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RESEARCH ARTICLE

Histological, histochemical and immunohistochemical studies on the regeneration of pulp tissue of male rats after exposure to low intensity laser therapy

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Abstract

The purpose of this study was to investigate the biomodulation effect of low intensity laser therapy (LILT) on regeneration process of exposed rat dental pulp after direct capping by Ca(OH)2. Forty healthy male albino rats ranged from 220-250 grams were used in this investigation. Animals were divided into 2 groups. Group (I): Animals of this group served as control group and were subjected to moderate cervical cavity preparations in middle thirds of lingual surfaces of upper incisors. This is followed by Ca(OH)2 as pulp capping agent and modified glass ionomer as permanent restorations. Group (II): Animals were subjected for similar procedure of group (I). This is followed by laser application using aluminum gallium arsenide (AlGaAs) diode laser source (660 nm, 3 mW, 18 J/cm2; Pocket Lase, Orotig. Med, S.r.I, Italy) at 0.5-1 cm distance from tooth surface. LILT was standardized at 4-second exposure per point; buccal, palatal, mesial and perpendicular to the tooth axis. (Godoy et al, 2007). After 5, and 30 days, the rats were sacrified and maxillae including prepared central incisors were removed. Then sections were prepared for histological, histochemical and immunohistochemical analysis.

Results obtained after 5 days of pulp exposure revealed that lased dental pulp was less damaged and degenerated figures were decreased when compared with non-lased dental pulp. Pulpal responses were significantly more accentuated and seemed to be restricted to the areas underneath the region submitted to cavity preparation and laser application when compared to other control group. One main difference between lased and non-lased group was the presence of intense vascularization at exposure sites, coronal and pulp cores with no evidence of large hemorrhagic areas as were found in control groups.

After 30 days, LILT exposure sites showed marked increase of active tertiary dentinogenesis. Matrix deposition by newly differentiated odontoblast-like cells and calcification areas were increased when compared with control group. In most of lased dental pulp specimens, tubular calcified tissues were observed more than those of non-lased group which exhibited osteodentin formation at exposure sites. In other words, formation of dentin-like cells after LILT rather than bonelike tissue secreted by osteoblast-like cells observed in control group.

Immunohistochemical demonstration of caspase-3, revealed marked increase of immunoreactivity of pulp tissue than that of non-lased control group.

Introduction

The pulp tissue provide nutrient supply for dentin-pulp metabolism to preserve tooth vitality through dentinogenesis during pulpal wound healing process after noxious stimuli such as caries, operative procedure, attrition, abrasion and trauma. Pulp tissue serve as sensory organ for prevention of deep caries, and also provide immunological response

to bacterial infiltration. The complex events following injury to the dentin-pulp complex are important determinants of the opportunities for regeneration and repair of these tissues. (Nakashima, 2005).

Three basic reactions tend to protect the pulp against injury: I) Decrease in dentin permeability, ii) tertiary dentin formation, iii) inflammatory and immune reactions. These responses occur concomitantly and their robustness is highly dependent on the aggressive nature of the injury. (Kenneth, 2011).

Evaluation of the restorative procedure effect on pulp-capping should be monitored through this triad. Examination of the responding pulp stem cells and their differentiation into odontoblast-like cells, evaluation of the released growth factors during healing periods and amount of dentin extracellular matrix scaffold formation should be considered as indication of pulp capping success. In this research we will utilize this triad to explore the role of calcium hydroxide application in conjunction with low intensity laser therapy on tertiary dentinogenesis.

Calcium hydroxide (Ca(OH)2) has been extensively and steadily used for direct and indirect pulp capping in modern clinical dentistry. As it was known to have potential to induce hard tissue repair, calcium hydroxide has been applied to the exposed dental pulp and the hard tissue is expected to be regenerated above the pulp. (YM et al, 2010).

The word laser is an acronym for "Light Amplification by Stimulated Emission of Radiation". A laser is a device consisting of solid, liquid or gas substances which produce a light beam when excited by a source of energy. This device can be classified into two categories: high-power lasers or surgical lasers, featuring thermal effects with cutting, vaporization and homeostasis properties, and low power lasers or therapeutic lasers, with analgesic, anti-inflammatory and biostimulation properties,(Barros et al ,2008) The last decade has seen an explosion of research work in the application of laser technology in general dental practice.

