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Expression of insulin-like growth factor-1 and proliferating cell nuclear antigen in human pulp cells of teeth with complete and incomplete root development

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Abstract

Caviedes-Bucheli J, Canales-Sánchez P, Castrillón-Sarria N, Jovel-Garcia J, Alvarez-Vásquez J, Rivero C, Azuero-Holguín MM, Diaz E, Munoz HR. Expression of insulin-like growth factor-1 and proliferating cell nuclear antigen in human pulp cells of teeth with complete and incomplete root development. *International Endodontic Journal*, **42**, 686–693, 2009.

Aim To quantify the expression of insulin-like growth factor-1 (IGF-1) and proliferating cell nuclear antigen (PCNA) in human pulp cells of teeth with complete or incomplete root development, to support the specific role of IGF-1 in cell proliferation during tooth development and pulp reparative processes.

Methodology Twenty six pulp samples were obtained from freshly extracted human third molars, equally divided in two groups according to root development stage (complete or incomplete root development). All samples were processed and immunostained to determine the expression of IGF-1 and PCNA in pulp cells. Sections were observed with a light microscope at 80× and morphometric analyses were performed to calculate the area of PCNA and IGF-1 immunostaining using digital image software. Mann–Whitney's test was used to determine statistically significant differences between groups ($P < 0.05$) for each peptide and the co-expression of both.

Results Expression of IGF-1 and PCNA was observed in all human pulp samples with a statistically significant higher expression in cells of pulps having complete root development ($P = 0.0009$).

Conclusion Insulin-like growth factor-1 and PCNA are expressed in human pulp cells, with a significant greater expression in pulp cells of teeth having complete root development.

Keywords: human pulp, insulin-like growth factor-1, proliferating cell nuclear antigen.

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Introduction

Morphogenesis and cytodifferentiation are events that take place during odontogenesis. The first consists of development and formation of coronal and radicular patterns as a result of proliferation, migration and organization of different layers of cell populations, including epithelial and mesenchymal cells. The latter consists of specific differentiation of cells, which will form dental tissues: enamel, dentine, cementum and pulp. Thus, cell proliferation and differentiation are two important processes during tooth development (Thesleff *et al.* 1995, Jernvall & Thesleff 2000).

Cell proliferation is observed during early phases of odontogenesis (dental lamina, bud and cap stages) and

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is characterized by a high mitotic activity of epithelial and mesenchymal cells. This process is controlled by numerous factors, including growth factors. On the other hand, cell differentiation is the result of structural and functional organization of cells after they have reached their final development in order to fulfil their synthesis and mineralization functions. Defence and reparative processes after pulp irritation also require cell proliferation and differentiation (Jernvall & Thesleff 2000, Thesleff 2003).

Insulin-like growth factor-1 (IGF-1) plays an important role during early stages of embryogenesis and regulates specific functions in several tissues and organs during postnatal growth (Baker *et al.* 1993). IGF-1 is essential in the signalling process for initiating cell growth, inducing cell proliferation, promoting differentiation and specialized functions in several cells (D'Ercole 1996). It is an anabolic peptide that mediates the biological effects of growth hormone (Laron 2001).

Several growth factors act as mediators in odontogenesis, regulating cell–extracellular matrix communications and epithelial–mesenchymal interactions (Thesleff & Mikkola 2002, Thesleff 2003, Yamashiro *et al.* 2003). Different levels of IGF-1 immunoreactivity have been detected in ameloblasts and odontoblasts of rat incisors according to developmental stage, suggesting that this growth factor participates in the formation and mineralization of dental tissues (Joseph *et al.* 1993). Exogenous IGF-1 added to mouse pulp cultures has stimulated mineralization, mitotic activity and cell differentiation, promoting ameloblast and odontoblast development, consequently leading to enamel and dentine formation (Caton *et al.* 2001). Polymerase chain reaction (PCR) studies have demonstrated that these functions are mediated by induction of enamel mineralizing specific genes (Caton *et al.* 2005).

