NOS EVALUATIONS IN HUMAN DENTAL PULP-CAPPING WITH MTA AND CALCIUM-HYDROXIDE

C. D'ARCANGELO, F. DI NARDO-DI MAIO, C. PATRONO[1] and S. CAPUTI

Department of Stomatology and Oral Sciences, University of Chieti, Chieti;

[1]Department of Pharmacology, University of Rome La Sapienza, Rome, Italy

The aim of this study is to compare mineral trioxide aggregate (MTA) with calcium hydroxide when used as pulp-capping material in human teeth. 40 teeth were divided into groups based on clinical diagnosis: healthy and hyperaemic. The teeth were pulp capped with MTA and calcium hydroxide. We localized the eNOS and iNOS by immunohistochemistry, tested their mRNA expression by RT-PCR and protein levels by western blots. The evaluation of the samples was based on the cell inflammatory response and on the pulp tissue organization. In particular, evaluation of eNOS and iNOS differences between the various groups and the cellular evolution after the first 7 days from the treatment, and at a distance of 28 days. Our results suggest that there are differences in localization and expression between eNOS and iNOS in dental pulp. Our study has helped us to better understand the effects that calcium hydroxide and MTA have on pulp tissue.

The procedure used in pulp-capping is very important as, on one hand, it must be able to maintain the vitality of the pulp, which would otherwise be subject to necrosis, and on the other, it must avoid mechanical-physical consequences from subsequent endodontic treatment, among which is a high risk of fractures (1). During the capping procedure it is necessary to take into consideration various factors, such as age, paradontal conditions and the state of the root formation (2). The size of exposure, its nature (traumatic, mechanical or carious) and microbial contamination are decisive factors for a successful pulp capping (2). However, the importance of these factors has been challenged. Calcium hydroxide is the material generally used for pulp capping even though it is often the cause of zones of obliteration (tunnel defects, dental bridges) (2-5). Subsequent to pulp capping with this conventional alkaline agent, the adjacent pulp tissue is usually completely deranged and distorted, forming a zone of obliteration. A weaker chemical effect on the subjacent, more apical tissue, results in a zone of coagulation necrosis. This layer causes sufficient stimulation to the vital pulp tissue to respond. Mineral trioxide aggregate (MTA) has been used in pulp-cap procedures in animals, demonstrating remarkable success compared to calcium hydroxide (6-7). MTA is a material that has been used worldwide, in several clinical applications such as apical barriers in teeth with immature apices (8), repair of root perforations (6), root-end filling (9-10), direct pulp capping and pulpotomy (3). This material has been tested thoroughly *in vivo* through implantation tests (8) and usage tests (6). Because this material comes into contact with vital tissues, it must be biocompatible and should favour the regeneration of the tissues involved, back to their pre-diseased status (8).

Only a few studies have evaluated MTA as a pulp-capping agent in human teeth (11-12). The purpose of this study is to compare the properties of MTA with calcium hydroxide in human tooth pulp-capping treatment, evaluating the enzymatic changes of the endothelial and inducible nitric oxide synthases (eNOS and iNOS).

MATERIALS AND METHODS

The local Ethics Committee approved the experimental protocol, and the patients participated after providing informed consent. For this study, forty (n=40) teeth were selected (VIII upper and lower, all protruding into the bucal cavity) which had to be extracted for orthodontic reasons, from patients between 24 and 30 years old. After being informed of the applied therapy, all the patients signed the consent giving authorization to use the teeth.

Before beginning the work protocol, periapical X-rays were carried out using an X-ray (de Gotzen long cone) set at 15mHs, 70kV, 60 Hz and 0,6 sec exposure. Tests for vitality to heat/cold and electrical tests were also performed.

It was therefore possible to divide the teeth into two groups:

- 20 healthy teeth (without caries or paradontal diseases);
- 20 teeth without paradontal diseases, showing carious lesions that gave a state of reversible hyperaemia when stimulated by the vitality test. In the X-rays they showed caries of the enamel and dentin that reached almost to the pulpal horn without touching the marginal crest.

After giving local anesthetic (Articaina 3% Septanest) and applying the rubber dam, each tooth was cleaned with a nylon brush and a polishing paste (clean-polish), then, finally, washed with a 70% alcohol solution.

