

## Carbon dioxide laser irradiation stimulates mineralization in rat dental pulp cells

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### Abstract

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**Aim** To examine the effect of carbon dioxide laser irradiation on mineralization in dental pulp cells.

**Methodology** Rat dental pulp cells were irradiated with a carbon dioxide laser at 2 W output power for 20, 40 and 60 s, and were cultured in ascorbic acid and  $\beta$ -glycerophosphate containing media. Cell viability was examined 24 h after laser irradiation by a modified MTT assay. Alizarin Red S staining was performed 10 days after laser irradiation. The amounts of secreted collagen from the cells after irradiation were quantified following Sirius Red staining. The expression levels of collagen type I and HSP47, collagen-binding stress protein, were analysed by real-time PCR. HSP47 protein expression was examined by Western blotting. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test.

**Results** The cell viability was not affected by laser irradiation at 2 W for up to 40 s. However, it was significantly decreased by 20% at 60 s ( $P < 0.05$ ). The amount of mineralization after 10 days of irradiation at 2 W for 40 s was significantly increased in comparison to the other conditions ( $P < 0.05$ ). The extracellular collagen production was significantly increased by 73% on day 2 and 38% on day 4 after laser irradiation ( $P < 0.05$ ). Although collagen type I gene expression was not changed by laser irradiation, HSP47 gene and protein expression was induced within 12 and 24 h, respectively.

**Conclusions** These results suggested that carbon dioxide laser irradiation stimulated mineralization in dental pulp cells. The laser irradiation also increased HSP47 expression but not collagen gene expression.

**Keywords:** carbon dioxide laser, collagen, dental pulp cells, heat shock protein 47, mineralization.

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### Introduction

Reparative dentine forms in the dental pulp in response to various external stimuli such as caries and abrasion (Kamal *et al.* 1997, Lee *et al.* 2006). Direct pulp capping with calcium hydroxide has been advocated

to accelerate reparative dentine formation on the exposed pulp surface. However, calcium hydroxide is highly alkaline and causes an inflammatory response. In addition, it has not always been clinically highly efficacious in the uniform formation of reparative dentine (Scarano *et al.* 2003). Recently, the use of mineral trioxide aggregate (MTA) in direct pulp capping has been reported (Aeinehchi *et al.* 2003, Chacko & Kurikose 2006). MTA is superior to calcium hydroxide for pulp capping of mechanically exposed human teeth; however, a variety of histological responses were still observed (Caicedo *et al.* 2006). Furthermore, no data from long-term clinical results

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are yet available. In an ideal situation, the exposed pulp surface should be covered promptly with reparative dentine and the dental pulp should not demonstrate an inflammatory response.

The application of lasers has expanded into various fields and has also been frequently used in clinical dentistry (Pearson & Schuckert 2003, Parker 2007). In cultured cells, irradiation by low power laser, such as a diode laser, has been reported to accelerate cell differentiation and mineralization in calvarial and dental pulp cells (Ozawa *et al.* 1998, Ohbayashi *et al.* 1999, Ueda & Shimizu 2003). On the other hand, Moritz *et al.* (1998a,b) reported that the utility of a high power laser, such as a carbon dioxide laser, is useful on exposed pulp surfaces in direct pulp capping experiments. Furthermore, Melcer *et al.* (1987) observed that a neo-dentine bridge was formed in the pulp tissue after carbon dioxide laser irradiation on teeth, suggesting that this laser is effective in mineralization. However, the mechanism by which carbon dioxide laser irradiation stimulates mineralization in direct pulp capping treatment is not fully elucidated. Laser irradiation may affect the collagen production in dental pulp cells, because the collagenous network plays an important role in mineralization (Linde 1989).

Heat shock proteins (HSPs) are induced by stress from heat and chemical stimuli (Noda *et al.* 2002). HSPs have been known to suppress the aggregation of denatured protein (Guzhova & Margulis 2006). They are also constitutively expressed in normal cells, and are associated with important functions such as protein synthesis and intracellular transport (Eisenberg & Greene 2007). In particular, HSP47 is a collagen-specific molecular chaperone. It specifically binds to collagen and plays an essential role in collagen production (Masuda *et al.* 1994, Koide *et al.* 2002).

