UNIVERSIDADE DE UBERBA
PROGRAMA DE MESTRADO EM ODONTOLOGIA

André Oliveira Naufel de Toledo

Expression evaluation of VEGF at pulp - dentin complex after action of different cementation agents

Artigo submetido ao programa de Mestrado em Odontologia.

Orientador: Prof. Dr. José Bento Alves.

UBERABA- MG
2012

Abstract:

The objective of this study was to evaluate the response of the complex dentin - pulp, using resin cements for cementation of ceramic after cavities prepares, made by KG Sorensen #3131, through histological and immunohistochemical analysis for VEGF in pulp cells of extracted third molars, immediate at 7 and 30 days, of patients after preparation and cementation of ceramic. Although inflammatory responses observed in the group of immediate extraction after analysis, we can observe a standard of normalcy in pulp responses after 7 and 30 days, suggesting a cellular reorganization after the injury by the cavity prepare.

O objetivo do presente estudo é avaliar a resposta do complexo dentina – polpa, frente a cimentos resinosos utilizados para cimentação de cerâmicas, através de analises histológicas e imunohistoquimicas para VEGF ,de células pulpares de terceiros molares preparados com a fresa KG Sorensen #3131 e extraídos, imediatamente , 7 e 30 dias, de pacientes após preparo e cimentação das cerâmicas. Apesar de respostas inflamatórias observadas no grupo de extração imediata, após analises conseguimos observar um padrão de normalidade nas respostas pulpares após 7 e 30 dias ,sugerindo uma reorganização celular após após a agressão sofrida pelo preparo cavitário.

Key Words:

- Cavity prepare, Dentin – pulp complex, Inflammation, Immunohistochemical, Resin cements, VEGF.

Introduction:

The search for dental materials that are biocompatible with the dentin–pulp complex, and suitable concerning physical, mechanical and esthetic properties, has guided the development of several commercial products. The possibility of materials adhesion to dental hard tissues has enabled modern approach to operative dentistry with minimal intervention and very conservative preparations to preserve tooth structure[1]. In order to innovate products and techniques, the dentistry market has launched improved versions of materials. New products and updates to those already marketed are intensely released by industry and normally presented to the dentists as differentiated from existing products. However, they must be thoroughly tested to validate and prove their properties[2].

Another important aspect to consider is the restorative procedure, Independent of the type of restoration when performing a preparation, nearly 2 million dentinal tubules (30,000 to 40,000 dentinal tubules per mm^2) can be exposed. This procedure leads to risks of damage for the pulp after preparation. Such damage can be more or less severe depending on the heat generated by a rotating device, amount of remaining dentin, dentin permeability, provisional restoration, the final cement type and degree of leakage[3]. According to Al-Dawood[4], there are two reasons for the occurrence of pulp inflammation after restorative procedure: the toxicity present in the composition of cement and a possible bacterial infection.

Modern concepts of indirect restoration have permitted more sophisticated applications of adhesive techniques, providing better esthetic and biomechanical characteristics of the restorative work[5]. Adhesive system, has been considered the best choice for both direct techniques and cementation of indirect restorations and application of low viscosity resins (resin coating) on the prepared teeth showing good biomechanical behavior[6]. However, the interface quality is constantly being questioned about bonding strength and marginal adaptation[7,8].

Pulpal responses for these restorative procedures are not yet clarified. An important aspect to be considered in evaluating the biocompatibility of a material is the potential for repair[9].

Vascular endothelial growth factor (VEGF) is a glycoprotein that shares homology with platelet-derived growth factor and is a potent inducer of microvascular permeability. VEGF is considered as an essential factor for differentiation of the vascular system[10]. It is a potent pro-inflammatory mediator[11], an important mediator of angiogenesis and osteogenesis[12].

Response capacity of the pulp, therefore, depends on the integration of numerous cellular and extra-cellular factors. Some reinforced ceramic systems are susceptible to conditioning with hydrofluoric acid to 10%. These systems are known as siliceous ceramic system, which contain a network of SiO_2 in its composition.

The conditioning produces micro retentions, which are effective in the interaction with the resin cement. However, these ceramics are joined to the resin luting agents by condensation, using silanization agent. The reinforced ceramics that do not
contain SiO2 composition are not capable of being conditioned and have no chemical union resin luting agents, even with the use of silane. Thus, this experiment aims to evaluate the pulp response against different adhesive cementation techniques in indirect inlay ceramic restorations.

