Carbon dioxide laser irradiation stimulates mineralization in rat dental pulp cells

Y. Yasuda[1], E. Ohtomo[1], T. Tsukuba[2], K. Okamoto[2] & T. Saito[1]

[1]Division of Clinical Cariology and Endodontology, Department of Oral Rehabilitation,

School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan;

[2]Division of Oral Pathopharmacology, Department of Medical and Dental Sciences,

Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Running title: Effect of laser irradiation

Key words: Carbon dioxide laser, heat shock protein 47, mineralization, dental pulp cells, collagen

Coresponding author: Yoshiyuki Yasuda, DDS, PhD

Division of Clinical Cariology and Endodontology, Department of Oral Rehabilitation, School

of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu,

Hokkaido, 061-0293 Japan. Tel: +81-133-23-2841, Fax: +81-133-23-1423, E-mail address:

yasuda@hoku-iryo-u.ac.jp

Abstract

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 β-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 analyzed 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

1

(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 h 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.

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 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, 1998b) 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 penicillin (Invitrogen,

Grand Island, NY, USA), and 10 mg/mL streptomycin (Invitrogen) at 37°C in a humidified

atmosphere of 5% CO_2.

Laser irradiation

 A CO_2 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 x 10^4 cells/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^2.

Irradiated or non-irradiated (control) cells were cultured in DMEM containing 50 µg/ml

ascorbic acid (AA, Sigma) and 10 mM β-glycerophosphate (β-GP, Sigma) for 10 days.

Cell viability assay

Rat dental pulp cells were cultured in DMEM containing 50 µg/mL AA and 10 mM β-GP for 24

h after irradiation or non-irradiation (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 μg/mL AA and 10 mM β–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 mM 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 three

times with distilled water and then rinsed with PBS for 15 min. Dye was extracted from fixed

cells by treatment with 500 µL 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 Arizarin Red S standard curve.

Quantitative analysis of extracellular secreted collagen

 The amount of extracellular secreted collagen was measured on day 2 to day 10 using the

method described by Ohbayashi *et al.*(1999). One hundred microlitres of conditioned media

were dispensed into wells of 96 well plates, and plates were incubated at 37°C for 24 h until dry. After rinsing with distilled water, 0.2% Sirius Red (Sigma) in saturated picric acid (wt/vol) 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 μL containing 1.5 μL (50 ng) of cDNA and 13.5 μL of master mix containing 7.5 μL of mix (SYBR Green PCR Master Mix, Invitrogen), 0.75 μL of each primer (10 pmol/L), and 4.5 μL 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'-TTGACCCTAACCAAGGATGC-3', reverse 5'-CACCCCTTCTGCGTTGTATT-3';

HSP47, forward 5'-GTGCGCTCCCTCAGTAACTC-3', reverse

5'-CCACATCCTTGGTGACCTCT-3'; Control primers specific for GAPDH were: forward

5'-TCCACCACCCTGTTGCTGTA-3', reverse 5'-ACCACAGTCCATGCCATCAC-3'. The

program was set at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of denaturation

at 95 °C for 15 s and annealing at 60 °C for 60 s. SYBR green fluorescence was monitored

after each elongation period. The threshold was set above the non-template 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: $\Delta C_T = C_T$ (Target) - C_T (GAPDH). The comparative $\Delta\Delta C_T$

calculation involved finding the difference between ΔC_T of irradiated cells and the mean value

of the ΔC_T from the control cells. Fold increase in the expression of specific mRNA in

irradiated cells compared to control cells was calculated as $2^{-(\Delta\Delta CT)}$. The data are expressed as

RQ (relative quantity) 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 mM Tris-HCl (pH 7.2) containing 150 mM NaCl, 0.1 mM DTT/EDTA, 0.1%

Triton X-100]. The protein concentrations were determined using protein assay kit (Bio-Rad).

Twenty micrograms of protein were 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 mM Tris-HCl

(pH8.0), 150 mM 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 1:2,000 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 ± 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-second 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-second or 60-second 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 (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 h, 24 h, 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, which is applied when making incisions in soft tissue and obtaining haemostasis. It also has a photochemical 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 (2W, 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), while 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 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 postcoitus 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 2-fold 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.

Figure Legends

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 MMT 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 β-GP for 10 days. Representative

photographs of Alizarin Red S staining are shown (Original magnification 200x). (C)

Quantification of Alizarin Red S staining. Bar represents the mean ± SD (n=3). The data were

analysed using one-way ANOVA: * $p<0.05$ vs. control.