In medicine and dentistry, diode lasers have been used predominantly for applications which are broadly termed low level laser therapy (LILT) or 'biostimulation'; however, there is controversy surrounding the effectiveness of some of these procedure.(Goldman,1987).

Immunohistochemistry involve the use of antibodies to locate antigens in tissues. Because these molecules are restricted in distribution to specific cell types, the ability to identify molecules through antigenic sequences has become a powerful technique in diagnosis surgical pathology **(Bhaskar, 1990).**

Apoptosis is one of the most studied mechanism of cell death. It is a genetically regulated process of cell elimination, and plays important roles in both morphogenesis and pathogenesis of multicellular organogenesis **(Song et al, 2010).**

Apoptotic cell death occur in 2 phases, first a commitment to cell death, followed by an execution phase characterized by dramatic morphologic changes in cell structure, suggesting the presence of common execution machinery in different cells **(Cohen, 1997).**

Caspase-3 is one of the key executioners of apoptosis. The 'c' denoting a cysteine protease-serine residue that is common to many other proteases is replaced by a cysteine residue- and the 'aspase' referring to the ability of these enzymes to cleave target proteins at sites next to aspartic acid residues **(Cohen, 1997).**

Caspase-3 has many cellular targets and when it is activated, produces morphologic features of apoptosis **(Jang et al., 2002).** To date, 14 caspases have been implicated in the apoptotic pathway cascade. Among these, caspase-3 is considered to be a major execution protease **(Nicholson, 1999).**

In this present study, we will try to explore histologically and immunohistochemically the influence of low intensity laser therapy as a conjunctive treatment with calcium hydroxide in treatment of pulp exposure and assessing the degree of apoptosis.

Materials and methods

Experimental animals

Fourty healthy male albino rats, were used in this investigation. Their body weight ranged from 200-220 grams. Animals were housed in clean and clear polycarbonates cages covered with stainless steel wire cover. Rats were

procured from laboratory animal colony, Faculty of Medicine, Cairo University. Animals were kept in a controlled environment in the animal house of Faculty of Medicine, Cairo University. Cleaning of cages as well as changing of bedding was performed once per week. All the animals had free access to tap water and soft rat food through the entire experiment. Animals were equally divided into 2 groups as follow:

Group (I)
Animals of this group served as control group and were subjected to moderate cervical cavity preparations on middle thirds of lingual surfaces of upper central incisors without pulp exposure. Class (I) cavities were prepared by using sterile low speed 1/4 round dental bur. Endodontic stoppers were used to standardize the size and depth of the cavity. This is followed by Ca(OH)2 as pulp capping agent and modified glass ionomer (Medental, USA) as permanent restorations with high abrasion ability, very low solubility and good bonding to dentin and enamel. All procedures were performed under anesthesia using an inrtaperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). After operative procedures, animals of this group (20) were equally divided into two subgroups housed in separate cages according to the two scarification periods (5 and 30 days).

Group (II)
After animals of this group were anesthetized (20 animals), they were subjected for similar procedure as group (I). This is followed by laser application using aluminum gallium arsenide (AlGaAs) diode laser source (660 nm, 3 mW, 18 J/cm2; Pocket Laser, Orotig. Med, S.r.I, Italy) at 0.5-1 cm distance from tooth surface. LILT was standardized at 4- second exposure per point; buccal, palatal, mesial and perpendicular to the tooth axis. (Godoy et al, 2007). Direct pulp capping were then performed by Ca(OH)2 against the exposure sites and openings were sealed by glass ionomer. Animals of this group were then divided into 2 subgroups as done in the previous control groups.
After 5 and 30 days, the rats were sedated and scarified using an overdose of ethanol and perfused with 10% neutral buffered formalin. The maxillae including prepared central incisors were removed, separated and fixed in neutral buffered formalin for 48 h. The specimens were decalcified in buffered 10 % EDTA. The specimens were rinsed under running tap water for 5 hours followed by dehydration with ascending concentrations of alcohol and then embedded in a butyl methacrylate paraffin wax. Five micrometer thick sections were prepared for histological and immunohistochemical analysis as follow:

I-Histological examination
It was done by routine Haematoxylin and Eosin as well as Masson trichrome stain, alizarin red and von-kossa stains for demonstration of calcium deposits and toulidine blue stain for detection of mast cells **(Drury & Wallingition, 1980).**

