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Pulp Responses After CO_2 Laser Irradiation of Rat Dentin

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Abstract

Objective: The purpose of this study was to investigate pulp responses after CO_2 laser irradiation of rat molars using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical methods. ***Background data:*** Many kinds of lasers have been used for the treatment of dentin hypersensitivity. However, the immunohistochemical details of the responses of dentin-pulp complex cells after CO_2 laser irradiation through the dentin are still not clear. ***Methods:*** Adult male Sprague–Dawley rats were used in this study. A CO_2 laser (wavelength of 10.6 μm, 2 W in the super pulse mode, pulse 0.6 ms) and total laser energy of 4 J (density, 203.84 J/cm^2) was used. The temperature change in the pulp was measured using a super fine sheath thermocouple. ***Results:*** The temperature at the pulp side after the CO_2 laser irradiation increased 22.5°C. The expression of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1-α mRNAs was significantly higher at 6, 12, and 24 h after laser irradiation than in the control group ($p < 0.05$). Histologically, a slight degeneration of the pulp tissue was observed immediately after the laser irradiation. Immunohistochemically, heat shock protein (HSP)-70-positive cells in the pulp horn were observed immediately after irradiation and cells positive for vascular endothelial growth factor (VEGF) in the subodontoblast layer were observed after 5 days. There were no nestin-positive cells immediately, but after 5 days nestin-positive cells were recognized in the deeper pulp cells. Immediately after irradiation, neurofilament protein (NFP)-positive nerve fibers were observed but they disappeared in the pulp horn after 5 days. ***Conclusions:*** These results suggest that 203.84 J/cm^2 CO_2 laser irradiation of pulp tissue through the dentin, such as is used in the clinic, induces inflammatory and pathological cytokine pathways to repair the damaged pulp tissue.

Introduction

Dentin hypersensitivity is characterized by brief but sharp pain arising from the exposed dentin in response to various types of stimuli, typically thermal, evaporative, tactile, osmotic, and/or chemical, which cannot be ascribed to any other form of dental defect or pathology.[1] The most common factors responsible for dentin hypersensitivity are attrition, abrasion, fraction, erosion, anatomic predisposition, and cavities, as well as improperly controlled dentinal acid conditioning.[2] Any treatment that reduces the dentinal permeability must diminish the dentinal sensitivity. The closing of dentinal tubules leads to the reduction of dentinal permeability and, proportionally, also decreases the degree of dentinal sensitivity.[3] The effectiveness of dentin desensitization agents is directly related to their capacity to promote the sealing of dentinal canaliculi, and they have been used in clinical settings.[4] Recently, many kinds of lasers have also been used to close dentinal canaliculi. However, several laser systems, such as argon (Ar), CO_2, helium-neon (He-Ne), neodymium: yttrium-aluminum-garnet (Nd:YAG), and erbium (Er):YAG, cause different tissue reactions, according to the active substance, wavelength, power density, and the optical properties of the target tissue.[5] The enormous and unregulated generation of heat during laser irradiation can result in severe damage causing necrosis of the dental pulp. The reasons for this include inadequate knowledge of the physical properties of the lasers and the complex interactions of the cytokine networks of dental pulp cells with the laser energy. It is known that the wavelength of the CO_2 laser is 10.6 μm; it is almost completely absorbed in the superficial layers of tissues, is extremely short, and its effects on dentin hypersensitivity are caused by the closing or narrowing of dentinal tubules.[6] However, the immunohistochemical details and the expression patterns of inflammatory cytokines that influence dentin-pulp complex cells after the CO_2 laser irradiation through the dentin are still not clear. The purpose of this study was to investigate the expression of inflammatory-related mRNAs

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that are involved in the immune and pathological cytokine pathways of inflammatory responses in the pulp tissue, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1-α, after CO_2 laser irradiation of rat molar pulp tissue through the dentin. Further, we characterized the expression of a repair-related protein, heat shock protein-70 (HSP-70), whose expression is increased when cells are exposed to elevated temperatures or other stress, vascular endothelial growth factor (VEGF), whose function is essential to endothelial cell proliferation and migration, and nestin, which is an intermediate filament protein most related to neurofilaments of stem cells. Neurofilament protein (NFP) was also investigated to clarify the regeneration of nerve fibers in rat dental pulp after irradiation with a CO_2 laser.