The purpose of this study was to examine the effect of a carbon dioxide laser on mineralization in rat dental pulp cells. Moreover, the amount of extracellular secreted collagen and the HSP47 expression levels were examined to clarify the stimulatory effects of carbon dioxide laser irradiation on mineralization of dental pulp cells.

## Materials and methods

### Cells and cell culture conditions

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Health Sciences University of Hokkaido, and experiments were carried

out under the control of the University's Guidelines for Animal Experimentation. The dental pulp cells were isolated from incisors of Wistar rats (female, 5-week-old) as described previously (Yokose *et al.* 2000). The cells were cultured in Dulbecco's modified eagle medium (DMEM; Sigma, St Louis, MI, USA) supplemented with 10% foetal bovine serum (Sigma), 10 000 U mL<sup>-1</sup> penicillin (Invitrogen, Grand Island, NY, USA), and 10 mg mL<sup>-1</sup> streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% carbon dioxide.

### Laser irradiation

A carbon dioxide laser apparatus (Bel Luxar LX-20SP, Takara, Kyoto, Japan) with a wavelength of 10.6 µm and a power output of 2.0 W (A4 mode, 10 pps, average power output of 0.3 W) was used. Rat dental pulp cells ( $5 \times 10^4$  cells per well) were seeded out in 24-well plates and cultured for 24 h, and then serum-starved for 24 h. After withdrawal of medium, the cells were irradiated at 2 W output power for 20, 40 and 60 s using the scanning method as applied in clinical laser irradiation. The tip was moved gradually at a constant rate, avoiding concentrating laser light on one site, and the whole area was irradiated. The laser beam was delivered by a ceramic tip (0.8 mm diameter) with the distance from the tip of the fibre to the cell layer being 2 cm (irradiation diameter approximately 2 mm). The total energy of irradiation time of 40 s was 382.2 J cm<sup>-2</sup>. Irradiated or nonirradiated (control) cells were cultured in DMEM containing 50 µg mol L<sup>-1</sup> ascorbic acid (AA, Sigma) and 10 m mL<sup>-1</sup> β-glycero-phosphate (β-GP, Sigma) for 10 days.

### Cell viability assay

Rat dental pulp cells were cultured in DMEM containing 50 µg mL<sup>-1</sup> AA and 10 mmol L<sup>-1</sup> β-GP for 24 h after irradiation or nonirradiation (control). Cell viability was determined by a modified MTT assay (WST-8 assay: Dojindo, Kumamoto, Japan), and data are presented as a percentage of viability values seen under control culture conditions. The assay is based on the cleavage of tetrazolium salt WST-8 to formazan by cellular mitochondrial dehydrogenase. The amount of the dye generated by activity of dehydrogenase is directly proportional to the number of living cells. For the WST-8 assay, a 10-µL quantity of WST-8 dye solution was added directly to 100 µL of culture medium per well. The absorbance of the dye was

measured at 450 nm using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

### Quantitative Alizarin Red S staining

Rat dental pulp cells were cultured as before in  $50 \mu\text{g mL}^{-1}$  AA and  $10 \text{ mmol L}^{-1}$   $\beta$ -GP-containing media for 10 days. Cells were fixed in 70% ice-cold ethanol for 1 h and rinsed with distilled water. Cells were stained with  $40 \text{ mmol L}^{-1}$  Alizarin Red S (Sigma), pH 4.2, for 10 min with gentle agitation. Alizarin Red S staining is specific for calcium deposition. Cells were rinsed thrice with distilled water and then rinsed with PBS for 15 min. Dye was extracted from fixed cells by treatment with  $500 \mu\text{L}$  10% cetylpyridinium chloride (Nakarai Tesque., Kyoto, Japan) for 20 min with gentle agitation. The absorbance of the extracted dye was measured at 570 nm using a Model 680 microplate reader. The amount of Alizarin Red S was determined according to an Alizarin Red S standard curve.

