Pulpal Response to Nano Hydroxyapatite, Mineral Trioxide Aggregate and Calcium Hydroxide when Used as a Direct Pulp Capping Agent: An *in Vivo* study

Swarup SJ* / Rao A** / Boaz K*** / Srikant N**** / Shenoy R*****

Nano hydroxyapatite (Nano-HA) and Mineral Trioxide Aggregate (MTA) because of its better qualities can be used as an alternative to calcium hydroxide in direct pulp capping procedures. The aim of the study was to compare the response of exposed human pulp to Nano-HA, Mineral Trioxide Aggregate and calcium hydroxide. ***Study design****: The study was done on 30 premolars, ranging from patients between 11-15 years. Intentional pulp capping was done using one of the experimental materials. The extracted teeth were then subjected to staining procedure and evaluated for dentin bridge and pulpal response after 15 and 30 days. Intragroup comparisons of the observed values were analyzed using Chi-square test.* ***Results****: Nano-HA and MTA produced continuous dentin bridges. Dentin bridge that was formed in MTA group had regular pattern of dentinal tubules but no tubules were seen in the nano-HA group. Dentin bridge was not observed in Dycal group for the 15 days period in majority of the sample and by 30 days dentin bridge was observed that were both continuous and interrupted in equal number of samples. The initial inflammatory response and necrosis was more with Nano-HA and calcium hydroxide which reduced with time.* ***Conclusion****: MTA showed no inflammatory changes in majority of the samples in both the study periods. Necrosis was least observed in MTA group followed by Nano-HA. Vascularity increased in Nano-HA group in the initial periods which reduced with increasing time. Based on the ability of nano-HA to produce complete dentinal bridges, favorable cellular and vascular response, the material could be considered as an substitute and could be tried used as a direct pulp capping agent.*

Keywords: *Nano Hydroxyapatite, MTA, Calcium Hydroxide, Direct pulp capping agents*

INTRODUCTION

The dental pulp has a well-documented ability to form hard tissue barriers called reparative dentin (Dentin Bridge) following direct pulp capping. The materials used should stimulate the formation of dentin bridge, cause minimum or no tissue damage and thereby restore the function and structure of pulp tissue. Calcium hydroxide, a long-favored and much used direct pulp capping agent induces a calcified barrier; however it is also associated with tissue necrosis and inflammation during the initial period of placement, and irregular dentin repair which is of poor quality.[1,2]

* Shanti J Swarup, Ex Post Graduate Student, Paedodontics and Preventive Dentistry.
** Arathi Rao, Professor and Head, Paedodontics and Preventive Dentistry.
*** Karen Boaz, Professor and Head, Oral Pathology.
**** Srikant N, Associate Professor, Oral Pathology.
***** RamyaShenoy Associate Professor, Public Health Dentistry.
Manipal College of Dental Sciences, Mangalore

Send all correspondence to: Dr. Arathi Rao, Manipal College of Dental Sciences, Manipal University, Light House Hill Road, Mangalore 575001, Karnataka State, India

E-mail: arathi_rao@hotmail.com
arathi.rao@manipal.edu

Literature indicates that materials containing calcium ions and high pH promote the formation of hard tissue barrier[2,3]. Hydroxyapatite and Mineral Trioxide Aggregate (MTA) are capable of releasing calcium ions into an environment with high pH and enabling regeneration of hard tissues. This has prompted dental researchers to evaluate the possibility of its use as an alternative to calcium hydroxide in direct pulp capping procedures.

Synthetic hydroxyapatite (HA) with a stoichiometric composition as $Ca_{10}(PO_4)_6(OH)_2$ has excellent biocompatibility with human teeth and bone, making it very attractive for biomedical applications.[4] It is one of the few materials, classified as a bioactive material that supports bone in-growth and osteointegration when applied in orthopedic, dental and maxillofacial scenarios.