Materials and Methods

Animals

All animal studies were conducted in compliance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College (Approval Number 223206). Sixty-five male Sprague–Dawley rats weighing ~200 g each were used in this study. Animals were distributed into three groups: a control group without any CO_2 laser irradiation, a group in which the animals were killed immediately after the CO_2 irradiation, and a group in which the animals were killed 5 days after the CO_2 laser irradiation.

The animals were fed a standard laboratory diet during the experimental time periods. The animals were anesthetized with sodium thiopental (Ravonal®; Tanabe, Japan).

Laser irradiation protocol

A CO_2 laser (Panalas CO5 Sigma; Panasonic Dental Co., Osaka, Japan) was used to irradiate the occlusal surface of the upper first molar dentin where there was no covering enamel due to because of the natural physical attrition phenomenon in rats. A Type 1A tip (defocus, diameter; 0.15 cm) was used in direct contact with the tooth surface and the exposure time was 8.8 sec. The parameters for the CO_2 laser were: wavelength of 10.6 μm, 2 W in the super pulse mode, pulse 0.6 ms, and total laser energy of 4 J (density; 203.84 J/cm^2). A peak power of 5 W was used in this study. An air flow system was provided at the tip of the hand piece. These laser conditions were based on clinical irradiation standards[7,8] and on preliminary experiments that established a slightly strong condition in which there were differences between the experimental group and the control group.

Temperature measurement after the CO_2 laser irradiation

After euthanasia of the rats, five upper first molars were extracted to measure the temperature changes in the pulp after CO_2 laser irradiation. A thermocouple thermometer 0.1 mm diameter was used in this study for the measurement of temperature changes in the pulp. Teeth were sliced transversely at the enamel cementum junction, and pulp was removed mechanically from the crown portion of the teeth. This 0.1 mm thick thermocouple thermometer was too big for temperature measurement to insert directly into the pulp chamber. The thickness of the dentin on the five teeth was ~0.8 mm on the top to the pulpal side. A super-fine mineral insulated thermocouple, type K (Okazaki Manufacturing Co., Kobe, Japan), 0.10 mm in outside diameter of the sheath, was in direct contact without any intermediate material on the pulp horn, and the CO_2 laser was used exactly as described previously. The thermo electromotive force change was recorded using a thermocouple thermometer SR6513 (Graphitic Corp., Yokohama, Japan). The recorded value was converted to temperature (°C) using a conversion chart (mV to °C = X(mV) × 25).

Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

For quantitative RT-PCR, five animals were sacrificed killed with an overdose of sodium thiopental at 6, 12, and 24 h after CO_2 laser irradiation. The upper first molar was extracted and washed in alpha-minimal essential medium (α-MEM, GIBCO, Carlsbad, CA) containing 10% gentamycin and 1.2% fungizone for 5 min. The pulp was then removed mechanically. Total RNA was extracted from each specimen using the acid guanidium thiocyanate/phenol-chloroform method as follows. The cells were homogenized in Trisol® Reagent (Invitrogen, Carlsbad, CA) after rinsing each sample with phosphate-buffered saline (PBS). Each solution was transferred to a tube containing chloroform and was mixed. The solutions were centrifuged at 14,000 rpm at 4°C for 20 min, after which they were incubated in 70% isopropanol at −80°C for 1 h. After centrifugation, the mRNA pellets were washed with 70% cold ethanol, and were then dissolved in RNAase-free (diethylpyrocarbonate [DEPC]-treated) water. Total RNAs were reverse transcribed and amplified using an RT-PCR kit (Takara Biomedicals, Shiga, Japan). RT-PCR products were analyzed by quantitative real-time RT-PCR using TaqMan® Gene Expression Assays for three target genes, TNF-α (Rn 99999017-ml) and IL-1-α (Rn00566700-ml), to confirm the appearance at the mRNA level of inflammatory cytokines, and β-actin (4352340E) as an endogenous control (Applied Biosystems, Foster City, CA), to determine variations in the amount of each RNA. All PCR reactions were performed using the real time PCR 7500 Fast System (Applied Biosystems). Gene expression quantitation using TaqMan® Gene Expression Assays was performed as the second step in a two-step RT-PCR. Assays were performed in singleplex reactions containing TaqMan® Fast Universal PCR Master Mix, TaqMan® Gene Expression Assays, distilled water and cDNA according to the manufacturer's instructions (Applied Biosystems). Reaction conditions consisted of primary denaturation at 95°C for 20 sec, and cycling for 40 cycles at 95°C for 3 sec and at 62°C for 30 sec. The experiments were conducted in triplicate, and data were analyzed using one-way ANOVA ($p < 0.05$) and using Scheffé's test for multiple comparison.

