

Effect of CO₂ Laser Irradiation on Hormesis Induction in Human Pulp and Periodontal Ligament Fibroblasts

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Abstract. *Background:* We have recently reported that a low level of CO₂ laser irradiation induced growth stimulation (hormesis) of both human gingival fibroblast (HGF) and oral squamous cell carcinoma cell line (HSC-2), but the extent of hormetic response was much smaller than that previously reported for toxicants and radiation in other experimental systems. Here we investigated the extent of hormetic response induced by CO₂ laser irradiation in human pulp cells (HPCs) and periodontal ligament fibroblast (HPLF). *Materials and Methods:* HPC and HPLF cells were established from the periodontal tissues of the first premolar extracted tooth. Cells were cultured for 24, 48 or 72 hours after exposure to various irradiation powers, and the viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. *Results:* CO₂ laser irradiation induced biphasic effects on the growth of both HPC and HPLF cells. The maximum hormetic response was less than 50%. The hormetic response was found within the energy density of 7.98-79.77 J/cm², and cytotoxicity emerged at powers over 132.96 J/cm². Combining with our previous report, HPCs showed the highest hormetic response, followed by HPLFs and then HGFs. Both HPLFs and HGFs showed similar time-course of hormesis response, increasing response with incubation time. *Conclusion:* The hormetic response may be the common survival mechanism by which cells escape from radiation-induced injury. Higher hormetic response of HPCs may reflect their potential for differentiation into one of the components in dentin.

Different laser modalities have been used in the treatment of various disorders (1-4). However, as compared with Er:YAG laser therapy, there is insufficient evidence of safety and effects to support the clinical application of CO₂, Nd:YAG, or diode laser (5, 6). Many toxicants, chemotherapeutic agents, hormones, metals and radiations have been reported to show bi-phasic growth-modulating effects: growth stimulation (hormesis) at lower concentrations and growth inhibition at higher doses (7, 8). Similarly, high level laser treatment induced cell membrane and DNA damage (9), whereas low level laser treatment induced growth stimulation and wound healing (10-12). It has been reported that hormesis may be detectable only under optimal condition (in terms of the dose and the treatment time) (7). On the other hand, we found that sodium fluoride (13), gefitinib (14), 2-aminotropone derivatives (15) and herbal extracts (16) induced little or no hormetic effects on cultured human oral normal cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] and human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4). These conflicting results suggest that the extent of hormesis induction may depend on the type of cell or the type of irradiation conditions. Most previous *in vitro* studies have been carried out with laser modalities other than CO₂ laser. Based on this background, we initiated an investigation of the effect of CO₂ laser irradiation on oral cells. We recently found that both the range and magnitude of hormetic response of human oral normal (HGF) and tumor (HSC-2) cells was very narrow and small (17). To explore more efficient application of CO₂ laser irradiation in dentistry, here we investigated the extent of hormetic response induced by CO₂ laser irradiation in HPC and HPLF cells.

Materials and Methods

Cell culture. HPC and HPLF cells were established from an extracted first premolar tooth in the mandible and periodontal tissues of a twelve-year-old girl, according to the guideline of the Intramural Board of Ethics Committee (no. A0808) (18). HPCs and HPLFs had an *in vitro* life-span of 43 and 43 cumulative cell population doubling

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Key Words: CO₂ laser irradiation, hormesis, cytotoxicity, pulp cell, periodontal ligament fibroblast.