HA, as a pulp capping agent, caused inflammation and necrosis of pulp in few cases.[5,6] Focus on overcoming the limitations and improving biological properties via exploring the unique advantages of nanotechnology has led to the development of nano hydroxyapatites (Nano-HA). Biomimetic and bio-inspired nano hydroxyapatites have been used extensively in regeneration of skeletal tissues. These molecules have been found to have similarity to those seen in dentin and enamel.

This study aims to analyze the response of exposed human pulp to bio-resorbable Nano hydroxyapatite powder and compare the response with that of Mineral Trioxide Aggregate and calcium

Table 1. Sample distribution

Group	Period of study	Sample size
Group 1 (Nano HA)	Group IA - 15 days	5
	Group IB - 30 days	5
Group 2 (MTA)	Group IIA - 15 days	5
	Group IIB - 30 days	5
Group 3 (Calcium Hydroxide)	Group IIIA - 15 days	5
	Group IIIB - 30 days	5
	Total	**30**

hydroxide when used as direct pulp capping agents.

MATERIALS AND METHOD

The present study, a double blind *in vivo* study, aimed at evaluating the dentinal and pulpal changes in response to three different direct pulp capping materials namely, nanohydroxyapatite *(Medical grade, low temperature sintered (300°C±5°C), particle size : < 100nm, Orthogran,Top-Notch, Aluva, Kerala*), MTA *(Angelus industria de produtosodontologicos S/A, Londrina, Brasil)* and calcium hydroxide *(Dycal, Dentsply Caulk, Milford, DE*) was carried out in the Department of Paedodontics and Preventive Dentistry in collaboration with Department of Oral Pathology and Microbiology, Manipal College of Dental Sciences, Mangalore. Institutional Ethics Committee clearance was obtained prior to the study.

The study was based on a total selected sample of thirty intact, developmentally sound, non-carious, vital human premolars, scheduled to be extracted for orthodontic reasons from medically fit patients between the ages of 11-15 years after obtaining consent from their parents/guardians.

Inclusion criteria

Non-carious vital premolars indicated for extraction for orthodontic reasons

Exclusion criteria

Defective and/or fractured teeth. Teeth with caries / restorations and medically compromised children

The teeth thus selected were subjected to multistage random sampling in which initially they were divided into three groups based on the material to be used, with further subdivision of each group into two subgroups based on the time period of the study (i.e., 15 days and 30 days) (Table 1).

Lignocaine hydrochloride (2%) with epinephrine (1:2,00,000) (Astra Zeneca, Pharma India Ltd, Bangalore, India) was used to anesthetize the selected tooth. Following rubber dam application, a cavity was prepared on the occlusal surface of the premolars using sterile diamond burs at high speed under air-water spray coolant. The prepared cavities were 3.0 mm to 3.5 mm in depth and 3.0 mm in diameter. Pulp exposure was performed at the center of the cavity by means of a high speed sterilized round diamond bur (ISO 001/012 Dia-Burs, Mani Inc, Tochigi, Japan) with water coolant. Hemostasis was achieved by applying a sterile cotton pellet soaked in saline on the exposure site. On achievement of complete hemostasis, the cavity was dried and the exposure site was covered with one of the three chosen pulp capping materials. Nano hydroxyapatite powder which is available as a white powder was mixed with saline (One

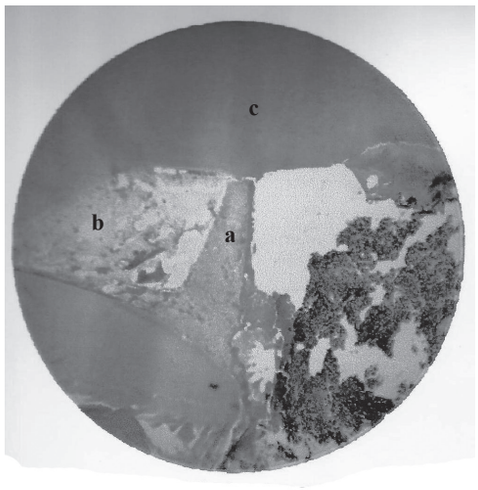

Figure 1. A thick continuous dentin bridge (a) seen in the tooth capped with Nano-HA after 30 days; b:Pulp tissue; c: Dentin (x100)

