INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

IN VIVO STUDY OF THE EFFECT OF EXOGENOUS FIBRONECTIN ON RAT DENTAL PAPILLA CELLS

Athraa Y. Al-Hijazi[1], Shatha S. Mohammed[1] and Najat A. Hasan[2*]

[1]Department of Oral Diagnosis, College of Dentistry, University of Baghdad, Baghdad-Iraq.
[2]Department of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University, Baghdad-Iraq.

ABSTRACT

Background: Odontogenesis is a complex and highly regulated process leading to tooth initiation, morphogenesis and cell-differentiation with eventual formation of enamel, dentin and cementum matrices. fn molecule account for its interaction with certain discrete extracellular substances ,thus it serves as a promoter of cell-cell adhesive contacts. **Aim:** To investigate the *in vivo* effect of fibronectin (fn) on rat tooth development. **Materials and Methods**: A randomized comparative study was implemented. Fibronectin was purified from the blood of adult Winster rats by series of gel filtration and affinity chromatography columns. A 50 µl of 100µg/ml of purified homologous rat plasma fn was injected at the maxillary molar tooth germ areas of the one day old Wister rats (n=25). Light and scanning electron microscopic examination was carried out after 15 days as the first and second molar crowns are fully developed. **Results:** Fn renders the odontoblast cells to be more packed and elongated as compared to those of the control rats who were injected with 0.05 ml of 0.9% NaCl .The pulp exhibits a network of fibrils of fn that are adherent to cells. **Conclusion:** the interaction of fn with the odontoblasts promotes cell to cell adhesion and regulates odontoblasts differentiation such changes are important for dentinogenesis.

Keywords: Fibronectin, molar tooth germ, odontoblasts.

INTRODUCTION

The microenvironment surrounding cells can exert multiple effects on their biological responses. In particular the extracellular matrix surrounding cells can profoundly influence their behavior[1] . During tooth root development, all functional hard tissues are formed by three kinds of cells: HERS, dental papilla mesenchymal and dental follicle cells, which form developing apical complexes[2].

Signaling molecules expressed in dental epithelium and captured in the basement membrane control the differentiation of pulp cells into odontoblasts. Odontoblasts produce the extracellular matrix components found in dentin and are implicated in dentin mineralization[3]. Like the tooth crown, tooth root development involves the interaction of the dental epithelium and the cranial neural crest-derived mesenchyme[4].

Fibronectin is a glycoprotein that is present on many cells surfaces, in extracellular fluids, in the connective tissue, and in most basement membranes[5]. Fn is secreted by variety of cell types including fibroblasts, endothelial cells, and hepatocytes[6]. The presence of the numerous binding sites on fn molecule account for its interaction with certain discrete extracellular substances such as heparin, fibrin, and collagen and with cell surface structures[7]. Moreover, it serves as a promoter of cell-cell adhesive contacts, in changing the cellular morphology, spreading, locomotion, and the organization of extracellular materials[8].

Fn present in an insoluble form on most cells and in a soluble form in the plasma and in the interstitial fluid. In the oral cavity, Fn has been demonstrated in saliva, crevicular fluid, tooth buds, dental pulp, oral mucosa, and periodontal tissue[9]. Fibronectin and integrins play crucial roles in a variety of morphogenetic processes, in which they mediate cell adhesion, migration, and signal transduction.

They induce hierarchical transmembrane organization of cytoskeletal and signaling molecules into multimolecular complexes of more than 30 proteins[10].

The present work is designed to investigate for the first time the possible biological significance of the exogenous fibronectin on the developing rat tooth germ in vivo.

MATERIALS AND METHODS

A. Purification of rat plasma fibronectin

The research protocol was approved by the institutional review board at the College of Medicine,Alnahrain University ,Baghdad-Iraq in 2001.The blood of ten albino Wister adult rats was used for the extraction of fn using series of column chromatographic techniques . In brief, the citrated rat plasma was mixed with serine protease inhibitor (ε- amino-α-hexanoic acid) at a concentration of 0.04 M .Then the plasma was applied to a column of sepharose-4B (2.5 x 12 cm) equilibrated with 0.05 M tris – HCl buffer (pH= 7.5) containing 0.04 M ε - amino - α - hexanoic acid and 0.02 % (W/V) sodium azide .The column was eluted with the same buffer and proteins that appear in the void volume were applied to a column of CNBr-activated gelatin-sepharose 4B (2.5x 10 cm) that was equilibrated with the above buffer .This column was first washed with the equilibrating buffer then with 50 ml of 0.2 M arginine in 0.05M tris- HCl buffer (pH=7.5) .Following dialysis ,the Fn containing eluate was applied to a second affinity column of arginine- sepharose(2.5x 10cm) equilibrated with 0.05 M tris-HCl buffer (pH= 7.5). After washing this column with the equilibrating buffer, Fn was eluted with 0.1 M NaCl in the above buffer[11].

Protein concentration of the purified extract was determined by the Lowery method, 1951[(12)]. The purity and molecular weight of the extracted Fn was checked by sodium dodecyl sulphate (SDS-PAGE) polyacrylamide gel electrophoresis[13] under denatured conditions. The gel was fixed with methanol acetic acid and stained with ammoniacal silver stain[14] and photographed (Figure 1) .The identity and the purity of the extracted fn was checked by the immunodiffusion test using rabbit anti fn antibody. The concentration of Fn in solution was determined by assuming that the extinction coefficient of pure fn at 280 nm is 12.8[15.]

B. Application of Fibronectin in vivo

Twenty-five one-day old albino rats were used at a time where their tooth germs in the bell stage (figure 2A, and 2B). Each neonate was injected on the right side of maxillary molar tooth germ with 0.05 ml of 100 µg /ml of the purified rat plasma fn. The other side which served as a control was injected with 0.05 ml of 0.9% NaCl, These rats was decapitated at an age of 15 days as the crowns of the first and second molars was fully formed.

C. Processing for light and scanning electron microscopical studies

For scanning electron microscopical studies: The heads of embryos were dissected and fixed with Karnovisky Fixative (3% formaldehyde, and 2% paraformaldehyde) for at least 24hours, decalcified with 10% nitric acid for 3-4 days. Washed specimens were incubated in 0.05M cocodylate buffer solution for 16 hours at 4 ◦C. The tooth germs removed after dissecting the maxillary ridge in the vertical plane, were kept in osmium tetrachloride for 1.5 hr at 4 C◦. Wash and dehydrate the specimens with serial acetone dilutions then with absolute acetone solution. The specimen were coated with gold and examined under scanning electron microscope[16].

For light microscopical studies: The specimens were fixed with Karnovisky Fixative, decalcified with 10% nitric acid for 3-4 days then gradually dehydrated with series of increasing percentages of alcohol and embedded in a melted paraffin. Sections of 4µm were cut by the microtome , mounted on glass slides, stained with hematoxylin and eosin stain and were examined by Leitz light microscope [(17)].

