



Histopathological Analysis and Comparison between Experimentally Fabricated Hydroxyapatite from Nile Tilapia Bone and Mineral Trioxide Aggregate as A Direct Pulp Capping Agents on Dog Pulp (*In Vivo* Study)

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Abstract

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AIM: The aim of this study was to study response of exposed dental dog pulp to the HP from Nile Tilapia bone and MTA on induction of reparative dentin formation and to inflammatory response in pulp tissue.

MATERIALS AND METHODS: Four male mongrel dogs 1 year old with total of 20 teeth from each dog were selected and divided into two groups that each group has 40 teeth according to the material used as pulp capping agents. The maxillary teeth and the mandibular teeth for each dog were capped by both materials alternatively; the cavities were then sealed by glass-ionomer cement. Each group divided in two groups according to 2-time intervals (4 weeks and 8 weeks). Animals were sacrificed and teeth were collected for histopathological analysis.

RESULTS: Specimens capped by fish bone powder after 4 weeks, which showed non-significantly higher of inflammatory cell scores than that capped by MTA. It showed thin (score 1) to moderate (score 2) thickness of dentine bridge formation, mostly a-tubular dentin, in all specimens. After 8 weeks, significant decrease in inflammatory cell infiltration scores for both groups were found, but it was significant in case of fish bone powder. Furthermore, dentine bridge became more significant for both materials with slight histological change in the group capped by fish bone powder.

CONCLUSIONS: HA from Nile Tilapia bone could be considered as a direct pulp capping material. Furthermore, more studies needed on the fabrication of this material to give better result in shape and the pattern of reparative dentine formed.

Introduction

Dental decay has always exposed all the involved dental components to great risk of trauma and destruction of different degrees throughout the tooth life. Therefore, in recent year's, regenerative dentistry become a main branch in conservative treatment in dentistry, which focused on several dental pathologies as tooth destructive disease such as dental caries and pathological or traumatic pulpal exposure [1]. Pulp regeneration or pulp repair depend mainly on the formation of newly reparative dentine with adequate thickness and correct biological structure [2].

The primary goal of pulp capping procedure is protection of underlying pulp vitality by formation of reparative dentine which increases the expected tooth life [3]. For this purpose, for many years, different protocols and methods for formation of a dentine have the same histological and biological characteristics as dentinal tissue using different materials as dental pulp capping (DPC) agents [4].

Calcium hydroxide ($\text{Ca}(\text{OH})_2$) used to be the standard of choice as pulp capping agent. It has an antimicrobial properties and induce the formation of reparative dentine, which created by its alkalinity that leads to formation of reparative dentine [5], but it have many drawbacks as tunnel defects or incomplete dentin bridge formation that affect the integrity of reparative dentin. The high pH of $\text{Ca}(\text{OH})_2$ is very toxic to odontoblast cells, cause inflammation, and incomplete cell regeneration also high solubility of this material compromise the integrity of permanent seal which allows bacterial leakage. Indeed, after long-term of clinical observations of $\text{Ca}(\text{OH})_2$, it seems that it became no longer to be the best pulp capping agent that could be used [6].

Many proposals were used in maximizing reparative dentin formation, such as improvement of direct capping techniques, increase the biocompatibility of the materials, and enhance the biological responses of the pulp tissues [7]. Recent researches in pulp treatment materials have been concentrated bioactive materials These biomaterials enhance dentin regeneration and formation. One of these biomaterials was Mineral

trioxide aggregate (MTA) a material which appears in the last decade. MTA is composed of tricalcium and dicalcium silicate and has been used successfully for pulp capping. Researches have reported that MTA was biocompatible and could maintain pulpal health as well as promote healing and pulp regeneration [8].

Mineral trioxide aggregate (MTA) is a bioactive material pioneered by Torabinejad *et al.* in the early 1990s with favorable physical properties proven to induce mineralization under the exposed pulp and have the ability of maintaining pulp vitality. MTA, available on the market for two decades, proven its biocompatibility and its success to induced reparative dentine with same histological structure as the dentinal tissue. However, it has a drawback of its high cost, and long setting time [9].