II-Immunohistochemical examination for detection of caspase-3
The sections were deparaffinized with xylene, hydrated in a series of descending grades of alcohol, and then rinsed briefly with tap water and phosphate buffered saline (PBS) (pH 7.4). They were incubated in 0.3% H2O2 in methanol for 10 min. Nonspecific protein binding was blocked with normal serum for 10 min. For the primary antibodies, the sections were incubated for 12 h at 4 C with polyclonal anti-caspase-3 **(Santa Cruz Biotechnology, Santa Cruz, CA, USA).** They were then rinsed with PBS three times for 5 min each time and incubated with biotinylated secondary antibodies for 10 min and with streptavidin-enzyme conjugate solution for 5 min. The antibody- localized antigen was then detected by peroxidase activation of 3, 3'-diaminobenzidine for 10 min to give it brownish discoloration. Finally, the sections were counterstained lightly with haematoxylin **(Romos – Vora, 2005).**

Results and discussion
In the current study, examinations of dental pulp after 5 days of injury were carried out to assess the inflammatory phase of healing. This phase can be broken down further into clot formation, early inflammation and late inflammation. In exposed dental pulp group (I) our results showed focal accumulation of inflammatory cells and phagocytosis of degenerated odontoblasts at exposure sites were observed. Some studies have been implicated that clot formation after exposure, survived odontoblasts, dendritic cells and injured endothelial cells can release chemotactic factors to initiate the inflammatory response and recruit the inflammatory and progenitor cells for the initiation of the healing process. They added that subsequent influx of immune effector cells is composed of lymphocytes, macrophages, neutrophils, mast cells and plasma cells. (Izumi et al,1995)

Low intensity laser therapy on exposed dental pulp after 5 days showed decrease of degenerated cells and tissue debris at the exposure sites when compared with non-lased exposure sites. Increase activities of inflammatory cells, and good survival rates of pulp cells after laser application could be the reasons of decreased vacuolization or degeneration phenomenon at the exposure sites. Fig (1) these findings were in agreement with Walsh (1997) who postulated that low level-laser enhances the phagocytic and chemotactic activity of human leukocytes. In the process of wound repair, activation of lymphocytes by laser radiation can make them more responsive to stimulatory mediators present in injured tissues. Still in accordance with the author's citations, therapeutic-laser treatment increase phagocytic activity of macrophages during the tissue repair, facilitating cleaning of the wound and establishing the conditions needed for the subsequent proliferative phase.

As laser therapy enhance the activities of lymphocytes and macrophages, our results showed the positive role of laser therapy in increase production of other important inflammatory cells i.e. mast cells by toulidine blue examinations. LILT increased mast cells concentrations after 5 days of pulp exposure when compared with non-lased group. Our results were parallel with Walsh study (1997) which postulated that mast cells play a pivotal role in controlling leukocyte traffic, modulation of mast cell functions by LILT can be of considerable importance in treatment of inflammation sites in the oral cavity. Furthermore, LILT can exert vasoactive effects by its actions on mast cells.

One of the interesting finding in the present study after LILT is the intense vascularization inside rat pulp engorged with blood cells. Our results showed increase of blood vessels concentration inside dental pulp. At areas under exposure sites, close associated blood capillaries to degenerated areas and secreting cells providing rapid transport of immune cells and provide nutrition needed for active dentinogenesis were observed. Blood vessels were found between odontoblasts, subodontoblastic layer and pulp cores. These results in agreement with other studies results that confirmed low-power laser stimulate proliferation of endothelial cells, resulting in formation of numerous blood vessels, and also stimulate both local microcirculation and vascular smooth muscle relaxation, thus contributing to the analgesic and anti-inflammatory effects of laser therapy. (Kreisler et al,2003 and Desmet et al,2006).

Results of lased dental pulp revealed increase of survival rates of subjacent injured odontoblasts at pulp exposure sites when compared with abundant degeneration areas of non-lased group and subjacent odontoblastic areas. Figs (2) these results obtained from current study, confirm other investigation (Godoy, 2007) which revealed that odontoblasts damage were not observed after LILT at pulp-dentin interface under cavity preparation without pulp exposure.

For all periods of lased group, cell-rich zones were more pronounced when compared with that of non-lased groups, presenting active fibroblasts, UMC, and inflammatory cells. LILT was positively modulated adjacent odontoblastic layers to exposure sites which showed normal pseudostratified appearance, with basophilic cytoplasm, open-faced nucleus and in an active tertiary dentinogenesis behavior on sides of exposure sites. These findings were consistent with those of earlier studies (Brugnera et al, 2003) and which can be explained through biostimulation effect of LILT.

