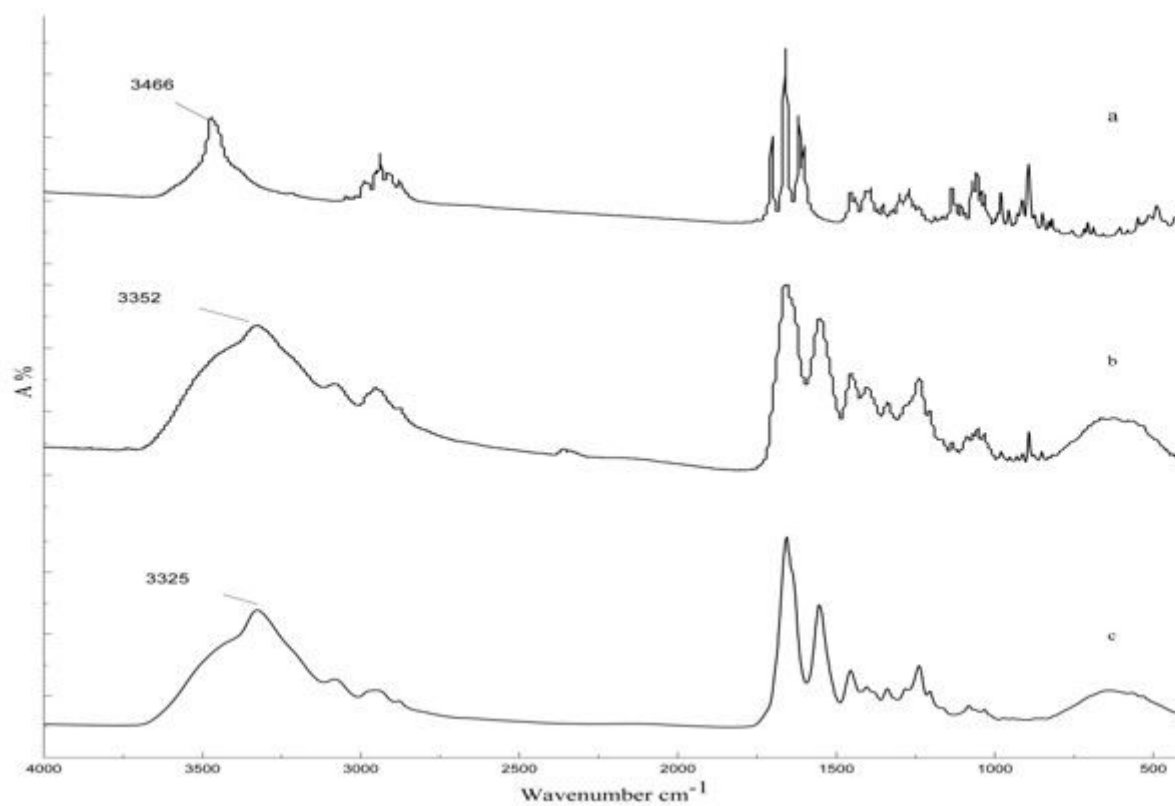
Figure 3

FT-IR for, a: dexamethasone, b: dexamethasone-loaded collagen, c: collagen film.
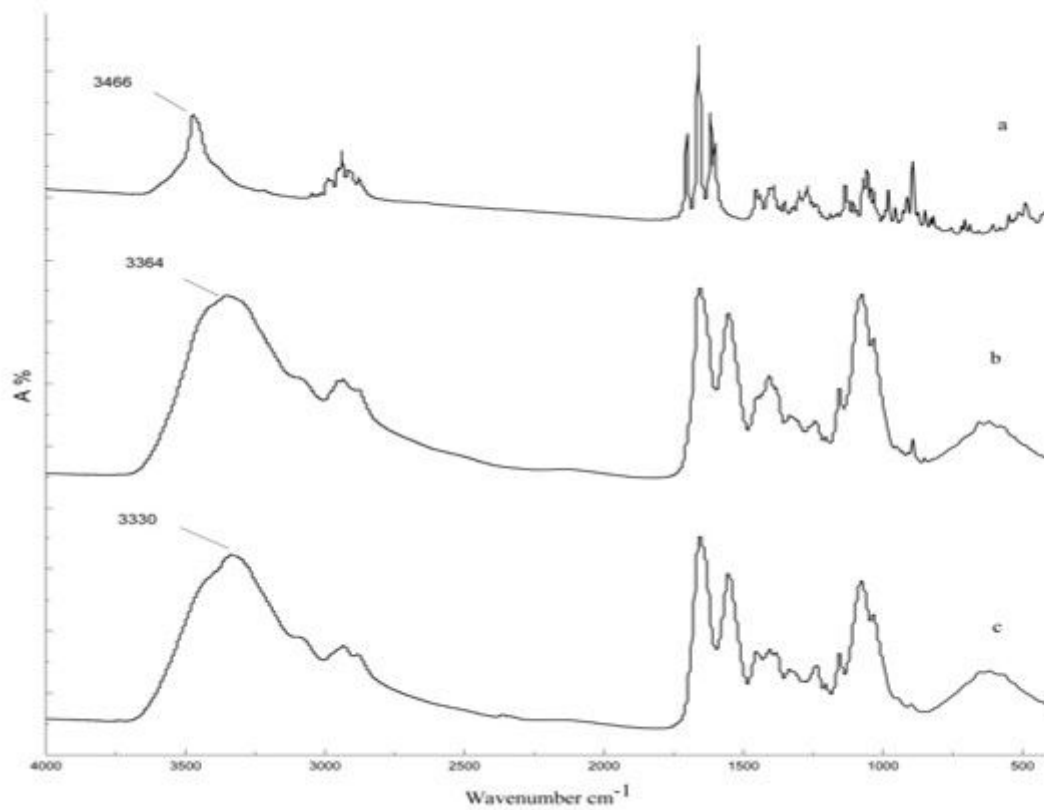