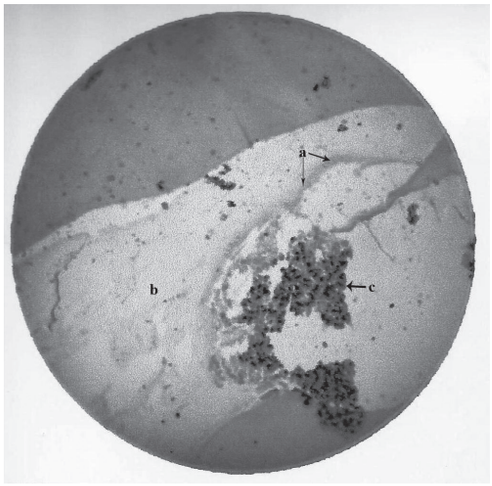

Figure 2. Continuous dentin bridge formation (a) seen between the pulp tissue (b) and MTA (c) after 30 days with no inflammatory cells or necrosis. (x100)

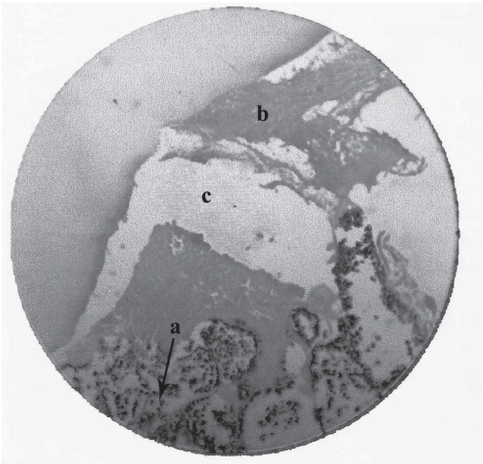

Figure 3. Pulp capped with calcium hydroxide (a) after 30 days showing extensive areas of necrosis beneath the material with continuous, porous dentin bridge (b) capping the exposure site. Void formation (c) can be noticed between the area of necrosis and the bridge. (x100)

Table 2. Modified evaluation criteria used for the assessment of pulpal response

	Dentin bridge formation	Quality of dentin formation in the bridge	Location of dentin bridge	Tissue reaction to the material	Inflammatory cell response	Necrosis
Score 0	No bridge formation	No tubules present	At the interface of exposed pulp	No macrophages/giant cells adjacent to the material	No scattered inflammatory cells in the pulp area corresponding to the pulp-exposed characteristic tissue.	Absent
Score1	Interrupted bridge formation	Irregular pattern of tubules	Not at the interface of exposed pulp	Mild infiltration of macrophages/ giant cells.	Slight inflammatory cell infiltrate with PMNs or MNLs.	Denaturation of proteins. autolysis
Score 2	Continuous bridge formation	Regular pattern of tubules	Combination	Moderate infiltration of macrophages/ giant cells.	Moderate inflammatory cell infiltrate involving the coronal third of the radicular pulp.	--
Score 3	--	--	--	Severe infiltration of macrophages / giant cells.	Severe inflammatory cell infiltrate involving the coronal third of the radicular pulp.	--

scoop powder to one drop saline) using a metal spatula on a glass slab to form a uniform paste of thick consistency.

Other two materials were also manipulated as per the manufacturer's instructions and carried to the exposure site by means of a carrier and condensed with a condenser.

All the prepared cavities were then restored with type II glass ionomer cement (GC Universal Restorative Powder, GC Corporation, Tokyo, Japan).The teeth belonging to groups 1 A, 2 A, and 3 A were observed for a period of 15 days and teeth allocated to groups 1B, 2B, and 3B were observed for a period of 30 days. Extraction of the teeth were carried out at the end of the observation period under local anesthesia consisting of 2% lignocaine with epinephrine (1: 2,00,000) using premolar extraction forceps.