RESULTS

Molar Tooth Germ Area in the Controls:

Figures 2A & 2B reveal the maxillary molar area of one day old albino rat, the time at which the tooth germ was in the bell stage. This area was injected with normal saline (0.05 ml of 0.9% NaCl) to serve as a control whereas the opposite side was injected with extracted rat plasma fibronectin (0.05 ml of 100 µg /ml). After 15 days, the animal is decapitated and the tooth germs from both sides were processed as mentioned and subjected to microscopical examination.

In figure 3A, the odontoblast cell bodies under light microscope appear columnar in shape with large oval nuclei which were elongated along the apical-basal axis and fill the basal part of the cell .The differentiated odontoblasts have secreted predentin matrix. Odontoblasts look short, barrel pseudo stratified with space in between when they were examined under scanning electron microscope (Figure 3B). Under the light microscope, the pulp showed numerous fibroblast cells which were the most

abundant cell type and appear spindle in shape with a large nucleus. Pulp fibroblasts were connected by desmosome-like and gap junctions (arrows in figure 3A). Undifferentiated mesenchymal cells were also detected as polyhedral in shape (Figure 3A). In the scanning electron micrograph, the collagen fibers appear scattered throughout the pulp. At higher magnification, the pulp showed scattered cells with obviously twisted collagen fibers (Figures 3 C and 3D).

Experimental Molar Tooth Germ Area (treated with fibronectin):

Odontoblast cell bodies under the light microscope were shown to be more elongated, closely packed together as compared to the controls (Figure 4 A). The width of the predentin and shape of the odontoblasts in those treated with fibronectin were not different from the controls. Yet, these cells when examined under the scanning electron microscope were observed to be elongated, packed with extracellular structure that appears as a patchy like material on their surfaces (Figure 4B). Odontoblasts aligned at the pulp periphery or included in the dentin matrix. Odontoblast cell body elongation was moving toward the pulp core. The pulp showed packed cells where fn deposited as fibrillar structures on the cell surfaces and in between some cells (Figure 4C). The collagen fibers are packed together and are adherent to the cells and they are less twisted in comparison to those of the control group (Figure 4D).

In figure 4E, the fibronectin was found to be expressed at the apical surface of dental mesenchyme cells. Fibronectin is deposited on the surfaces of cells as fibrillar network, as dense fibers that link between cells, and surrounding the odontoblast cell surface. Some odontoblasts express lateral branching.

DISCUSSION

This study has provided the first direct evidence concerning the in vivo effect of fibronectin on the differentiation of odontoblast in rat tooth development. The developing tooth is an excellent model for studying the molecular mechanisms of morphogenesis[18]. Tooth morphogenesis in mice starts at embryo day (E) 11.5, when the oral ectoderm invaginates into the underlying mesenchyme[19]. Continuation of this process results in the formation of epithelial tooth buds at E13.5. Ectomesenchymal cells surrounding the bud form the dental papilla, which later develop into dentin-secreting odontoblasts and the tooth pulp. Following the bud stage, the tooth germ develops to the cap and bell stages, and dental epithelium differentiates into polarized and elongated enamel secreting ameloblasts[18].

Odontoblast differentiation is characterized by a sequence of events: withdrawal from the cell cycle, cytological polarization, and secretion of predentin/dentin[2]. All these steps are achieved within six hours in the mouse, and most of them are cytoskeleton-dependent[20, 21].

Our result showed that injection of exogenous fn affect the development of dental papilla cells specially odontoblasts causing their packing and elongation. This finding is consistent with those of Tziafas et al.,1992[(22)] who found formation of an elongated cell layer that contacts the implant Millipore filler supplemented with 1 mg /ml bovine plasma fn in dental pulp of dog. Tziafas[23] observed an initiation of reparative dentinogenesis in the dental pulp of young dog treated with fn . Concerning fibronectin, a weak or negative localization is seen in the condensed mesenchyme surrounding the dental lamina. In the cap stage, different patterns of the distribution between tenascin and fibronectin are evident in the human tooth germ. Strong tenascin accumulation is present in the dental papilla under the basal membrane, preodontogenic layer and osteogenic tissue of alveolar bone. However, tenascin is immunohistochemically negative in the dental follicle, the fibroblastic layer developing into the periodontium[24].

Thesleff et al. (1987) reported that when purified plasma fibronectin was added at 50 µg /ml to the culture medium, it became incorporated as fibrillar matrix on the surfaces of dental papilla cells. This indicates that the cells are not deficient in cell-surface receptors or other surface-associated molecules which bind fibronectin. When the cells were disaggregated and cultured at high cell density, the cells in the central area of the pellet were covered by fibronectin containing fibrillar structures which were lost as the cells spread out. This indicates that the maintenance of close contacts between the dental papilla cells is required for the assembly of fibronectin into the extracellular matrix [(25)].

Odontoblasts synthesize and secrete several collagenous and noncollagenous proteins to form the unique extracellular matrix (ECM) of dentin[26]. The cells in contact with the basement membrane elongate polarize, and start to synthesize predentin and then dentin components such as type I, type V, and type VI collagens. Dentine is a biologically active, convoluted tissue secreted by specialized odontoblasts. Whilst collagen forms the bulk of its matrix, non-collagenous proteins have also

been identified, which may have an important role in guiding cytodifferentiation, secretion of matrix and its mineralization. For instance, dentine phosphoproteins and dentine sialoproteins have been suggested to be involved in mineralization process [27].

Fibronectin transitorily accumulates at the apical surface of dental mesenchyme cells and polarizing preodontoblasts and at later stages, surrounds the odontoblast cell surface[28,29]. These fibronectin expression patterns in developing teeth agree with a previous report[30]. Zhang et al.(2007)reported that FN was located in the epithelium and dental mesenchyme on bud stage, but on bell stage, the FN was found at the region of differentiating odontoblasts and in the inner enamel epithelium, and also BMP-2, 4 were abundant mainly at the brisk region of differentiating odontoblasts[31].

We found that Pulp fibroblasts were connected by desmosome-like and gap junctions, which facilitate intercellular communication. This also implies that if the cells move or translocate, this communication may be broken. Goldberg and Smith (2004) reported that lateral branching may contribute to cell-cell communication and cell-matrix communication and may possibly be involved in peritubular dentin formation. Odontoblasts, like many other cells, may use intermediary filaments to provide spatial information .Functionally, the secretory odontoblast can be considered to consist of two distinct parts: the cell body involved in the synthesis and control of cellular and extracellular proteins, and the process whereby secretion and limited re-internalization occur. The odontoblast process consists of one main trunk with numerous lateral branches along its length. The process is limited by a plasma membrane and contains predominantly cytoskeletal components[32].

In the present study, some of the extracellular materials on odontoblast surface are organized as small patches rather than being in fibrillar form. These findings coincide with the results of Hedman et al[33] who showed that the fibroblast cell have small patches of amorphous fn containing material on the cell membrane. Fibronectin is re-distributed during odontoblast polarization and interacts with the cell surface. Lesot and coworkers reported that fibronectin transitorily accumulates at the apical surface of dental mesenchyme cells and polarizing preodontoblasts and at later stages, surrounds the odontoblast cell surface[20, 21]. An in vivo study conducted on dog dental pulp tissue to determine immunolocalization of fibronectin after interaction of pulp with Ca(OH)2-containing material showed that fibronectin had a strong association with micro crystals formed at the surface of the cement [(33).] Following its adsorption on the cement, a new biochemically active surface is formed serving the same role which the basement membrane plays for cellular mechanics of odontoblasts during developmental stages. There is evidence that Ca++ stimulates synthesis of fibronectin in dental pulp cells[34.]