Figure 4

FT-IR for, a: dexamethasone, b: dexamethasone-loaded chitosan:collagen (1:1) film, c: chitosan:collagen (1:1) film.
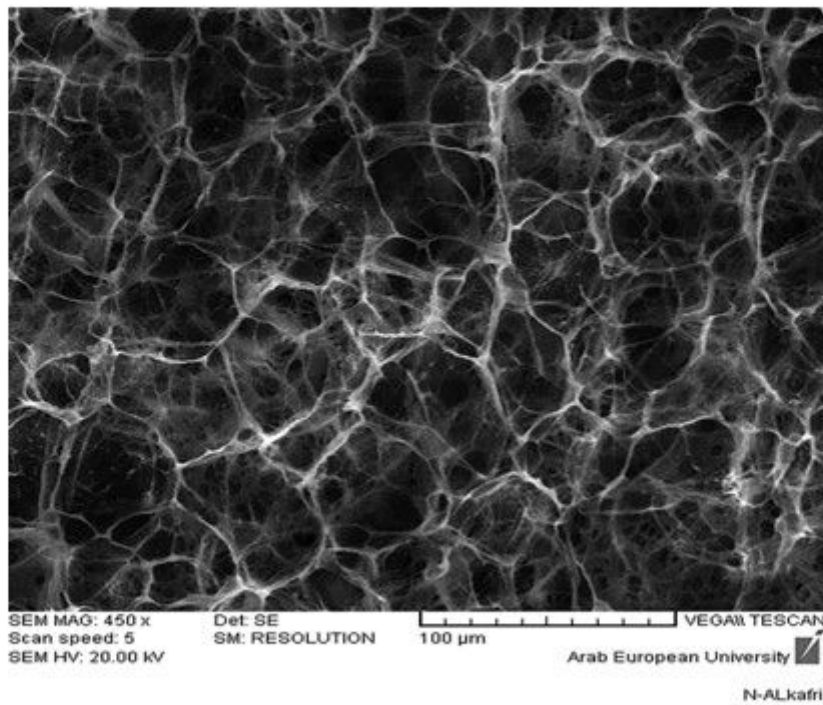
Figure 5