Previous studies (Caviedes-Bucheli *et al.* 2004, 2007) have shown that there are differences in IGF-1 expression and its respective receptor between pulps from teeth with complete or incomplete root development. When analysing these differences, it is interesting to note that there was a statistically significant lower IGF-1 expression in pulps from teeth with incomplete root development, whilst a radioreceptor analysis showed a statistically significant higher expression of IGF-1 receptor in a group of pulps with the same characteristics. From these results, it was hypothesized that locally produced IGF-1 in pulps from teeth having incomplete root development is rapidly bounded to receptors in pulp cells due to high tissue metabolism. This hypothesis would also explain the statistically significant higher expression of IGF-1 in pulps from teeth with complete root development and the lower expression of its receptor in the same group of pulps.

It has been shown that IGF-1 induces the mRNA expression for bone morphogenetic proteins (BMPs)-2 and -4 in human pulp fibroblasts (Lesot *et al.* 1994, Li *et al.* 1998). BMP-4 is involved during early stages of odontogenesis, whilst BMP-2 participates in later stages (Thesleff *et al.* 1995, Cheifetz 1999). Both peptides have been associated with proliferation and differentiation of mesenchymal pulp cells into odontoblasts (Li *et al.* 1998, Lovschall *et al.* 2001). It seems that one of the roles of BMPs in the root development is to modulate the production of bone sialoprotein (BSP), as it is co-expressed with BMP-2, -3 and -7 in the differentiated odontoblast (Yamashiro *et al.* 2003). Therefore, IGF-1 could be associated with differentiation and mineralization process of pulpodentine complex.

Insulin-like growth factor-1 induces proliferation and differentiation of dog dental pulp cells into odontoblast-like cells in serum-free cultures (Onishi *et al.* 1999). Furthermore, it increases the proliferate activity of human pulp cells in primary cultures (Denholm *et al.* 1998). This evidence supports the hypothesis that this growth factor plays a significant role in growth and differentiation of pulp cells, and thus in pulp reparative process.

Specific markers have been used to analyse cell proliferation during odontogenesis in animals and humans, including proliferating cell nuclear antigen (PCNA) and bromo-deoxyuridine (BrdU) (Casasco *et al.* 1995, Kaneko *et al.* 1999, Matulová *et al.* 2002). PCNA is essential for cell growth; it acts as an accessory protein of DNA-polymerase and is expressed in the late G1 and S phases of the cell cycle (Gao *et al.* 2001, Matulová *et al.* 2002), therefore correlating with cell proliferate state. PCNA is present at varying levels, detected immunohistochemically, throughout cell growth. For these reasons, labelling for PCNA indicates that a cell is in a dividing state and not in a G0 phase. PCNA detection has been used to reveal proliferation of fibroblasts in human dental pulp and gingival tissue (Casasco *et al.* 1996, Ebensberger *et al.* 2002).

The purpose of this study was to identify by immunohistochemistry, the co-expression of IGF-1 and PCNA in human pulp cells from teeth with complete or incomplete root development, to support the specific role of IGF-1 in cell proliferation during tooth development and pulp repairing process.

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Materials and methods

A descriptive comparative study was performed according to Colombian Ministry of Health recommendations regarding ethical issues in research involving human tissue. Written informed consent was obtained from each patient participating in the study. Human pulp was obtained from 26 freshly extracted noncarious and unrestored third molars from different patients (15 female patients and 11 male patients/18–35 year old). Half of the samples were from teeth having incomplete root development and the other half from teeth with complete root development. Determining root development stage was done both visually and radiographically following the same criteria used in previous studies (Caviedes-Bucheli *et al.* 2004, 2007). All of the teeth included in the incomplete root development group had an apical orifice diameter larger than 3 mm mesiodistal or buccolingual. The teeth included in the complete root development group had an apical orifice diameter no larger than 0.5 mm. Teeth with an apical orifice diameter between 0.5 and 3 mm were discarded.