### Quantitative analysis of extracellular secreted collagen

The amount of extracellular secreted collagen was measured on day 2–10 using the method described by Ohbayashi *et al.* (1999). Conditioned media ( $100 \mu\text{L}$ ) were dispensed into wells of 96 well plates, and plates were incubated at  $37^\circ\text{C}$  for 24 h until dry. After rinsing with distilled water, 0.2% Sirius Red (Sigma) in saturated picric acid (w/v) was placed in each well for 30 min. The plates were washed with 0.5% NaOH. The eluted stain was then drawn up and down several times in a pipette and placed into a second plate. Absorbance was read at 540 nm in a Model 680 microplate reader, and the amount of extracellular secreted collagen was estimated from a standard curve.

### Real-time PCR

The mRNA expression of collagen type I, HSP47 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined by real-time PCR with rat-specific primers. Total RNA was extracted using RNeasy (Qiagen Inc., Chatworth, CA, USA) and was digested with DNase I (Sigma), according to the manufacturer's instructions. Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was performed on a volume of  $15 \mu\text{L}$  containing  $1.5 \mu\text{L}$  (50 ng) of cDNA and  $13.5 \mu\text{L}$  of master mix containing  $7.5 \mu\text{L}$  of mix

(SYBR Green PCR Master Mix, Invitrogen),  $0.75 \mu\text{L}$  of each primer ( $10 \text{ pmol L}^{-1}$ ), and  $4.5 \mu\text{L}$  of diethyl pyrocarbonate-treated water using an ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The sequences of the rat-specific primers were as follows: collagen type I, forward 5'-TTGACCCTAACCAAG GATGC-3', reverse 5'-CACCCCTTCTGCGTTGTATT-3'; HSP47, forward 5'-GTGCGCTCCCTCAGTAACTC-3', reverse 5'-CCACATCCTTGGTGACCTCT-3'; Control primers specific for GAPDH were: forward 5'-TCCACC ACCCTGTTGCTGTA-3', reverse 5'-ACCACAGTCCAT GCCATCAC-3'. The program was set at  $50^\circ\text{C}$  for 2 min and  $95^\circ\text{C}$  for 10 min followed by 40 cycles of denaturation at  $95^\circ\text{C}$  for 15 s and annealing at  $60^\circ\text{C}$  for 60 s. SYBR green fluorescence was monitored after each elongation period. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted  $C_T$ ).

Samples were amplified in triplicate, averages were calculated, and differences in  $C_T$  data were evaluated by Sequence Detection Software V1.3. (Applied Biosystems). For each primers set, validation experiments demonstrated that the efficiencies of target and reference gene amplification were approximately equal; the absolute value of the slope of log input amount versus  $C_T$  was  $<0.1$ . For data analysis, we used the comparative  $C_T$  method ( $\Delta\Delta C_T$  method) with the following formula:  $\Delta C_T = C_T (\text{Target}) - C_T (\text{GAPDH})$ . The comparative  $\Delta\Delta C_T$  calculation involved finding the difference between  $\Delta C_T$  of irradiated cells and the mean value of the  $\Delta C_T$  from the control cells. Fold increase in the expression of specific mRNA in irradiated cells compared to control cells was calculated as  $2^{-(\Delta\Delta C_T)}$ . The data are expressed as relative quantity (RQ) and differences are shown in the figures as the expression ratio of the normalized target gene according to the software results.

