J Periodont Res 2008; 43: 322–327

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01032.x

Cellular responses of rat periodontal ligament cells under hypoxia and re-oxygenation conditions *in vitro*

H. Amemiya[1], K. Matsuzaka[1,2], E. Kokubu[1], S. Ohta[1], T. Inoue[1,2]
[1]Department of Clinical Pathophysiology and [2]Oral Health Science Center HRC7, Tokyo Dental College, Chiba Japan

Amemiya H, Matsuzaka K, Kokubu E, Ohta S, Inoue T. Cellular responses of rat periodontal ligament cells under hypoxia and re-oxygenation conditions in vitro. *J Periodont Res 2008; 43: 322–327. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard*

Background and Objective: The aim of this study was to investigate the responses of periodontal ligament cells under hypoxia and re-oxygenation conditions *in vitro*.

Material and Methods: Periodontal ligament fibroblasts were isolated from rat incisors. In the hypoxia group, cells were incubated in 2% O_2 for 1–3 d. In the re-oxygenation group, cells were first incubated under the same conditions as the hypoxia group for 24 h and then were returned to normoxic conditions and cultured for 1–2 additional days.

Results: Proliferation ratios increased in all groups in a time-dependent manner. Proliferation ratios in both the hypoxia and re-oxygenation groups were significantly higher than in the control group on days 2 and 3. Alkaline phosphatase activity was significantly higher in the hypoxia group than in the control and the re-oxygenation groups. The expression of bone sialoprotein mRNA was significantly higher in the hypoxia group than in the control group on days 1 and 2. The expression of vascular endothelial growth factor mRNA was significantly higher in the hypoxia group than in the control group on days 1 and 2. In the re-oxygenation group, the level of expression of bone sialoprotein mRNA and vascular endothelial growth factor mRNA were similar to those of the control group. The expression of heat shock protein 70 mRNA in the hypoxia group was similar to that in the control group, whereas in the re-oxygenation group it was statistically higher than in the other groups.

Conclusion: These results suggest that periodontal ligament cells maintain their osteogenic ability in hypoxia and re-oxygenation conditions *in vitro*.

Professor Takashi Inoue, Department of Clinical Pathophysiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba, 261–8502, Japan
Tel: +81 43 2703582
Fax: +81 43 2703583
e-mail: inoue@tdc.ac.jp

Key words: hypoxia; *in vitro*; periodontal ligament; re-oxygenation

Accepted for publication July 6, 2007

It is known that hypoxia, which disrupts oxygen homeostasis because of a low oxygen supply, has grave and critical consequences in the brain and heart, which are most susceptible to circulatory disturbance. Many reports on astrocytes in the brain (1,2) or myocardial cells in the heart (3) have shown the effects on those cells of ischemia or ischemia-reperfusion injury.

During complete ischemia in the brain, oxygen, glucose and glycogen are rapidly depleted in the blood and brain tissues, and ATP levels are reduced by 10–20% for 2–3 min followed by severe ATP depletion (4). There have been many investigations of ischemia and angiogenesis at the

molecular level. Angiogenesis can be stimulated by lowered physiological oxygen or by hypoxia (5–8).

Heat shock proteins have been considered to be involved in hypoxia because their expression is induced by various types of stress, such as ischemia, anoxia and re-oxygenation (9,10). It has been reported that increased expression of heat shock protein 70 occurs in cardiomyocytes (3), pulp cells (11) and rat gastric epithelial cells (12) after hypoxia re-oxygenation, which suggests that the up-regulation of heat shock protein 70 represents a protective response against hypoxic injury.

Vascular endothelial growth factor is an angiogenic growth factor that elicits cellular responses to hypoxia and has recently also attracted much attention as a potent inducer of vascular permeability and angiogenesis (13–15).

It is known that physiological blood flow in the periodontal ligament (16) is slightly lower than either pulpal or cerebral blood flow (17), and blood flow is easily reduced by occlusal forces. Moreover, in cases of tooth transplantation or replantation, periodontal tissues containing osteogenic fibroblasts, which may differentiate into either cementoblasts or osteoblasts, decrease during severe ischemia. If the periodontal ligament is subjected to a dry environment after tooth extraction for more than 30 min (18–20), its ability to differentiate and to proliferate is lost. However, little is known about the behavior of periodontal ligament cells under conditions of hypoxia or re-oxygenation *in vitro*.