Materials and methods

This *in vivo* study was deemed to be ethical according to the Brazilian Guidelines (Resolution 196 of the National Health Council, 1996), and the protocol was approved by the Research Ethics Committee of the University of Uberaba (protocol 026/11). Signed consent was given by patients or their parents after they had received a thorough explanation about the study.

Thirty-two healthy third molars showing no clinical or radiographic impairment, with complete formed roots and indicated for extraction were selected for this study. All clinical procedures were executed by a single professional.

Teeth were randomly divided into 3 groups according to the materials used (Table 1): control groups: Positive control (PC) and negative Control (NC) and experimental groups: group I : Teeth received cementation of ceramic e-max (Ivoclar Vivadent, Liechtenstein) with Rely X™ Luting 2 Cement (3M Dental Products, St. Paul, MN); and Group II Teeth received cementation of ceramic e-max with Rely X™ U100 Cement (3M Dental Products, St. Paul, MN). Teeth from the experimental groups were extracted after 7 or 30 days for immunohistochemical analysis.

Using a radiographic examination, the size of the occlusal surface between dentin and the pulp chamber was assessed in the radiographic image. The teeth were polished with a rubber cup and low abrasion prophylactic paste (Odahcam; Dentsply,Petrópolis, Brazil).

After local anesthesia, the teeth were prepared using a high-rotation sterile diamond 3131# bur (KG Sorensen , Barueri, SP,Brazil) under water irrigation to reduce the maximum aggression generated by the friction produced between the drill with the tooth surface. This preparation followed dimensions of the drill, 3 mm in depth, 2.5 mm of this / mesial and 2.5 mm vestibule / lingual (Fig.1). The taper of the surrounding walls was approximately 12 degrees as the angle of tip of the drill used as recommended by SEGRETO, Raimundo Dario[13].

The depth of the cavities was assessed with measuring instruments and radiographic examination, in order to control the remaining pulp dentin wall about 1mm (Fig. 2).

The internal surface of the e-max (Ivoclar Vivadent) ceramic restoration was treated with 9.5% hydrofluoric acid for one minute, received application of silane (Monobond-S, Ivoclar Vivadent) and was left to dry for 5 minutes.

Group I (Rely X ™ Luting 2) - Equal amounts of base and catalyst pastes were dispensed into the pad of paper for a device, called by the manufacturer.

Then, the manipulated cement was applied to the treated surface of the ceramic, which was placed into the cavity by manual pressure.

Group II (U100 Rely X ™) - Equal amounts of catalyst and base pastes were discharged on the pad exclusively provided by the manufacturer and the mixture was applied to the internal surface of the ceramic restoration. Then the restoration was seated in the cavity by manual pressure. The details of the cement composition and description are presented in Table 2.

After tooth extraction, the teeth were sectioned transversally and fixed in 10% buffered neutral formalin for 48h. Demineralization was carried in 10% ethylene diaminotetraacetic acid (Sigma, St. Louis, MO) solution (pH 7.3) at room temperature for a period ranging from 120 to 180 days. The tissue was dehydrated in ascending series of ethanol, immersed in xylene, and embedded in paraffin using conventional procedures. Sagittal sections of 6 µm were mounted on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma) and submitted to immunohistochemical analysis.

For immunohistochemical procedure**,** the slices were deparaffinized in xylene, rehydrated in 100%, 90%, and 70% alcohol, in distilled water and washed in Tris-buffered saline (TBS). The sections were then immersed in 0.3% hydrogen peroxide for 1 hour to block the endogenous peroxide activity. The slides were then incubated with monoclonal antibodies for

VEGF for 60 minutes at room temperature and rinsed with TBS for 3 minutes 3 times. Next, the secondary biotinylated antibody was applied to the sections, incubated for 30 minutes and rinsed again with TBS. The streptavidin-biotin-peroxidase complex (Vector) was then applied to the slides, incubated for 30 minutes, rinsed in TBS and counterstained with Mayer hematoxylin. Staining specificity was ascertained by omission of primary antibodies.

At least 10 representative sections of each specimen were analyzed under light microscope (BX50; Olympus, Tokyo, Japan). Immunohistochemical analysis was performed individually in a blind fashion by 2 calibrated examiners (kappa index 0.91). Relative staining intensity was assessed for each molecule at the odontoblast layer, predentin layer, and pulp tissue. Samples were scored as follows: 0 - no immunoreactivity; 1 - weak but visible staining intensity; 2 - moderate staining intensity; and 3 - strong staining intensity.

Statistical analysis

The collected data were statistically tested by using Tukey test.
All groups were evaluated three times and significance was considered when $p<0,05$.