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/well) was measured by the Sirius Red staining

method. Bar represents the 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.

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 the 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.

References

Aeinehchi M, Eslami B, Ghanbariha M, Saffar AS (2003) Mineral trioxide aggregate (MTA)

and calcium hydroxide as pulp-capping agents in human teeth: a preliminary report.

International Endodontic Journal **36**, 225-31.

Caicedo R, Abbott PV, Alongi DJ, Alarcon MY (2006) Clinical, radiographic and histological

analysis of the effects of mineral trioxide aggregate used in direct pulp capping and

pulpotomies of primary teeth. *Australian Dental Journal* **51**, 297-305.

Chacko V, Kurikose S (2006) Human pulpal response to mineral trioxide aggregate (MTA): a

histologic study. *Journal of Clinical Pediatric Dentistry* **30**, 203-9.

Eisenberg E, Greene LE (2007) Multiple roles of auxilin and hsc70 in clathrin-mediated

endocytosis. *Traffic* **8**, 640-6.

Guzhova I, Margulis B (2006) Hsp70 chaperone as a survival factor in cell pathology.

International Review of Cytology **254**, 101-49.

Hamajima S, Hiratsuka K, Kiyama-Kishikawa M et al. (2003) Effect of low-level laser

irradiation on osteoglycin gene expression in osteoblasts. *Lasers in Medical Science* **18**, 78-82.

Kamal AM, Okiji T, Kawashima N, Suda H (1997) Defense responses of dentin/pulp complex to experimentally induced caries in rat molars: an immunohistochemical study on kinetics of pulpal Ia antigen-expressing cells and macrophages. *Journal of Endodontics* **23**, 115-20.

Koide T, Takahara Y, Asada S, Nagata K (2002) Xaa-Arg-Gly triplets in the collagen triple helix are dominant binding sites for the molecular chaperone HSP47. *Journal of Biological Chemistry* **277**, 6178-82.

Lamandé SR, Bateman JF (1999) Procollagen folding and assembly: the role of endoplasmic reticulum enzymes and molecular chaperones. *Seminars in Cell & Developmental Biology* **10**, 455-64.

Lee MW, Muramatsu T, Uekusa T, Lee JH, Shimono M (2008) Heat stress induces alkaline phosphatase activity and heat shock protein 25 expression in cultured pulp cells. *International Endodontic Journal* **41**, 158-62.

Lee YL, Liu J, Clarkson BH, Lin CP, Godovikova V, Ritchie HH (2006) Dentin-pulp complex responses to carious lesions. *Caries Research* **40**, 256-64.

Linde A (1989) Dentin matrix proteins: composition and possible functions in calcification.

Anatomical Record **224**, 154-66.

Masuda H, Fukumoto M, Hirayoshi K, Nagata K (1994) Coexpression of the collagen-binding stress protein HSP47 gene and the α 1(I) and α 1(III) collagen genes in carbon tetrachloride-induced rat liver fibrosis. *Journal of Clinical Investigation* **94**, 2481-8.

Melcer J, Chaumette MT, Melcer F (1987) Dental pulp exposed to the CO_2 laser beam. *Lasers in Surgery and Medicine* **7**, 347-52.

Mizuno M, Fujisawa R, Kuboki Y (2000) Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction. *Journal of Cellular Physiology* **184**, 207-13.

Mizuno M, Kuboki Y (2001) Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. *Journal of Biochemistry* **129**, 133-8.

Moritz A, Schoop U, Goharkhay K, Sperr W (1998a) The CO_2 laser as an aid in direct pulp capping. *Journal of Endodontics* **24**, 248-51.

Moritz A, Schoop U, Goharkhay K, Sperr W (1998b) Advantages of a pulsed CO_2 laser in direct pulp capping: a long-term in vivo study. *Lasers in Surgery and Medicine* **22**, 288-93.

Nagai N, Hosokawa M, Itohara S et al. (2000) Embryonic lethality of molecular chaperone hsp47 knockout mice is associated with defects in collagen biosynthesis. *Journal of Cell Biology* **150**, 1499-506.

Noda M, Wataha JC, Kaga M, Lockwood PE, Volkmann KR, Sano H (2002) Components of dentinal adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentrations. *Journal of Dental Research* **81**, 265-9.

Ohbayashi E, Matsushima K, Hosoya S, Abiko Y, Yamazaki M (1999) Stimulatory effect of laser irradiation on calcified nodule formation in human dental pulp fibroblasts. *Journal of Endodontics* **25**, 30-3.