In this study, we examined the cellular responses of rat periodontal ligament cells under conditions of hypoxia and re-oxygenation, in terms of cell proliferation ratio and alkaline phosphatase activity. Furthermore, we also evaluated the expression of mRNAs encoding vascular endothelial growth factor, heat shock protein 70 and bone sialoprotein.

Material and methods

This study was conducted in compliance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College (approval number 01-63).

Animals and cell culture

Periodontal tissues were obtained from the upper incisors of 16 anesthetized male Sprague–Dawley rats, 4 wk of age, each weighing $\approx$100 g (Sankyo Labo Service, Tokyo, Japan), according to the method of Inoue *et al.* (19). Periodontal ligament cells were then incubated in a humidified atmosphere of 95% air, 5% CO_2, at 37°C for primary culture, using α-minimal essential medium (Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (Sigma, St Louis, MO, USA), supplemented with 10 mg of gentamicin (Sigma). The medium was changed every 48 h and cells in the fifth subculture were used for the experiments.

For setting the oxygen concentrations, a BL-40M CO_2 incubator (Juuji-Field Labo; Bio Labo, Tokyo, Japan) was used, which can regulate the concentration of oxygen in the air to nitro-oxygen.

In the control group, cells were incubated in a humidified atmosphere at normoxic conditions of 20% O_2, 5% CO_2 and 75% N_2 for 1–3 d at 37°C.

In the hypoxia group, cells were incubated in a humidified atmosphere of 2% O_2, 5% CO_2 and 93% N_2 for 1–3 d at 37°C.

In the re-oxygenation group, cells were first incubated under the same conditions as the hypoxia group for 24 h and then returned to normoxic conditions and cultured for 1–2 additional days. All data of the hypoxia group on day 1 were the same as the re-oxygenation group at day 1.

Media were not changed during the experimental period.

Proliferation assay

Cultured cells ($\approx 1 \times 10^5$) were seeded in 35-mm cell-culture dishes (Corning Costar, New York, NY, USA) and were incubated for 1–3 d at each of the culture conditions described above. Cells were detached using 0.125% trypsin at each time-point, and the numbers of detached cells were counted using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

Alkaline phosphatase activity

Alkaline phosphatase activity was measured using a colorimetric assay kit (Alkaline Phosphatase Opt; Roche Diagnostics Japan, Tokyo, Japan). Cultured cells (1×10^5) were seeded in 35-mm cell-culture dishes and incubated for 1–3 d. Cells were washed with calcium- and magnesium-free phosphate-buffered saline at each time-point, harvested with demineralized H_2O (Milli Q; Millipore, Billerica, MA, USA) and then homogenized in distilled water for 60 s using a sonicator (Sonifier 250D; Branson, Rochester, MI, USA) on ice.

Each homogenate was centrifuged at 800 *g* for 5 min and the supernatants were used for assay. One millilitre of premixed solution (1 M diethanolamine buffer, pH 9.8, with 0.5 mM $MgCl_2$ and 10 mM *p*-nitrophenylphosphate, kept at 37°C) was added to 10 μL of supernatant. Absorption at 405 nm for *p*-nitrophenol was measured using a spectrophotometer (Ultrospec 3000; Amersham Pharmacia Biotechnologies, Rochester, NY, USA), 1, 2 and 3 min after addition of the premixed solution.

To determine the specific activity of alkaline phosphatase, protein concentrations in each lysate were determined using the Pierce bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). A volume of 100 μL of each cell lysate was added to 100 μL of bicinchoninic acid working reagent (kept at 37°C for 30 min). Absorbance was measured at 595 nm using a microplate reader, and the average absorption per min was calculated. Alkaline phosphatase activity was calculated according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction

After 1, 2 and 3 d of culture, cells were detached and homogenized using TRIZOL (Invitrogen, Carlsbad, CA, USA) and total RNAs were extracted. Suspensions to which chloroform had been added were centrifuged at 16,000 *g* for 20 min. The supernatants were recov-

ered and mixed with isopropyl alcohol, then frozen at −80°C overnight. Subsequently, the samples were centrifuged at 16,000 *g* for 20 min and total RNA pellets were recovered and washed with 75% ethanol. Finally, the total RNA pellets were dissolved in RNAase-free water and stored at −80°C until used.

