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The role of inducible nitric oxide synthase and haem oxygenase 1 in growth and development of dental tissue'

Lorenza Speranza[1]*, Mirko Pesce[1], Sara Franceschelli[1], Filiberto Mastrangelo[2], Antonia Patruno[3], Maria Anna De Lutiis[1], Stefano Tetè[2], Mario Felaco[1] and Alfredo Grilli[1]

[1]*Department of Human Movement Sciences, University G. D'Annunzio, Chieti, Italy*
[2]*Department of Oral Sciences, University G. D'Annunzio, Chieti, Italy*
[3]*Department of Drug Sciences, University G. D'Annunzio, Chieti, Italy*

In this study, the activity of the antioxidant enzyme network was assessed spectrophotometrically in samples of dental pulp and dental papilla taken from third-molar gem extracts. The production of nitric oxide by the conversion of L-(2,3,4,5)-[3H] arginine to L-(3H) citrulline, the activity of haem oxygenase 1 (HO-1) through bilirubin synthesis and the expression of inducible nitric oxide synthase (iNOS), HO-1 proteins and messenger RNA by Western blot and reverse-transcribed polymerase chain reaction were also tested. The objective of this study was to evaluate the role of two proteins, iNOS and HO-1, which are upregulated by a condition of oxidative stress present during dental tissue differentiation and development. This is fundamental for guaranteeing proper homeostasis favouring a physiological tissue growth. The results revealed an over-expression of iNOS and HO-1 in the papilla, compared with that in the pulp, mediated by the nuclear factor kappa B transcription factor activated by the reactive oxygen species that acts as scavengers for the superoxide radicals. HO-1, a metabolically active enzyme in the papilla, but not in the pulp, seems to inhibit the iNOS enzyme by a crosstalk between the two proteins. We suggest that the probable mechanism through which this happens is the interaction of HO-1 with haem, a cofactor dimer indispensible for iNOS, and the subsequent suppression of its metabolic activity. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—inducible nitric oxide; haem oxygenase 1; oxidative stress; development tissue

INTRODUCTION

Oxidative stress is defined as an imbalance between the production of oxidizing agents and the antioxidant defence mechanism. The term 'oxygen free radicals' is frequently, but mostly wrongly, used to indicate all reactive intermediates, including molecular forms that are not radicals (e.g. hydrogen peroxide).[1] For this reason, the term 'reactive oxygen species' (ROS) is more appropriate to use because it indicates their effects on the organism. ROS are constantly formed in living organisms, and the presence of an endogen defence mechanism is established.[2] In a physiological environment, various ROS are formed by different generating systems, whereby they exert their physiologic actions. The cells produce energy by reducing molecular oxygen into water in addition to four electrons.[3] Small amounts of ROS are formed during this process, as inevitable products of mitochondrial respiration. The defence system can tolerate the toxic radical products by incomplete reduction of oxygen to water.[2] Some unstable radicals may also intervene in biological intracellular pathways, for example in vascular endothelium, thus exerting crucial physiologic roles.[4] This study evaluates the nuclear factor kappa B (NF-κB) expression as a transcription factor that has a fundamental role in response to various stimuli, such as oxygen induction by the lipopolysaccharide (LPS) endogenous inductors such as inflammatory cytokines [interleukin 1, tumour necrosis factor alpha (TNF-α)] and oxidative stress, against which the NF-κB levels rise rapidly.[5] NF-κB is present in the cytosol as an inactive inhibitor of kappa B (Iκb)–NF-κB complex. This complex is phosphorylated by an IκB kinase, through the activation of the above-mentioned stimuli, which facilitate the translocation of the free form of NF-κB from the cytosol to the nucleus, determining the induction of the expression of the two inducible proteins: inducible nitric oxide (NO) synthase (iNOS) and haem oxygenase 1 (HO-1).[6,7] In the vascular endothelium and in other systems, NO radicals are produced from L-arginine by the NO synthase (NOS) family enzymes.[8] The enzyme exists in three isoforms: endothelial NOS, iNOS and neuronal NO.[9] Of the three isoforms, iNOS is expressed in many cell types after various stimuli, such as cytokines, endotoxins and physiopathological conditions.[10] Compared with the other NOS enzymes, the inducible enzyme generates quantities of NO for longer periods.[11,12] NO reacts with the superoxide anion (O_2^-) to yield the