The main source of bioactive components for therapeutic and pharmaceutical use came mostly from nature. These natural ingredients are generally preferred over synthetic ones due to their availability, safety, affordability, biocompatibility, and broad spectrum of biological activities [10]. During the last decades, many efforts have been devoted for obtaining alternative natural sources for biomaterials used as pulp capping agent.

The potential of hydroxyapatite (HA) extracted from bones of animals, as fish, has been studied, due to the high biocompatibility, stability, and high percentage in calcium carbonate called biphasic calcium phosphate (comprising 67.6% of β -calcium pyrophosphate (β -CPP) and 32.1 % of β -tricalcium phosphate (β -TCP) and osteogenic ability) [11].

However, the process of changing waste bone fish into HA is an environmentally friendly process and a good chance of lowering costs with minimal impact into the environment, all those advantages made hydroxyapatite (HA) extracted from fish bone that is a good natural alternative biomaterial needed to studied and evaluated to be used as pulp capping agent [12].

For this purpose, the aim of this study was to compare MTA as a pulp capping agent with tilapia bones powder and evaluate its capability to induce reparative dentine formation with minimal inflammation.

Materials and Methods

Materials used in this study are summarized in Table 1.

Ethical regulation

The research was approved by the Research Ethics Committee, Faculty of dentistry, Beni-Suef University (FDBSU-REC)

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Experimental procedure

Hydroxyapatite powder (from Nile Tilapia fish bone) preparation

Nile Tilapia fish bones were collected from local market, which washed with running water, after that putting in boiling water until all organic debris and meat separated from bone, removed from boiling water, and cleaned; then, left until it became completely dry at room temperature, after that entered into an electric furnace at 900°C for 5 h [13]. Left for cooling about 15 min, fish bone ashes grounded, milled, and sieved to a powder friction range between 100 and 250 μ m in certain milling machine, collect in form of white color powder in sterile container and tested on for confirm and recorded the size and shape of particles. Finally, preserved for use as pulp capping agent.

Animals grouping and pulp capping procedures

Four mongrel healthy male dogs approximately 1-year old (detected from numbers and the color of teeth) weighing 23.5–30 kg were selected and used in the study. The dogs were housed in the animal house of faculty of Dentistry, Minia University. The dogs had balanced diet under good conditions of temperature, humidity, and lighting. All animal experiments conformed to the Guidelines for Animal Experiments of ethics committee. The dogs were weighted to receive the correct anesthetic dose for each one. No food for 12 hours and no drink for 6 hours were provided prior to the dental procedure, they were injected prior to anesthetic drug to minimized salivation with atropine sulphate 0.04 mg/kg body weight. Mixture of Xyla-ject solution 0.1 mg/kg and ketamine hydrochloride 5.0 mg/kg were injected as anesthetic solution through cannula of gauge fixed in the cephalic vein for each dog. The injection was delivered slowly and steadily, to ensure best distribution of drugs.

Each dog was placed in rest position on the operating table with the head in tilted position to keep respiration airway opened. The working parts were disinfected by 0.2% chlorhexidine solution (Ultra dent, USA). Rubber dam used to achieve intraoral dry field.

Total of 20 teeth in each dog was prepared on the buccal surface for class V with minute exposure, including six of maxillary incisors and two upper canines, and two premolars. Six mandibular incisors and two lower canine and two premolars in the dog were intentionally exposed using a sterile size two round tungsten carbide bur (Hager and Meisinger GmbH, Germany) mounted onto low-speed hand piece under copious amount of air water spray until exposure was performed. For each ten cavities in the same dog restored by the same capping agent, one round bur was used and sprayed by antiseptic agent between each cavity preparation to avoid any infection. Bleeding was controlled by cotton

pellets moistened with sterile saline with gentle pressure until physiologic hemostasis occurred.