Morphological observations

For morphological observations, the animals were killed immediately and at 5 days after the CO_2 laser irradiation with an overdose of sodium thiopental. The upper molar with the maxillary bone of each rat was removed and fixed in 10% buffered formalin and dehydrated and then was embedded in paraffin. Paraffin sections were cut and observed with hematoxylin and eosin (HE) staining.

Immunohistochemical observations

For immunohistochemistry, the streptavidin-biotin immunoperoxidase method was employed using a Histofine SAB-PO (MULTI) kit (Nichirei Co., Ltd., Tokyo, Japan). Paraffin sections were deparaffinized with xylene, then were washed with 100% alcohol, and then were washed with distilled water. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 in methanol for 30 min. They were then microwaved for 30 min at 65°C in 0.01 M citrate buffer (pH 6.0), cooled to room temperature and then washed in PBS three times for 5 min each. To prevent nonspecific reactions, sections were incubated with 10% serum for 10 min. The HSP-70 antibody, which was used to confirm the expression of HSP-70 heat shock protein (at a dilution of 1:200, Abcam, Nihonbashi, Tokyo, Japan), the VEGF antibody, which was used to confirm the change of tissue restoration (at a dilution of 1:200, Abcam), the nestin antibody, which was used to confirm the appearance of odontoblast function (at a dilution of 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the NFP antibody, which was used to confirm the existence of neural fibers (at a dilution of 1:200, DAKO, Glostrup, Denmark) were used as primary antibodies. They were reacted at 4°C overnight. As a negative control, PBS was used instead of the primary antibody. After the primary antibody reaction, the sections were rinsed in PBS three times for 5 min each. The secondary antibody (biotinylated anti-mouse immunoglobulin G [IgG] or anti-rabbit IgG) was reacted at room temperature for 30 min. After washing in PBS three times for 5 min each, 3,3′-diaminobenzidine-tetra-hydrochloride in Tris-HCl buffer (pH 7.6) was used to visualize the reaction. Finally, sections were counter-stained with Mayer's hematoxylin. Specimens were examined using a light microscope (BX41, Olympus, Shinjuku, Japan) and were photographed.

Results

Temperature change caused by the CO_2 laser irradiation

The temperature change at the pulp side caused by the CO_2 laser irradiation increased ~0.9 mV, which was ~22.5°C as an average (Fig. 1). The temperature increases for each of the five samples were 20°C, 22.5°C , 25°C , 22.5°C, and 22.5°C.

Quantitative RT-PCR analysis

The expression of TNF-α and IL-1-α mRNAs was significantly higher at 6, 12, and 24 h after the CO_2 laser irradiation compared with the control group ($p < 0.05$), as shown in Figs. 2 and 3. The expression of those mRNAs increased in a time-dependent manner and was highest 24 h after the CO_2 laser irradiation. However, there were no significant differences in the expression of TNF-α or IL-1-α mRNA at 48 h after the irradiation.

Morphological observations

Histologically, small vacuoles were observed in the cells immediately after the CO_2 laser irradiation (Fig. 4a). In another specimen, a slight hyaline degeneration in the pulp horn was observed underneath the odontoblast layer (Fig. 4b).