*Correspondence to: Lorenza Speranza, Department of Human Movement Sciences, University G. D'Annunzio, Via dei Vestini, 31, 66123, Chieti, Italy.
E-mail: l.speranza@unich.it

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secondary oxidized products: nitrite, nitrate and dinitrogen trioxide. Antioxidant enzymes [superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx)] are endogenous defence mechanisms that balance the production of O_2^- generated during normal metabolism, as well as in response to specific external stimuli.[13–15] Mammalian cells can produce other endogenous gaseous molecules such as carbon monoxide, produced by the microsomal HO activity during the breakdown of the haem into biliverdin.[6] So far, three isoforms have been identified. The first HO-1 is an inducible protein stimulated by numerous stimuli that catalyse the first and rate-limiting step in the degradation of the protoporphyrin ring of tetrapyrrole from effete red blood cells, issuing an equimolecular amount of biliverdin IXa, carbon monoxide and iron. The biliverdin is converted into bilirubin, and the iron is taken away as ferrite.[16] HO-1 uses the haem as a protein group as well as a substrate.[17] The second HO isoform (HO-2) is a constituent form and is generally insensitive to the inductors of HO-1.[18] The third HO isoform (HO-3) catalyses the degradation of the haem much less than the other isoforms.[16] In a tissue undergoing growth and differentiation, oxidative stress causes apoptosis, the elimination of cells that are no longer useful to the harmonious development of tissues.[19,20] The role of apoptosis during embryonic development is not only to guarantee cell turnover but also to guarantee the correct development of a specific organism. In fact, if the organs or tissues with transitory roles were not eliminated, development would not be correct.[21] Dental embryogenesis is a complex model because the mesenchymal and ectodermal tissues together contribute to the differentiation and formation of different tissues and structures. Tooth embryogenesis comprises various phases of tissue development, passing from a germ stage to a cap stage, followed by a bell stage.[22,23] Dentin sialophosphoprotein (DSPP) is an extracellular matrix protein of primary importance for tooth formation and is expressed by odontoblasts and preameloblasts. DSPP is particularly expressed in the early stages of matrix mineralization, while successively its expression decreases, demonstrating the specific role of this protein during dentin and enamel mineralization and the significance of evaluating DSPP expression in dental tissues to better understand its role during biomineralization and odontogenesis.[24] During these phases, the ectoderm and the mesenchyme tissues show morphological and structural variations with the formation of different cellular types, among which ameloblastic cells.[25,26] The objective of this work is to evaluate the role of two proteins, iNOS and HO-1, through NF-κB in response to oxidative stress present in tissues during the phases of growth and differentiation.

MATERIALS AND METHODS

The experimental protocol, approved by the local ethics committee, was composed of a group of 30 patients from a university dental clinic, with an average of 13 years of orthodontic treatment for class II and III malocclusion, overcrowding and hyperdivergent coverage SNO^GoGn value of 38.5°. After an accurate case history, an objective examination and informed consent, the patients underwent surgical removal of the tooth gems; 30 dental papilla and 30 dental pulps were obtained. All samples were fixed in liquid nitrogen and placed at −80 °C. Before screening, we analysed all samples histochemically to distinguish the samples of the two tissues: dental papilla and dental pulp.