The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remains of periodontal ligament that could contaminate the pulp sample. The teeth were then sectioned using a Zekrya bur (Dentsply Tulsa Dental, Tulsa, OK, USA) in a high-speed handpiece irrigated with saline solution. The pulp tissue was obtained using a sterile endodontic excavator and fixed in 10% neutral buffered formalin for 24 h.

After fixation, the tissue block was embedded in paraffin, and four longitudinal sections for each sample were cut in a microtome (Leica Microsystems, Wetzlar, Germany) at 3-μm thickness and affixed onto positively charged slides. Once mounted, the slides were dried to remove any water that may get trapped under the section. This was done by leaving the slides at room temperature overnight.

Before proceeding with the staining protocol, the slides were deparaffinized at 60 °C and rehydrated with xylene washes, followed by 100%, 95%, 70% and 50% ethanol washes and final rinsing with running cold tap water. Heat-induced antigen retrieval was performed using a steamer and a water bath in an antigen retrieval solution (Ref. 7119; Novocastra Laboratories, Newcastle, UK) at 90 °C for 30 min.

Quenching of endogenous peroxide activity was achieved by incubating the sections for 10 min in 3% hydrogen peroxide in methanol. Slides were dried using paper tissue and sections were immediately covered with blocking reagent (PowerBlock; BioGenex Laboratories, San Ramon, CA, USA) and incubated for 10 min in a humidified chamber.

Immunohistochemistry

Monoclonal antibodies were added at the concentrations recommended by the manufacturer. For PCNA: PC-10 (Ref. NCL-PCNA; Novocastra Laboratories) was used and left in incubation for 60 min in a humidified chamber at room temperature. Then, the sections were washed with IHC WASH (Novocastra Laboratories) for 3 min and biotintylated rabbit anti-mouse was applied as a secondary antibody, left in incubation for 30 min and washed again with IHC WASH. As a final point, diaminobenzidine (DAB) was applied to each section as a chromogen, left in incubation for 10 min and finally, sections were washed thoroughly in distilled water.

For IGF-1, Anti-IGF-1 (Ref. 22568; AbCam Inc, Cambridge, MA, USA) was used following the same protocol described for PC-10, except for the use of a fast-red chromogen instead of DAB for a better visualization of IGF-1. Finally, haematoxylin was used as a counterstain.

Sample analysis

After sample processing, sections were observed with a light microscope (Axiophot; Zeiss, Oberkochen, Germany) at 80×, and captured with a CCD video camera connected to a computer. Morphometric analyses were performed to calculate the area of PCNA and IGF-1 immunostaining using digital image software (KS300; Kontron Elektronik Imaging System, Zeiss). For each sample, two sections were randomly selected for separate measurement of PCNA and IGF-1 staining, and one section for the co-expression of both.

Statistical analysis

Values are presented as μm^2 of immunostained area by field of section studied. Mean and maximum/minimum values are presented for each group and for each peptide. Mann–Whitney's *U*-test was performed for establishing statistically significant differences ($P < 0.05$) between the groups of pulps from teeth having incomplete and complete root development for each peptide and the co-expression of both.

Results

All of the human pulp samples were positive for IGF-1 and PCNA immunostaining. Mean IGF-1 immuno-

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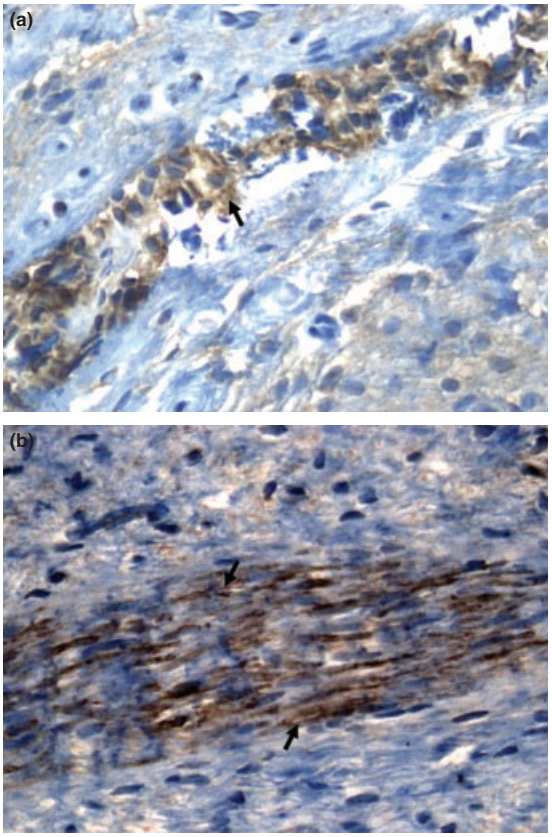