Split mouth technique was used to give the same opportunity for both capping materials to have the same circumstances. The 20 pulp exposed teeth were capped by MTA directly on the exposure site according to manufacture instructions, the material was mixed and applied in maxillary teeth of on dog and on mandibular teeth of another one, using Liner Placement Instrument (caulk, Dentsply). The experimental HP extracted from fish bone was mixed with distilled water to slurry and applied in the same manner as MTA. All cavities after capping with capping agents restored using high viscous glass-ionomer cement applied according to manufacture instruction. Then, a total of 80 teeth were divided randomly and equally into two groups 40 teeth for each one. Group (A) were sealed using MTA as pulp capping agent. Group (B) had their exposure sealed with experimental fish bone powder. Further division in to sub-groups was done according to time interval 4 weeks and 8 weeks which was also the time for scarifying by dogs.

Animal care

The dogs were housed in separate kennels, adequately ventilated, sprayed daily with antiseptic solution. Furthermore, all dogs were injected with Ivermectin to avoid any parasitic infection (subcutaneously in a dose of 0.1 mg/kg). They reserved soft diet of mixture of milk, bread, mashed potato, and rice and also pain killer of Meloxicam 0.1 mg IM. The dog care was guided by the protocol of Canadian Council on Animal Care and in coherence protocol, with the Three Rs (replacement, reduction, and reinforcement) of animal ethics [14].

Animal scarification

The dogs were scarified using standard protocols after 4 and 8 weeks, respectively. The teeth and all the bone surrounding them were dissected in blocks and all the blocks were immersed in 4% paraformaldehyde solution mixed with PBS buffer for histological preparation and examination.

Sample preparation for histological evaluation [15]

Standard protocol for preparation of all samples was used started by fixation of the tissue: putting all samples in in fixative 10% formalin form 48 to 72 h.

After that, all samples were washed under running water to remove the remaining of fixing solution from the samples. Then, EDTA was used for decalcification step for about 10 days. After that, dehydration was done by immersing the samples in ascending grade of ethyl

Table 1: Materials in this study

Material	Description and manufactures and lot number
MTA	Powder: 75% type I Portland cement, 20% bismuth oxide, 5% calcium sulfate dehydrate. Liquid: Tricalcium aluminate and water. Manufacture: Angelus, Londrina, PR, Brazil Lot number: powder 12002493 Liquid 120407
Experimentally fabricated hydroxyapatite from Nile Tilapia fish bone High viscus glass-ionomer (Ketac Molar)	Powder: Powdered fish bone extracted from Nile Tilapia bone and fabricated according to well-known protocol Liquid: distilled water Manufacture: Al-Azhar University Faculty of Science (for boys) Powder: Al-Ca-La fluorosilicate glass, 5% copolymer acid (acrylic and maleic acid) Liquid: Polyalkenoic acid, tartaric acid, water Manufacture: 3M ESPE, Seefeld, Germany Lot number :82229

MTA: Mineral trioxide aggregate.

alcohol; 50%, 70%, 90% and 100% alcohol respectively. Cleaning was done using cleaning agent xylene which is miscible with both alcohol and paraffin. All samples embedded in melted paraffin and cutting serially using microtone in a buccolingual direction, parallel to the tooth and through the prepared cavity and pulp, the sections have a thickness of 5 microns thickness showing the deepest part of the cavity and the underlying pulp. Finally, mounting of samples was done by that the short length of paraffin ribbon soaked in a pan of warm water (about 20°C). The prepared slide was slipped under the ribbon and then lifted from the water with the ribbon, which contained the tissue sections arranged on its upper surface. The slide was placed on a table to dry at a temperature about 37–42°C.

The staining procedures

Hematoxylin-eosin stain was used [16] after that all slide stained and examined under a light microscope to record and score the inflammatory condition of the pulp tissue and reparative dentine barrier formed for both groups.

Examination procedure

All the slides obtained from the teeth were marked according to the groups and subgroups and were blindly sent to two pathologists to evaluate individually, then, the two reports were evaluated for each slide and the results were recorded and tabulated for the groups.

Imaging and analysis

All images were taken by digital camera connected to light microscope (BX60, Olympus, Japan), the images were arranged according to the criteria, as presented in Table 2 [17].