Preparation of the extracted tooth for histological analysis

The extracted teeth were immediately immersed in 10% formalin solution and allowed to remain for ten days to facilitate fixation.

The teeth were then decalcified using 10% formal formic acid. (100ml of 85% formic acid [Merck Specialties Private Limited, Worli, Mumbai] was added to 50ml of formalin and 850ml of distilled water to obtain the decalcifying solution of desired concentration). The specimens were immersed in the solution for a period of 5 days and the demineralization was checked with serial radiographs to ensure complete demineralization.

Once decalcified, the specimens were placed under running water to neutralize the effects of decalcification and then subjected to routine histological tissue processing and sectioned to 5micron thickness followed by routine staining with Hematoxylin and Eosin.

The H&E-stained slides were evaluated for pulpal response to the pulp capping materials as per the evaluation criteria (modified from previous studies)[2,7] and described in Table 2.

The statistical analysis was done using SPSS.11 software. Intragroup comparisons of the observed values were analyzed using Chi-square test.

During the study period no abnormal clinical signs and symptoms were present in any of the samples.

RESULTS

Analysis of the tissue changes were done under microscope with magnification of x100. (Table 3 and 4), (Figures 1 to 3).

Table 3. Results of analysis of the Hard Tissue Barrier

	Dentin bridge formation						Quality of Dentin bridge						Location of Dentin Bridge					
	15 days			30 Days			15 days			30 Days			15 days			30 Days		
Scores →	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
HA	1	4	-	-	1	4	5	-	-	4	-	1	5	-	-	5	-	-
MTA	-	4	1	-	1	4	2	-	3	1	-	4	5	-	-	5	-	-
Dycal	4	1	-	2	1	2	4	1	-	3	2	-	5	-	-	5	-	-
Level of Significance P<0.05	P = 0.032			P = 0.261			P = 0.006			P = 0.015			P = 0.011					

Table 4. Results of Pulpal Response

	Tissue reaction								Inflammatory cell response								Necrosis			
	15 days				30 Days				15 days				30 Days				15 days		30 Days	
Scores →	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	0	1
HA	3	2	-	-	4	1	-	-	-	-	3	2	3	2	-	-	3	2	4	1
MTA	5	-	-	-	5	-	-	-	3	2	-	-	4	1	-	-	5	-	5	-
Dycal	5	-	-	-	4	1	-	-	-	-	4	1	-	4	1	-	2	3	2	3
Level of Significance P<0.05	P = 0.080				P = 0.412				P = 0.003				P = 0.040				P = 0.060		P = 0.059	

Dentin Bridge Formation

The interface between the exposed pulp and the direct pulp capping material was analysed for dentin bridge formation, with all samples in MTA group showing dentin bridge formation. In the 15 days group the bridge was interrupted in 4 samples, but was continuous in 30 days sample. A layer of pseudo stratified columnar odontoblast-like cells were found abutting the layer of predentin-like matrix that was formed capping the exposure site. The matrix deposition was found to be in intimate contact with the material.

Four of the five samples of nano-HA group developed interrupted dentin bridge in 15 days and continuous bridge formation in 30 days period. The osteoid-like deposition was found to be a non-continuous layer between the material and the pulp. An increased recruitment and activity of pulpal fibroblasts was also observed next to the material. Globular pattern of calcification was found on the osteoid with the mineral matrix being laid down surrounding the material. However, no dentin bridge was evident in four and two samples of the calcium hydroxide group in the 15 day period and 30 day period respectively. All cases that showed formation of dentin bridge did so only at the interface of the material and the pulp.

The difference in the ability of the materials to produce a reparative dentin bridge in 15 days was found to be statistically significant (P=0.032), while the difference between the materials in 30 days group was not significant (P=0.261).