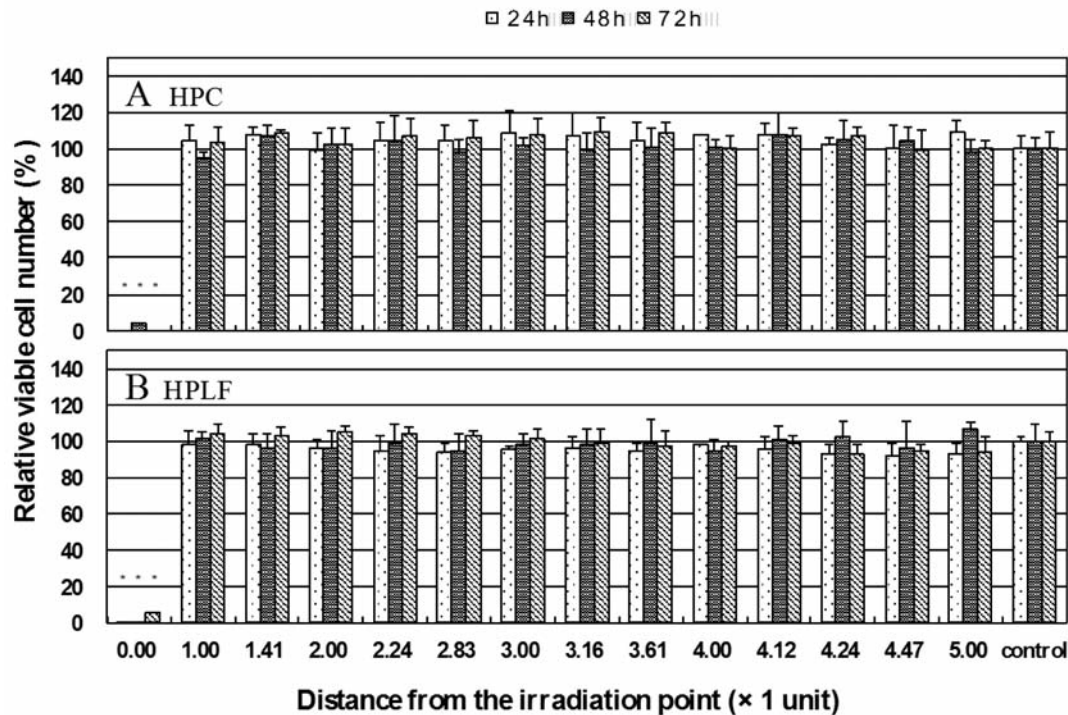


Figure 1. Ripple effect of laser irradiation on the viability of cells grown on the adjacent wells. HPC (A) and HPLF (B) cells were inoculated on a 96-microwell plate, and incubated for 48 hours to achieve complete attachment. A tip of CO₂ laser (Opelaser PRO LA12) was set just 52 mm above the cell surface, and the cells were exposed to CO₂ laser irradiation (5 W with dispatch mode CW, 30 s) without removing the medium (100 µl). After medium change, the cells were further incubated for 24 (left: dotted bars), 48 (center: gray bars) or 72 (right: slashed bars) hours, and the number of viable cells (expressed as percentage of un-irradiated control cells) was then determined by MTT method. The distance between one well to the adjacent well was defined as '1 unit'. Each value represents the mean±S.D. of 12 samples. **p*<0.01.

level (PDL) (18). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), under a humidified 5% CO₂ atmosphere. The cells at 10-15 PDL were used in this experiment.

Assay for cytotoxicity of laser irradiation. Cells (1.5×10^3) were inoculated on 96-microwell plate (37.6 mm²) (Becton Dickinson, Franklin Lakes, NJ, USA), and incubated for 48 hours to allow complete attachment. The medium was changed for new medium, and the cells were placed at 52 mm distance from the CO₂ laser source (Opelaser PRO LA12; Yoshida, Tokyo, Japan) (17). All the cells were evenly irradiated with a specially manufactured tip, under the following conditions: irradiation power (0.5, 1, 2, 3 or 5 W), irradiation time (0.5, 1, 1.5, 3, 5, 10, 15, 20, 30 s) and dispatch mode (CW, SP1, SP2). After incubation for 24, 48 or 72 hours in fresh culture medium, the viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. In brief, cells were incubated for a further 4 hours with 0.2 mg/ml MTT reagent in DMEM/10% FBS at 37°C, and dissolved with 0.1 ml of dimethyl sulfoxide (DMSO) to determine the absorbance at 540 nm with a plate reader (17).

Statistical analysis. The mean values and standard deviations were calculated. The average values were compared by one-way ANOVA and Student's *t*-test.

Results

Ripple effect of CO₂ laser irradiation. When either HPC and HPLF cells were exposed to laser irradiation (5 W, with a dispatch mode of CW, 30 s) from 52 mm distance, almost all of the cells under the irradiation point died after incubation for 24, 48 and 72 hours in regular culture medium (Figure 1). On the other hand, the cells that had been inoculated at the wells adjacent to those exposed to laser irradiation all appeared to survive, judging from cell growth comparable with that of control (unirradiated cells) (Figure 1). This finding made it possible for us to perform sequential CO₂ laser irradiation without damaging the viability of the cells grown on the adjacent wells.

Effect on HPC cells. We investigated the effect of irradiation time and power on the viability of HPC cells (Figure 2). Lower power irradiation (0.5-1 W) for up to 30 s did not significantly affect the viability of HPC cells, regardless of dispatch mode and incubation time. Higher power irradiation [2 W (20-30 s); 3 W (15-30 s); 5 W (10-30 s)] reduced the viable cell number by more than 60%. The range of