Statistical analysis

All data were tabulated and statistically analyzed using the Statistical Package for the Social

Table 2: Scoring system for the inflammatory reaction, and dentine bridge formation

Grade	Characterization
Scoring Grade for inflammation	
0	No inflammation or slight inflammatory response limited to the exposure area
1	Mild-to-moderate inflammation: < 10 inflammatory cells with or without dilated blood vessels
2	Moderate inflammation under the exposure area, 10–25 inflammatory cells with dilated blood vessels
3	Severe inflammation > 25 inflammatory cells under exposure area with dilated blood vessels
Scoring Grade for dentin bridge formation	
0	No hard tissue deposition
1	Mild hard tissue deposition beneath the exposed area or partially formed hard tissue 1 to 149 μm
2	Moderate hard tissue deposition beneath the exposed area 150 to 249 μm
3	Thick hard tissue deposition beneath the exposed area Above 250 μm

Science (SPSS version 16) [18]. In the present study, the data assumed non-parametric distribution. Subgroups comparison of this study was determined using Kruskal–Wallis test followed by Mann–Whitney U-test for pair-wise comparison with Bonferroni correction. The main groups' data (Two materials and two time intervals) were analyzed using two-way ANOVA to show the effect of both materials (MTA and fish bone powder) and both time intervals (2 weeks and 8 weeks) and all the interaction of different parameters (Inflammation and calcific bridge formation), with level of significance at p value of ($p \leq 0.05$).

Results

Histopathological evaluation for specimens:

Statistical data according to Mann–Whitney U-test distribution test revealed that:

Inflammatory cell infiltration

In 2 weeks, application specimens capped by MTA showed inflammatory reaction grade 2 which was found in 90% of specimens, and grade 3 inflammatory cell response in 10% of specimens (Figure 1a), while in specimens capped by fish bone powder 82% of specimens showed score 2 and only 18 % of specimens showed score 3 (Figure 1b).

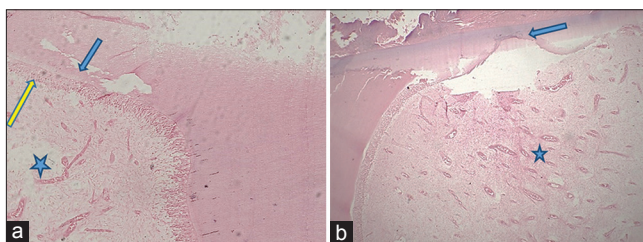


Figure 1: Photomicrograph showing reaction after 4 weeks. (a) Represent cavity lined by MTA, showed score 2 inflammatory reaction (star) with score 2 dentine bridge formation (blue arrow) and ameloblast-like cells (yellow arrow). (b) Represent cavity lined by fish bone powder, where inflammation was more profound score 3 (star), with score 1 (thin dentine bridge) (H&E $\times 40$)

Specimens capped by fish bone powder after period of interval for 4 weeks showed non-significantly higher of inflammatory cell scores than that capped by MTA.

In 6 weeks, application significant decreases in inflammatory cell infiltration scores for both materials were found. About 95% of all specimens capped by any of the capping agents showed score 1, while the other 5% of specimens showed score 2 (Figure 2a and b).

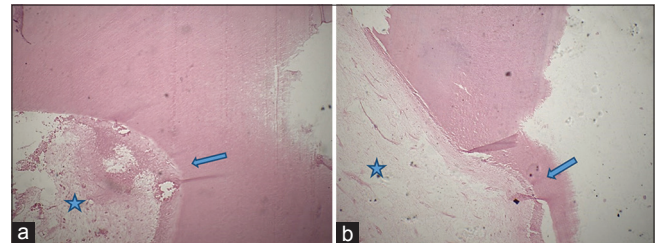


Figure 2: After 8 weeks MTA capped cavity (a) regular thick dentine bridge formation score 3 while in cavity applied by fish powder, (b) thick atubular dentine bridge score 3 (arrows) with morphologically normal pulp without inflammatory reaction in both (a) and (b) (star) score 1 (H&E $\times 40$)

After 8 weeks, significant decrease in inflammatory cell infiltration scores for both materials were found, and it was highly significant in case of fish bone powder.