As stated by Kenneth (2011) pulp reacts towards injury through three ways. Inflammatory reactions, decrease of dentinal tubules permeability and tertiary dentin formation. After 30 days of cavity preparations and with or without pulp exposure, pulp regained its normal appearance, and inflammatory reactions were subsided. Intratubular calcifications as stated by Cohen is a combination of an increased deposition of intratubular dentin and direct deposition of mineral crystals into the dentinal tubules to decrease the dentin permeability. He added that intratubular calcification is the first defense to caries and is called dentin sclerosis. Our findings not only showed presence of calcium deposits stained by Alizarin red and Von Kossa inside dentinal tubules but also inside dental pulp tissue related to newly secreted matrix and wall of blood vessels. Fig (3). These findings were in parallel with other studies (Adams et al, 2006) which revealed that biological response of Ca(OH)2 has also been attributed to Ca++ released from the applied material. They added that concentration is highest at the site of application and goes on decreasing as the distance increases from the material, thus forming a gradient. It must be mentioned here that such a Ca++ gradient is capable of activating cells such as stem cells, osteoblasts and fibroblasts and bring about migration.

In lased group, intratubular calcifications and decreasing on dentinal tubules diameters were observed earlier in subjacent and areas of pulp exposure in comparison to non lased groups. As stated by previous authors, (Lansdown,

2002) increase of calcium deposits in lased dentin-pulp organ can also explain increase of stem cells migration and differentiation. These findings could be discussed through biostimulation effect (Conlan et al, 1996) of LILT on odontoblastic cells which were histologically appeared as active cells and exhibited open-faced nuclei.

After 30 days of exposed dental pulp group dentin bridge formation partially or completely mineralized were formed at exposure sites. Fig (4) These findings were in parallel with Lu et al (2008) who showed appearance of dentin matrix around 30 days after Ca(OH)2 capping on exposure sites once the cellular and vascular inflammatory events began to fade and new cells with secretory potential arrived at the region to effect repair. Incomplete mineralized dentin bridge formation can also be influenced by pulp-capping materials, degree of mechanical injury, and the creation of dentin debris during operative procedures. Inflammation and bacterial leakage also negatively influence dentin bridge formation. (Murray et al, 2002) Inclusion of secreting cells inside the reparative dentin were observed in this group in comparison with lased dental pulp. This phenomenon was commonly observed in other previous studies (Hosoya et al, 2012) which named it as osteodentin.

After 30 days of laser application on exposed dental pulp, sites of exposure showed reparative dentin formation with no cellular inclusions and seemed to be secreted by odontoblast-like cells rather than osteoblast-like cells found in non-lased group. Et al one of the important differences between lased and non-lased group is the secretion of organized tubular matrix observed in lased group after 30 days Figs (5). Odontoblast-like cells were attached to the tubular matrix and were secreted tubular reparative dentin pattern. It certainly raises the question as to whether the nature of regenerative responses has biological or clinical significance. Murray et al (2002) have explained that the primary dentin matrix secreted during tooth formation has a characteristic tubular structure through which the odontoblast maintains communication with its extracellular matrix environment and which is also implicated in the transmission of sensation through the tissue. Thus, loss of this tubular structure in instances of tubular dentine regeneration would be expected to impact on tissue function.

As mentioned previously, migration of progenitor cells to the sites of injury for differentiation into a new generation of odontoblast-like cells will be an important event for cell recruitment during regeneration when the vitality of the primary odontoblasts is compromised. Evidence that such migration occurs is provided from the wealth of studies (Schro ,1985) reporting that reparative dentinogenesis and dentin bridge formation secreted during pulp capping procedures after differentiation of migrated stem cells. They added that if the necessary chemotactic signals for specific cell populations can be determined, this could be harnessed for directed recruitment of those cells to provide greater specificity to the tissue response. Whilst the precise mechanism remains unclear, the presence of a stable mechanical support seems to be as essential prerequisite for initiation of reparative dentine formation. (Nakashima , 2005).