Semi-quantitative reverse transcription polymerase chain reaction for iNOS, HO-1 and DSPP

Semi-quantitative reverse-transcribed polymerase chain reaction (RT-PCR) was used to determine messenger RNA (mRNA) levels of the iNOS. Total RNA was extracted using 1-ml ULTRASPEC-RNA (Biotecx, Lab., Inc. Houston, TX, USA), as recommended by the manufacturer. RNA was dissolved in diethyl-pyrocarbonate-treated water and quantified spectrophotometrically at 260 nm. First-strand complementary DNA was generated by adding RNA (1 μg) to a mixture containing 1 mM deoxynucleoside triphosphates, 1 U μl^{-1} ribonuclease inhibitor, 2.5 U μl^{-1} Moloney murine leukaemia virus reverse transcriptase, 2.5 μM oligo-dt, 5 mM $MgCl_2$, 10× PCR buffer in a final volume of 20 μl. Reverse transcription was performed at 42 °C for 1 h followed by heat inactivation of reverse transcriptase at 92 °C for 10 min. β-Actin was amplified from the same amount of RNA to correct for variation of different samples. PCR amplification was performed using a programmable thermal controller (MJ Research, Inc. MA, USA). The PCR solution contained 10 μl of first-strand complementary DNA, 4 μl 10× PCR buffer and 2 mM $MgCl_2$. The following primer pairs were used: sense 5′-CGT AAA GAC CTC TAT GCC AA-3′ and antisense 5′-AGC CAT GCC AAA TGT CTC AT-3′ for iNOS, sense 5′- CAG GCA GAG AAT GCT GAG TTC-3′ and antisense 5′- GCT TCA CAT AGC GCT GCA-3′ for HO-1 primers and 5′-GTC AGA CTC CCC TTG CTT TGG G-3′ (antisense) for human DSPP. 18S primers, 0.15 mM of both sense 5′-TAC GGA GCA GCA AAT CCA C-3′ and antisense 5′-GAT CAA AGG ACT GCA GCC TG-3′, 2 U *Thermus aquaticus* DNA polymerase (Celbio, Milan, Italy) and water with a final volume of 50 μl were also used. These samples were overlaid with mineral oil and subjected to 35 cycles at 95 °C for 60 s, 60 °C for 60 s and one cycle at 72 °C for 7 min for iNOS and 40 cycles at 95 °C for 60 s, 58 °C for 60 s and one cycle at 72 °C for 7 min for HO-1. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under ultraviolet light. Bands on the gel were scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).[6]

Western blot analysis for iNOS, HO-1 and NF-κB

Determination of iNOS and HO-1 proteins was performed in two series of protein extracts by Western blotting. Equal amounts of protein (50 μg), quantified by spectrophotometric

assay (HP 8452A, CA, USA) using the Lowry method, from human peripheral adherent mononuclear cells were separated by electrophoresis in a 7.5% sodium dodecyl sulphate polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred at 4 °C to nitrocellulose membrane (Bio-Rad, Hercules, CA) in glycine–methanol buffer. Nitrocellulose was then blocked in Tris-buffered saline milk and incubated overnight, with various primary antibodies: anti-human iNOS (dilution 1:1000, Santa Cruz Biotech, CA), anti-human HO-1 (dilution 1:1000, Stressgen) and anti-human NF-κB (dilution 1:1000 Santa Cruz Biotechnology). The nitrocellulose was then washed in Tris-buffered saline and incubated with horseradish-peroxidase-conjugated secondary antibody (dilution 1:10 000, Pierce) for 1 h, washed again and developed. β-Actin was used as an internal standard. The nitrocellulose was scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).

Immunohistochemical localization of iNOS and HO-1

The immunohistochemical localization of iNOS and HO-1 was performed with primary rabbit anti-human iNOS and anti-human HO-1 (1:100) antibodies (Santa Cruz Biotech Inc., Santa Cruz, CA, USA) in accordance with a previous study.[6]

Citrulline synthesis (NO activity)