Regarding the effect of time, the inflammation scores decreased significantly at 8 weeks interval compared to 4 weeks interval for fish bone powder, while, in MTA, there were no-significant difference in both time intervals.

Dentin bridge thickness

In the first-time, interval represented by 4 weeks:

MTA specimens have moderate thickness (score 2) of dentine bridge formation, of high quality. Fish bone powder showed thin (score 1) to moderate (score 2) thickness of dentine bridge formation, mostly a-tubular dentin, in all specimens (Figure 1a and b). There were significant differences between both capping agents at that time interval.

In the second time, interval represented by 8 weeks:

Fish bone powder specimens showed score 2 dentin bridge formation in 66% of the specimens and 32% representing score 3. Only 2% of dog pulp capped with fish bone powder for 6 weeks have score 1 with no tubules formation (Figure 2b). About 80% of specimens capped by MTA showed score 3 dentin bridge formation and 20% of specimens have score 2 (Figure 2a). Regarding to time, both specimens in both groups showed significant increase of mean dentin bridge thickness. Furthermore, according to materials, MTA showed significant increase in dentine bridge

thickness and quality than those capped with fish bone powder for the same capping period.

Data analysis

Descriptive statistics for the study are illustrated in the graphs, as presented in Figure 3a and b.

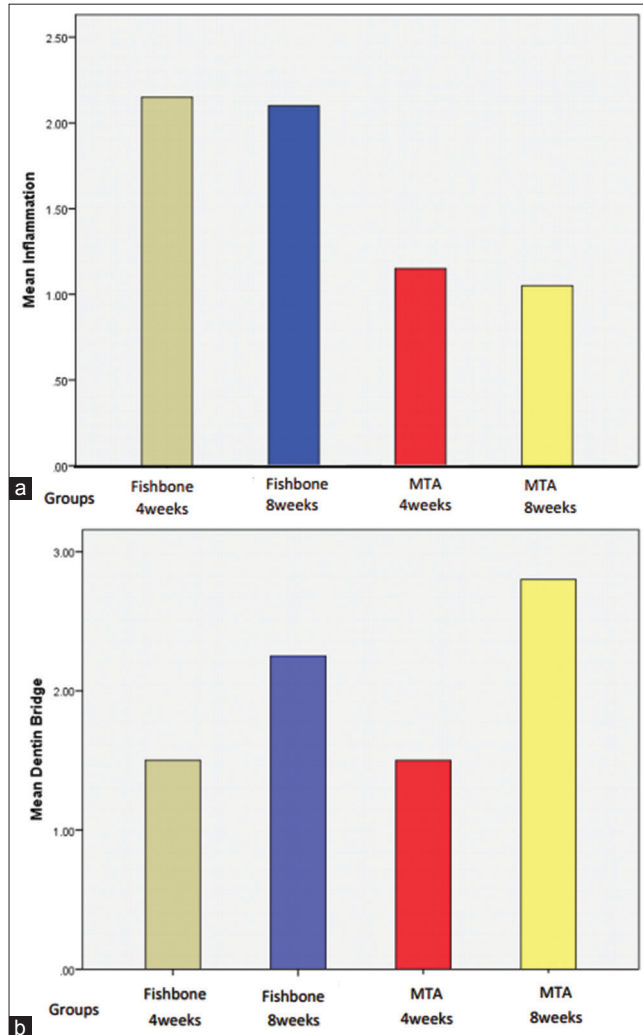


Figure 3: Graph illustrating the mean inflammatory reaction between groups (a) and the dentin bridge formation (b)

Subgroups comparison of this study was determined using Kruskal–Willies test which showed significance Table 3.