Cavities (I class) were performed on the occlusal surface of the 40 teeth, using diamond cylindrincal cutters # 6835 (Komet)

Key words: dental pulp, nitric oxide synthase, mineral trioxide aggregate, calcium hydroxide

Mailing address: Dr. Camillo D'Arcangelo,
Department of Endodontic and Operative Dentistry,
University of Chieti,
Via dei Vestini 31, 66100 Chieti, Italy
Tel/Fax: ++3908713554030
e-mail: cdarcang@unich.it

0394-6320 (2007)

mounted on a high speed turbine with sufficient water cooling. The cavities were rectangular, 4 mm long in the mesiodistal sense and 2.5 mm wide in the bucal vestibile sense. The initial depth was of 2.5 mm; in the teeth with caries all the softened dentin was removed using a round bur angled cutter (# H1SE Komet), and then, on all the teeth the mesial pulpal horns were uncovered using a ball diamond cutter (# 6830 Komet) and about 1 mm^2 of the chamber roof was removed . The pulpal hemorrhage was blocked using sterile cotton pellets, with a saline solution.

At this point the 40 teeth were divided as shown below:

1. 10 teeth without caries were capped with calcium hydroxide (Stromidros). After mixing the powder and liquid the material was delicately applied directly into the pulp, and then sterile cotton was applied and a temporary filling material;
2. 10 teeth without caries were capped with Pro-Root MTA (Dentsply). After homeostasis of the opening, MTA was applied to the exposed area, about 2-3 mm thick, a pellet of damp cotton and temporary filling material were applied;
3. 10 teeth with caries where calcium hydroxide was applied on the pulp;
4. 10 teeth with caries on which MTA was applied.

On all the teeth, permanent fillings were applied after 48 hours using Esthet-X (Dentsply) compound. 5 teeth from each group were extracted, after local anesthetic, after 7 days; while the remaining teeth were extracted after 28 days (Table I).

After extraction, the dental pulps were harvested by tooth fracturing, immediately frozen and kept at –80°C for 24-48 hours. Then longitudinal serial sections of about 7 µm were cut with a cryostat (Reichert-Jung Frigocut 2800). For histopathological analysis 3 slides from 5 pulps in each group were stained with hematoxylin-eosin. These slides were used to measure the arteriolar diameter and to localize the odontoblasts and the inflammatory cells.

Biochemical identification of eNOS and iNOS. The immunohistochemical localization of eNOS and iNOS was performed in 3-3 slides of 5 pulps from each group with primary rabbit anti-eNOS (1:100) or primary rabbit anti-iNOS (1:100) antibodies (Santa Cruz Biotech Inc., Santa Cruz, California) as previously described (13). The eNOS and iNOS mRNA were detected from homogenates of 5 dental pulps of each group by RT-PCR using 5'-TGTCTGTCTGCTGCTAG-3' (sense) and 5'-CTCTCCAGGCACTTCAGGC-3' (antisense) for human eNOS and 5'-AGTGATGGCAAGCACGACTTC-3' (sense) and 5'-TCTGTCACTCGCTCACCACGG-3' (antisense) for human iNOS primer pairs, as published earlier by Felaco et al. (13). The eNOS and iNOS protein expressions were detected from equal amounts of protein (50 µg, obtained by homogenizing 5 third molar pulps from each group with lysis buffer (Sigma-Aldrich Co., St. Louis, MO., USA) by Western blots with primary anti-eNOS or iNOS antibodies (Santa Cruz Biotech Inc., Santa Cruz, California) as described in detail in Felaco et al. (13).

Image processing, image analysis and statistical evaluation. The stained sections of the pulps were examined with Leitz Dialux 22, LEICA (Heidelberg, Germany) microscope. The quantitative evaluation of the immune reactions were performed by determination of the integrated optical density (I.O.D.) changes with digital image analysis. Three investigated areas were randomly selected and recorded on 3-3 slides/5 pulps in each group. For data processing each experimental frame was digitized into 512x512 pixels by a Sony video camera connected to a LEICA Quantimet 500 plus (LEICA Cambridge Ltd, Cambridge, England) and the change in I.O.D. was determined using ISO Transmission Density (Kodak CAT 152-3406, Eastman Kodak Company, Rochester, U.S.A.) as a standard.