Total RNA concentrations were measured using a NanoDrop® (ND-1000 Spectrophotometer; Scrum, Tokyo, Japan). Using total RNA as a template, reactions were conducted using a reverse transcription-polymerase chain reaction kit (RNA-PCR kit version 2.1; Takara Biomedicals, Shiga, Japan) to synthesize cDNA. Quantitative PCR was then conducted using specific primers for bone sialoprotein, heat shock protein 70 and vascular endothelial growth factor, and β-actin as a housekeeping gene, with a LightCycler™ using the double-stranded DNA dye SYBER Green I (Roche Diagnostics Japan). The PCR conditions and primer sequences used are shown in Table 1.

Quantification was performed by comparing the levels obtained with standardized samples. Melting curve analyses were also performed after PCR amplification to confirm the absence of the primer dimer in each PCR product. Relative quantification of gene expression with real-time PCR data was calculated relative to β-actin.

Data were averaged from five dishes in each group.

Statistical analysis

The results were analyzed using one-way analysis of variance and were then compared by Scheffe's test.

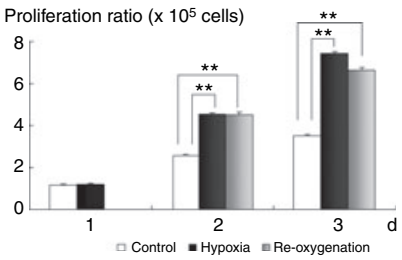

Fig. 1. Proliferation ratios increase in all groups in a time-dependent manner. Proliferation ratios in both the hypoxia and re-oxygenation groups were significantly higher than in the control group on days 2 and 3. Data are expressed as means ± standard deviation; $^{**}p < 0.01$ compared with the control group; $n = 5$.

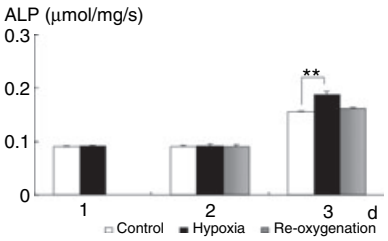

Fig. 2. Alkaline phosphatase activity. There was no significant difference in alkaline phosphatase activity in any of the groups on days 1 or 2. However, alkaline phosphatase activity was significantly higher in the hypoxia group than in the control group or the re-oxygenation group on day 3. There was no significant difference between the re-oxygenation group and the control group on day 3. Data are expressed as means ± standard deviation; $^{**}p < 0.01$ compared with the control group; $n = 5$. ALP, alkaline phosphatase.

Results

Proliferation ratios

Cell proliferation ratios increased in all groups in a time-dependent manner (Fig. 1). Proliferation ratios in the hypoxia and the re-oxygenation groups were significantly higher than in the control group on days 2 and 3 ($p < 0.01$).

Alkaline phosphatase activity

There was no significant difference in alkaline phosphatase activity in any of the groups on days 1 or 2 (Fig. 2). However, alkaline phosphatase activity was statistically higher in the hypoxia group than in the control group on day 3 ($p < 0.01$). There was no significant difference between the re-oxygenation group and the control group on day 3.

MRNA expression

Bone sialoprotein — The expression of bone sialoprotein mRNA in the hypoxia group was statistically higher than in the control group on days 1 ($p < 0.01$) and 2 ($p < 0.05$) (Fig. 3). There was no significant difference between the re-oxygenation group and the control group on days 2 or 3.

Heat shock protein 70 — The expression of heat shock protein 70 mRNA in the hypoxia group was similar to that in the control group at each time-point. However, the expression of heat shock protein 70 mRNA in the re-oxygenation group was statistically higher than in the control group on day 3 ($p < 0.01$) (Fig. 4).

Table 1. Polymerase chain reaction (PCR) primers used for LightCycler™ -assisted analysis

	Primer sequences (5′ to 3′)	PCR conditions	Product size (bp)
BSP	Forward: TCC CAC AGG AGA AGA TTG TC Reverse: CCT TCT GGA TCT TCC TCT GT	95°C (10 s), 55°C (6 s), 72°C (7 s)	132
HSP70	Forward: GTG TGC AAC CCG ATC ATC AG Reverse: CAC CAG CAG CCA TCA AGA GT	95°C (10 s), 60°C (10 s), 72°C (7 s)	201
VEGF	Forward: TAC CAG CGC AGC TAT TGC CGT Reverse: TTT GGT GAG GTT TGA TCC GCA TG	95°C (10 s), 54°C (5 s), 72°C (8 s)	180
β-Actin	Forward: CCT GTA TGC CTC TGG TCG TA Reverse: CCA TCT CTT GCT CGA AGT CT	95°C (10 s), 62°C (10 s), 72°C (10 s)	260

BSP, bone sialoprotein; HSP70, heat shock protein 70; VEGF, vascular endothelial growth factor.