Results:

The Scan Electron Microscope (SEM) images of the diamond tips # 3131 (Sorensen kg) used in the study demonstrated the loss of diamond particles and also accumulation of waste from the cavity (Fig. 3).

No pain or particular symptoms were reported by the patients during the study. The radiographic evaluation of the teeth demonstrated no periapical pathology prior to the clinical procedures or extraction.

Histological Results

Due the difficulty of working with 3rd molars, the mandibular arch position and occlusion often deficient, some histological sections of the region in the final preparation showed distances to the pulp near to 0.5 mm. (Fig. 4). The individual mean Remaining Dentin Thickness (RDT) values associated with each material and evaluation period are given in Table 3.

The histological analyses of the healthy tooth revealed pulp tissue with normal histological characteristics. It can be noted the tubular dentin associated with a homogeneous predentin layer which is underlined by at the continuous odontoblast monolayer (Fig. 5).

The histological results of group NC (prepared tooth immediately extracted) exhibited slight disruption of the odontoblast layer related to the cavity floor and presence of many small vessels among the odontoblast cells characterizing the disruption of the odontoblast layer. However, the pulp tissue exhibited a defined cell-rich zone in which small vessels can be noted. In the central layer large vessels can be observed (Fig. 6)

Group I and II in general, most of the specimens of group I and II, both at 7 and 30 days, exhibited unchanged morphology. Their histological features showed that the pulp response from groups I and II were quite similar. The samples exhibited pulp tissue normally organized with no inflammatory response or dentin matrix deposition (Fig. 7).

Immunohistochemistry study

Results revealed a strong immunostaining (Fig.8) in the group NC (prepared tooth immediately extracted). However, at days 7 and 30 days in the group I and II a weak immunostaining similar to the not prepared tooth were observed (Fig. 9) The score of relative staining intensity are showed in table 4.

Discussion

The ideal way to evaluate the biocompatibility of different materials for Dentistry, would be to analyze the responses of apical and periapical tissues by histopathological studies performed in humans. Some human studies aim to understand the healing characteristics of pulp cells[14.]

In the present study, based on previous articles, it was possible to obtain and analyze samples of human dental tissue, which allowed us to obtain tissue response at a more precise way and to obtain more precise tissue.

The evaluation of tissue compatibility concerning to different dental materials is important as dentin and pulp are considered as one body (dentin-pulp complex), because of the intimate relationship between the cellular content of dentinal tubules and pulp tissue[15].

Dentin has an average of 65 to 75,000 tubules per mm^2 near the pulp, 30 to 35000 at medium portion and 10 to 25,000 tubules in the periphery. Close the pulp, the number of tu-

bules whit larger diameter ranging between 2.5 to 3.0 microns, is larger while on the periphery it reaches a diameter smaller than 1.0 micrometer[16].

Thus, the diffusion of substances through dentin may facilitate their contact with the pulp and cause pathological changes, depending on the size of the molecule, the components of the product used, the area available for diffusion, the permeability of dentinal tubules and the remnant width of the dentin[17.]

The direct application of an adhesive resin on sites of pulp exposure was shown to induce an increase of pulp inflammation and vascularization[18].VEGF is produced by several cell types, such as keratinocytes, macrophages, mast cells and fibroblast. It was also observed that VEGF increased vascular permeability and was involved in the pathobiology and progression of inflammation[19.]Several publications address the potencial role of VEGF in the biology of the dentin-pulp complex. VEGF has been shown to be present in the dentin matrix, Which suggests a contribution to the overall reparative response of the dentin-pulp complex[20]. VEGF expression has been reported in stromal cells of healthy pulps[21.]

The pulp reaction to cavity preparation can range from a discreet inflammatory response associated with a slight tissue disorganization, to a pulp necrosis or a complete pulp collapse. It is expected that these factors might cause a more intense pulp response with reduced remaining dentin thickness. In the present study all the cavities were prepared by one clinician who had relevant clinical experience. Moreover, the burs were replaced after every two cavity preparations and the burs were verified by SEM (Fig.3).

Despite all these precautions in group NC, we found little disorganization of odontoblast layer with increased vascularity without inflammatory infiltrate.

In the groups I and II, the pulp tissue showed no histological changes and no significant inflammatory infiltrate at 7 and 30 days. These data suggest that the ceramics and the cementation process did not injury the pulp tissue, and were in contrast, able to protect the pulp tissue by creating conditions for tissue repair installed immediately after preparation and cementation of ceramics.