The measurement of the conversion of L-arginine to L-citrulline is a standard assay method currently used for quantitative NOS activity. Briefly, 10 µl of radioactive arginine L-(2,3,4,5-^{3}H) arginine monohydrochloride (64 Ci mM^{-1}, 1 µCi $µl^{-1}$) (Amersham, Arlington Heights, Illinois, USA), 50 µl of NADPH (10 mM) and 50 µl of $CaCl_2$ (6 mM) (Calbiochem, CA USA) were added to each cell homogenate sample and incubated for 30 min at room temperature. After incubation, the reactions were stopped with 400 µl of stop buffer (50 mM HEPES, pH 5.5, 5 mM ethylenediaminetetraacetic acid), and the equilibrated resin was added to each sample. The equilibrated resin bound unreacted arginine. After centrifugation, the radioactivity corresponding to L-(^{3}H)-citrulline was measured with liquid scintillation spectrometry. Calcium was omitted from these incubations to favour the determination of the calcium-independent iNOS isoform.[9]

Assay for HO activity

HO activity assay was performed as described previously by Srisook *et al.*[27] Briefly, microsomes from harvested cells were added to a reaction mixture containing 0.8 mM NADPH (Sigma), 2 mM glucose 6-phosphate (Sigma), 0.2 U glucose-6-phosphate dehydrogenase (Sigma), 2 mg rat liver cytosol prepared from a 105 000 3 *g* supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4) and 20 mM hemin (Sigma). The reaction was conducted at 37 °C in the dark for 1 h and then placed on ice for 2 min to terminate the reaction. Bilirubin was determined by calculation from the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO activity was expressed as nanomoles of bilirubin formed per milligramme of microsomal protein per hour. The total protein content of confluent cells was determined using a Bio-Rad DC protein assay (Bio-Rad, Herts, UK) by comparison with a standard curve obtained with bovine serum albumin.

Cu,Zn-SOD activity

SOD activity was determined as described by Sun and Zigman.[28] The assay mixture contained 50 mM sodium carbonate buffer, pH 10, 0.1 mM epinephrine (Sigma) and tissue fraction (containing about 1–50 µg of protein) in a final volume of 2.5 ml. The inhibitory effect of SOD on the autoxidation of epinephrine, with the use of 1.25 mM KCN to discriminate the CN^--insensitive MnSOD from the CN^--sensitive Cu,Zn-SOD, was assayed spectrophotometrically at 480 nm at 25 °C. Percentage inhibition values were converted into activities by using a purified Cu,Zn bovine SOD as standard (Sigma). One unit of SOD is the amount of enzyme required to halve the rate of substrate auto-oxidation.

CAT activity

CAT activity was measured spectrophotometrically.[29]The decomposition of H_2O_2 was monitored continuously at 240 nm. The assay mixture in a final volume of 3 ml contained 10 mM potassium phosphate buffer, 10 mM H_2O_2 and 1.5–11 µg protein of enzymatic extract. CAT units were defined as 1 µmol H_2O_2 decomposed per minute at 25 °C.

Glutathione peroxidase

Quantification of GPx activity was evaluated using the Paglia and Valentine method as modified by Di Ilio *et al.*[30,31] The activity of the Se-dependent GPx was measured with H_2O_2 (0.25 mM) as substrate. The oxidation of NADPH was followed at 25 °C on a Hewlett and Packard spectrophotometer at 340 nm. One unit was defined as 1 µmol of glutathione oxidized per minute.

Statistical analysis

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

RESULTS

iNOS, HO-1 and NF-κB protein levels

The Western blot technique was used to investigate the amounts of iNOS, HO-1 and NF-κB in dental pulp and dental papilla tissues; we detected their protein levels. The results of our experimental study demonstrate that the presence of iNOS and HO-1 was consistent in dental papilla. Figure 1A,C shows the marked expression of the two proteins compared with that of the pulp. The NF-κB expression