### Western blotting

For investigating the expression of HSP47 protein in dental pulp cells, immunoblot analysis was performed. The extracts were prepared from irradiated or non-irradiated cells using lysis buffer [ $100 \text{ mmol L}^{-1}$  Tris-HCl (pH 7.2) containing  $150 \text{ mmol L}^{-1}$  NaCl,  $0.1 \text{ mmol L}^{-1}$  DTT/EDTA, 0.1% Triton X-100]. The protein concentrations were determined using protein assay kit (Bio-Rad). Protein ( $20 \mu\text{g}$ ) was loaded onto

10% SDS-PAGE gel. After electrophoresis, the SDS-PAGE separated proteins were transferred to nitrocellulose membrane at 60 V for 2 h. The membrane was blocked with 10% bovine serum albumin in TBST [10 mmol L<sup>-1</sup> Tris-HCl (pH8.0), 150 mmol L<sup>-1</sup> NaCl, 0.05% Tween 20] for 30 min, and incubated with a 1 : 1000 dilution of polyclonal rabbit IgG against human HSP47 (Stressgen, Ann Arbor, MI, USA) in TBST for 1 h. Then, the membrane was incubated with a 1 : 2000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) for 1 h. Horseradish peroxidase activity was detected using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

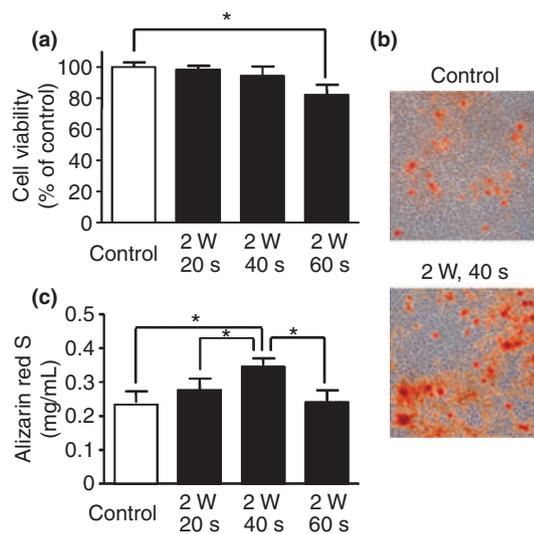
### Statistical analysis

Statistical analysis was performed with data obtained from three independent experiments. The data are expressed as mean  $\pm$  SD and analysed using one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. Statistical significance was accepted at  $P < 0.05$ .

### Results

Rat dental pulp cells were irradiated with a carbon dioxide laser at 2 W output power for 20, 40, and 60 s. Thereafter, the cell viability was measured 24 h after irradiation (Fig. 1a). There was no difference in the cell viability between the control and the cells which were irradiated 20 or 40 s. However, it was significantly decreased by 20% in the cells which were irradiated for 60 s in comparison to the controls ( $P < 0.05$ ). Next, the effect of laser irradiation was examined on the mineralization in dental pulp cells. The cells which were irradiated for 40 s had a clearly increased number and total area of calcified nodules stained by Alizarin Red S (Fig. 1b). In addition, when the mineralization was determined quantitatively on day 10, the cells with 40-s irradiation had significantly increased the degree of mineralization in comparison to the other conditions ( $P < 0.05$ ). However, no significant differences were observed between the controls and the cells with 20- or 60-s irradiation ( $P > 0.05$ ; Fig. 1c).

Next, the culture media were collected every 2 days up to 10 days and the amount of extracellular secreted collagen was determined quantitatively after Sirius Red staining. The amount of secreted collagen significantly increased after laser irradiation in comparison to the controls 73% and 38% on day 2 and 4, respectively



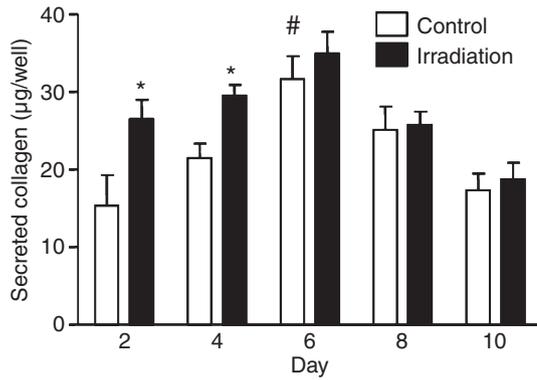
**Figure 1** Effect of laser irradiation on cell viability and mineralization of dental pulp cells. (a) Cell viability after carbon dioxide laser irradiation at 2 W for 20, 40 and 60 s was analysed by a modified MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. (b) After laser irradiation at 2 W for 20, 40 and 60 s, rat dental pulp cells were cultured in DMEM containing AA and  $\beta$ -GP for 10 days. Representative photographs of Alizarin Red S staining are shown (Original magnification 200 $\times$ ). (c) Quantification of Alizarin Red S staining. Bar represents mean  $\pm$  SD ( $n = 3$ ). The data were analysed using one-way ANOVA: \* $P < 0.05$  versus control.