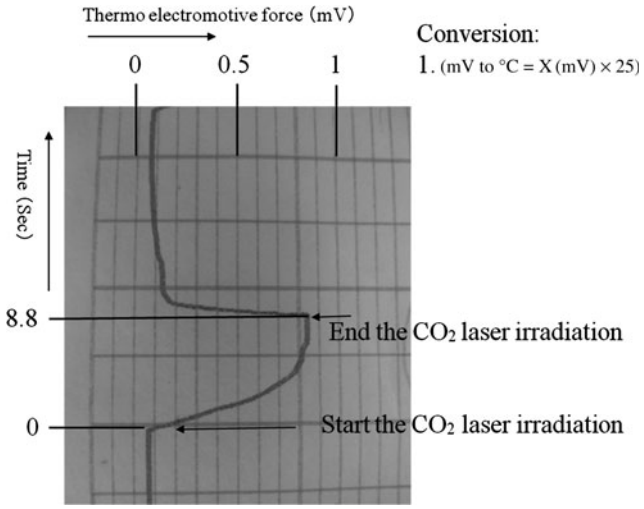

FIG. 1. Measurement record of the thermocouple thermometer The electromotive force rises upon exposure to the irradiating CO_2 laser and returns to the initial value when the irradiation stops. Recorded values were converted to temperature (°C) using the conversion chart (mV to°C = X(mV) × 25).

Immunohistochemical observations

Positive reactions for HSP-70 were observed both in odontoblasts and in pulp cells just beneath the odontoblast layer immediately after the CO_2 laser irradiation (Fig. 5b). Five days after the irradiation, HSP-70-positive reactions became weaker (Fig. 5c), but were still stronger than the control (Fig. 5a).

Positive reactions for VEGF were recognized in dental pulp cells just beneath the odontoblast layer and in odontoblasts and pulp cells just beneath the odontoblast layer

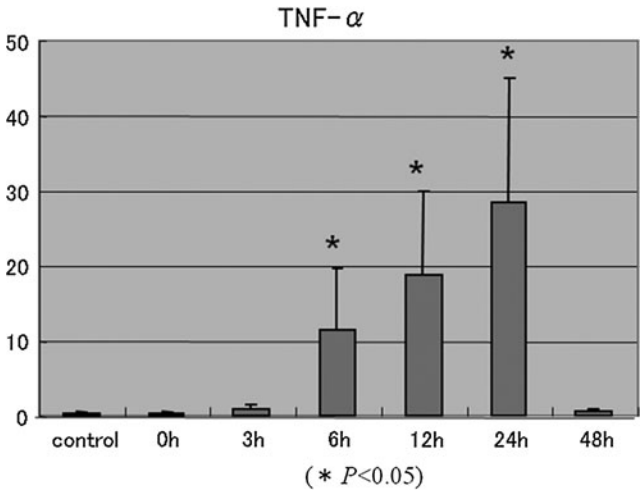

FIG. 2. Expression of tumor necrosis factor-alpha (TNF-α) mRNA after CO_2 laser irradiation. The expression of TNF-α mRNA was significantly higher at 6, 12, and 24 h after the CO_2 laser irradiation compared with the control group ($p < 0.05$). The expression of TNF-α mRNA increased in a time-dependent manner and was highest 24 h after irradiation. However, there are no significant differences in the expression of TNF-α mRNA at 48 h after irradiation.

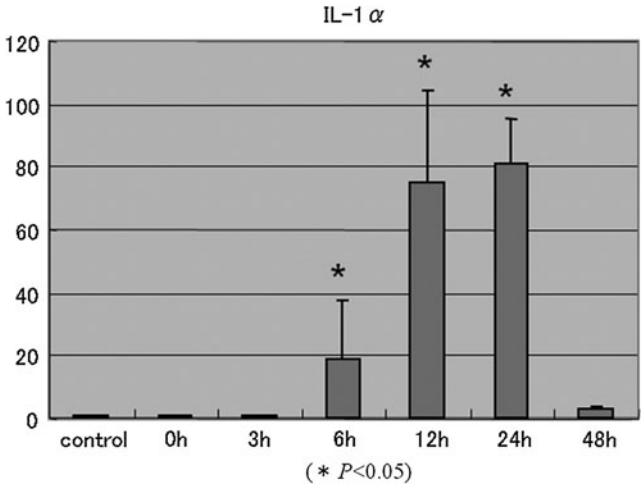

FIG. 3. Expression of interleukin (IL)-1-α mRNA after CO_2 laser irradiation. The expression of IL-1-α mRNA was significantly higher at 6, 12, and 24 h after the CO_2 laser irradiation compared with the control group ($p < 0.05$). The expression of IL-1-α mRNA increased in a time-dependent manner and was highest 24 h after irradiation. However, there are no significant differences in the expression of IL-1 mRNA at 48 h after irradiation.

immediately after the irradiation (Fig. 5e). The positive reaction for VEGF became weaker at 5 days (Fig. 5f).