Quality of dentin bridge

The reparative dentin that was formed was analyzed to determine the quality of the bridges. In all the specimens of nano-HA group, no tubules were seen both in the 15 days and 30 days group except one sample in the 30 day group which showed regular pattern of tubules. Majority of the samples of MTA group showed regular pattern of tubules, while those in the calcium hydroxide group showed tubular pattern with tunnel defects and porosities.

Statistical analysis using Chi square test revealed significant difference (P=0.006 and 0.015) in the quality of dentin bridges formed in three groups during both the observational periods.

Cellular reaction of the pulp to the material

The ability of the material to evoke a foreign body response in the pulpal tissue was assessed by the appearance of giant cells or macrophages in the area adjacent to the capping material. All the specimens showed histological features either of abscess or mild infiltrate of macrophages/giant cells. All the samples in MTA group showed total absence of these cells in both 15 and 30 days period.

This difference in the cellular reaction between the groups was not significant (P=0.080 and 0.412).

All the groups showed increased inflammatory cell infiltration in the 15 day period compared to the 30 day period. MTA group showed the least and calcium hydroxide the maximum infiltration. It appears that inflammatory cell infiltration reduced as the days increased in all the groups.

The difference between the groups in evoking an inflammatory cell response was statistically significant (P=0.003 and 0.040)

Necrosis was identified based on the denaturation of proteins and autolysis of the tissue adjacent to the capping material. None of the samples of MTA group showed necrosis in the tissue adjacent to the material. Maximum of calcium hydroxide samples showed necrosis.

The difference between the groups in 30 days group was marginally statistically significant (P=0.059).

DISCUSSION

Recent investigations have opened newer vistas in understanding pulpal healing following capping with various capping materials. It has been suggested that wounded dental pulps require capping with bioactive materials which could ensure consistent dentin bridge formation.[8,9]

The attributes of nano-HA namely (a) porous to allow ingrowth of cells and growth factors, (b) allows effective transport of nutrients, oxygen, and waste, (c) biodegradable, leaving no toxic byproducts, (d) replaced by regenerative tissue while retaining the shape and form of the final tissue structure, (e) biocompatible, (f) adequate physical and mechanical strength and (g) antibacterial efficacy, has made the possibility of the material being used as a scaffold in tissue regeneration in endodontics and eventually as a pulp capping agent.

The particle size of nano-HA used in this study had a length of about 50 to 100 nm and a width of about 20 to 40 nm similar to biologic apatites found in the human dentin. Moreover these particles when applied in physiologic medium were found to agglomerate forming tight microclusters with adequate space in between the microclusters to allow the ingrowth of tissue. The molar Ca/P ratio of the material was found to be 1.75, slightly higher than that of naturally occurring stoichiometric hydroxyapatite, Ca10(PO4)6(OH)2 (1.67).[10,11]

Hard tissue barrier

It is established that MTA showed better ability to form tubular dentin bridge over the exposed pulp when compared to the other two materials. Collagen producing cells were observed under higher magnification beneath the dentinal bridges that were formed. Few of these cells were morphologically similar to odontoblasts, which

were responsible for the production of tubular type of reparative dentin seen in specimens capped with MTA and few specimens of calcium hydroxide. Globular type of dentin or osteodentin that was observed frequently in the pulps capped with HA could be produced by pulpal fibroblasts. Increased activity of fibroblasts was noticed immediately subjacent to the capping material, the differentiation of which could have been induced by the material.[2]

Another possible explanation for the increased frequency of osteodentin formed under nano-HA is the osteo conductive property of the material that should have accounted for the increased recruitment of osteoblasts eventually resulting in osteoid deposition by these cells. Previous studies have shown that human pulpal fibroblasts in contact with hydroxyapatite release alkaline phosphatase and serve as a mediator in cell-to-cell interaction, and are related to the functional differentiation of cells for tissue repair including pulpal calcification.[12]

The formation of osteodentin could be considered favorable as one of the studies pointed out that it may provide a suitable microenvironment and scaffold for odontoprogenitor cells. It should also be mentioned that the presence of atubular dentin has been found to be important as it provides a "barrier effect" against penetration of noxious agents.[13] Few authors[8,14] have also speculated that the initially formed atubular dentin or osteodentin can become progressively lined with tubules forming tubular dentin. This result was mirrored in our study as no tubular dentin formation was observed in nano-HA group after 15 days; however one of the sample demonstrated a regular pattern of tubules after 30 days of capping.