Ozawa Y, Shimizu N, Kariya G, Abiko Y (1998) Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. *Bone* **22**, 347-54.

Parker S (2007) Low-level laser use in dentistry. *British Dental Journal* **202**, 131-8.

Pearson GJ, Schuckert KH (2003) The role of lasers in dentistry: present and future. *Dental Update* **30**, 70-4.

Posten W, Wrone DA, Dover JS, Arndt KA, Silapunt S, Alam M (2005) Low-level laser

therapy for wound healing: mechanism and efficacy. *Dermatologic Surgery* **31,** 334-40.

Razzaque MS, Hossain MA, Kohno S, Taguchi T (1998) Bleomycin-induced pulmonary fibrosis in rat is associated with increased expression of collagen-binding heat shock protein (HSP) 47 *Virchows Archiv* **432**, 455-60.

Scarano A, Manzon L, Di Giorgio R, Orsini G, Tripodi D, Piattelli A (2003) Direct capping with four different materials in humans: histological analysis of odontoblast activity. *Journal of Endodontics* **29**, 729-34.

Ueda Y, Shimizu N (2003) Effects of pulse frequency of low-level laser therapy (LLLT) on bone nodule formation in rat calvarial cells. *Journal of Clinical Laser Medicine and Surgery* **21**, 271-7.
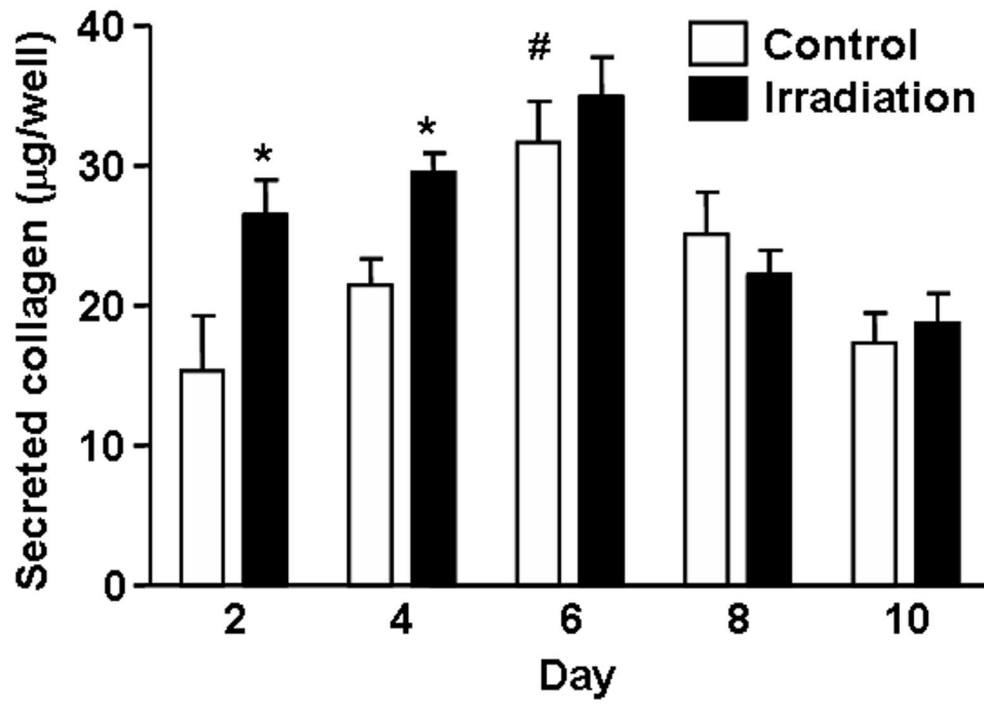
Yokose S, Kadokura H, Tajima Y et al. (2000) Establishment and characterization of a culture system for enzymatically released rat dental pulp cells. *Calcified Tissue International* **66**, 139-44.
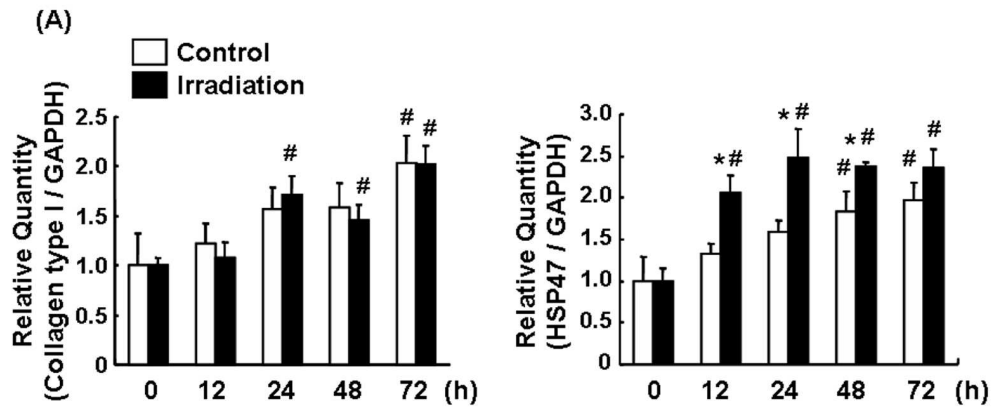
Fig. 1