Positive reactions for nestin were observed in pulp cells just beneath the odontoblast layer, in odontoblasts, and in the dentinal canaliculi immediately after the laser irradiation (Fig. 5h) and in the control pulp (Fig. 5g). At 5 days after irradiation, cells positive for nestin were not observed in the odontoblast layer, but were observed in deeper areas of the dental pulp (Fig. 5i).

Positive reactions for NFP were observed in the pulp horn area immediately after the laser irradiation (Fig. 5k) and in the control pulp (Fig. 5j), and were observed in the deeper pulp cells 5 days after the laser irradiation (Fig. 5l).

Discussion

In our morphological observations, attrition was observed on the occlusal surface of rat molar teeth where the dentin was exposed without covering enamel. As has been reported previously, commercial food pellets for laboratory animals are abrasive and increase tooth wear in rats compared with a powdered diet,[9] and the attrition is not only caused by the abrasive hard food but because the animals also spend more time chewing it.[10,11] That is why we consider that the molar teeth of rats are suitable experimental models for dentin hypersensitivity caused by natural physical attrition.

Many fundamental studies about the treatment of dentinal hypersensitivity with various types of laser irradiation, such as the Nd: or Er:YAG laser, the diode laser (8.5 J/cm^2 for 60 sec)[12] and the CO_2 laser (irradiation condition: energy: 0.3 J, 0.5 J, 0.9 J, 1.5 J, 2.5 J; application: one exposure/mm, five times within 5 mm of gingiva[13,14]) has been reported to have extremely good clinical success. Zhang et al.[15] reported that after CO_2 laser treatment of dentinal hypersensitivity, all patients were immediately free from sensitive pain. In another study, all patients showed absolutely identical perfusion indices immediately before and after the CO_2 laser treatment as well as 1 week after treatment.[16] Furthermore, the CO_2 laser treatment reduced dentinal hypersensitivity to an air stimulus, and all teeth remained vital with no adverse effects at 3 months after the irradiation.[15]

However, the thermal effects of laser irradiation on oral tissues have been of concern.[17–19] Irradiation that causes rises in temperature exceeding the threshold of pulpal tolerance will cause thermal injury to the dental pulp. Previous studies have demonstrated that healthy pulp tissue is not injured thermally if the laser equipment is used within appropriate parameters so that any temperature rise within the dental pulp remains $< 5°C$.[20] Using a CO_2 laser, no damage was

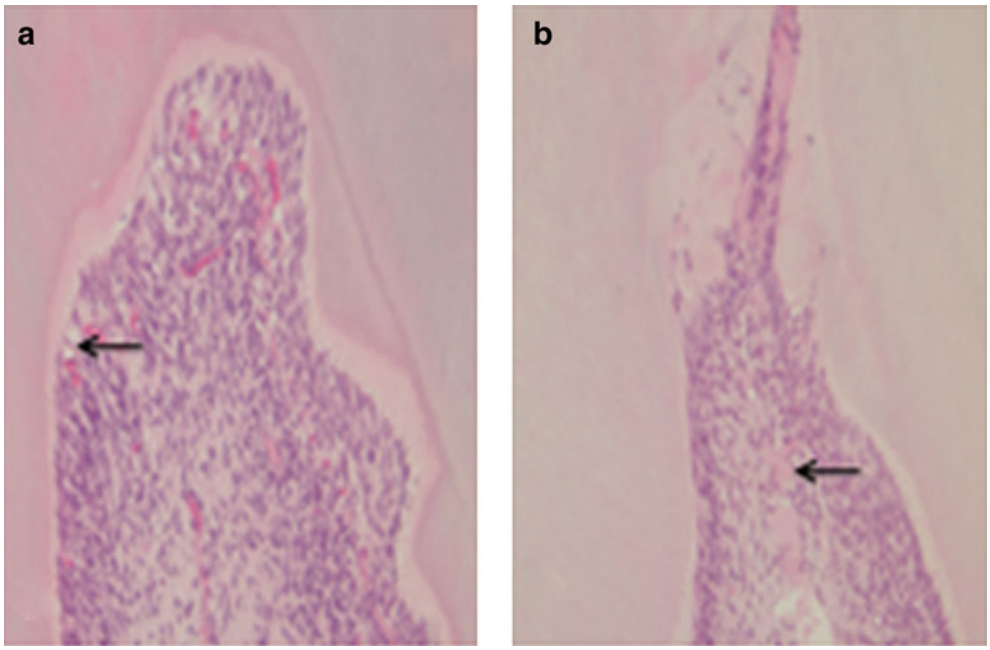

FIG. 4. Hematoxylin-eosin staining. **(a)** Immediately after the CO_2 laser irradiation, small vacuoles were observed in the cells. **(b)** In another specimen, slight hyaline degeneration in the pulp horn was observed underneath the odontoblast layer.