Tissue reaction

Intracellular and extracellular biodegradation processes occur after implantation of hydroxyapatite, which is considered to be the essential step before the replacement of the material by the hard tissue. The presence of macrophages in contact with nano-HA in the present study could thus be explained on the grounds of biodegradable property of the material. It has been hypothesized that the intracellular solubilization of phagocytosed nano-HA granules could release a high concentration of free calcium and phosphate ions into the matrix that is produced by the collagen producing cells. Furthermore the tissue fluid is locally supersaturated with nano-HA promoting a physical mineral deposition.[15] The appearance of macrophages has also been attributed to be a sign of healing as they play an important role in scavenging superficial necrotic tissues. The absence of macrophages in the calcium hydroxide-capped pulps until 30 days could explain the continued presence of necrotic tissue beneath the material and a delayed healing response. The absence of macrophages in MTA group could be attributed to the non-biodegradable property and excellent biocompatibility of the material.

Moderate to severe inflammation was seen in the samples capped with nano-HA in the initial days of placement of the material, however there was a statistically significant decrease in the inflammation when observed after 4 weeks. It could be inferred that nano-HA causes a phase of acute inflammation following which there is a healing or recovery period during which restoration of pulpal morphology is attempted.

Necrosis of the pulpal beneath the layer of calcium hydroxide was a consistent outcome evident in most of the specimens after 15 days. Few specimens of the 30 day group demonstrated dentin bridge formation beneath this necrotic layer capping the exposed pulp, with the necrotic layer degenerating and forming a void between the capping material and the bridge. The increased inflammation that was noticed in these specimens suggests that these voids facilitated the entry of irritants through the dentin bridges to the pulp via the tunnel defects.[16-19]

Necrosis was perceived in 2 samples after 2 weeks of placement of nano-HA, nevertheless the necrosed areas were seen only in one sample after 4 weeks. This observation suggests that though the material caused necrosis due to its higher content of calcium and high alkaline pH (pH 11), the material has become more receptive by the pulpal tissues by the progressive loss in calcium to the physiologic fluids, and the necrotic tissue is seen to be scavenged progressively with the normal pulpal morphology being restored. MTA samples showed no areas of necrosis and could be considered as having a favorable pulpal response during the initial periods of pulp capping when compared with nano-HA and calcium hydroxide.

Though extensive histologic serial sectioning of the exposure site has been done to rule out the inconsistency in the bridges and the presence of cellular inclusions between the bridges, the present study was carried out under ideal conditions where the pulps were relatively healthy when exposed. The extrapolation of these results into clinical scenario must be done after taking into consideration that the response is likely to be different in a tooth pulp that is compromised as a result of caries.

CONCLUSIONS

The inference that could be derived from the present study is that:

1. Nano-HA is capable of producing continuous dentin bridge at the interface between the pulp and the material similar to MTA. Calcium hydroxide differed as it displayed only attempts of bridge formation.
2. Osteodentin formation was observed with nano-HA without any tunnel defects or cellular inclusions. MTA samples exhibited regular pattern of dentinal tubules.
3. All the samples showed favorable tissue reaction with initial inflammatory response and necrosis. The response was more with nano-HA and calcium hydroxide when compared to MTA. With time nano-HA showed much reduction in tissue reaction compared to the other materials.

Based on the ability of nano-HA to produce complete dentinal bridges and evoke favorable cellular and vascular response, the material could be considered as a substitute agent for direct pulp capping. Extrapolation of these results to clinical scenario would require further studies utilizing larger sample size and longer periods of observation to ascertain the long term pulpal reaction to these materials.

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