LILT enhanced the dentinogenic specificity of reparative dentinogenesis through variety of mechanisms. Secretion of tubular dentin matrices containing specific bioactive molecules at exposure sites play pivotal role in recruitment of component precursor cells and directed their differentiation towards dentinogenic tissue secretion. Tubular matrices secretion guided the differentiation of odontoblast-like cells in contrast to non-lased group which showed a tubular dentin matrices formation and thus secretion of bone-like tissue was observed. Bioactive molecules involved in osteoid tissue formation differs than those of tubular matrices and thus different cytodifferentiation may be occurred

Some investigators (Masuda et al, 2012) showed that in highly vascularized tissue, after transplantation of progenitor cells, tubular dentin production is observed. They suggested that good supply can enhance differentiation and fusion of odontoblast-like cells to form specialized mineralized tissue. As mentioned in current study, LILT stimulated angiogenesis which in turn provided the secreting cells by good blood supply and increase delivery of UMC to the exposure sites.

After 5 days, immunoreactivities for caspase-3 were observed in extracellular matrices subjacent and close to pulp exposure sites, dentinal tubules, pulp cells and odontoblastic layers. Immunohistochemical examinations revealed negative immunoreactivities for caspase-3 in control groups (Fig.6), while in lased group marked increase of positive immunoreactivities after pulp exposure were observed.

Results after 30 days showed marked increase of positive immunoreactivity for antigen-antibody complex of caspase-3 of control group (Fig.7) sequestrated on extracellular matrix secreted by pulp cells. In lased group intense reactions were observed on ground substances, dentinal tubules of pulp cells. (Fig.8).

Dental pulp is a special type of loose connective tissue characterized by dental pulp cells and abundant amorphous ground substance which is glycosaminoglycans (Inoki et al, 1990). Caspase-3 is the most studied member of the caspase family, involved in apoptosis is important for maintaining tissue homeostasis and dentinogenesis. Our study revealed that caspase-3 activity was increased significantly in dental pulp tissue of lased group. LILT might be capable of inducing both apoptosis and regeneration in dental pulb tissue as stated by Song et al (2010).

From the above mentioned data, we can conclude that LILT accelerates the regeneration process of exposed dental pulp. We recommend the use of LILT as a conjunctive treatment in operative fields to stimulate active tertiary dentin formation associated with pulp capping procedures and as prophylactic treatment. Further experiments should be done to investigate the exact effect LILT on carious teeth with and without cavity preparation and deep cavities.

Legend of figures

Fig.1. Photomicrograph of lased rat dentin pulp organ after 5 days showing good survival rates of odontoblasts and inflammatory cells infilteration. H&E X400.

Fig.2. Photomicrograph of non-lased group after 5 days showing inflammatory cells and fibroblasts in dental pulp. Masson's trichrome X400.

Fig.3. Photomicrograph of lased dental pulp after 30 days showing red calcium deposits in dentin-pulp organ. Alizarine red X400.

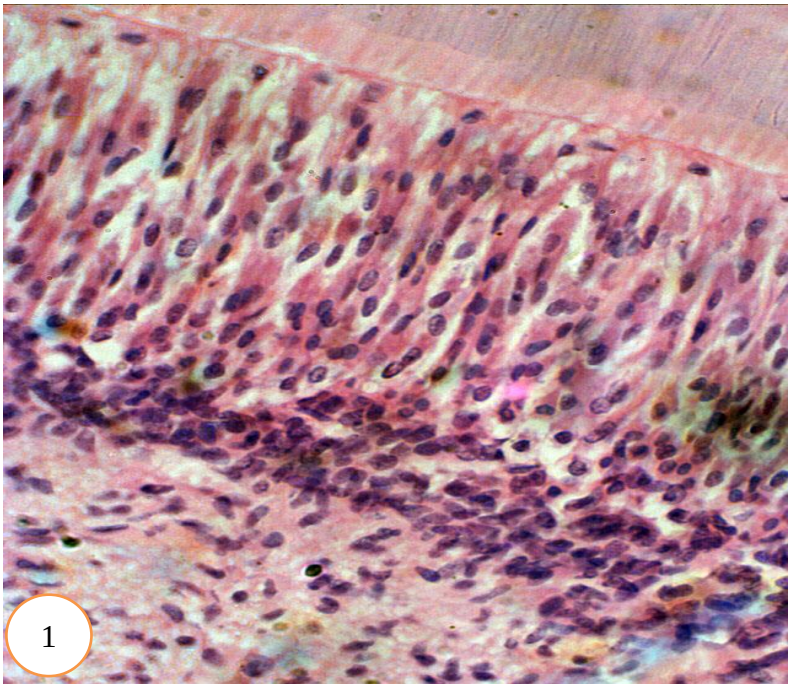
Fig.4. Photomicrograph of non-lased dentin-pulp organ after 30 days showing bone-like tissue formation at exposure site. H&E X100.