Table 3: Results for subgroups comparison using Kruskal–Willies test

Test statistics	DentinBridge	Inflammation
Parameters		
χ^2	44.291	61.340
Df	3	3
Asymptotic significant	0.000*	0.000*
Monte Carlo Significant		
Significant	0.000*	0.000*
99% CI		
Lower bound	0.000*	0.000*
Upper bound	0.000*	0.000*

* Significant: < 0.05. CI: Confidence interval.

Subgroups comparison of this study was determined using Kruskal–Willies test which showed significance then Mann–Whitney U-test for pair-wise

comparison with Bonferroni correction and Chi-square test using both Asymp.sign and Monte Carlo sign (Table 4), to show the effect of both materials (MTA and fish bone powder) and both time intervals (4 weeks and 8 weeks) and all the interaction of different parameters (Inflammation reaction, Dentine bridge formation). The results showed that the materials had significant effect on all tested parameters, where the MTA group had lower inflammation reaction, and thicker dentine bridge formation with high quality compared to fish bone group (Figures 1 and 2). Regarding the effect of time, the inflammation scores decreased significantly at 8 weeks interval compared to 2 weeks interval for fish bone powder, while, in MTA, there was no-significant difference in both time intervals.

Discussion

Direct pulp capping (DPC) is a procedure by which a biocompatible medicament is placed over the exposed pulp to induce reparative dentinogenesis, attempts have been made to introduce new materials for DPC [19], [20]. Evidence shows that the reaction of pulp toward application of demineralization agents, that is, hydroxyapatite improves the remineralization process of remaining dentine and induces reparative action in the pulp in form of reparative dentine formation [21].

Calcium and phosphate ions which are lost due to demineralization can be returned back to the tooth structure [22]. Both calcium and phosphate can be obtained by applying hydroxyapatite on the tooth structure which can be obtained from high-cost synthetic or low-cost natural materials such as egg shell, fish bone, and other sources [23].

Hydroxyapatite is the main biomineral component found in human hard tissues such as teeth and bone. It is believed that fish which is widely consumed all over the world due to its high protein content and low fat may have therapeutic value due to its high content of calcium and phosphate [24]. Fish waste such as bone, skin, head and scale contain high biological components that can be turned into useful products [25]. The extraction process of hydroxyapatite (HA) from natural resources are better than synthetic for its biocompatibility, using uncomplicated method on production, and low cost [26]. It has been demonstrated that fish sources are safe and present low risks of disease transmission [27]. Many studies have been reported on the production of HA from fish source as a raw material for HA extraction; also, all cytotoxicity tests done on hydroxyapatite extracted from fish bone showed no cellular necrosis [28].

The aim of the present investigation was to evaluate pulp capping efficiency of Nile Tilapia fish bone compared to MTA which is used as a gold standard as

Table 4: p value analyzed using Kruskal–Wallis, Mann–Whitney U-test with Bonferroni correction and Chi-square test using both Asymptotic sign and Monte Carlo sign for all groups

Dentin bridge	Asymptotic significant (two-tailed)	Exact significant (2* [one-tailed significant])	Inflammation	Asymptotic significant (two-tailed)	Exact significant (2* [one-tailed Significant])
Fishbone 4 weeks	0.000*	0.001*	Fishbone 4 weeks	0.000*	0.000*
Fishbone 8 weeks			Fishbone 8 weeks		
Fishbone 4 weeks	1.000	1.000	Fishbone 4 weeks	0.000*	0.000*
MTA 4 weeks			MTA 4 weeks		
Fishbone 8 weeks	0.002*	0.005*	Fishbone 8 weeks	0.000*	0.000*
MTA 8 weeks			MTA 8 weeks		
MTA 4 weeks	0.000*	0.000*	MTA 4 weeks	0.534	0.779
MTA 8 weeks			MTA 8 weeks		

* Significant: < 0.05. MTA: Mineral trioxide aggregate.

pulp capping agent despite of its disadvantages such as high cost and longtime setting.

This study was performed on dogs due to a similarity in their teeth composition and growth to that of humans, large sized teeth with good accessibility, and large number of teeth that minimize the number of used animals in the study [29], [30].