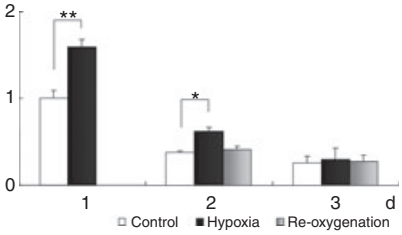

Fig. 3. Expression of bone sialoprotein mRNA in the hypoxia group was statistically higher than in the control group on days 1 and 2. There was no significant difference between the re-oxygenation group and the control group on days 2 and 3. Data are expressed as means ± standard deviation; $*p < 0.05$ and $**p < 0.01$ compared with the control group; $n = 5$.

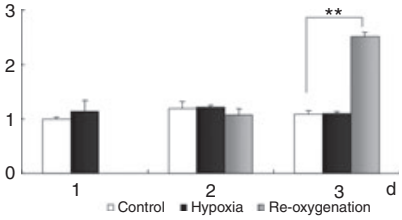

Fig. 4. Expression of heat shock protein 70 mRNA in the hypoxia group was similar to that of the control group at each time point. The expression of heat shock protein 70 mRNA in the re-oxygenation group was statistically higher than in the control group on day 3. Data are expressed as means ± standard deviation; $**p < 0.01$ compared with the control group; $n = 5$.

Vascular endothelial growth factor — The expression of vascular endothelial growth factor mRNA in the hypoxia group was statistically higher than in the control group on days 1 ($p < 0.01$) and 2 ($p < 0.05$) (Fig. 5). However, there was no significant difference in vascular endothelial growth factor mRNA expression between the re-oxygenation group and the control group on days 2 or 3.

Discussion

Proliferation ratios

In general, oxygen is necessary for cells to maintain their functions and is a source of energy during ATP synthesis in mitochondria. Vega *et al.* (1)

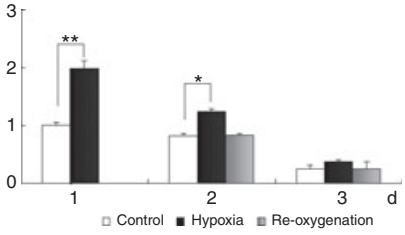

Fig. 5. Expression of vascular endothelial growth factor mRNA in the hypoxia group is statistically higher than in the control group on days 1 and 2. However, there is no significant difference in vascular endothelial growth factor mRNA expression between the re-oxygenation group and the control group on days 2 or 3. Data are expressed as means ± standard deviation; $*p < 0.05$ and $**p < 0.01$ compared with the control group; $n = 5$.

reported that hypoxia increased the amount of glucose transporter 1 in rat primary cortical astrocytes, and they suggested that glucose transporter 1 contributed to the hypoxic modulation of glucose uptake. The function and oxygen-glucose metabolism of periodontal ligament cells is regulated by oxygen or glucose as primary cellular energy sources, and it is assumed that reductions in these result in a crisis for cell survival. In this study, the cell proliferation ratio of rat periodontal ligament cells increased in the hypoxia group and in the re-oxygenation group.

Amemiya *et al.* (11) suggested that the mitochondrial function and proliferation of pulp cells under conditions of hypoxia increased glucose consumption. However, glucose consumption did not change under the condition of re-oxygenation compared with the control. Similar phenomena might occur in a conparable manner for periodontal ligament cells.

Expression of heat shock protein 70 mRNA

Heat shock protein 70 participates in the maintenance of cell homeostasis and is induced reversibly during disrupted energy metabolism, protein synthesis or calcium homeostasis in cells (17). During conditions of oxygen glucose block and following re-oxygenation, the expression of heat shock protein 70 significantly protects astrocytes in the brain (21). Heat shock protein 70 levels have also been analyzed in rat gastric epithelial cells (12,22) and in pulp cells (11). Heat shock protein 70 was reported to be regulated by re-oxygenation following hypoxia and was up-regulated during re-oxygenation following hypoxia, which suggests that heat shock protein 70 is involved in maintaining cell survival and in supporting cell function. In the present study, the expression of heat shock protein 70 mRNA in the hypoxia group was not up-regulated, but it was significantly up-regulated in the re-oxygenation group, which may support those suggestions.