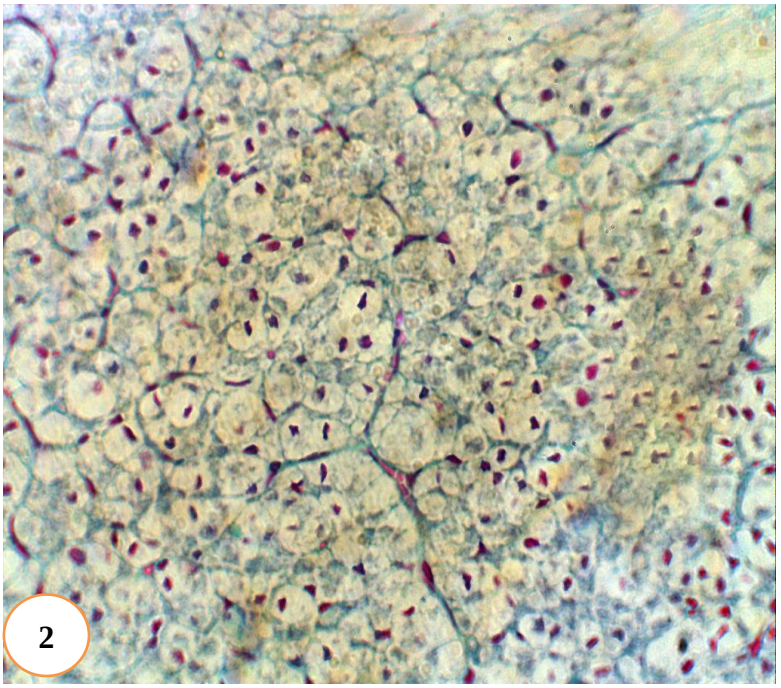
Fig.5. Photomicrograph of lased group after 30 days showing mature and organized collagen fibers near exposure site, increase vascularization and normal pulp tissue. Masson's trichrome. X400.

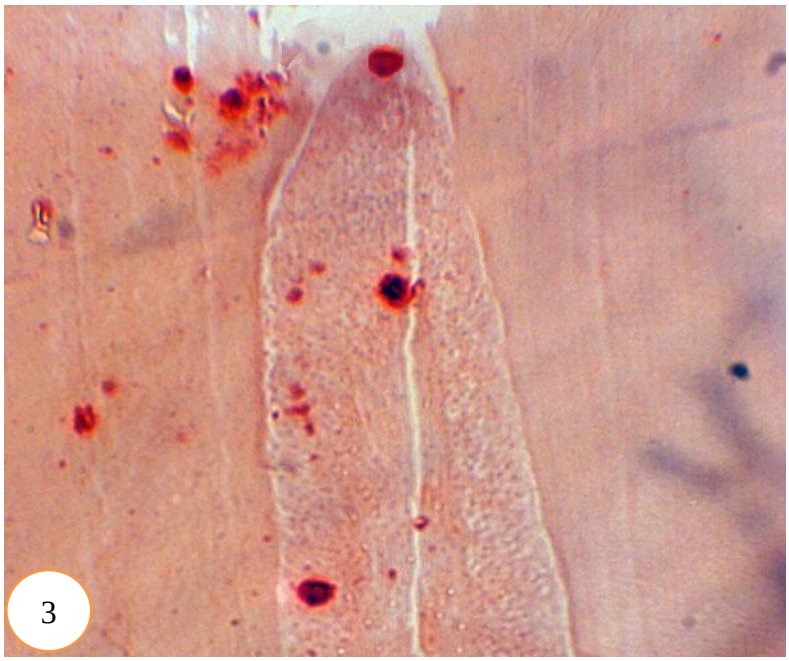
Fig.6. Photomicrograph of non-lased dental pulp after 5 days showing negative immunoreactions in extracellular matrix. Caspase-3 X400.

Fig.7. Photomicrograph of non-lased dental pulp after 30 days showing moderate immunoexpression in extracellular matrix and odontoblasts. Caspase-3 X400.

Fig.8. Photomicrograph of lased dental pulp after 30 days revealing intense reactivity in matrix and odontoblasts. Caspase-3 X400.