Figure 1 Histological sections showing cytoplasmic Insulin-like growth factor-1 (IGF-1) immunostained areas (reddish coloration, arrows) in human pulp cells. (a) Pulp of tooth with incomplete root development. (b) Pulp of tooth with complete root development.

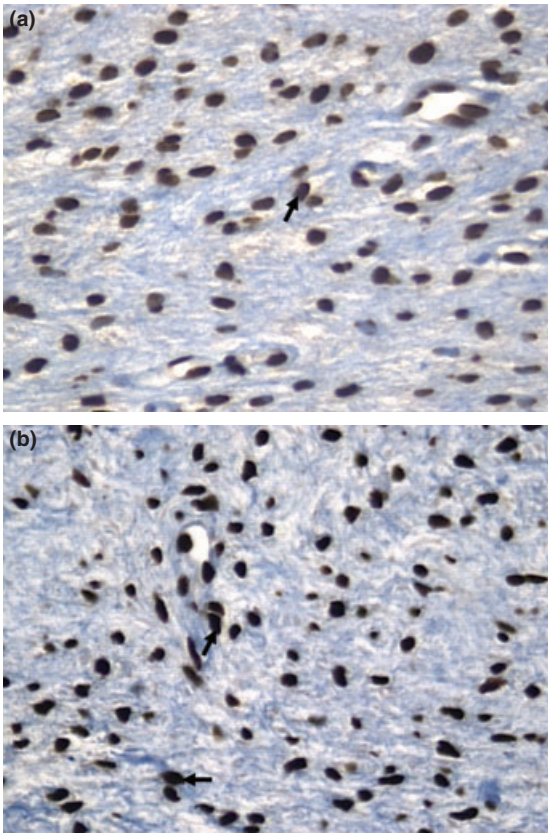

Figure 2 Histological sections showing intranuclear proliferating cell nuclear antigen (PCNA) immunostained areas (dark brown, arrows) in human pulp cells. (a) Pulp of tooth with incomplete root development. (b) Pulp of tooth with complete root development.

stained area (Fig. 1) for the group of pulps from teeth having incomplete root development was $3501.97 \pm 1075.69\ \mu m^2$. For the group of pulps from teeth having complete root development mean immunostained area was $4694.88 \pm 1627.09\ \mu m^2$. The maximum and minimum values and confidence intervals for IGF-1 expression are presented in Table 1. Mann–Whitney's *U*-test revealed a statistically significant difference between groups ($P = 0.02$).

Mean PCNA immunostained area (Fig. 2) for the group of pulps from teeth having incomplete root development was $2912.09 \pm 887.82\ \mu m^2$. For the group of pulps from teeth having complete root development mean immunostained area was $3880.92 \pm 729.29\ \mu m^2$. The maximum and minimum values and confidence intervals for PCNA expression are presented in Table 2. Mann–Whitney's *U*-test revealed a statistically significant difference between groups ($P = 0.003$).

Table 1 Insulin-like growth factor-1 immunostained area in human dental pulp cells[a]

Root development	*n*	Mean*	Standard deviation	95% Confidence intervals		Min	Max
				Lower limit	Upper limit		
Incomplete	13	3501.97	1075.69	2917.23	4086.71	1974.19	5003.39
Complete	13	4694.88	1627.09	3810.40	4694.88	2806.45	7796.00

[a]Values are presented in μm^2 of immunostained area by field of section analysed.
*Differences between groups are statistically significant ($P = 0.02$).