( $P < 0.05$ ; Fig. 2). However, there was no difference in comparison to the controls after day 6.

To clarify the mechanism of increased collagen secretion after irradiation, the effect of laser irradiation on the expression of collagen type I and HSP47 was examined by real-time PCR method. There was no significant difference in the expression of the collagen type I gene between the irradiated cells and the controls at any time point ( $P > 0.05$ ; Fig. 3a). Interestingly, the expression of the HSP47 gene in the irradiated cells was significantly increased compared to the controls by 54%, 57% and 24% at 12, 24 and 48 h, respectively ( $P < 0.05$ ). In addition, Western blot analysis showed that HSP47 protein with a molecular weight of 47 kDa was increased in the cells 24 h after irradiation compared to that in control cells (Fig. 3b).

### Discussion

A carbon dioxide laser has a photothermal effect that is applied when making incisions in soft tissue and obtaining haemostasis. It also has a photochemical

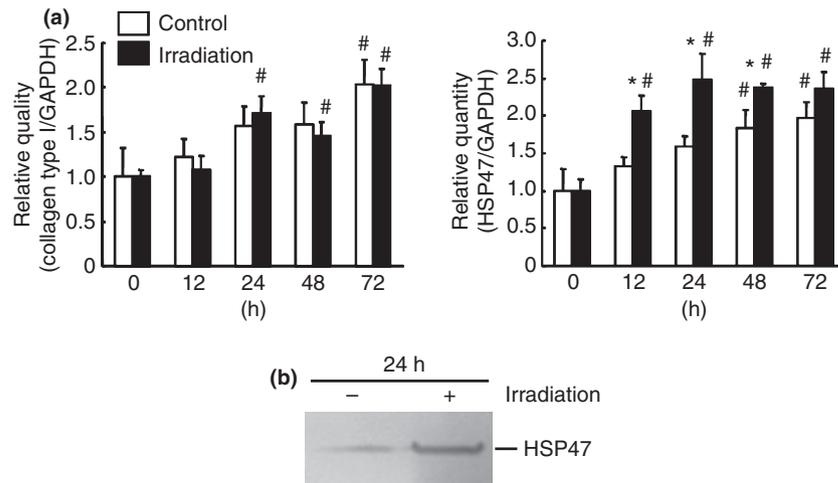


**Figure 2** The amount of collagen secreted into the culture media of dental pulp cells. The dental pulp cells were irradiated at 2 W for 40 s and cultured in DMEM supplemented with AA and β-GP for 10 days. Conditioned media from control and irradiated cells were collected every 2 days. The amount of collagen (µg per well) was measured by the Sirius Red staining method. Bar represents mean ± SD (*n* = 3). The data were analysed using one-way ANOVA. Significantly different from the control at each time point: \**P* < 0.05. Significantly different from the day 2: #*P* < 0.05.

effect used in alleviating pain (Posten *et al.* 2005). Melcer *et al.* (1987) reported that neo-dentine bridge formation was observed in pulp tissue after carbon dioxide laser irradiation on teeth of dogs and monkeys. This finding indicates that carbon dioxide laser may be

useful for the induction of mineralization. The current study showed a decrease of cell viability for irradiation at 2 W for 60 s using a carbon dioxide laser. In other words, if the irradiation time is long, then the energy density is increased even with a low power laser and injuries to cells occur. Therefore, the effect of carbon dioxide laser on mineralization, collagen secretion and HSP47 expression was examined under conditions (2 W, 40 s) that did not injure the cells.