We used the same analysis system to measure the diameter of 20 randomly chosen arterioles in the hematoxylin-eosin stained coronal pulps of the groups studied. The examination was based on more than one slide/5 pulps/group. The blood vessels selected were of homogenous appearance, free of apparent artifact, and perpendicular to the plane of section.

All results were expressed as mean ± standard deviation (SD). Repeated measure ANOVA was performed to compare means between groups. Probability of null hypothesis of <5% ($p<0.05$) was considered as statistically significant.

RESULTS

The histological evaluation of the samples was based on the cell inflammatory response and on the pulp tissue organization. In particular, a careful microscopic examination highlighted the differences between the various groups and the cellular evolution after the first 7 days from the treatment, and at a distance of 28 days.

In group 1, where the healthy teeth were capped with calcium hydroxide, after 7 days we found a coagulative necrosis of the cell layer in proximity to the area of application calcium hydroxide, a modest inflammatory infiltration characterized by neutrophils and PMN. In particular, the presence was noted of numerous fibroblasts concentrated in the coronal area of the pulp with a very large nucleus (mitotic activity). After 28 days the tissue, still characterized by numerous fibroblasts, did not present inflammatory cells and altered vascular phenomena.

After 7 days, the *eNOS* values were notably increased, mainly localized at the level of the endothelial, fibroblast and odontoblast cells that were in mitotic activity; this was slightly reduced after 28 days. The *iNOS* was present in a slight form after 7 days, and reduced after 28 days.

In group 3, where there were carious lesions, a coagulative necrosis showing intense fibroblast activity, pronounced vascular phenomena (vasodilation) and the presence of infiltrated inflammation (macrophages, giant cells) was observed after 7 days. After 28 days it was observed that the macrophages and giant cells had disappeared and that there were neutrophils, lymphocytes and numerous fibroblasts. Vascular phenomena were normal. The odontoblastic layer was disarranged, but with very large nuclei (dentinogenic activity).

The levels of *eNOS* and *iNOS* in group E were high, and, above all the *iNOS,* reduced after 28 days (Fig. 1 A, B, C, D).

In group 2, where the capping of the pulps was performed with MTA, after 7 days there was an absence of vascular phenomena, inflammatory cells were scarce,

	Group 1	*Group 2*	*Group 3*	*Group 4*
eNOS 7 days	30 ± 2.625	20 ± 2	40.1 ± 1.969	14.9 ± 1.792
eNOS 28 days	20 ± 1.633	10.2 ± 1.476 [a]	30 ± 1.333	9.9 ± 1.524 [a]
iNOS 7 days	20 ± 1.826	5 ± 1.491	30 ± 1.633	10.1 ± 1.524
iNOS 28 days	15 ± 1.155	5.2 ± 1.751	10 ± 1.826	3 ± 1.155

Table I. *The changes of the I.O.D. determined on a surface of 512x512 pixel, using an ISO standard transmission density, have graphically demonstrated how the enzyme expression varies in the different groups. The results were expressed through a mean ± standard deviation (SD). Pairwise comparisons indicated statistical significance at P<0.05. Superscript letters indicate means that are not significantly different (P> 0.05).*

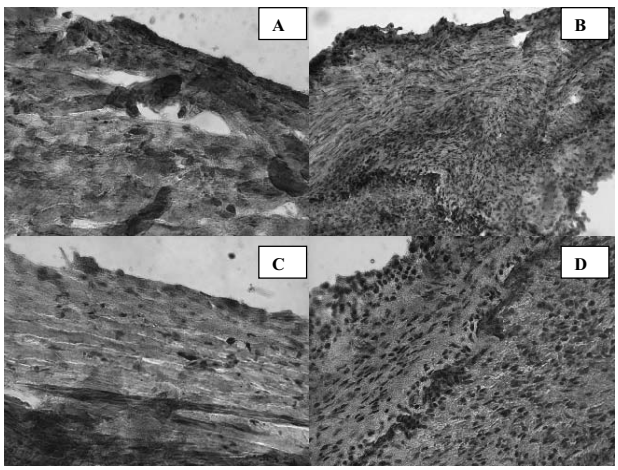

Fig. 1.*Group 3 where the carious lesion teeth, were capped with calcium hydroxide. (Magnification 100x). A. eNOS evaluation after 7 days. B. iNOS evaluation after 7 days. C. eNOS evaluation after 28 days. D. iNOS evaluation after 28 days.*