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Table 2 Proliferating cell nuclear antigen immunostained area in human dental pulp cells[a]

Root development	*n*	Mean**	Standard deviation	95% Confidence intervals		Min	Max
				Lower limit	Upper limit		
Incomplete	13	2912.09	887.82	2429.47	3394.71	1713.89	4403.03
Complete	13	3880.92	729.29	3484.48	4277.36	2902.34	4790.24

[a]Values are presented in μm^2 of immunostained area by field of section analysed.
**Differences between groups are statistically significant ($P = 0.003$).

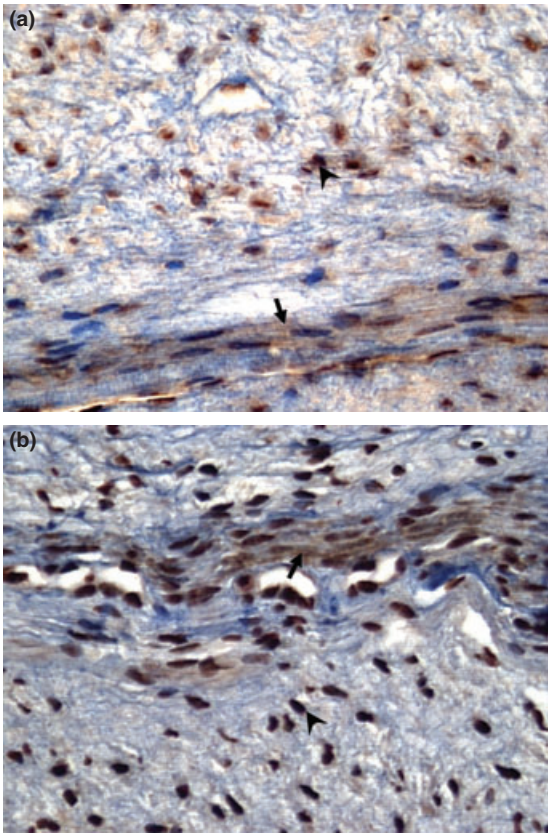

Figure 3 Histological sections showing insulin-like growth factor-1 (IGF-1) (arrows) and proliferating cell nuclear antigen (PCNA) (arrowheads) immunostained areas in human pulp cells. (a) Pulp of tooth with incomplete root development. (b) Pulp of tooth with complete root development.

Mean immunostained area for the co-expression of IGF-1 and PCNA (Fig. 3) in the group of pulps from teeth having incomplete root development was $1468.91 \pm 759.39\ \mu m^2$. For the group of pulps from teeth having complete root development mean immunostained area was $2645.69 \pm 932.55\ \mu m^2$. The maximum and minimum values and confidence intervals for IGF-1 and PCNA co-expression are presented in Table 3. Mann–Whitney's *U*-test revealed a statistically significant difference between groups ($P = 0.0009$).

Discussion

Cell proliferation and differentiation of both epithelial and mesenchymal cells are events that occur during odontogenesis. They are guided by epithelial–mesenchymal and cell–extracellular matrix interactions (Thesleff & Mikkola 2002, Thesleff 2003, Yamashiro *et al.* 2003). These interactions are regulated by the expression and secretion of diverse growth and transcription factors (Thesleff *et al.* 1995, Jernvall & Thesleff 2000). Once the tooth is completely formed, the dental pulp maintains its potential to initiate these mechanisms in the event of pulpodentine complex injuries, inducing a reparative response (Thesleff & Mikkola 2002).

Insulin-like growth factor-1 is a highly mitogenic and differentiation factor, which exerts endocrine, paracrine and autocrine functions, to control pre- and post-natal growth and development (Baker *et al.* 1993, Caton *et al.* 2005). It is a potent stimulator of cell proliferation and differentiation, as well as mainte-

Table 3 Immunostained area for insulin-like growth factor-1 and proliferating cell nuclear antigen co-expression in human dental pulp cells[a]

Root development	*n*	Mean**	Standard deviation	95% Confidence intervals		Min	Max
				Lower limit	Upper limit		
Incomplete	13	1468.91	759.39	1056.11	1881.71	522.46	2639.41
Complete	13	2645.69	932.55	2138.76	3152.62	1510.25	4136.04

[a]Values are presented in μm^2 of immunostained area by field of section analysed.
**Differences between groups are statistically significant ($P = 0.0009$).