Collagen, which constitutes almost 90% of the dentine matrix protein, is synthesized by the odontoblasts and secreted into predentine, where collagen molecules are arranged into fibres. These fibres form the collagenous network in which the mineral crystals are deposited (Linde 1989). Furthermore, it has been reported that collagen type I time-dependently stimulates the expression of osteopontin and alkaline phosphatase (ALP), whilst also inducing the differentiation of bone marrow cells into osteoblasts (Mizuno & Kuboki 2001). The DGEA (Asp-Gly-Glu-Ala) domain of type I collagen binds with integrin on the cellular membrane. Differentiation is thought to be promoted through its binding with integrin (Mizuno *et al.* 2000). In this study, carbon dioxide laser irradiation significantly increased in the secretion of collagen into culture media on day 2 and 4. This finding suggests that increased collagen in culture media acted on the integrin of the cells, and mineralization was thus



**Figure 3** The expression of collagen type I and HSP47 in control and irradiated cells. (a) The dental pulp cells were irradiated at 2 W for 40 s and cultured in DMEM supplemented with AA and β-GP. The mRNA expression of collagen type I and HSP47 was analysed at the indicated time points by real-time PCR. Bar represents mean ± SD (*n* = 3). The data were analysed using one-way ANOVA. Significantly different from the control at each time point: \**P* < 0.05. Significantly different from time 0: #*P* < 0.05. (b) The HSP47 protein expression was examined by Western blotting.

stimulated. Most recently, Lee *et al.* (2008) reported that heat stress at 42 °C for 30 min significantly elevated ALP activity on days 7 and 14 in rat pulp cells compared to control groups, revealing the possibility that heat stress generated by laser elevated ALP activity, thereby stimulating mineralization.

HSP47 knockout mice cannot produce collagen with the correct triple helix. Therefore, they die by 11.5 days post-coitus due to apoptosis in various tissues and vascular ruptures because they cannot form collagen fibres and basement membranes (Nagai *et al.* 2000). In addition, HSP47 expression is induced by thermal stimuli, and its constitutive expression is closely coupled with the amount of the collagen matrix. For example, an increase in the HSP47 expression has been reported in pulmonary fibrosis in which there is increased production of collagen (Razzaque *et al.* 1998). Therefore, HSP47 expression, which has a close relationship with collagen production, was examined. The results clearly showed that the HSP47 gene was induced by laser irradiation within 12 h and HSP47 protein was induced within 24 h. The observation that the expression level of HSP47 was correlated with the amount of collagen secretion is consistent with the findings of a previous study (Razzaque *et al.* 1998). Although collagen gene expression was not altered by carbon dioxide laser irradiation, extracellular collagen secretion did increase. Regarding this discrepancy, increased HSP47 production by laser irradiation have led to efficient assembly of procollagen molecules prior to their secretion, thereby promoting extracellular collagen secretion (Lamandé & Bateman 1999).

To date, the studies of the mechanism of mineralization induction by laser have been conducted using a low power laser. Irradiation by a low power laser on osteoblasts resulted in increased expression of ALP and osteocalcin (Ozawa *et al.* 1998, Ohbayashi *et al.* 1999, Ueda & Shimizu 2003). It has been reported that these increases are one cause of mineralization induction. Hamajima *et al.* (2003) indicated that the gene expression of a bone-inducing factor called osteoglycin increased by twofold within 2 h when MC3T3-E1 osteoblast-like cells were irradiated by a low power laser. These findings indicate that the mechanism of mineralization induction might differ according to the cells, type of laser, and irradiation conditions.

## Conclusion

Carbon dioxide laser irradiation stimulated collagen production and calcified nodules formation on rat

dental pulp cells. Furthermore, laser irradiation enhanced HSP47 gene and protein expressions but not type I collagen gene expression. Further study will be needed to elucidate the role of HSP47 on laser-induced mineralization in dental pulp cells.

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