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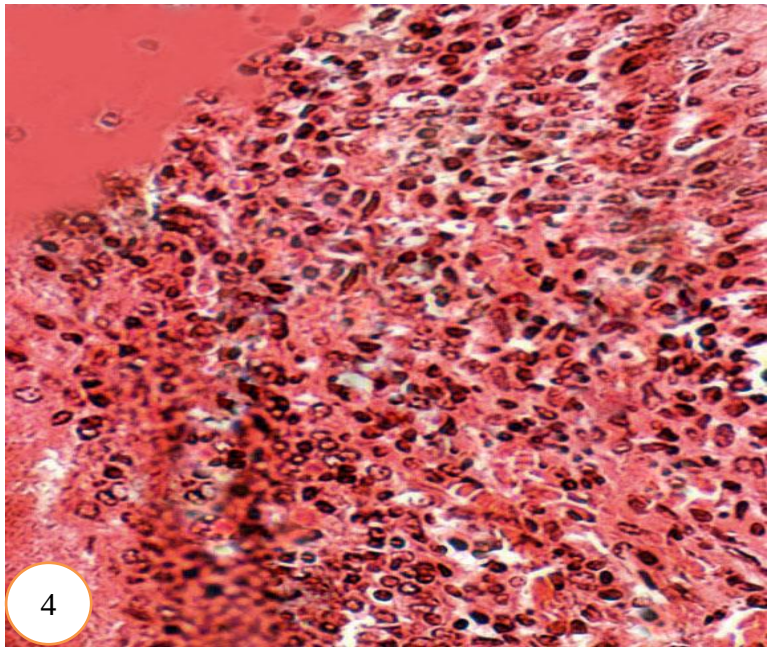
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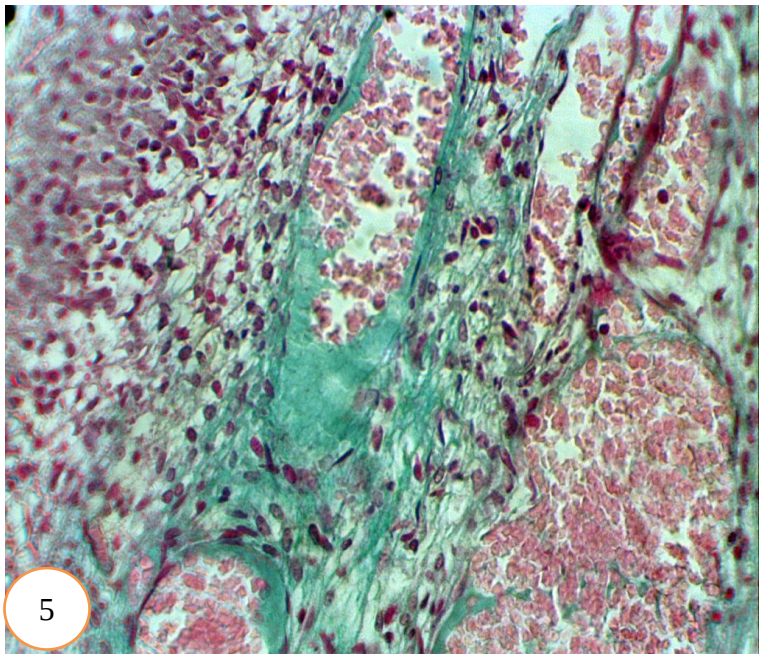
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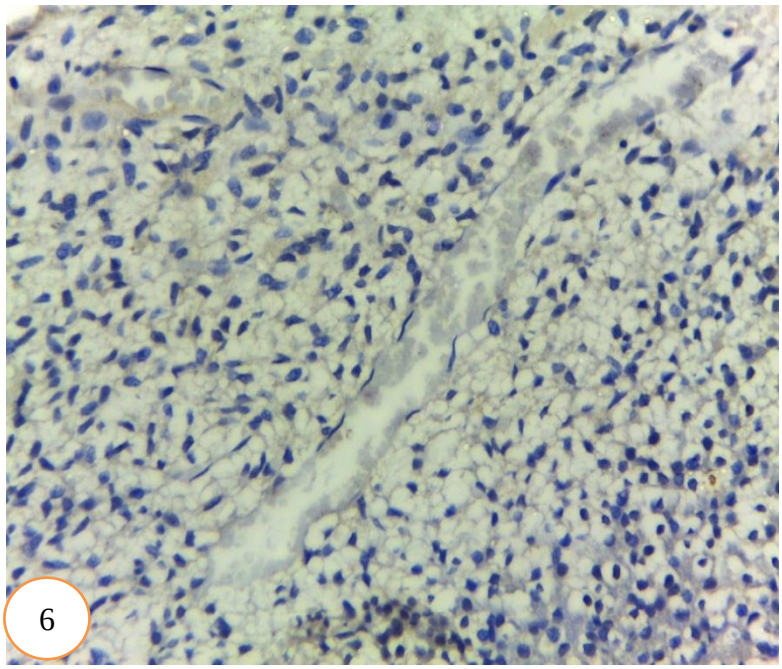
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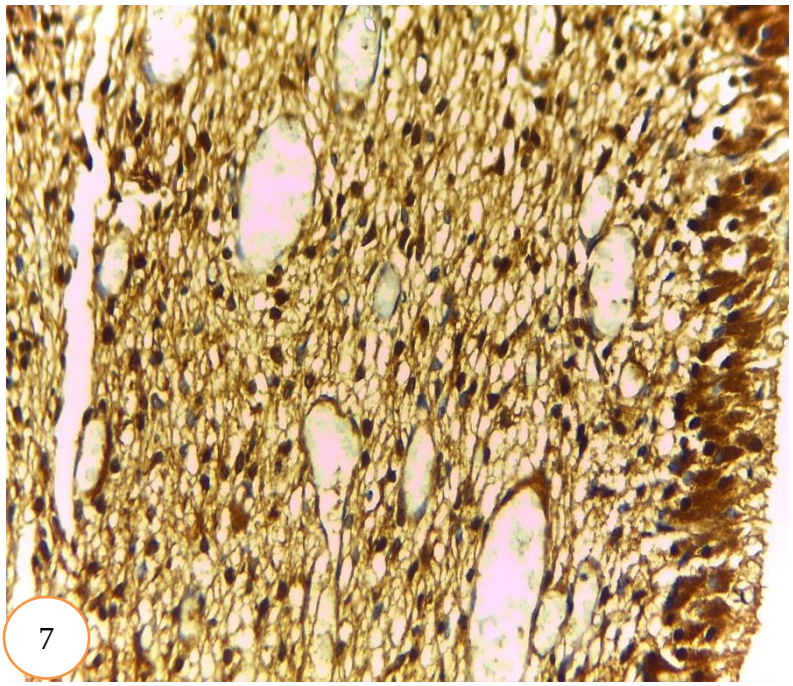