SEM for chitosans:collagen (1:1) sponge.
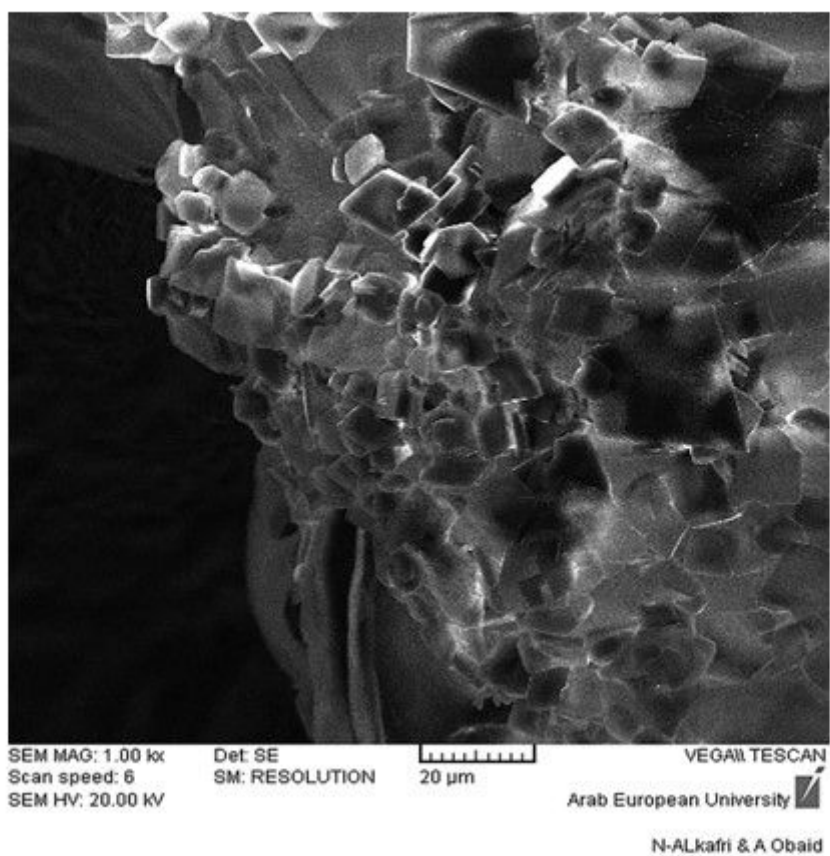
Figure 6

SEM for dexamethasone loaded chitosan: collagen (1:1) sponge.
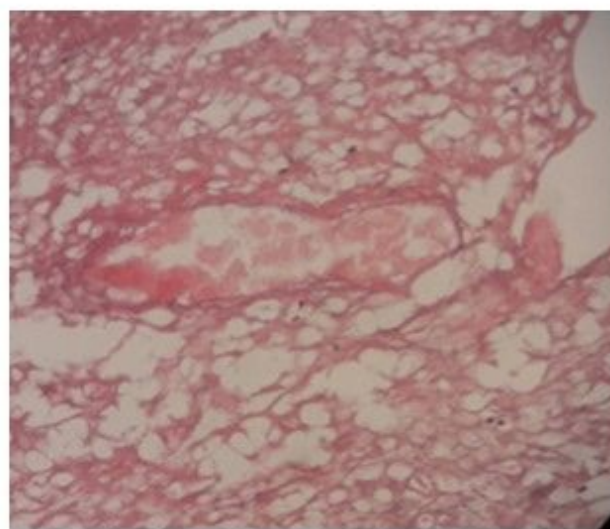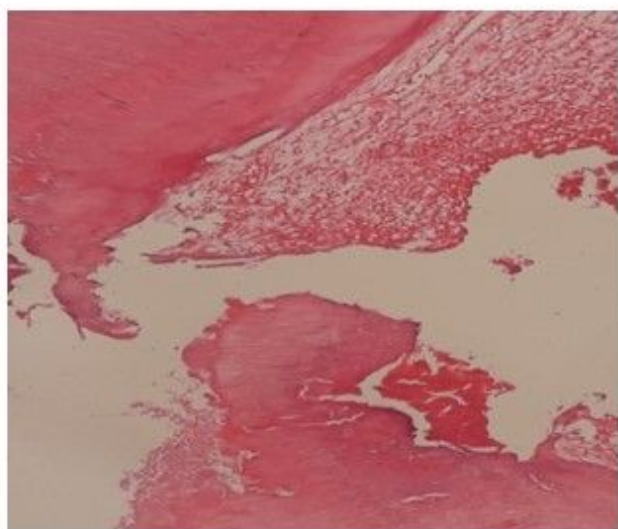
Figure 7