Ishikawa et al. (2010) indicated that following an injury, granular retaining dental pulp cells (transit-amplifying cells) present in the subodontoblastic layer first migrate and differentiate into odontoblast-like cells without proliferation to replace the lost original odontoblasts[35]

$Ca(OH)_2$ has been shown to increase recruitment, migration, proliferation and mineralization of dental pulp stem cells[36,] thus facilitating the whole process of reparative dentinogenesis in such cases.An in vitro study[34] was carried out by treating human dental pulp cells with high concentration of Ca^{++}. The authors concluded that raised concentration of Ca^{++} in pulp tissue achieved owing to its release from applied $Ca(OH)_2$-induced fibronectin synthesis in pulp cells, followed by accretion of fibronectin in necrotic tissue adjacent to the healthy pulp. This accumulated fibronectin might then induce differentiation and secretion in odontoblasts. The mRNA levels of BMP-2 have also been found to increase under elevated Ca^{++} culture conditions in a study that suggested the Ca^{++} from $Ca(OH)_2$ specifically modulated BMP-2 levels in pulp tissue to effect repair process[37.] Alkaline conditions in culture medium also raise expression of mRNA for BMP-2 in human dental pulp cells[38.] Tada et al. (2010) conducted a research on cultured human dental pulp cells suggested that modulation of gene expression for BMP-2 through elevated extracellular Ca^{++} from $Ca(OH)_2$ may be a crucial mechanism for induction of dentinogenesis[39.] Expression of fibronectin has been demonstrated in vivo in dental pulps following pulp capping with $Ca(OH)_2$[40].

In conclusion, the interaction of fn and the odontoblasts promotes cell to cell adhesion and regulates odontoblasts differentiation such changes are important for dentinogenesis and may be exploited in the treatment of tooth development disorders and human periodontal defects.

ACKNOWLEDGMENTS

We wish to express our deep thanks to Professor Mahmood Hayyawi Hammash(department of embryology, College of

Medicine, AL- Nahrain University, Baghdad-Iraq)for his guidance concerning tissue culture aspects and to Miss Mayson abdulameer(department of human Biology, College of Medicine, AL- Nahrain University, Baghdad-Iraq) for her valuable technical help and support in conducting the scanning electron microscopical(SEM)examination.

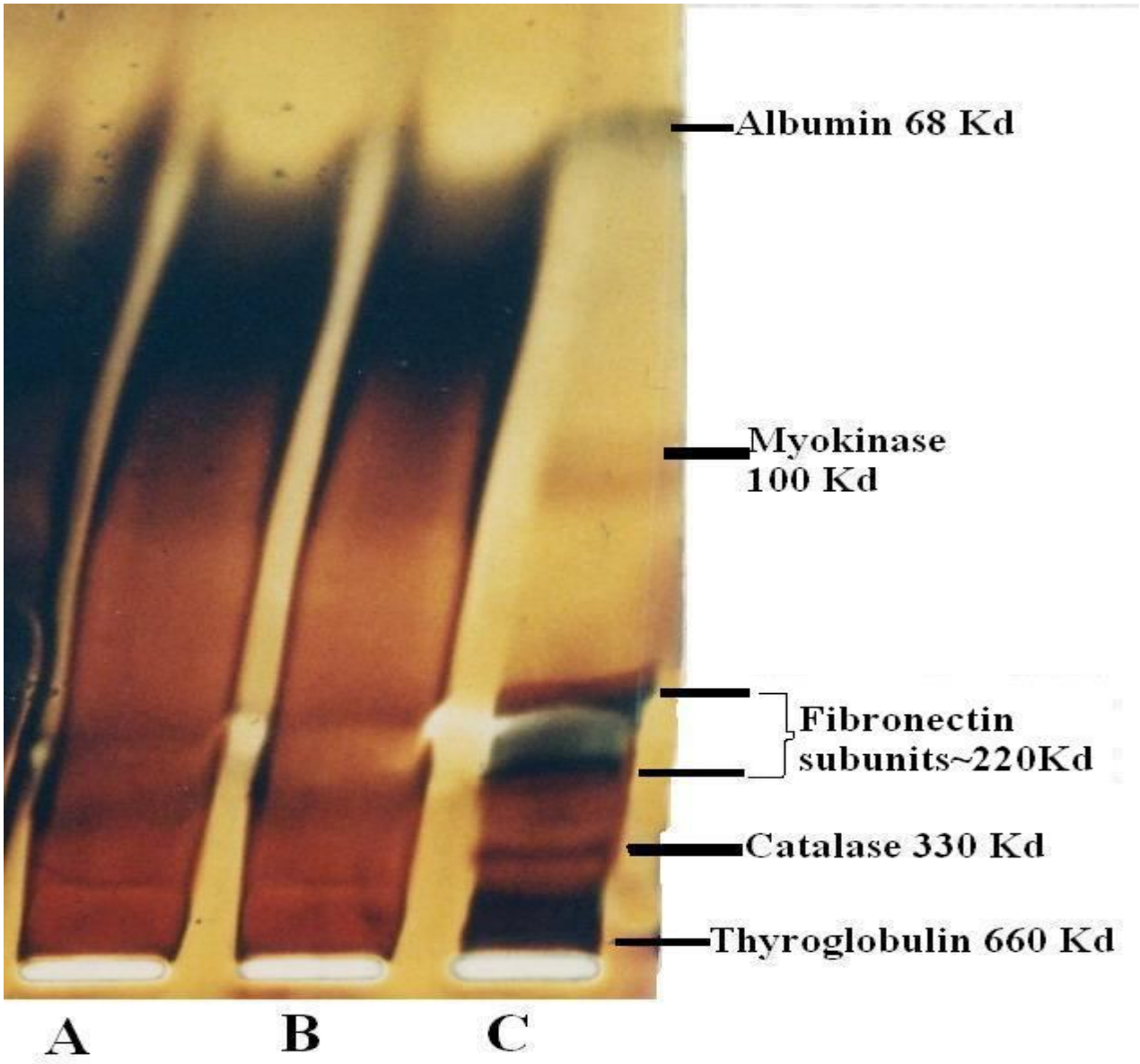

Fig. 1: Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the extracted fibronectin from the rat plasma. *Lane A and B*: rat plasma fibronectin (one µg), *lane C*: molecular weight markers (5 µg):-bovine serum albumin 68kd, myokinase 100kd, lyophilized fibronectin (subunit) 220kd, catalase 330kd, and thyroglobulin 669kd