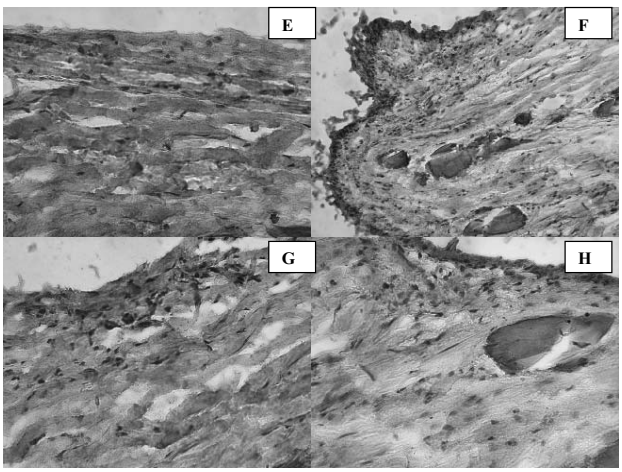

Fig. 2. *Group 4 where the carious lesion teeth, were capped with MTA. (Magnification 100x). E. eNOS evaluation after 7 days. F. iNOS evaluation after 7 days. G. eNOS evaluation after 28 days. H. iNOS evaluation after 28 days*.

and the odontoblasts presented proliferative activity. After 28 days, except for an architectural alteration of the odontoblastic layer, the tissue was healthy. The *eNOS* were increased in respect to the healthy tissue, mainly localized on a fibroblast and odontoblast cell level. After 28 days the *eNOS* had returned to normal levels. After 7 days the *iNOS* showed a low intensity that was reduced after 28 days.

In group 4, after 7 days an infiltrated inflammation formed by neutrophils and PMN was observed, as well as slight vasodilatation and numerous cells in proliferative activity. After 28 days a slight disorganization of the pulp tissue was observed in the coronal portion, but there was a reduction of the above described phenomena, with an absence of inflammatory infiltration. The intensity, after 7 days, of *eNOS* in group F compared to group C taken as reference, results increased as the vascular phenomena are higher, after 28 days there was a reduction of this intensity. After 7 days there was only a small quantity of *iNOS* localized in the inflammatory cells and this decreased after 28 days (Fig. 2 E, F, G, H). The results of the rt-PCR (Fig. 3) biochemical evaluation and the western blots (Fig. 4) for the *eNOS* and *iNOS* enzymes, confirmed the localization and expression of enzymes obtained by the immonohistochemical technique. The highest values of eNOS were obtained after 7 days in group 3; after 28 days there was a reduction in all the groups except for group 3.

The changes of the I.O.D. determined on a surface of 512x512 pixel, using an ISO standard transmission density, have graphically demonstrated how the enzyme