Alkaline phosphatase activity and expression of bone sialoprotein mRNA

Alkaline phosphatase, which has been associated with early osteoblastic differentiation (23), and bone sialoprotein, which has been associated with mineral crystal formation and is localized specifically in mineralized tissues in several pathologies (24), are considered to be the most useful markers of early mineralization.

The effects of hypoxia on osteoblastic differentiation in various osteogenic cells have been reported. Increases of alkaline phosphatase activity in human periodontal ligament fibroblasts by 10% O_2 (25) and in dog pulp cells by 2% O_2 (11) have been reported, whereas decreases of alkaline phosphatase activity of human osteoblastic cells by 5% O_2 (26) and of alkaline phosphatase activity and the expression of bone sialoprotein mRNA of human stromal cells from bone marrow (MIAMI cells) by 3% O_2 (27) have been demonstrated. In the present study, increases of alkaline phosphatase activity and of the expression of bone sialoprotein mRNA in rat periodontal ligament cells by 2% O_2 were found. These contrasting results suggest that alkaline phosphatase activity and the expression of bone sialoprotein mRNA depend on different oxygen tensions, on species and on cell type.

Expression of vascular endothelial growth factor mRNA

Vascular disruption has been shown to lead to the formation of hypoxic areas of ischemic injury. Angiogenesis is vital for the delivery of oxygen, nutrients and growth factors necessary to initiate the reparative process of ischemic tissue healing.

Vascular endothelial growth factor is regulated by the hypoxia-mediated control of gene transcription (6–9). Vascular endothelial growth factor induces angiogenesis as well as the permeabilization of blood vessels, and is involved in the regulation of vasculogenesis *in vivo*. Vascular endothelial growth factor is a highly specific mitogen for vascular endothelial cells in response to hypoxia. Vascular endothelial growth factor induces endothelial cell proliferation and promotes cell migration in a paracrine manner. Moreover, the induction of vascular endothelial growth factor mRNA has been found in periodontal ligament cells (28,29). A study has demonstrated that hypoxia regulates vascular endothelial growth factor expression and cellular differentiation (30). An investigation on the response of astrocytes to hypoxia showed that vascular endothelial growth factor mRNA and protein levels increased significantly (31,32). Amemiya *et al.* (11) also reported that vascular endothelial growth factor production in pulp cells increased under conditions of hypoxia. In the present study, the expression of vascular endothelial growth factor mRNA in the hypoxia group was statistically higher than in the control group. However, there was no significant difference between the re-oxygenation group and the control group. Therefore, these results suggest that the expression of vascular endothelial growth factor mRNA is up-regulated in periodontal ligament cells in response to hypoxia, which results in the promotion of cellular proliferation and differentiation in an autocrine manner.

The results of the present study suggest that the acitivities of periodontal ligament tissues injured during tooth extraction in the case of transplantation or replantation, are not impaired.

Conclusion

Periodontal ligament cells maintain cell homeostasis during hypoxia and re-oxygenation and promote cell proliferation and cell differentiation during hypoxia at an early stage *in vitro*.

Acknowledgements

We would like to thank Miss Kaori Takano for her technical assistance. This research was supported by Oral Health Science Center Grant HRC7 from the Tokyo Dental College (and) by a 'High-Tech Research Center' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2006-10.