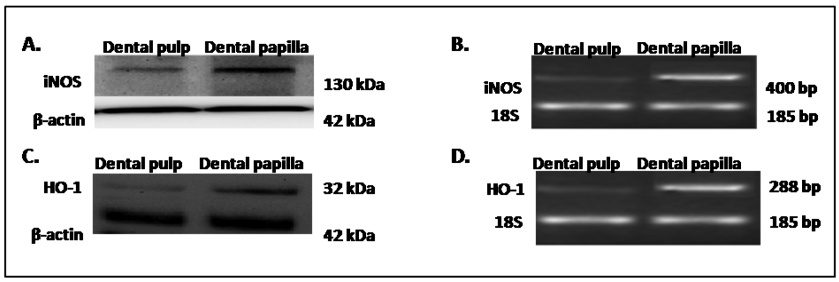

Figure 1. Western blot analysis of (A) iNOS and (C) the HO-1 protein obtained from human dental pulp and dental papilla. The proteins were stained by antibodies against human iNOS antigen (130 kDa) and HO-1 antigen (32 kDa). β-Actin is used for control (42 kDa). A significant increase of iNOS protein and HO-1 protein was detectable in the human dental papilla. The expression of (B) iNOS, 400 bp, and (D) HO-1 gene, 288 bp, evaluated by RT-PCR in human dental pulp and dental papilla. The standard band is expressed as 18S, 185 bp. The presence of iNOS of 400 bp is more evident in the human dental papilla than in the human dental pulp whereas the presence of HO-1 of 288 bp is more evident in the human dental papilla than in the human dental pulp

shown in Figure 2A clearly shows how this factor is significantly expressed in the papilla compared with that in the pulp.

iNOS, HO-1 and DSPP mRNA levels by RT-PCR analysis

RT-PCR was performed to assess the expression of the iNOS (400 bp), the HO-1 (288 bp) and the DSPP (472 bp). For the iNOS and HO-1 mRNA, the results obtained follow the same expression as the proteins. Figure 1B,D shows an increased expression of iNOS and HO-1 gene in dental papilla compared with that in the pulp. For the evaluation of DSPP mRNA, in Figure 2B, we show the increase of DSSP mRNA in the dental papilla as a marker of dental tissue development.

Activity of iNOS through citrulline synthesis

Basal L-[^{3}H] citrulline production from L-[^{3}H] arginine was detectable in homogenates obtained from each sample. No significant difference was observed between the samples regarding iNOS activity in human dental papilla compared with that in the pulp (Figure 3A). This enzyme activity is not in relation to increased levels in the iNOS mRNA and iNOS proteins observed previously (Figure 1A,B).

Activity of HO-1 through bilirubin synthesis

HO activity as expressed from production of bilirubin was detectable in homogenates obtained from each sample, and a significant difference was observed between the samples. In fact, the activity of this enzyme was increased in the papilla compared with that in the pulp (Figure 3B). This enzyme activity was related to an increased level of the HO-1 mRNA and HO-1 proteins observed previously (Figure1C,D).

Antioxidant network

The antioxidant network studied included SOD, CAT and GPx and demonstrated an increase in the levels of ROS during growth and development of dental pulp. In fact, as shown in Figure 4, the activity of these enzymes considerably increased in the papilla tissue compared with that in the pulp.

Immunohistochemical analysis

Immunohistochemical analysis as presented in Figure 5 shows that the protein level, iNOS and HO-1 are more evident in the dental papilla than in the dental pulp.

DISCUSSION

Embryonic development is dynamic, encompassing a continuous progression of cell division, movement, differentiation and death.[23] One of the most important facets of the developing tissue is the crosstalk and interaction of the cells that are constantly changing from a stage of totipotency to a stage of determination. Cell suicide programmes may also be activated in response to abiotic stimuli.[32] Many diverse triggers of cell death, such as TNF, heat shock, viruses, protein synthesis inhibition, oxidative stress, hypoxia or NO can induce apoptosis. NO has been shown to have variable consequences in relation to either promoting or preventing apoptosis, likely because of its many interactions with other biological molecules.[33] Although the concentration