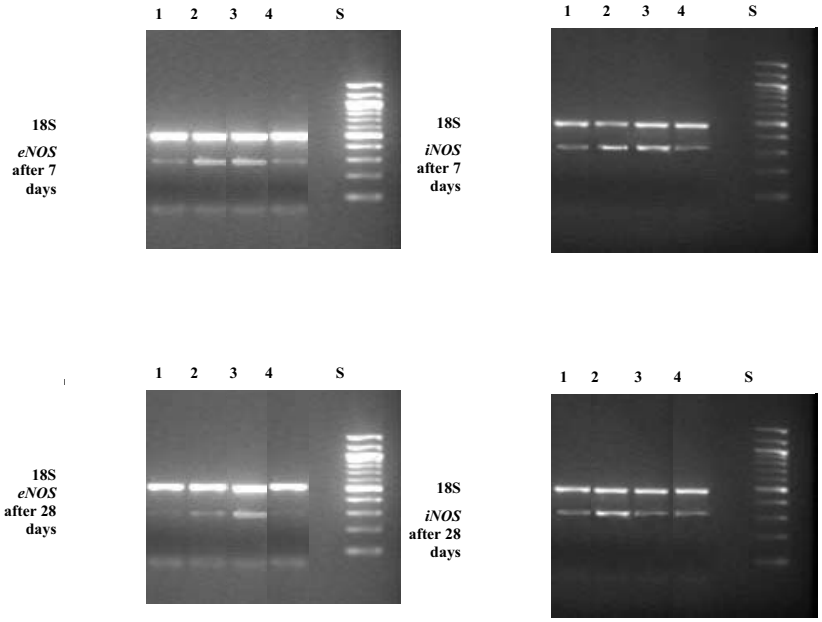

Fig. 3. *Occurrence of mRNA of eNOS in 300 bp and iNOS in 340 bp by RT-PCR in healthy teeth capped with calcium hydroxide (1), in the carious lesion teeth, capped with calcium hydroxide (2), in the healthy teeth capped with MTA (3), in the carious lesions teeth, capped with MTA (4). The standard band is on the right lane (S 488 bp). 18S (488 bp) is the internal standard.*

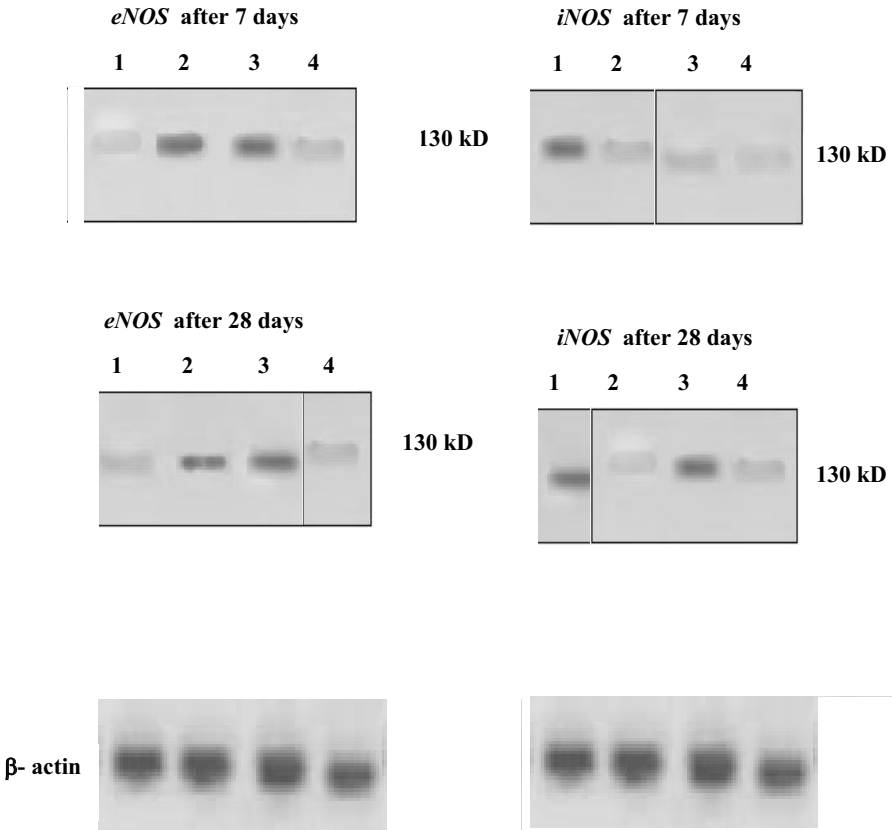

Fig. 4. *Western blot analysis of eNOS and iNOS proteins obtained from healthy teeth capped with calcium hydroxide (1), in the carious lesions teeth capped with calcium hydroxide (2), in the healthy teeth were capped with MTA (3), in the carious lesions teeth, capped with MTA (4). The pulpal proteins were stained by antibodies against human eNOS antigen (133 kD) and iNOS antigen (130 kD).* β-*actin is used for control.*

expression varies in the different groups. The results were expressed through a mean ± standard deviation (SD), and are shown in Table I.