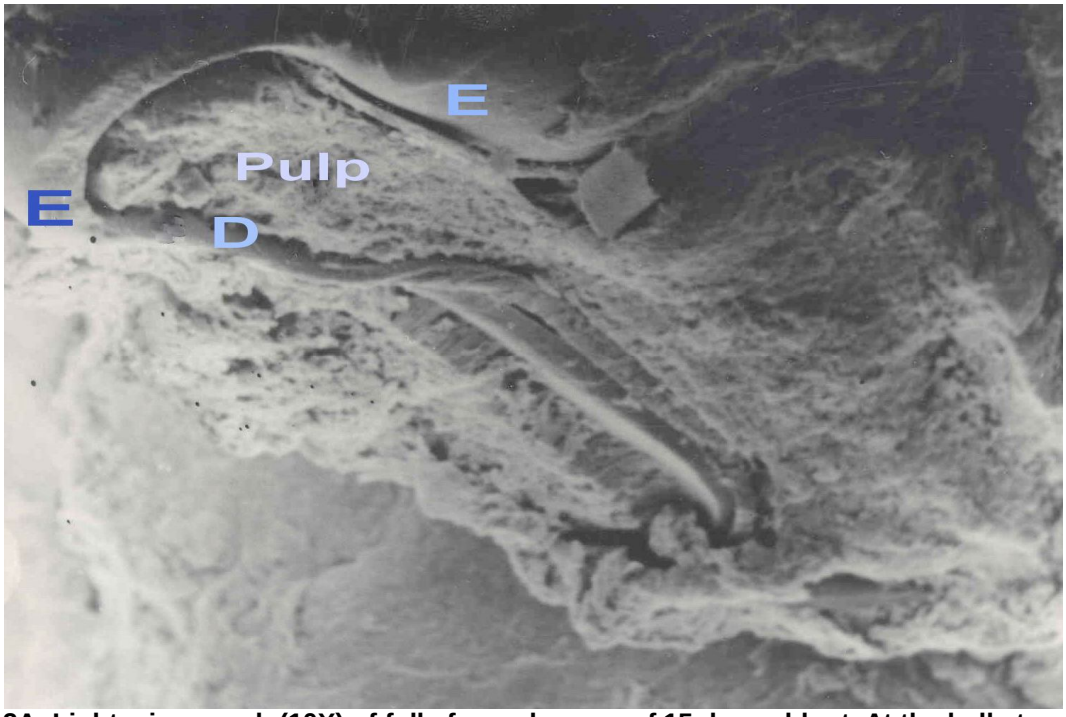

Fig. 2A: Light micrograph (10X) of fully formed crown of 15 days old rat. At the bell stage the form of the tooth crown is established, and odontoblasts and ameloblasts differentiate and start to secrete dentin (D) and enamel matrices (E), respectively. (P) Dental pulp .The differentiated odontoblasts have secreted predentin matrix and the epithelial cells are polarizing into ameloblasts

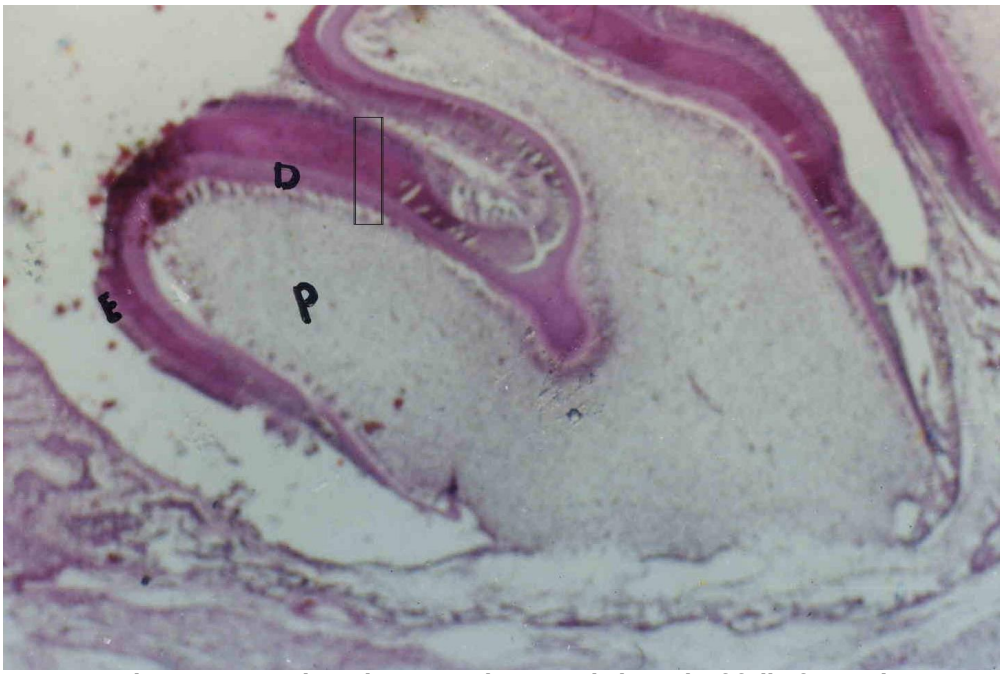

Fig. 2B: Scanning electron micrograph (600X) of fully formed crown of 15 days old rat. (E) Enamel, (D) dentin

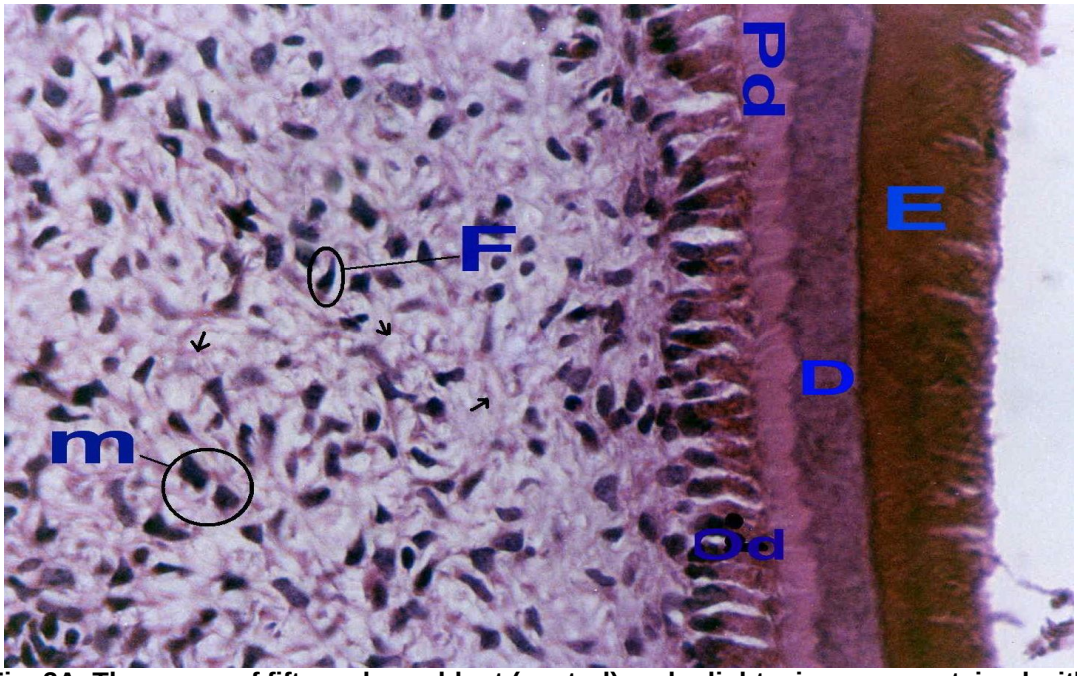

Fig. 3A: The crown of fifteen days old rat (control) under light microscope, stained with hematoxylin and eosin.(40X) shows odontoblast cells(Od),fibroblast(F),and undifferentiated mesenchymal cell(m)