It has been reported that odontoblast-like cells and undifferentiated pulp cells express VEGF, these pulp cells may be an important source of VEGF in the dental pulp for maintenance of pulp vascularization[22].

Our results showed increased expression of VEGF in the group NC (Immediately prepared tooth extracted). These data are consistent with results from Mantellini et al[23], by which VEGF was involved whit regulation of pulp neo vascularization. We believe that after the preparation and cementation of resin and immediate extraction of dental elements, the pulp

tissue was influenced for VEGF secretion and increases the vascularization. At 7 and 30 days after the operating procedures, pulp tissue recovers its normal standard, as shown by histological and immunohistochemical results.

Conclusion:

Cavite prepare cause inflammatory process at the local of the injury. Rely X Luting 2 and Rely X U100 showed no pulp responses, after 7 and 30 days, therefore can be used for cementation of deep and very deep ceramic restorations.

References:

1 . Costa Cas, Mesas Na, Hebling J. pulp response to direct capping with an adhesive system. Am J. Dent 2000;13:81-87

2 . Osorio R; Pisani-Proenca J; Erhardt Mcg. Resistance of ten Contemporary adhesives to resin–dentine bond degradation. J Dent,2008. v.36, p.163-169.

3 . Pashley, D. H.; Sano, H.; Yoshima, M. *et al.* Dentin, a dinamic bonding substrate: the effect of dentin variables on resin adhesion. Quintessence Int,1996 v. 12, n. 4, p. 24-43.

4 . Al-Dawood, A.; Wennerberg, A. Biocompatibility of dentin bonding agents. Endod , Dent Traumatol.1993,v.9, p.1-7.

5 . Derand T., Molin M., Kleven E., Haag P., Karlsson S. Bond strength of luting materials to ceramic crowns after different surface treatments. Eur J Prosthodont Restor Dent,2008. v.16, n.1, p.35-38.

6 . Andrade Os; De Goes MF; Montes M. Marginal adaptation and microtensile bond strength of composite indirect restorations bonded to dentin treated with adhesive and low-viscosity composite. Dent Mater.2007, v.23, p.279-287.

7 . Balkenhol M; Huang J; Wortmann B; Hannig M. Influence of solvent type in experimental dentin primer on the marginal adaptation of Class V restorations. J Dent, v. 35, 2007, p.836-844.

8 . Abdalla AI; Feilzer AJ. Four-year water degradation of a total-etch and two self-etching adhesives bonded to dentin. J Dent,2008. v.36, n.6, p.611-617.

9 . Cheung GS; Lai SC; NG RP. Fate of vital pulps beneath a metal-ceramic crown or a bridge retainer. Int Endod J, v.38, n.8, p.521-530, 2005.

10 . L. Tram-Hung, P. Laurent, J. Camps. About. Quantification of angiogenic growth factors released by human dental cells after injury. archives of oral biology 5 3 (2008) 9 – 1 3.

11 . Gert Mayer. Capillary rarefaction, hypoxia, VEGF and angiogenesis in chronic renal disease. Nephrol Dial Transplant. 2011. 26: 1132–1137

12 . Wanjin Tang, Fan Yang, Yang LI, Transcriptional Regulation of Vascular Endothelial Growth Factor (VEGF) by Osteoblast-specific Transcription Factor Osterix (Osx) in Osteoblasts. JBC Papers in Press. 2011, M111.288472.

13 . SEGRETO, Dario Raimundo. Influencia da modulação da energia luminosa e da densidade energetica sobre a resistencia da união de restaurações de compositos fotoativados por LED ou luz de lampada halogena. 2005. Dissertação (Mestrado em Odontologia) Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba, Piracicaba, SP.

14 . Fernandes Alexandre M., Direct capping of human pulps with a dentin bonding system and calcium hydroxide: an immunohistochemical analysis.Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:385-90.

15 . Mjor IA. “Dentin-predentin complex and its permeability: pathology and treatment overview.” J Dent Res. 1985 Apr; 64 Spec No:621-7.

16 . Garberoglio R. Branstrom M.: S. E. M. "Investigation of human dentinal tubules". Arch Oral Biol.1976.21: 355-362.

17 . Rivera, Daniela. Cimentos Resinosos: Resposta Tecidual em dentes de cães. 2010. Dissertação Mestrado em Odontologia, Faculdade de Odontologia de Ribeirão Preto da Universidade de São Paulo. Ribeirão Preto, SP.