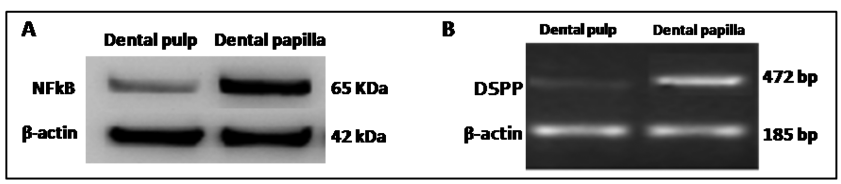

Figure 2. (A) NF-κB expression detected in human dental pulp and dental papilla. The protein was stained by antibodies against human NF-κB antigen (65 kDa). β-Actin is used for control (42 kDa). The expression of this transcriptional factor is more evident in human dental papilla than in human dental pulp. (B) DSSP mRNA, used as a development tissue marker, is more evident in human dental papilla than in human dental pulp

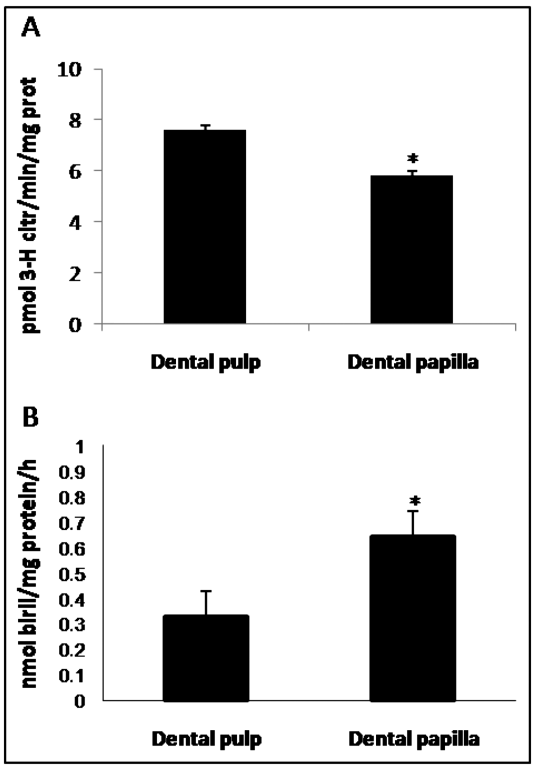

Figure 3. Basal L-[^{3}H] citrulline production from L-[^{3}H] arginine detectable in homogenates obtained from human dental pulp and dental papilla; this enzyme activity is not reported to increase in the iNOS mRNA and iNOS protein observed in Figure 2A,B. In this case, we observed the reduction of iNOS activity in human dental papilla (A). HO activity (B) was expressed from production of bilirubin, detectable in homogenates obtained from human dental pulp and dental papilla. This enzyme activity was related to an increase in the HO-1 mRNA and HO-1 protein as observed in Figure 2C,D

and source of NO can determine the response of the cell to NO exposure, pro-apoptotic and anti-apoptotic response to NO appear to be specific to the type of cells that are involved.[11] Such proteins include growth factor receptors, protein kinases, protein phosphatases and G proteins, as well as a number of important transcription factors, including activator protein 1, NF-κB and protein 53, some of which are thought to be involved in the activation of apoptosis.[34]

During recent years, a large body of evidence has suggested that ROS can activate programmed cell death.[35,36] Apoptosis, or programmed cell death due to DNA fragmentation, is a distinctive form of eukaryotic cell death characterized by a series of morphologic and biochemical changes that result in the elimination of cells from the tissues without eliciting an inflammatory response. Oxidative stress, as a result of an imbalance in the rate of production and removal of ROS, is known to induce apoptosis in various cell types.[37] To contrast the effect of the ROS, an endogenous system is activated, represented by antioxidant enzymes such as SOD, CAT and GPx. The SOD transforms the radical superoxide into hydrogen peroxide; in turn, hydrogen peroxide is reduced to oxygen and water by the enzyme CAT and GPx.[15,38] Oxidative stress, resulting from an imbalance between the production of ROS and the activity of the antioxidant system, is shown to be necessary to induce apoptosis in various cell types.[14] Many studies demonstrate that, during the embryonic development and during cell differentiation, there is an increase in the production of ROS.[23] The increase in the production of O_2^- is the beginning of a cascade of reactions, passing through the activation of the transcriptional factor NF-κB and leading to the synthesis of two molecules that function as antioxidants:

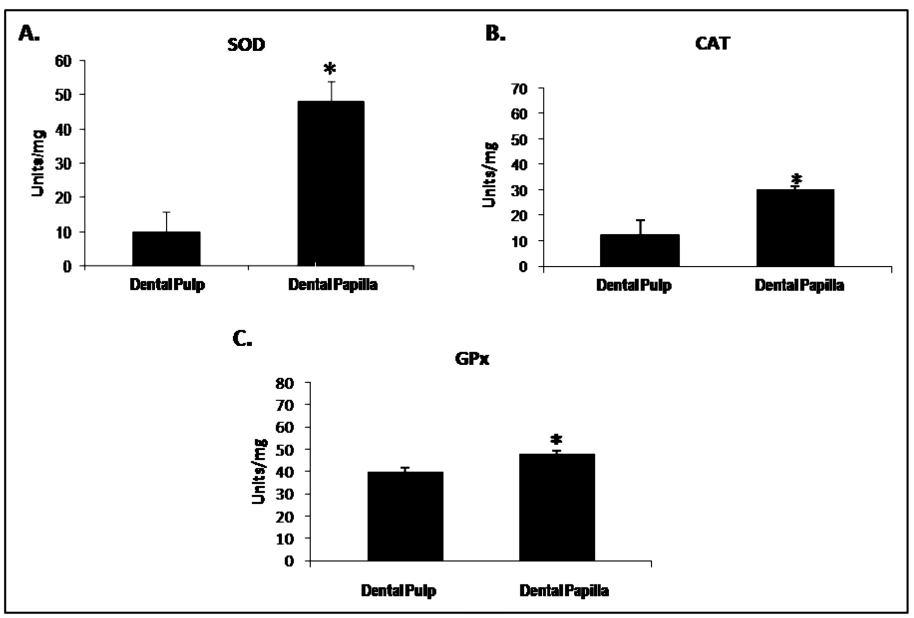

Figure 4. Activity of the antioxidant enzymes: SOD, CAT and GPx. SOD was assayed spectrophotometrically at 25 °C, at 480 nm (A), CAT at 240 nm (B); GPx at 340 nm (C)

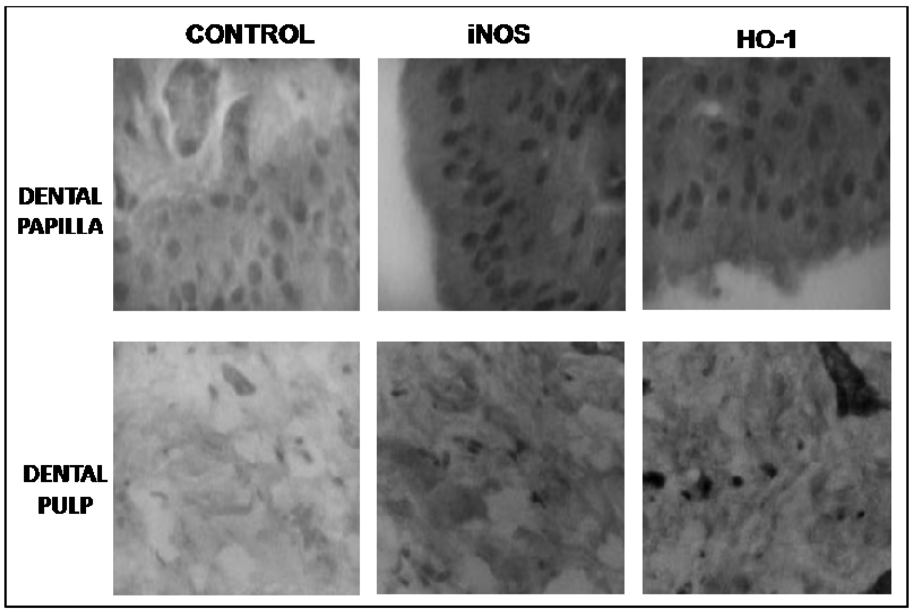

Figure 5. Immunohistochemical analysis of iNOS and HO-1 proteins confirmed the increase of both proteins in human dental papilla compared with that in human dental pulp (scale bar 100 μm)