DISCUSSION

In this work we evaluate the effect of two different capping materials (calcium hydroxide and mineral trioxide aggregate) on pulp tissue. The cellular alterations were evaluated not only by examining the inflammatory cells, the vasodilatation and the tissue architecture, but careful immunohistochemical and molecular biology analyses were made through expression of the *eNOS* and *iNOS* enzymes.

In literature it has been demonstrated that all the three isoforms of nitric oxide synthase (NOS) are expressed in pulp tissue (15-17). The constituent form of eNOS is localized in physiological conditions in the vascular endothelium, in the fibroblasts. The nNOS has been identified in the prevascular tissue and in the nerve fibre. The expression of iNOS is induced by the inflammatory processes (14). Law et al. demonstrated, in an experimental model, the pulp of rats, the increase of iNOS in passing from a healthy tissue to an inflamed one (16). The presence of iNOS has also been demonstrated in human pulp tissue in cases of hyperaemia and acute pulpitis (14).

The results of our studies have clearly demonstrated that MTA results the best material for pulp capping. Where the pulp was healthy, the inflammation was scarce, confirming what has been described in literature stating that MTA is extremely biocompatible in studies carried out *in vitro* on cells and tissues as well as those carried out *in vivo* with subcutaneous and bone applications. Pitt Ford et al. demonstrated the lack of cytotoxicity when MTA comes into contact with fibroblast and osteoblast cultures (6). The expression of endothelial nitric oxide synthase, in this group of samples (2 group), demonstrates how, after 7 days, the tissue presents a higher expression of the enzyme compared to a healthy pulp, this being due to an increase of the vascular phenomena within the pulp, caused by the mechanical exposure that had been performed. In fact, after 28 days the tissue was completely healed, the levels of eNOS were normal and there was only an architectural alteration of the odontoblasts. This agrees with the research by Torabinejad that demonstrates that MTA, when it comes into contact with the pulp, stimulates the dentinogenesis and the formation of dentin bridges (10-18). After 7 days iNOS is localized in the neutrophils and lymphocytes present in the pulp, but after 28 days, with the total disappearance of the white cells, this decreases to the same level found in healthy pulp, as already demonstrated in literature.

In group 4, where the capping was performed on teeth with caries and the pulp showed signs of reversible hyperaemia, after 7 days there were numerous inflamed cells, with high eNOS values and an increase of iNOS, as described in literature where it has been demonstrated that in the hyperaemic phase, the vascular effect and the inflammatory cells bring about an increase of the level of enzymes in the pulp. After 28 days there was an improvement in the situation, the inflammatory cells decreased, as did the vascular phenomena, and there was a decided decrease of eNOS and iNOS. This is due to the antiseptic action of MTA, even though it has been demonstrated that this material has a limited spectrum of action on bacteria.

In groups 1 and 3, that represent the samples treated with calcium hydroxide, there is a great cellular proliferation. For some years dental literature has described the biological sequence of calcium hydroxide on the pulp. The healing mechanisms observed are of dystrophic calcification, coagulative necroses, migration and differentiation of the competent pulp cells with synthesis and secretion of the dentinal matrix that brings about the formation of the dental bridge. However, these mechanisms are not yet completely clear, but it would seem that the strong alkaline influence of calcium hydroxide causes the solubilization and release of some proteins that function as factors in the dental growth. In the samples it could be clearly seen that the tissue was richly endowed with cells but disarranged. The eNOS values were high, not for the presence of strong vascular phenomena but because they are localized where there are phenomena of proliferative cell activities; the iNOS present, after 7 days, in groups 1 and 3, decrease after 28 days in both groups, showing that, as hypothesized in literature, the persistence of giant cells and macrophages is not to be attributed to a chronic inflammation but to a phagocytic mechanism of cell repair. If this did not occur then, at a distance of 28 days there would be an increase in the level of iNOS and not a decrease.

In conclusion we can say that our study has helped us to better understand the effects that calcium hydroxide and MTA have on pulp tissue. MTA results as being the better material, even though calcium hydroxide does not only activate the dentinogenisis and the healing process of the tissue, but is seen to have a strong antibacterial influence; for this reason some authors, in cases of capping in the presence of pulp pathology, advise first the application of calcium hydroxide and its removal and substitution with MTA after a few days .