References

1. Vega C, Sachleben RL Jr, Gozal E. Differential metabolic adaptation to acute and long-term hypoxia in rat primary cortical astrocytes. *J Neurochem* 2006; **97:**872–883.
2. Rayner BS, Duong TT, Myers SJ, Witting PK. Protective effect of a synthetic anti-oxidant on neuronal cell apoptosis resulting from experimental hypoxia re-oxygenation injury. *J Neurochem* 2006; **97:**211–221.
3. Suzuki K, Sawa Y, Kaneda Y, Ichikawa H, Shirakura R, Matsuda H. In vivo gene transfection with the heat shock protein70 enhances myocardial tolerances to ischemia reperfusion injury in rat. *J Clin Invest* 1997;**99:**1645–1650.
4. Lowry OH, Passonneau JV, Hasselberger FX, Schultz DW. Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *J Biol Chem* 1964;**239:**18–30.
5. Denko NC, Fontana LA, Hudson KM *et al.* Investigating hypoxic tumor physiology through gene expression patterns. *Oncogene* 2003;**22:**5907–5914.
6. Carmeliet P, Dor Y, Herbert JM *et al.* Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumor angiogenesis. *Nature* 1998;**394:**485–490.
7. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 2003;**9:**677–684.
8. Tang N, Wang L, Esko J *et al.* Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* 2004;**6:**485–495.
9. Ang D, Liberek K, Skowyra D, Zylicz M, Georgopoulos C. Biological role and regulation of the universally conserved heat shock proteins. *J Biol Chem* 1991;**266:** 24233–24236.
10. Neupert W, Hartl FU, Craig EA, Pfanner N. How do polypeptides cross the mitochondrial membranes? *Cell* 1990;**63:**447–450.
11. Amemiya K, Kaneko Y, Muramatsu T, Shimono M, Inoue T. Pulp cell responses during hypoxia and re-oxygenation in vitro. *Eur J Oral Sci* 2003;**111:**332–338.
12. Katada K, Naito Y, Mizushima K *et al.* Gene expression profiles on hypoxia and re-oxygenation in rat gastric epithelial cells: a high-density DNA microarray analysis. *Digestion* 2006;**73:**89–100.
13. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999;**13:**9–22.
14. Matsushita K, Maruyama I, Takada H, Torii M. Lipopolysaccharide enhances the production of vascular endothelial growth factor by human pulp cells in culture. *Infect Immun* 1999;**67:**1633–1639.
15. Ferrara N. Vascular endothelial growth factor. *Eur J Cancer* 1996;**32:**2413–2422.
16. Ohuchi M, Fujimura A. Changes of blood flow in pressure side of periodontal ligament tooth movement. *Dent J Iwate Med University* 2004;**29:**15–23.
17. Kim S. Regulation of pulpal blood flow. *J Dent Res* 1985;**64:**590–596.
18. Takizawa H. An experimental study of tooth preservation for tooth transplantation and replantation – especially, biological activity of periodontal ligament. *J Jpn Prosthodont Soc* 1991;**35:**723–737.
19. Inoue T, Deporter DA, Melcher AH. Induction of chondrogenesis in muscle, skin, bone marrow, and periodontal ligament by demineralized dentin and bone matrix in vivo and in vitro. *J Dent Res* 1986;**65:**12–22.
20. Nowak TS Jr. Synthesis of a stress protein following transient ischemia in the gerbil. *J Neurochem* 1985;**45:**1635–1641.
21. Massa SM, Swanson RA, Sharp FR. The stress gene response in brain. *Cerebrovasc Brain Metab Rev* 1996;**8:**95–158.
22. Sun Y, Ouyang YB, Xu L *et al.* The carboxy-terminal domain of inducible HSP70 protects from ischemic injury in vivo and vitro. *J Cereb Blood Flow Metab* 2006;**26:**937–950.
23. Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ. Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. *J Periodontol* 1997;**68:** 320–327.

24. Ganss B, Kim RH, Sodek J. Bone sialoprotein. *Crit Rev Oral Biol Med* 1999; **10:**79–98.
25. Kubota M. Study on proliferation and function of periodontal ligament fibroblasts and osteoblastic cells under hypoxia. *Kokubyo Gakkai Zasshi* 1989;**56:**473–484.
26. Matsuda N, Morita N, Matsuda K, Watanabe M. Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia and mechanical stress in vitro. *Biochem Biophys Res Commun* 1998;**249:**350–354.
27. D'Ippolito G, Diabira S, Howard GA, Roos BA, Schiller PC. Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. *Bone* 2006;**39:**513–522.
28. Oyama T, Sakuta T, Matsushita K, Maruyama I, Nagaoka S, Torii M. Effects of roxithromycin on tumor necrosis factor-α-induced vascular endothelial growth factor expression in human periodontal ligament cells in culture. *J Periodontol* 2000;**71:**1546–1553.
29. Suthin K, Matsushita K, Machigashima M, Torii M, Izumi Y. Possible roles of vascular endothelial growth factor in periodontal tissue. Expression of VEGF in human gingival and periodontal ligament fibroblasts and its effects on the cells in culture. *J Hard Tissue Biol* 2001;**10:**85–88.
30. Steinbrech DS, Mehrara BJ, Saadeh PB *et al*. Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro. *Plast Reconstr Surg* 1999;**104:**738–747.
31. Kaur C, Sivakumar V, Zhanq Y, Linq EA. Hypoxia-induced astrocytic reaction and increased vascular permeability in the rat cerebellum. *Glia* 2006;**54:**826–839.
32. Kaur C, Sivakumar V, Ang LS, Sundaresan A. Hypoxia damage to the periventricular white matter in neonatal brain. Role of vascular endothelial growth factor nitric oxide and excitotoxicity. *J Neurochem* 2006;**98:**1200–1216.