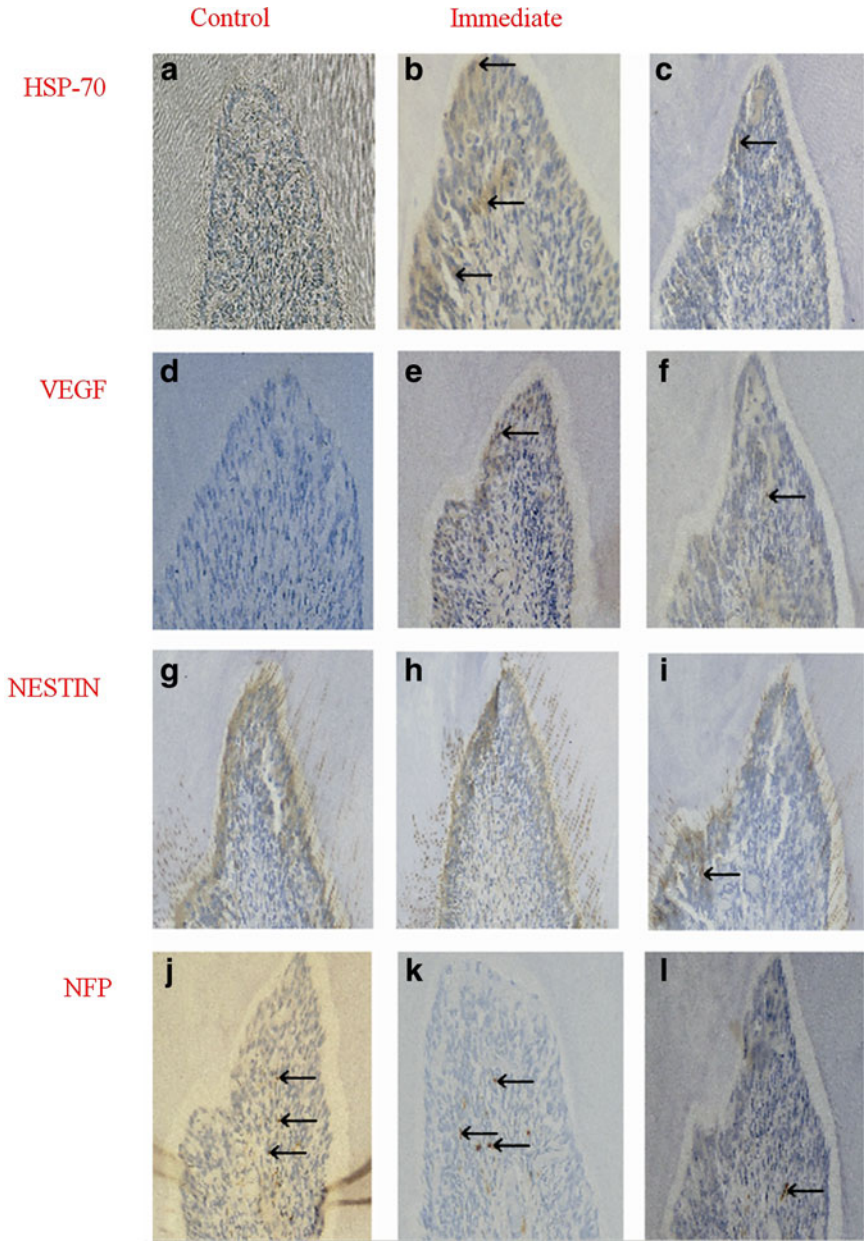

FIG. 5. Immunohistochemical staining. **(a)** Positive reactions for HSP-70 in the control group HSP-70 were not found either in odontoblasts or in pulp cells. **(b)** Positive reaction for heat shock protein (HSP)-70 was observed in odontoblasts and in pulp cells just beneath the odontoblast layer immediately after irradiation (arrows). **(c)** Five days after irradiation, HSP-70-positive reactions became weaker but were still stronger than the control (arrow). **(d)** Positive reactions for VEGF in the control group were not recognized in dental pulp cells. **(e)** Positive reactions for vascular endothelial growth factor (VEGF) were recognized in dental pulp cells just beneath the odontoblast layer and in odontoblasts and pulp cells just beneath the odontoblast layer immediately after the irradiation (arrow). **(f)** The positive reaction for VEGF became weaker at 5 days (arrow). **(g, h)** Positive reactions for nestin were observed in pulp cells just beneath the odontoblast layer, in odontoblasts and in the dentinal canaliculi immediately after the laser irradiation. **(i)** At 5 days after irradiation, nestin-positive cells were not observed in the odontoblast layer, but were observed in the deeper area of the dental pulp (arrow). **(j, k)** Positive reactions for neurofilament protein (NFP) were observed in the pulp horn area immediately after the laser irradiation (arrow, k) and in the control pulp (arrow, j). **(l)** NFP-positive reactions were only observed in the deeper pulp cells at 5 days after the CO_2 laser irradiation (arrow).

reported after pulpal exposure to 3 W of power for 2 sec in the continuous wave (CW) mode in monkeys and dogs. In our studies using a CO_2 laser, 2 W of power for 8.8 sec induced a slight pulpal degeneration, and this power of the CO_2 laser increased the temperature $\sim$22.5°C, causing pulpal degeneration. This is because in our study the dentine thickness of the rat molar teeth was less than the average depth of the cavities used for the pulpal wall radiations in the monkey and dog study.

HSP-70 has been shown to play a significant role in rescuing stressed cells by helping damaged proteins refold or by participating in the synthesis of new proteins to replace damaged proteins.[21,22] Amemiya et al.[23] reported that HSP-70 is expressed in dental pulp cells under the stress condition of hypoxia. Our immunohistochemical investigations showed that HSP-70-positive cells were observed strongly in pulp cells compared with the control immediately after irradiation with the CO_2 laser. This suggests that the increased temperature stresses the pulp cells causing slight morphological damage immediately after the laser irradiation within 5 days. This suggests that the remaining cells in the pulp tissue may react, and the expression of TNF-α and IL-1-α mRNAs may increases.