iNOS and HO-1.[6] In normal conditions, therefore, the activity of these molecules, contrasting the action of O_2^-, re-establishes an oxidant/antioxidant equilibrium.[39] In particular conditions, such as embryonic development or cell differentiation, it is necessary to maintain a high level of O_2^- to induce apoptosis for the normal re-modelling process and/or elimination of cells that are no longer necessary.[19] In this study, where the differentiation mechanism is fundamental for the passage from a slightly differentiated tissue, such as the papilla, to that of a more differentiated one, such as the pulp, we observed that oxidative stress increases considerably in the papilla. It was seen that the activity of the antioxidant enzyme in the papilla did not work efficiently. In fact, the SOD dismutases, hydroxyl radical into hydrogen peroxide, and the catalase and the GPx in the papilla tissue do not manage to transform this radical into water and oxygen molecules, determining in the slightly differentiated tissue a considerable oxidative stress (Figure 4A–C). In fact, when the cell has increased levels of SOD without a proportional increase in peroxidases such as GPx (Figure 4C), the peroxide can react with transitional metals and generate the hydroxyl radical. This condition activates a transcriptional factor, such as the NF-κB, that has a fundamental role in response to various stimuli, such as exogenous induction by LPS and endogenous inductors such as inflammatory cytokines (interleukin 1, TNF-α) and oxidative stress, to which the levels of NF-κB respond by rapidly increasing. NF-κB is present in the cytosol as the inactive complex Iκb–NF-κB. This complex is phosphorylated by an IκB kinase through the activation of the above-mentioned stimuli. These facilitate the translocation of the NF-κB free form of cytosol to the nucleus, determining the induction of the expression of inducible proteins. Because an increase of radical oxidization is evident during the process of cell differentiation (Figure4), it is possible to have the induction of various transcriptional factors such as NF-κB in the papillary tissue differentially compared with that in the pulp (Figure 2A), determining an increase of both iNOS and HO-1 mRNA and proteins (Figure 1A–D). However, whereas the activity of the HO-1 protein is increased in the samples (Figure 3B), the activity of the iNOS, on the contrary, shows a significant decrease (Figure 3A). In light of our results, we can postulate the fundamental role of these two proteins in restoring a metabolic equilibrium to contrast the toxic activity of ROS during the cell differentiation process. In fact, the iNOS protein produces large quantities of NO from its catalytic activity, which presents a high affinity with the superoxide radical, entering into competition with SOD. This may bring about the formation of further reactive species such as the peroxynitrite ($ONOO^-$). To avoid such formation, the HO-1 enzyme in combination with haem, the indispensible cofactor for the dimerization and activation of the iNOS, blocks the activity of this protein. These results lead us to hypothesize that the relationship between HO-1 and iNOS, during the cell differentiation phase, is able to compensate for the production of radicals to obtain a balanced tissue growth and does not activate a mechanism of wrong cell turnover. HO-1 and its capacity as the rate-limiting enzyme of haem levels limit the availability of haem for synthesis or the activity of haem-containing enzymes such as iNOS. In summary, our studies demonstrate a negative feedback interaction between iNOS and HO-1 during the development of dental tissue. The inhibition of the iNOS activity, probably due to the degradation of haem by HO-1, can, therefore, limit haem's availability for optimal activity of iNOS, blocking the production of NO. This leads to a massive presence of O_2^- in the papilla where apoptotic mechanisms may be triggered

through NF-κB, which permits re-modelling that is at the base of the differentiation process that gradually transforms the papilla into the pulp.

CONFLICT OF INTEREST

No conflict of interest.

ACKNOWLEDGMENT

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