A photomicrograph of dental pulp under Ca(OH)2: a) showing moderate inflammation deposition beneath the capping material in the coronary third and slightly extended to the middle third after one week of application of capping materials. b) Low degree of inflammation limited to the capping site after four weeks of application of capping materials.
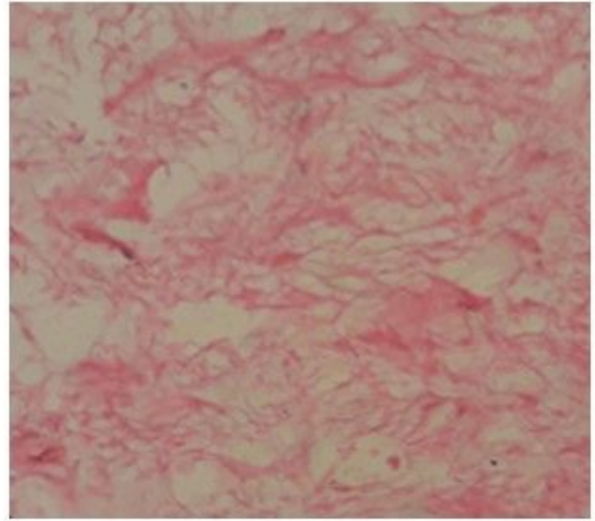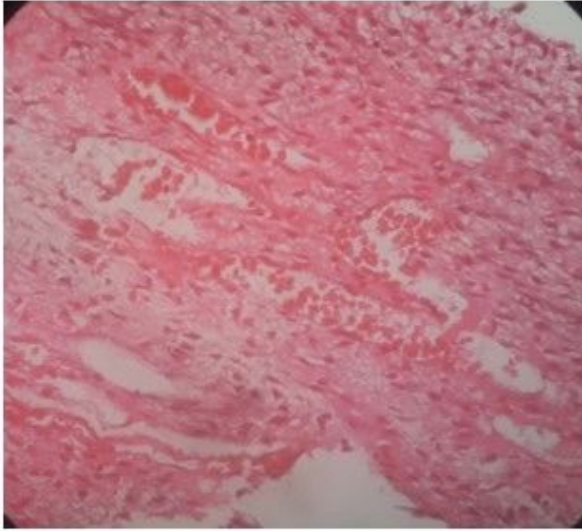
Figure 8

A photomicrograph of dental pulp in the control sample: a) showing sever inflammation Severe inflammation extending the whall pulp after one week. b) Necrosis of whole pulp appeared in the fourth week.
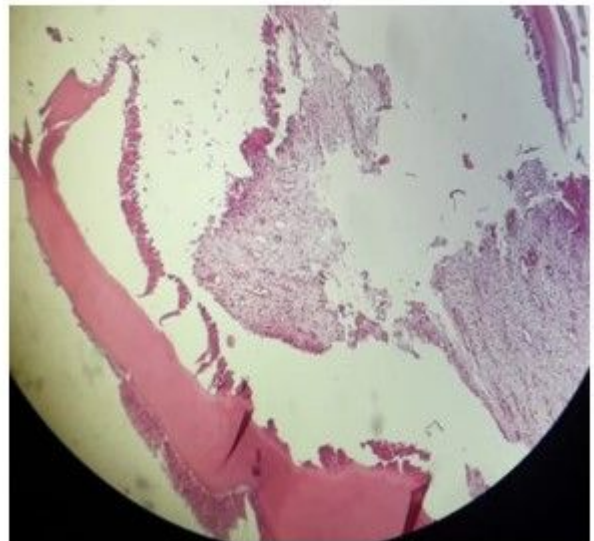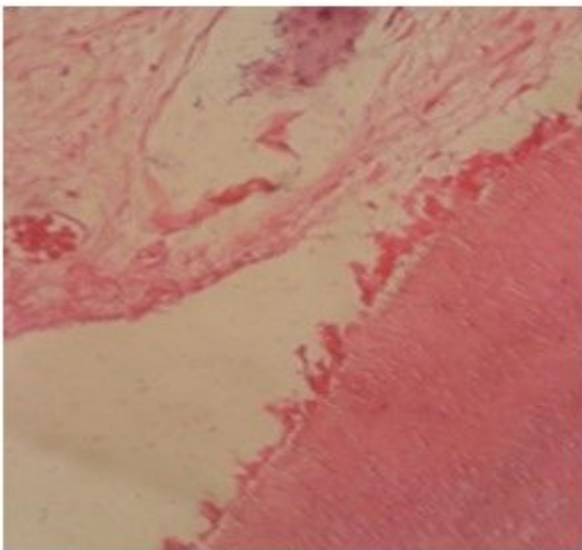