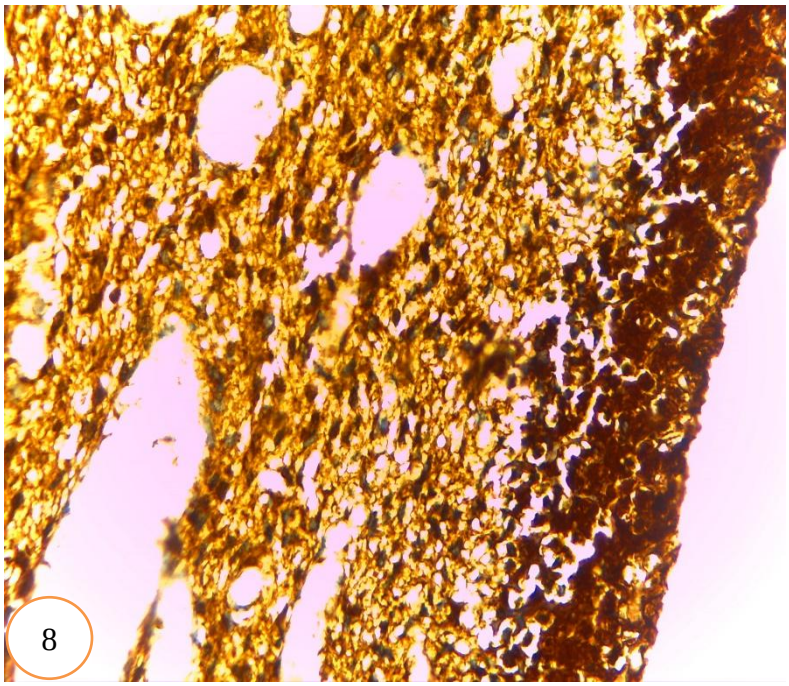

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