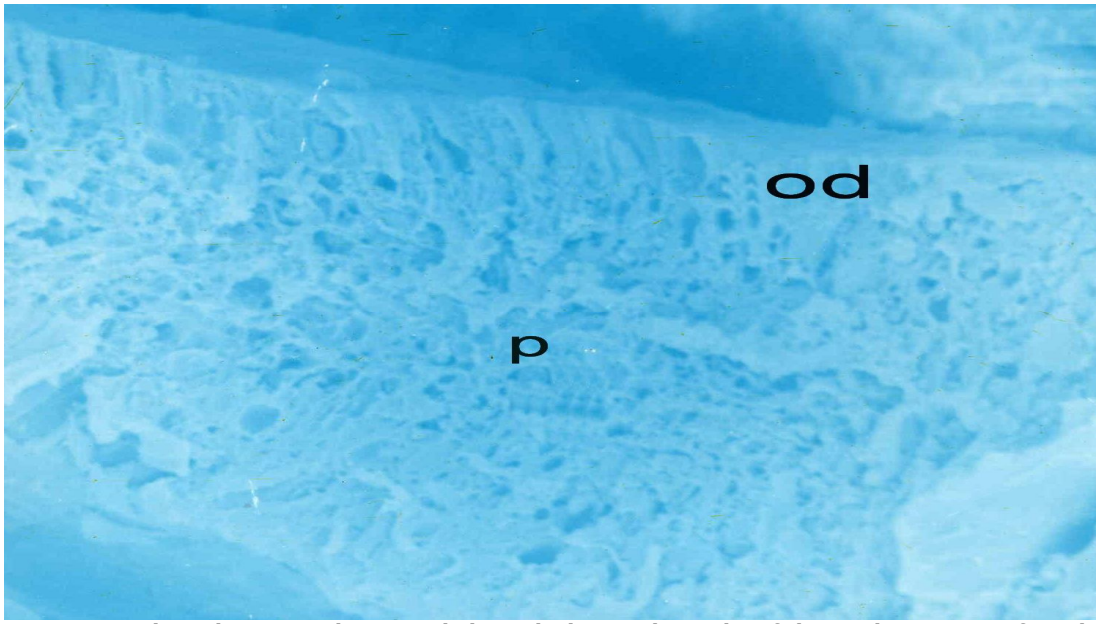

Fig. 3B: scanning electron micrograph (600X) shows the pulp of the molar crown of 15 days old rat (control).The odontoblast cells (od) are located at the tip of the cusp. (P) Dental pulp

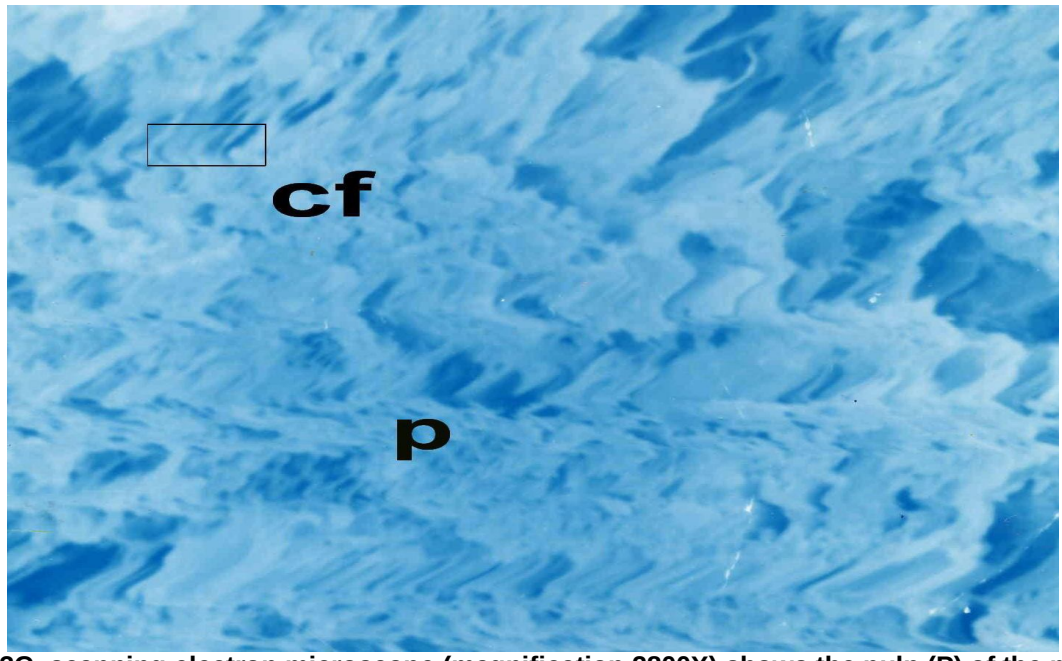

Fig. 3C: scanning electron microscope (magnification 2800X) shows the pulp (P) of the molar crown and collagen fibers (CF) of 15 days old rat (control)