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nance of specialized functions in several tissues (Werner & Katz 2004). IGF-1 belongs to a polypeptide family related to insulin, which includes two ligands (IGF-1 and IGF-2), two cell surface receptors (IGF-1R and IGF-2R), at least six high-affinity binding proteins (IGFBP-1 to IGFBP-6) and multiple IGFBP proteases (D'Ercole 1996, Gotz *et al.* 2006).

Insulin-like growth factor receptors, the binding proteins and the proteases regulate the activity of the ligands in several tissues. IGFBP's function as transport and storage peptides for IGF in the intravascular and extracellular environment, lengthening its half life and potentiating or inhibiting its activity by regulating its interactions with receptor (D'Ercole 1996, Werner & Katz 2004, Caton *et al.* 2005). They also allow the IGF-1 storage in the extracellular matrix (Mohan & Baylink 2002, Gotz *et al.* 2006). On the other hand, proteases cleave IGFBP's and therefore, modulate ligand accessibility (Collet-Solberg & Cohen 1996, Werner & Katz 2004, Gotz *et al.* 2006).

Insulin-like growth factor-1 is involved in the formation, maturation and reparation processes of several tissues, and almost all cell types of the human body express IGF-1 receptors during development stages or in tissue repair, when the tissues are experiencing a fast growth rate. All IGF-1 biological effects are mediated through interaction with its specific cell surface receptor, which is responsible of mitogenic signalling and most of the effects that IGF-1 promotes during tissue growth, development and repair (Baker *et al.* 1993, D'Ercole 1996). Previous studies have reported presence of IGF-1 receptor in human dental pulp (Caviedes-Bucheli *et al.* 2004, Gotz *et al.* 2006).

It has been demonstrated that IGF-1 regulates cell growth and differentiation in developing tissues and modulates some functions in mature cells (Joseph *et al.* 1993). This could explain IGF-1 presence in both groups of this study. Other immunohistochemistry and in situ hybridization studies have shown that IGF-1 acts in a paracrine/autocrine way during odontogenesis, participating in tissue mineralization (Joseph *et al.* 1994, 1996). Real-time PCR studies in mouse pulp cultures have also shown that IGF-1 enhances mineralization of enamel and dentine by inducing expression of specific genes (Caton *et al.* 2005).

The role of IGF-1 in pulp repairing processes has been demonstrated by adding this growth factor in pulp exposures of rat molars, showing better healing when comparing with control teeth (Lovschall *et al.* 2001). It also has been reported that IGF-1 could get trapped in the dentine matrix structure during its synthesis and mineralization, and could be released into dental pulp after an injury to the pulpodentine complex, stimulating repair processes (Finkelman *et al.* 1990, Mitsiadis & Rahiotis 2004).

Insulin-like growth factor-1 is essential in the signalling process for initiating cell growth, thus inducing cell proliferation and differentiation, participating actively in metabolic processes (Baker *et al.* 1993, D'Ercole 1996, Werner & Katz 2004). Some growth factors induce the synthesis of cyclin-dependent kinase (CdK), which is a basic component of cell cycle control system, which promotes the progression from G1 to S phase of cell cycle (Blagosklonny & Pardee 2002). In cultured fibroblasts of embryonary rat tissue, IGF-1 signalling regulates the CdK's level (Baker *et al.* 1993), suggesting that IGF-1 is required to pass the restriction point R, which is the previous step to the DNA replication or S phase.