18 . Costa Ca;Ribeiro Ap, Giro Em, Randall RC. Pulp response after application of two resin modified glass ionomer cements (RMGICs) in deep cavities of prepared human teeth. Dent Mater. 2011 Jul;27(7):e158-70.

19 . Ferrara N. The role of vascular endothelial growth factor . Eur J Cancer;
2413 -2422 ,1996.

20 . Roberts-Clark Dj, Smith AJ. Angiogenic growth factor in human dentin matrix. Archs Oral Biol 2000;45:1013-1016.

21 . Artese L, Corrado, Ferrero G, Fiorini M, Santinelle A, Piatelli A. Vascular endothelial growth factor (VEGF) expression in healthy and inflamed human dental pulps. J Endod 2001;28:20-23

22 .Botero Tm, Mantellini MG, Song W, Hanks CT, Nor JE. Effect osf lipopolysaccharides on vascular endothelial growth factor expression in mouse pulp cells and macrophages. Eur J Oral Sci 2003; 111:228-34.

23 .Mantellini mg, Botero T ,Yamann P, Dennison Jp , Hanks, Nör JE. Adhesive resin and the hydrophilic monomer HEMA induce VEGF expression on dental pulp cells and macrophages. Dent Mater. 2006 May;22(5):434-40. Epub 2005 Sep 22.

Tables:

Table 1:

Groups	Experimental Material	Tooth Extraction	N° of tooth / Subgroup	N° of tooth / Total
CP[1]	_	Immediate	4	4
CN[2]	_	Immediate	4	4
I	Rely X™ Luting 2	Immediate	4	12
		7	4	
		30	4	
II	Rely X™ U100	Immediate	4	12
		7	4	
		30	4	

[1]PC: Positive Control. – Healthy tooth

[2]NC: Negative Control – Tooth prepared without experimental material

Table 2:

Product	Manufacturer	Product Description	Product Composition *
Rely X® Luting 2	3M ESPE St. Paul, MN, USA	Glass ionomer cement modified by resinclicker	Paste A: fluorine-glass-aluminum silicate, reducing agent, opacifying agent, HEMA, water. Paste B: polycarboxylic acid metacrylic, BisGMA, HEMA, water, perssulfato potassium load of zirconia silica.
Rely X® U100	3M ESPE St. Paul, MN, USA	Self-adhesive resin cement in clicker	Paste base: glass fiber, phosphoric acid esters methacrylate,triethyleneglycol dimethacrylate, silane-treated silica, sodium persulfate. Catalyst paste: fiberglass dimethacrylate substitute treated silicasilane, p-toluenesulfonate, sodium calcium hydroxide.

* According to information from the manufacturer.

Table 3:

Table 3 - Remaining dentin thickness (RDT) in µm as a function of the material and period of evaluation.				
Period	Specimen	Groups		
		Group NC	Group I	Group II
7 days	1	459.5	461.4	459.3
	2	462.3	459.1	458.1
	3	458.4	462.8	467.2
	4	460.7	464.3	456.4
	Mean (SD)	460.2±1,67[a]	461.9±2.21[a]	460.2±4.14[a]
30 days	1	462.7	459.2	460.8
	2	460.2	455.1	467.1
	3	465.3	470.1	462.0
	4	464.1	466.2	459.2
	Mean (SD)	463.07±2.19[a]	462.25±6.19[a]	462.4±3.27[a]
Means identified with the same letter do not differ statistically.				

Table 4:

	Healthly tooth	Immediate	GI 7 days	GI 30 days	GII 7 days	GII 30 days
VEGF						
Odontoblast layer	1.23±0.03[a]	2.19±0.06[b]	1.17±0.05[a]	1.18±0.09[a]	1.17±0.02[a]	1.31±0.8[a]
Cell rich layer	1.42±0.08[a]	2.06±0.04[b]	1.22±0.04[a]	1.26±0.07[a]	1.27±0.05[a]	1.3±0.7[a]

Figures:

FIG.1

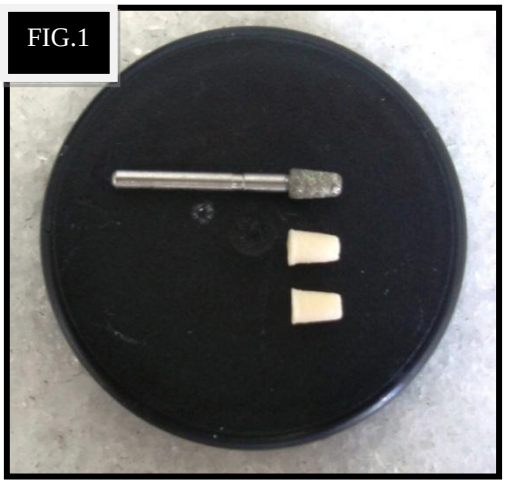


FIG.2

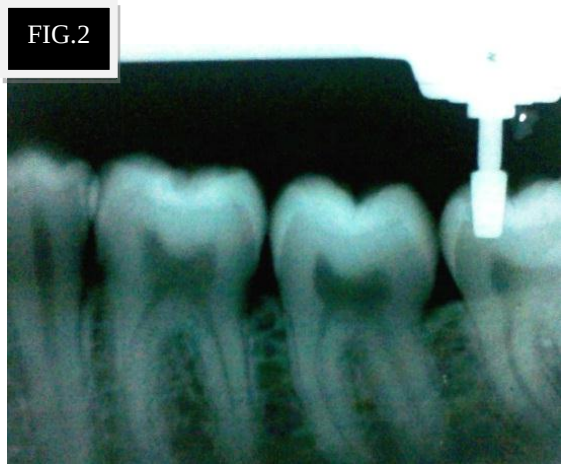


Fig. 3

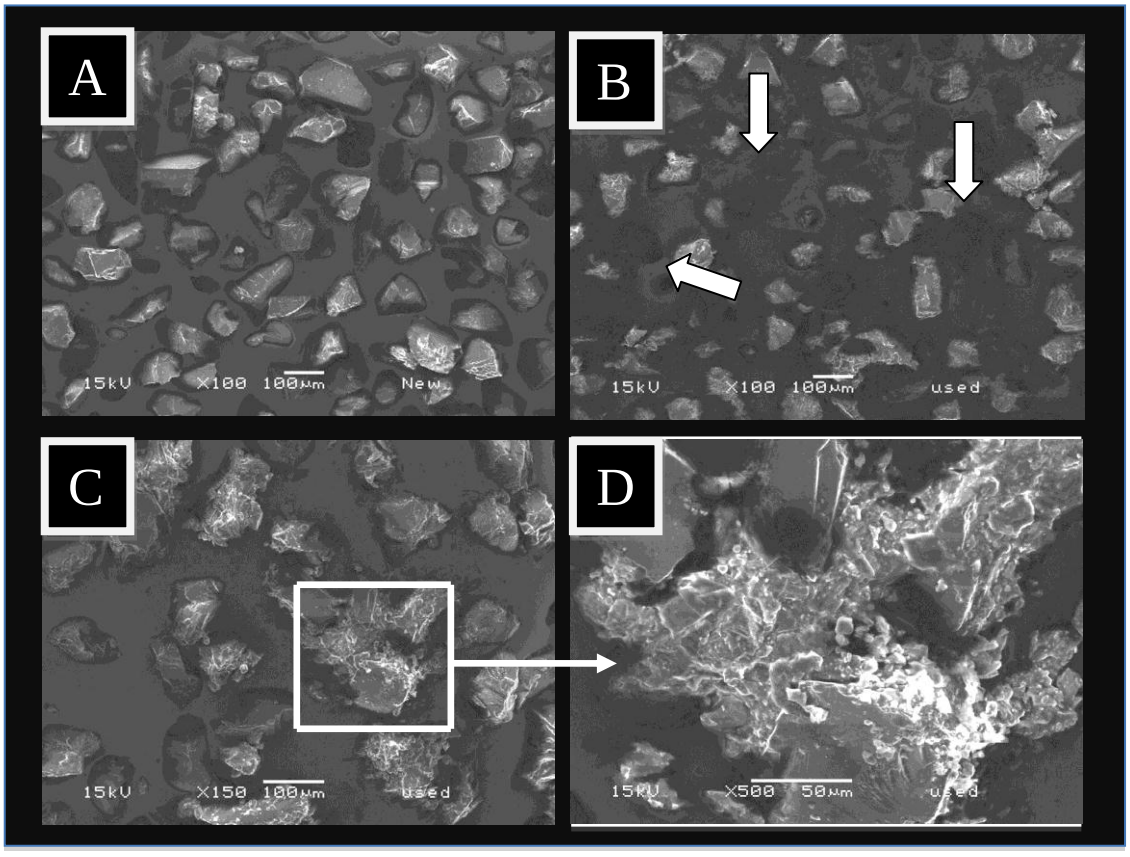


FIG.4

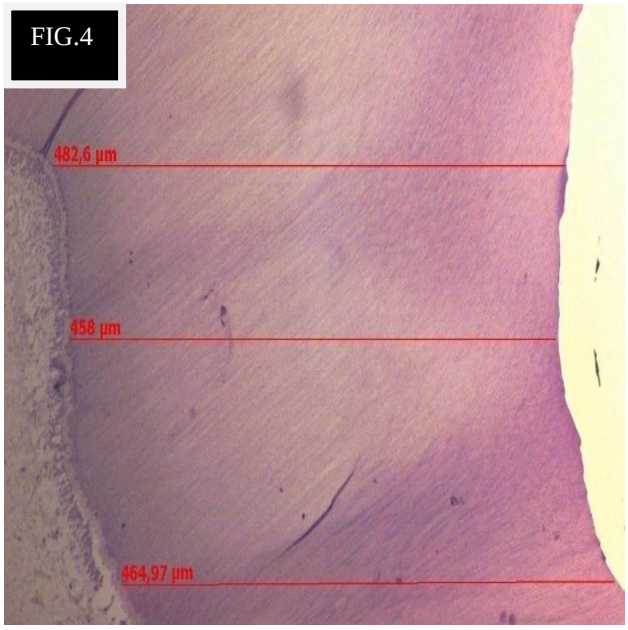


FIG.5

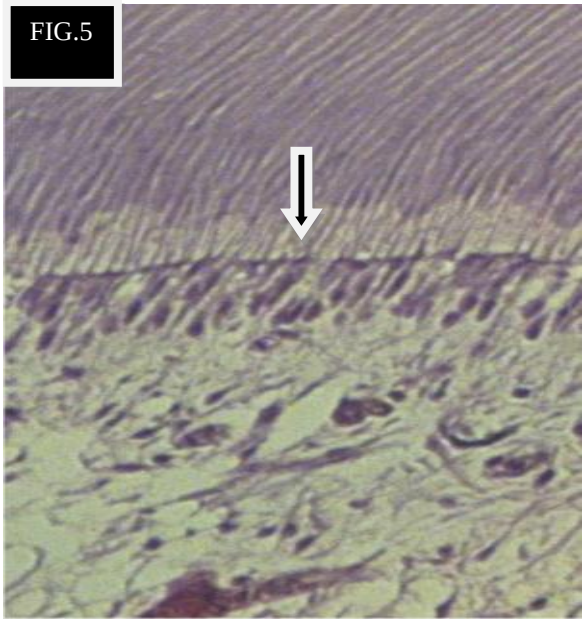


FIG.6

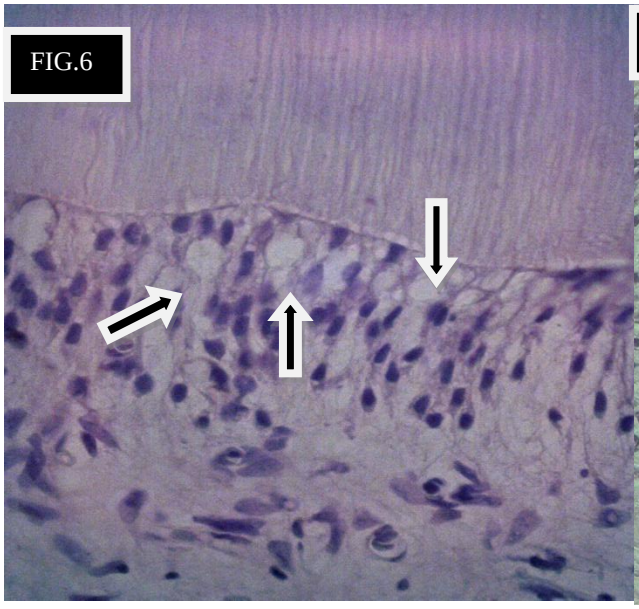
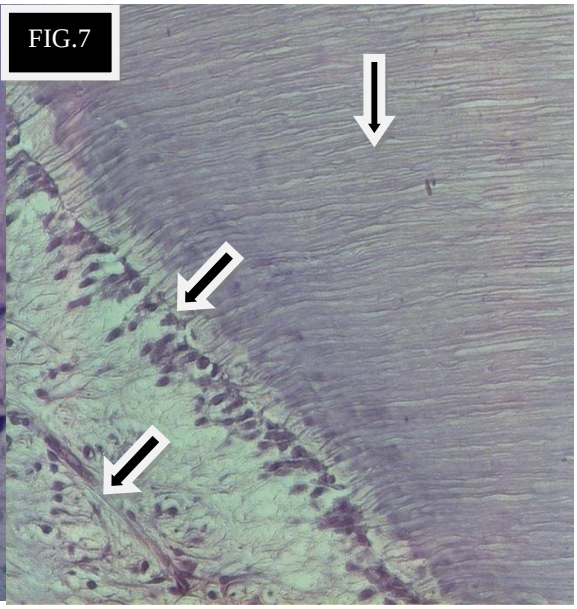
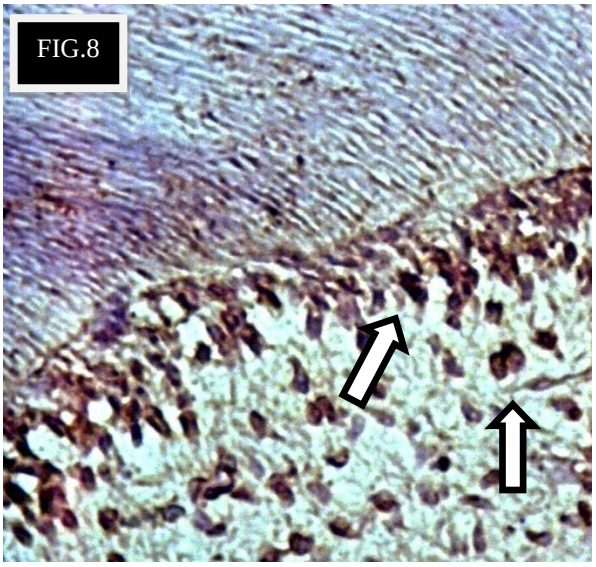
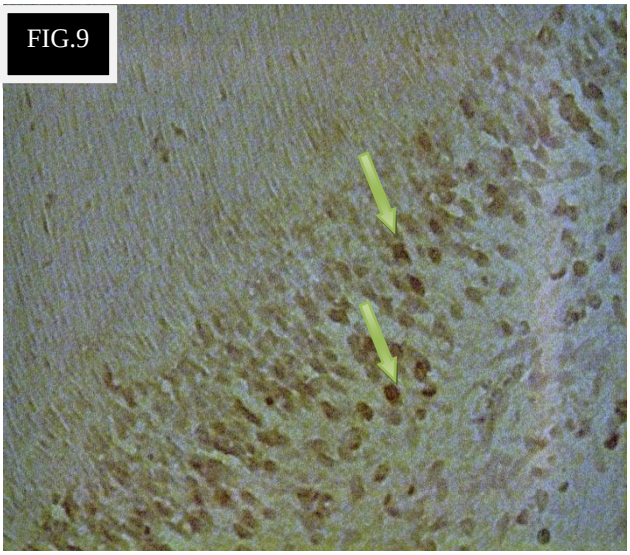


FIG.7







Legends of tables and figures:

Table 1: Details of the separation of samples groups

Table 2: Materials used, manufacturers and composition.

Table 3: Staining levels of VEGF at 7 and 30 days in the investigated areas.
Data are presented as median and standard deviation of the average staining of all sections analyzed per area.