(A)

(B)

Control

2 W, 40 s

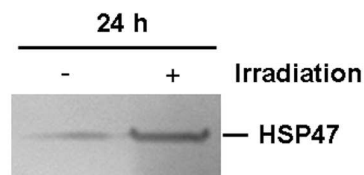
(C)
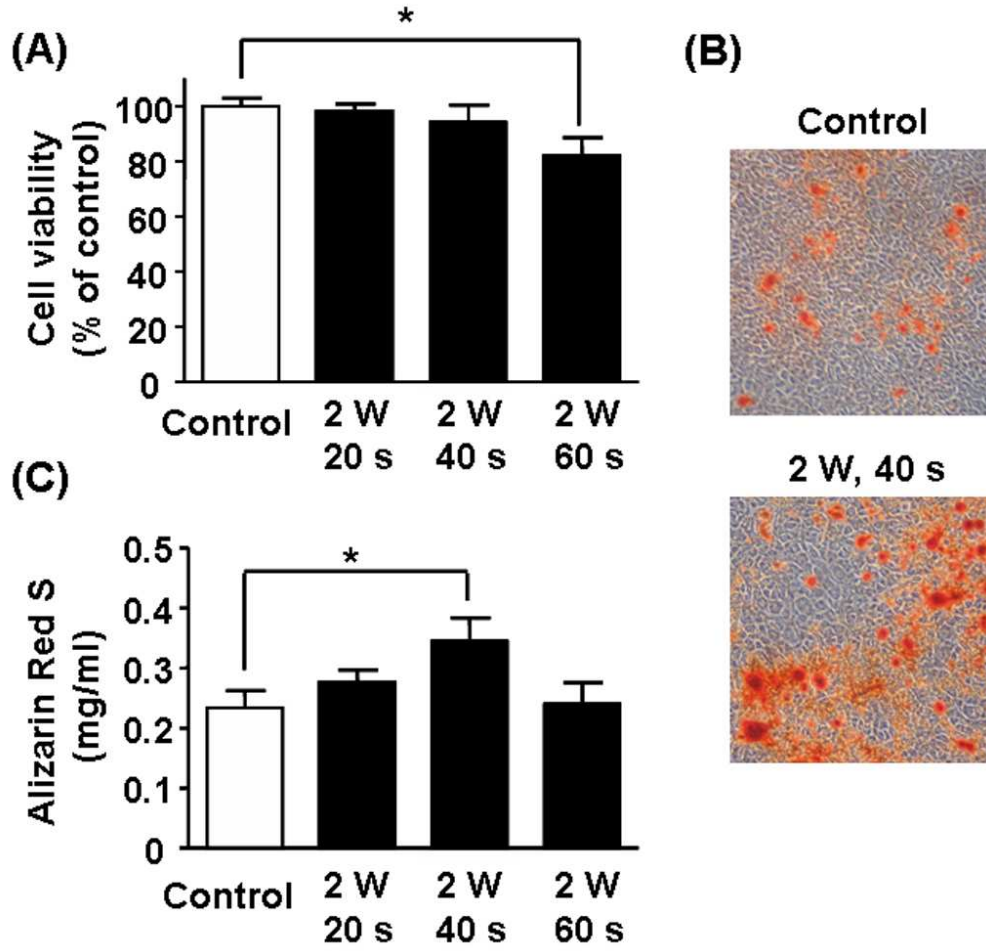
130x132mm (200 x 200 DPI)

Fig. 2

80x68mm (500 x 500 DPI)

Fig. 3

(A)

(B)

160x111mm (500 x 500 DPI)