Given the functions of IGF-1 during odontogenesis or repair processes, the present study was aimed to analyse the participation of this growth factor in human pulp cells from teeth having complete or incomplete root development, using immunohistochemistry with PCNA as specific marker for cell proliferation. It is important to point out that samples were analysed by longitudinal sections (including both coronal and radicular pulp). This information could be relevant as it has been shown that apical papilla from immature teeth is distinctive to the mature pulp in terms of containing less cellular and vascular components than those in the pulp (Sonoyama *et al.* 2008).

Proliferating cell nuclear antigen is a peptide present in proliferating eukaryote cells. It plays crucial roles during DNA replication in cell growth. PCNA allows detecting cell proliferation because it is increased specially during S phase of cell cycle, which is why it has been used as a biological or endogenous immunohistochemical marker to verify cell proliferation (Kobayashi *et al.* 1996, Matsuzaka *et al.* 2001, Ebensberger *et al.* 2002, Wildemann *et al.* 2003). PCNA is more practical to use than BrdU, which needs to be infiltrated *in vivo*, making it feasible to use it in studies with tissue samples (Matulová *et al.* 2002). Furthermore, PCNA has been used in odontogenesis studies both in animals and humans, being detected in a wide range of mesenchymal and epithelial cells (Casasco *et al.* 1995, Kaneko *et al.* 1999).

Immunohistochemical observations from this study showed a statistically significant higher IGF-1 presence in pulps from teeth with complete root development. This result confirms that IGF-1 is not only involved in

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embryonary formation processes, but it also has an active role during the proliferation and differentiation processes, that take place during reparative response after physical or chemical irritation of the pulpodentine complex during common clinical procedures. On the other hand, IGF-1 presence in human pulp cells from teeth with incomplete root development supports its role in the secretion and mineralization of dental tissues, generating a potential therapeutic basis for its potential use in cases were an induction for root-end formation is needed.

The results from this study are in accordance with a previous study that showed higher IGF-1 levels in human pulp tissue from teeth having complete root development when compared with pulp from teeth having incomplete root development (Caviedes-Bucheli *et al.* 2007). Another study, showed a statistically significant higher IGF-1 receptor expression in pulps from teeth having incomplete root development (Caviedes-Bucheli *et al.* 2004). From the above results, it could be hypothesized that locally produced IGF-1 in pulps from teeth having incomplete root development is rapidly bounded to receptors in pulp cells, due to its high cellular activity.

Immunohistochemical observations for PCNA in the present study showed expression for this protein in both experimental groups, having a statistically significant higher expression in the pulps from teeth having complete root development. Based on these results it can be hypothesized that pulp cell proliferation is constant and nondependent from root development stage. This fact is relevant to understand the tolerance and response capacity of the pulp to physical, chemical or mechanic aggression. This proliferation ability allows pulp cells to get in constant renovation, to keep tissue homeostasis or to form new hard tissue as a defence mechanism.

Immunohistochemical observations of the co-expression of IGF-1 and PCNA, showed a statistically significant higher expression of both peptides in pulps from teeth having complete root development, confirming the potential role of IGF-1 in proliferation and differentiation of pulpal cells in mature pulps, allowing them to respond to pulpodentine complex irritation. Lower co-expression of both peptides in immature pulps could be attributed to a higher proportion of cells going into differentiation processes than into proliferation, because teeth is experiencing a histodifferentiation stage.

This data suggest that IGF-1 could play an important role in homeostasis and reparative processes of the pulpodentine complex, when it is injured due to trauma, caries, or clinical operative procedures, inducing cell proliferation and differentiation to form reactionary and/or reparative dentine as a defence mechanism. However, further research is needed to ascertain this. In the same way, IGF-1 also participates actively in the cell proliferation and differentiation of developing teeth, being a crucial factor in the mineralizing process during odontogenesis, suggesting potential therapeutic use of IGF-1 in pulp tissue, both in immature and mature teeth when insulted by external irritants. Further studies about the interactions of this growth factor in the pulpodentine complex could lead to developing biological pulp therapies.

Conclusion

Insulin-like growth factor-1 and PCNA are expressed in human pulp cells. Their expression is significantly higher in pulp cells of teeth having complete root development when compared to pulp cells of developing teeth.

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