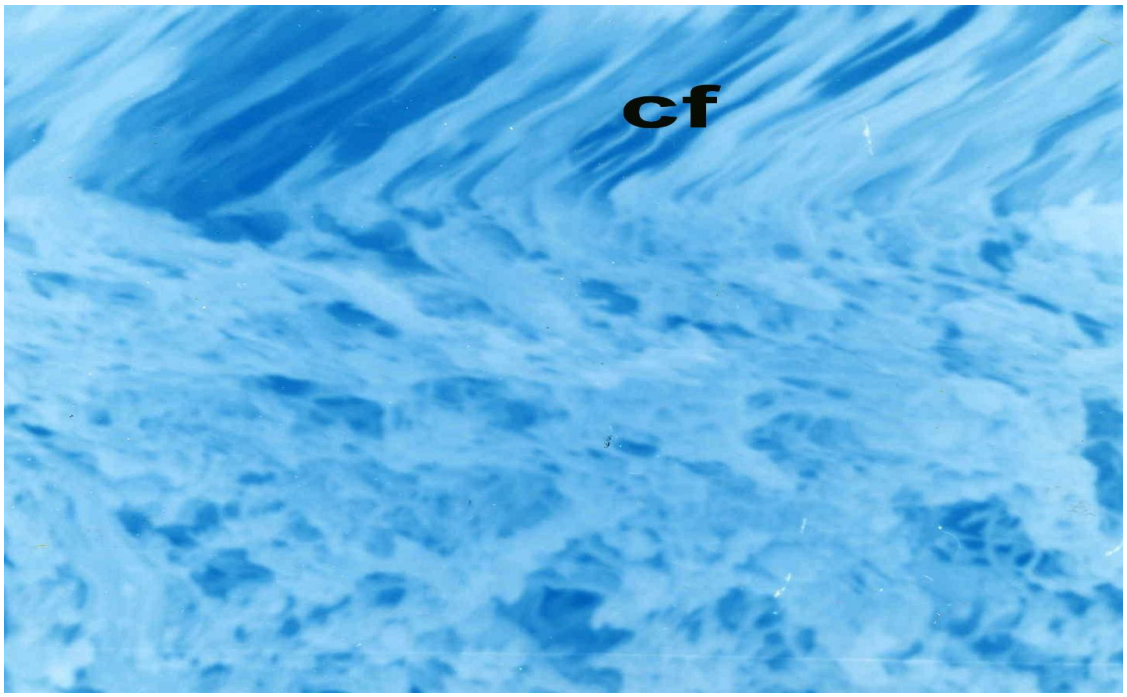

Fig. 3D: Higher magnification of figure 5 (magnification 5700X) shows the twisted collagen fibers (CF) of 15 days old rat (control)

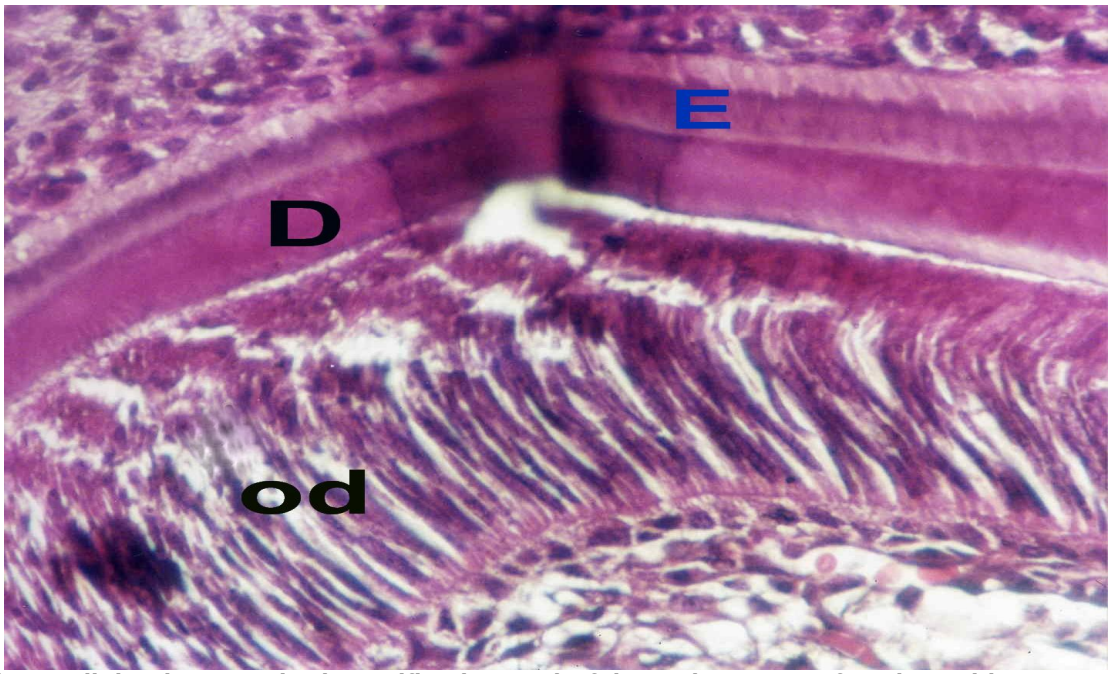

Fig. 4A: light photography (magnification 40X) of the molar crown of 15 days old rat treated with exogenous rat plasma fibronectin (100µg/ml) showing elongated odontoblast cells (Od)

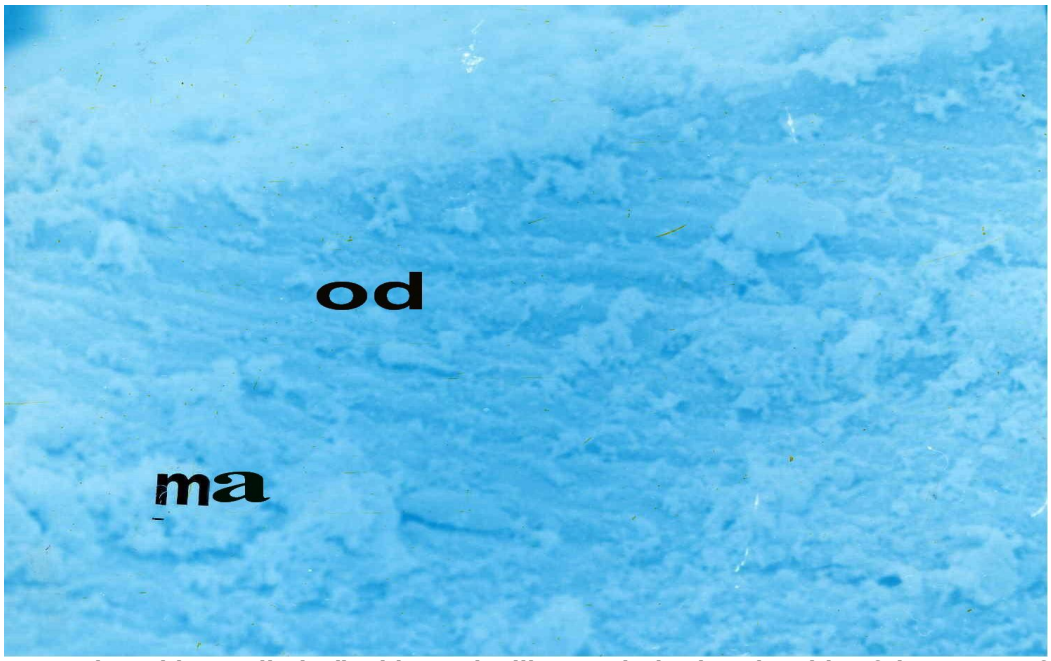

Fig. 4B: odontoblast cells (Od) with patchy like matrix (ma) at the side of the crown of rat treated with exogenous rat plasma fibronectin (100µg/ml). Magnification 1200X

Fig. 4C: scanning electron microscope (magnification 1200X) shows odontoblast cells (Od) at the tips of the cusp. The cells were treated with exogenous rat plasma fibronectin (100µg/ml) and processed after 15 days