Hydroxyapatite used in this study was extracted from Nile Tilapia fish, synthesized using calcination method at 900° to produce two types of calcium phosphate beta-tricalcium phosphate and tetra-tricalcium phosphate which revealed a high degree of purity. The hydroxyapatite powder produced was pure and stable. Furthermore, the calcination method is a simple, cheap, and effective method to extract HA [13], [31]. Two evaluation periods during this study, the first period (4 weeks) and the second is (8 weeks), were selected to give enough time to clearly evaluate effect of both materials used in this study on the pulp.

At 4 weeks, MTA found to be less toxic and causes less pulpal inflammation than Nile Tilapia fish bone. Moderate pulp tissue inflammation occurred in the pulp chamber of approximately 90% of MTA samples and in 82% of fish bone samples. Grade (3) inflammatory cell response was found in only 10% of MTA specimens and in 18 % of Nile Tilapia fish bone power capped specimens. Slight inflammatory reaction is important for generating chemical signals that will lead dental pulp progenitor cells to migrate to the affected area, proliferate, and differentiate to form a mineralized bridge that will separate the pulp tissue from the noxious stimuli to enable the pulp to heal [32], [33]. On contrast, severe inflammation is toxic to normal cells, which may cause incomplete cell regeneration [7]. That is why dentine bridge in some Nile Tilapia fish bone power capped specimens showed a-tubular dentin.

After 8 weeks, significant decrease in inflammatory cell infiltration scores for both materials was found. These results are in agreement with results of the previous studies [7], [20], [34]. Our results evidence that pulp capping with MTA, as well as with HA extract from fish bone, stimulates the formation of reactionary dentin. Dentine bridge formation was observed in all samples capped with MTA (mostly, score 2) and fish bone (score 1 and 2). There were significant differences between both capping agents at that time interval.

All results at 8 weeks interval confirmed the favorable outcome of MTA compared with fish bone in terms of thicker and more complete dentine bridge formation and presence of odontoblast like cell layer almost in all samples. That is may be because the odontoblast like cells need to adhere to proper surface for cell differentiation and new dentine bridge formation. As MTA is biocompatible cement with high sealing ability, odontoblasts can adhere to and produce fibrous connective tissue for further mineralization [35]. However, there are several disadvantages with MTA, high solubility, the long setting time, and also high cost which have urged the development of new materials to overcome these drawbacks [36]. The alternative materials must be more accessible, more cost effective and set in a shorter time. As an alternative, HP extract from Nile Tilapia fish bone was investigated in this study as a natural biological source for hydroxyapatite extraction used as direct pulp capping. A histological study confirmed that the application of both MTA and hydroxyl appetite extracted from fish bone has a direct effect on regeneration potential of the dental pulp and is associated with increase in TGF-β1 secretion from pulp cells [37]. This factor directs the progenitor cells' migration to the material-pulp interface and stimulates their differentiation to odontoblastic cells secreting reparative dentin; thus, it affects the quality of the induced hard barrier. These data indicated that both MTA and HP extract from fish bone promote the same desired cellular response resulting in formation of hard tissue barriers.

The favorable results of MTA compared with fish bone may be due to using calcination method in this study, which produced more large particles and less Ca/P ratio than other methods of hydroxyapatite extraction as dry methods, wet methods, high temperature methods, and combination methods which are slightly difficult and more complicated than calcination method [38], [39].

Conclusion

Regarding the use of experimental HA extract from Tilapia fish bone as a direct pulp capping agent and under the limitation of this study.

HA from Tilapia fish bone is a promising resource as direct pulp capping agent with commercial interest, many of researchers work on this target were funded by scientific institutions in their countries.

1. All biocompatibility tests done before on this material showed the high safety and efficacy.
2. Investigations on hydroxyapatite extracted from fish bone by calcination method and used it as pulp capping agent are still in their infancy (this study was the first one), and its potency as capping material used instead of many of expensive pulp agents found in marked need further studies.
3. Further studies on this material were recommended with using of different methods on manufacture of HA from fish bone.

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