Figure 9

A photomicrograph of dental pulp under Ca(OH)2: a) showing slight hard tissue deposition beneath the capping material with a mild inflammatory response after one week of application of capping materials.

b) showing heavy and irregular hard tissue deposition distant from the capping material after four weeks of application of capping materials.
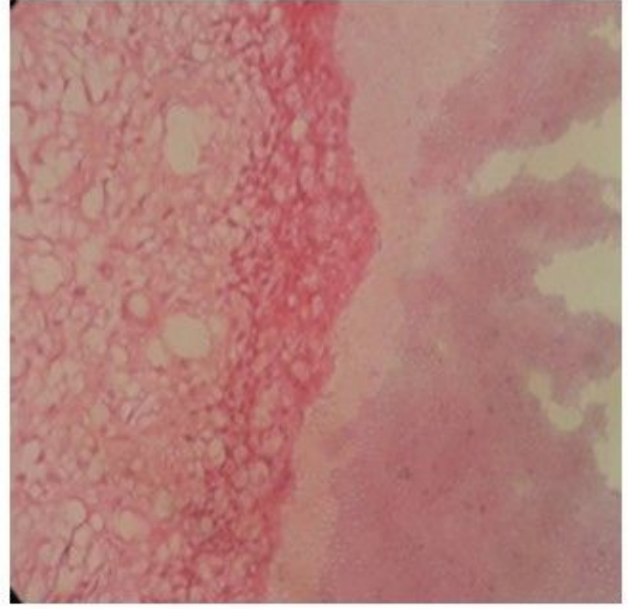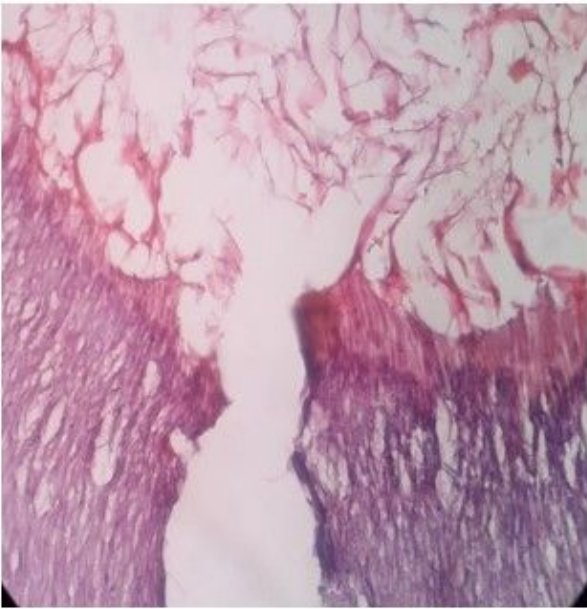

Figure 10

A photomicrograph of dental pulp under bio sponge capping materials: a) showing moderate hard tissue deposition beneath the capping material with no inflammatory response after one week of application of capping materials. b) showing heavy and regular hard tissue deposition at the exposure area at the interface of the grafting material after four weeks of application of capping materials.