In this study, a weakly positive reaction for VEGF was detected at the depth of dental pulp cells at 5 days after the CO_2 laser irradiation. VEGF is a signal protein produced by cells which stimulates the growth of new blood vessels, and is part of the system that restores the oxygen supply to tissues when the blood circulation is inadequate. VEGF function is essential to endothelial cell proliferation and migration. This phenomenon may represent the start of tissue repair after the CO_2 laser irradiation, at least up to day 5.

The expression of nestin at 5 days after the CO_2 laser irradiation decreased compared with the control in this study. Nestin is an intermediate filament protein most related to neurofilaments of stem cells. In injured teeth, nestin

expression is upregulated in a selective manner in odontoblasts surrounding the injury site, showing a link between tissue repair and competence. Nestin is also distributed in the processes of mature odontoblasts and takes the place of degenerated odontoblasts in the case of pulpal damage.[24] In our study, the expression of nestin was observed in deeper pulpal cells, but only weakly in odontoblasts at 5 days after the CO_2 laser irradiation. This suggests that pulpal stem cells in deeper areas probably begin migrating toward the degenerated pulp horn area.

It is known that neurofilament protein NFP is a special protein required for nerve fibers. In this study, the expression of NFP was seen in the irradiated pulp compared with the control 5 days after the CO_2 laser irradiation. This must be is another reason for the reduction of dentinal sensitivity in hypersensitive patients. In conclusion, these results suggest that 203.84 J/cm^2 of CO_2 laser irradiation, such as is used in the clinic, to the pulp tissue through the dentin, such as is used in the clinic, induces the inflammatory and pathological cytokine pathways to repair the damaged rat pulp tissue.

Conclusions

These results suggest that 203.84 J/cm^2 of CO_2 laser irradiation to the pulp tissue through the dentin, such as is used in the clinic, induces the inflammatory and pathological cytokine pathways to repair the damaged rat pulp tissue.

Author Disclosure Statement

No competing financial interests exist.

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