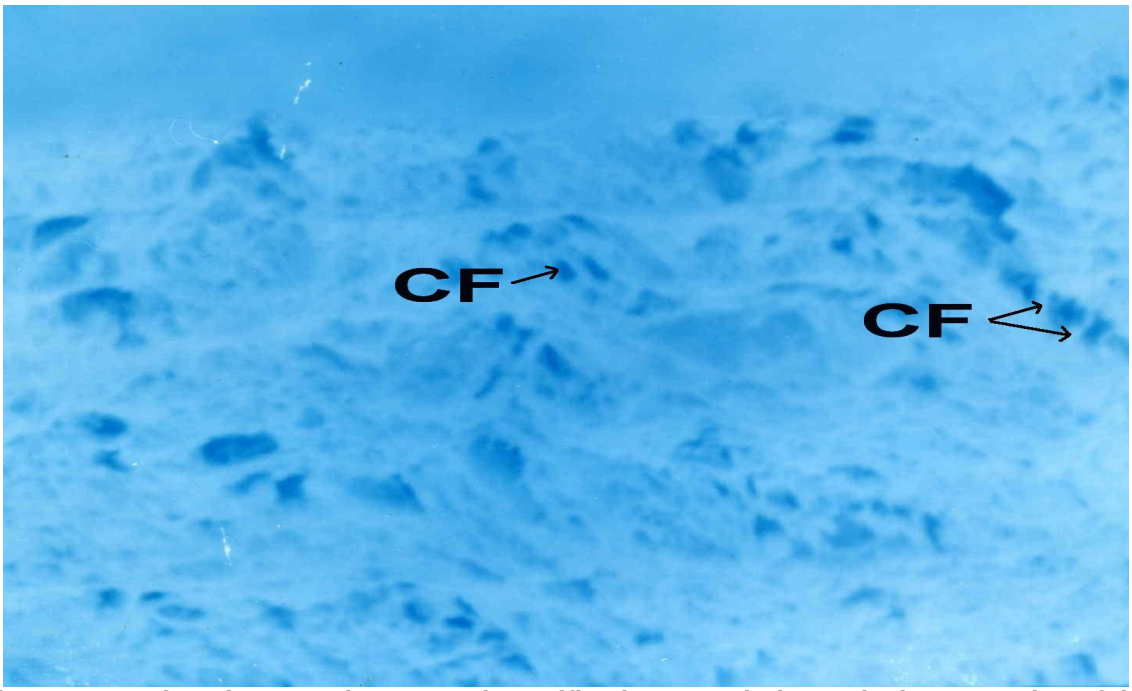

Fig. 4D: scanning electron microscope (magnification 2800X) shows the incorporation of the injected rat fibronectin in the pulp after 15 days .Cells(C), fibronectin (Fn). Fibronectin is deposited on the surfaces of cells as fibrillar network, as dense fibers that link between cells, and surrounding the odontoblast cell surface.

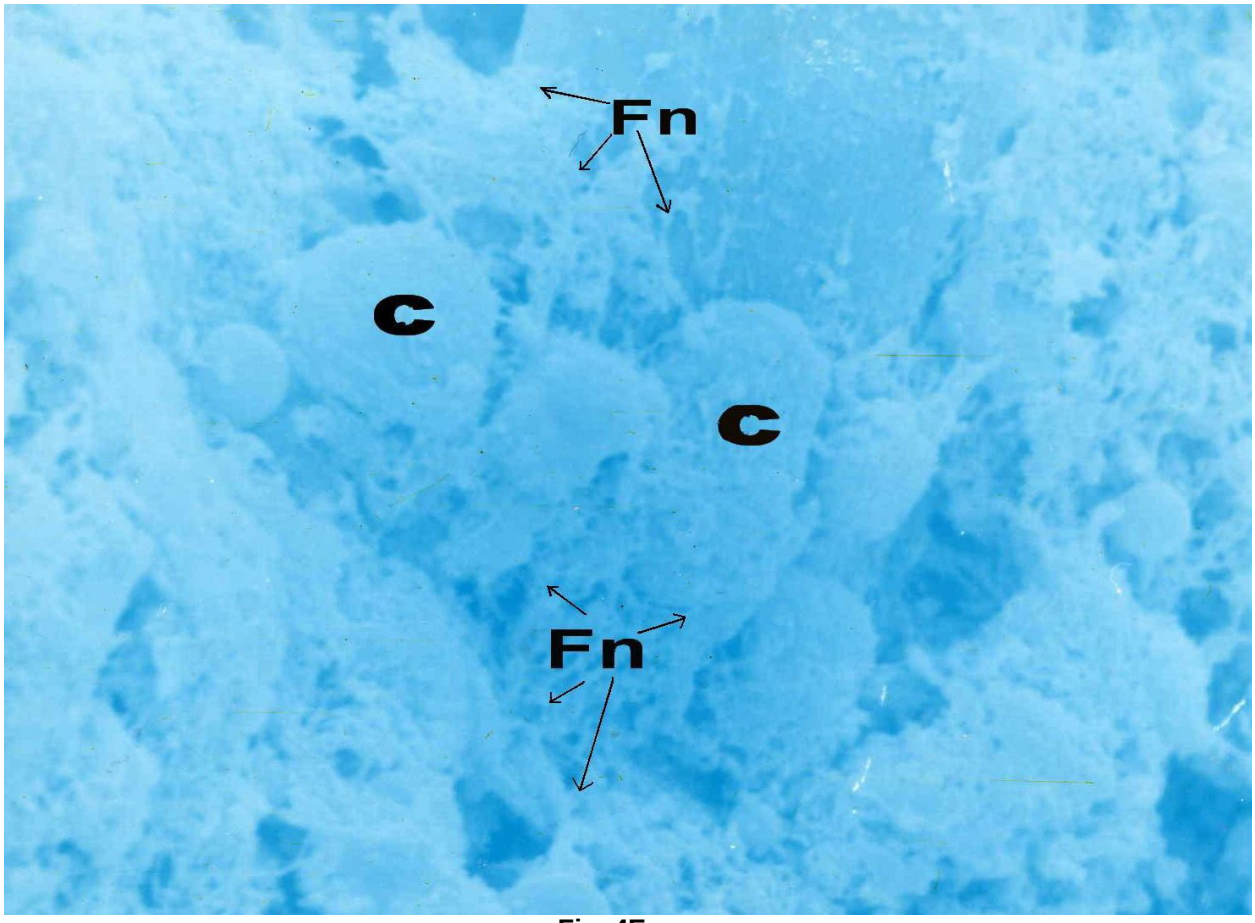

Fig. 4E:

REFERENCES

1. Addison CL, Nör JE, Zhao H, Linn St A, Polverini PJ and Delaney CE. The response of VEGF-stimulated endothelial cells to angiostatic molecules is substrate-dependent. BMC Cell Biol. 2005;6:38.
2. Feng J, Mantesso A, De Bari C, Nishiyama A and Sharpe PT. Dual origin of mesenchymal stem cells contributing to organ growth and repair. Proceedings of the National Academy of Sciences of the United States of America. 2011;108:6503–8.
3. Ruch Jv,Lesot H and Beguekirn C. Odontoblast Differentiation. Int J Develop Biol. 1995;39(1):51-68.
4. Xiao-Feng Huang and Yang Chai Molecular regulatory mechanism of tooth root development. International Journal of Oral Science. 2012;1–5.
5. IzumiT, Yamada K, Inoue H, Watanabe K and Nishigawa Y. Fibrinogen / fibrin and fibronectin in the dentin-pulp complex after cavity preparation in rat molars. Oral Surgery Oral Med, Oral Pathol Oral Radiol. 1998;86:587-91.
6. Zhu-Q, Safavi KE and Spangberg LS. Integrin expression in human dental pulp cells and their role in cell attachment on extracellular matrix proteins. J Endodontics. 1998;24:641-4.
7. Thurlow D, Kenneally A and Connellan M. The role of fibronectin in platelet aggregation. British J Haematol. 1990;75:549-556.
8. Couchman J, Austria M and Woods A. Fibronectin - cell interactions. J. Investig Dermatol. 1990;94:75-135.
9. Miyamoto S, Kathz B-Z, Lafrenie RM, and Yamada KM. Fibronectin and integrins in cell adhesion, signaling, and morphogenesis. Ann NY Academic Sci. 1998;857:119-129,.
10. Vuento M and Vaheri A. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. Biochem J. 1979;183:331-337.
11. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement

with folin phenol reagent. J Biol Chem. 1951;193:265-275,.
12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 1970;227: 680-685.
13. Merri CR. Gel staining techniques.In Methods in Enzymology,Guide to protein purification. Dentscher, M,P.(eds).Chapter 36,Volume 182.Academic press, London, 1990;482-483.
14. Mosesson MW, Chen AB and Huseby RM. The cold-insoluble globulin of human plasma: studies of its essential structural features. Biochim Biophys Acta. 1975;386 (2):509-24.
15. Gartner L and Hiatt J. Color texbook of histology. W.B: Saunders Company, Philadelphia, Pennsylvania. 1997;429-32.
16. Bhaskar SN and Jacoway JP. Pyogenic granuloma. Clinical features, incidence, histology, and result of treatment. J Oral Surg. 1966;24:391.
17. Fukumoto S, Miner JH, Ida H, Fukumoto E, Yuasa KI, Miyazaki H.,Hoffman MP and Yamada Y. Laminin _5 is required for dental epithelium growth and polarity and the development of tooth bud and shape. J Biol Chem. 2006;281:5008–5016.
18. Cohn SA. Development of molar teeth in the albino mouse. Am J Anat. 1957;101:295–319.
19. Lesot H, Kubler MD, Fausser JL and Ruch JV. A 165 kDa membrane antigen mediating fibronectin-vinculin interaction is involved in murine odontoblast differentiation. Differentiation. 1990;44:25-35.
20. Lesot H, Fausser JL, Akiyama SK, Staub A, Black D, Kubler MD and Ruch JV. Differentiation. 1992;49:109–118.
21. Tziafas D, Alvanou A and Kaidoglon K. Dentinogenic activity of allogenic plasma fibronectin on dog dental pulp. J Dent Res. 1992;71:1189-95.
22. Tziafas D. Induction of reparative dentinogenesis in vivo: a synthesis of experimental observation. Connect Tissue Res. 1995;32:297-301.
23. Thesleff I, Partanen AM, Kuusela P, and Lehtonen E. Dental papilla cells synthesize but do not deposit fibronectin in culture. J Dental Res. 1987;66:1107-1115.
24. Keisuke Nakano , Hitoshi Nagatsuka , Hidetsugu Tsujigiwa , Mehmet Gunduz, Naoki Katase, Chong Huat Siar, and Toshiyuki Kawakami. Immunohistochemical Characteristics of Odontogenic Neoplasms and Their Physiological Counterparts. Journal of Hard Tissue Biology. 2008;17(3):79-90.
25. Vega S, Iwamoto T, Nakamura T, Hozumi K, McKnight DA, Fisher LW, Fukumoto S and Yamada Y. J Biol Chemistry. 2007;282(42):30878–30888.
26. Matsuura T, Duarte WR, Cheng H, Uzawa K and Yamauchi M. Differential expression of decorin and biglycan genes during mouse tooth development. Matrix Biol. 2001;20: 367-373.
27. Suzuki S, Sreenath T and Haruyama N. Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization. Matrix Biology. 2009;28:221–9.
28. Fisher LW, Whitson SW, Avioli LV and Termine JD. Matrix sialoprotein of the developing bone. J Biol Chem. 1983;258:12723–7.
29. Chen J, Zhang Q, Mcculloch C and Sodek J. Immunohistochemical localization of bone sialoprotein in fetal porcine bone tissues - comparisons with secreted phosphoprotein-1 (spp-1, osteopontin) and sparc (osteonectin). Histochemical J. 1991;23(6):281-289.
30. D'Souza RN, Bronckers AL, Happonen RP, Doga DA, Farach-Carson MC and Butler WT. Developmental expression of a 53 KD dentin sialoprotein in rat tooth organs J. Histochem Cytochem. 1992;40: 359-366.
31. Zhang J, Tian WD, Nie X, Liu L, Tang W and Lin YF. Expressions of fibronectin and bone morphogenetic protein -2, 4 during development of mouse tooth germ. Sichuan Da Xue Xue Bao Yi Xue Ban. 2007;38(5):826-8
32. Bronckers AL, Gay S, Finkelman RD and Butler WT .Immunolocalization of Gla proteins (osteocalcin) in rat tooth germs: comparison between indirect immuno fluorescence, peroxidase-antiperoxidase, avidin-biotin-peroxidase complex, and avidin-biotin-gold complex with silver enhancement

.J Histochem Cytochem. 1987;35: 825-830.
33. Tziafas D, Panagiotakopoulos N and Komnenou A. Immunolocalization of fibronectin during the early response of dog dental pulp to demineralized dentine or calcium hydroxide-containing cement. Archives of Oral Biology. 1995b;40:23–31.
34. Mizuno M and Banzai Y. Calcium ion release from calcium hydroxide stimulated fibronectin gene expression in dental pulp cells and the differentiation of dental pulp cells to mineralized tissue forming cells by fibronectin. International Endodontic Journal. 2008;41:933–8.
.Ishikawa Y, Ida-Yonemochi H and Suzuki H. Mapping of BrdU label-retaining dental pulp cells in growing teeth and their regenerative capacity after injuries. Histochemistry and Cell Biology. 2010;134:227–41.
35. Ji YM, Jeon SH, Park JY, Chung JH, Choung YH and Choung PH. Dental stem cell therapy with calcium hydroxide in dental pulp capping. Tissue Engineering Part A. 2010;16: 1823–33.
36. Rashid F, Shiba H and Mizuno. The effect of extracellular calcium ion on gene expression of bone-related proteins in human pulp cells. Journal of Endodontics. 2003;29:104–7.
37. Okabe T, Sakamoto M, Takeuchi H and Matsushima K. Effects of pH on mineralization ability of human dental pulp cells. Journal of Endodontics. 2008;32:198–201.
38. Tada H, Nemoto E, Kanaya S, Hamaji N, Sato H and Shimauchi H. Elevated extracellular calcium increases expression of bone morphogenetic protein-2 gene via a calcium channel and ERK pathway in human dental pulp cells. Biochemical & Biophysical Research Communications. 2010;394: 1093–7.
39. Leites AB, Baldissera EZ and Silva AF.Histologic response and tenascin and ibronectin expression after pulp capping in pig primary teeth with mineral trioxide aggregate or calcium hydroxide. Operative Dentistry. 2011; 36:448–56.