REFERENCES

1. **Lawrence W.S.** 1999. Vital pulp capping: a worthwhile procedure. *J. Can. Dent. Assoc. 65:328.*
2. **Cox C.F., A.A. Hafez, N. Akimoto, M. Otsuki, S. Suzuki and B. Tarim.** 1998. Biocompatibility of primer, adhesive and resin composite systems on non-exposed and exposed pulps of non-human primate teeth. *Am. J. Dent. 11:55.*
3. **Hebling J., E.M. Giro and C.A. Costa.** 1999. Biocompatibility of an adhesive system applied to exposed

human dental pulp. *J. Endod. 25:676.*

4. **Estrela C., L.L. Bammann, C.R. Estrela, R.S. Silva and J.D. Pecora.** 2000. Antimicrobial and chemical study of MTA, Portland cement, calcium Hydroxide paste, Sealapex and Dycal. *Braz. Dent. J. 11:3.*
5. **Pashley D.H., B. Ciucchi, H. Sano, R.M. Carvalho and C.M. Russel.** 1995. Bond strength versus dentine structure: a modelling approach. *Arch. Oral Biol. 40:1109.*
6. **Pitt Ford T.R., M. Torabinejad, H.R. Abedi, L.K. Bakland and S.P. Kariyawasam.** 1996. Using mineraI trioxide aggregate as a pulp-capping material. *J. Am. Dent. Assoc. 127:1491.*
7. **Faraco I.M. Jr and R. Holland.** 2001. Response of pulp of dogs to capping with mineral trioxide aggregate or a calcium hydroxide cement. *Dent. Traumatol. 17:163.*
8. **Torabinejead M. and N. Chivian.** 1999. Clinical applications of mineral trioxide aggregate. *J. Endod. 25:197.*
9. **Torabinejad M., C.U. Hong, F. McDonald and T.R. Pitt–Ford.** 1995. Physical and chemical properties of a new root-end filling material. *J. Endod. 21:349.*
10. **Torabinejad M., C.U. Hong, F. McDonald and T.R. Pitt –Ford.** 1995. Physical and chemical properties of a new root-end filling material. *J. Endod. 21:349.*
11. **Smith A.J., C. Garde, N. Cassidy, I.V. Ruch and H. Lesot.** 1995. Solubilization of dentine extracellular matrix by calcium hydroxide. *J. Dent. Res. 74:829.*
12. **Aeinehchi M., B. Eslami, M. Ghanbariha and M.A.S Saffar.** 2003. Mineral trioxide aggregate (MTA) and calcium hydroxide as pulp-capping agents in human teeth: a preliminary report. ***Int. Endod. J.*** *36:225.*
13. **Felaco M., F. Di Nardo Di Maio, P. De Fazio, C. D'Arcangelo, M.A. De Lutiis and G. Varvara.** 2000. Localization of the e-NOS enzyme in endothelial cells and odontoblasts of healthy human dental pulp. *Lif. Sci. 68:297.*
14. **Di Nardo Di Maio F., Z. Lohinai and C. D'Arcangelo.** 2004. Nitric Oxide Synthase In Healthy and Inflamed Human Dental Pulp. *J. Dent. Res. 83:312.*
15. **Kerezoudis N.P., L. Olgart and L. Edwall.** 1993. Differential effects of nitric oxide synthesis inhibition on basal blood flow and antidromic vasodilatation in rat oral tissues. *Eur. J. Pharmacol. 241:209.*
16. **Law A.S., K.R. Baumgardner, S.T. Meller and G.F. Gebhart.** 1999. Localization and changes in NADPH-diaphorase reactivity and nitric oxide synthase immunoreactivity in rat pulp following tooth preparation. *J. Dent. Res. 78:1585.*
17. **Lohinai Z., I. Balla, J. Marczis, Z. Vass and A.G. Kovách.** 1995. Evidence for the role of nitric oxide in the circulation of the dental pulp. *J. Dent. Res. 74:1501.*
18. **Koh E.T., F. McDonald, T.R. Pitt Ford and M. Torabinejad.** 1998. Cellular response to mineral trioxide aggregate. *J. Endodont. 24:543.*