Table 4: Data are presented as median and standard deviation of the average staining of all section analyzed per area.
a,b letter, represent intergroup analysis. Different letters differs statistically (Tuckey, $p>0.05$)

Fig. 1: ceramic restorations with dimensions of the diamond tip 3131(KG Sorenssen).

Fig. 2: Control X-ray of the remaining dentin.

Fig. 3: The new diamond tip with a large amount of diamond particles distributed homogeneously over the surface. Fig. 3B there is the space left by the loss of diamond particles indicated by the arrows. Fig. 3C observe the waste from dental preparations which can be seen in an increase in Fig. 3D.

Fig. 4: Distance of the odontoblast layer after cavity preparation.

Fig. 5: PC, continuous odontoblast monolayer pointed by the arrow, healthy tooth revealed pulp tissue with normal histological characteristics.

Fig. 6: NC, Disruption of the odontoblast layer, whit the presence of many small vessels among the odontoblast cells pointed by the arrows.

Fig. 7: dentinal tubules, odontoblast layer and area rich in cells pointed by the arrows at this order, normal histological response for groups I and II (7 and 30 days).

Fig. 8\9:.Results revealed a strong immunostaining pointed by the arrows (fig.8) in the group NC (prepared tooth immediately extracted). At days 7 and 30 in the group I and II a weak immunostaining(pointed by the arrows) similar to the not prepared tooth were observed (Fig.9).