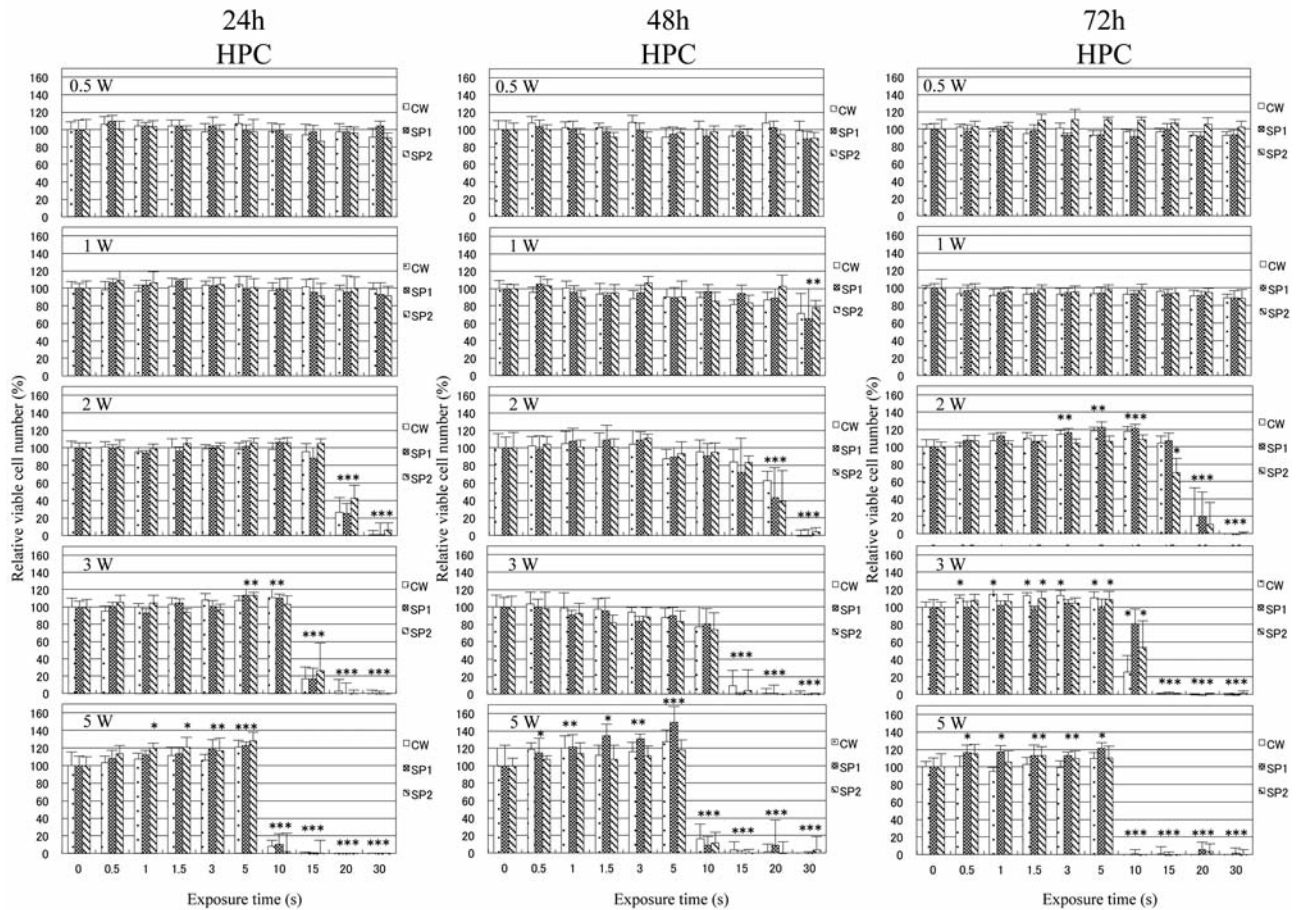


Figure 2. HPC cells were irradiated for 0 (control), 0.5, 1, 1.5, 3, 5, 10, 15, 20 or 30 sec at 0.5, 1, 2, 3 or 5 W with dispatch mode (CW, SP1, SP2), and then cultured for a further 48 hours in DMEM/10% FBS to determine the viable cell number. Each value represents the mean \pm S.D. of 12 samples. * $p < 0.01$.

cytotoxicity induced by higher power irradiation is indicated by gray shading in Table I. Cytotoxicity of CO₂ laser irradiation reached nearly a maximum level within 24 hours' incubation in culture medium (Figure 2), indicating the cytotoxicity induction by CO₂ laser irradiation in HPC cells reached a plateau level within 24 hours.

Slight, but significant ($p < 0.01$) growth stimulation was observed after incubation for 24–72 hours, using dispatch model of CW [2 W (72 hours), 3 W (24 and 72 h), 5 W (24 and 48 h)], SP1 [2 W (72 h), 3 W (24 and 72 h), 5 W (24, 48 and 72 h)] and SP2 [2 W (72 h), 3 W (24 and 72 h), 5 W (24, 48 and 72 h)] (Figure 2). The maximum hormetic response was 50.00%. The mean of the maximum hormetic response achieved with the use of either CW, SP1 or SP2 dispatch mode was 22.30, 31.6 and 20.38%, respectively, yielding the mean value of 24.79% (Table II).

Effect on HPLF cells. We next investigated the effect of irradiation time and power on the viability of HPLF cells

(Figure 3). Lower irradiation power (0.5–1 W) for up to 30 s was not cytotoxic to HPLF cells, regardless of dispatch mode and incubation time. Higher power irradiation [2 W (20–30 s); 3 W (15–30 s); 5 W (10–30 s)] reduced the viable cell number by more than 60%. The range of cytotoxicity induced by higher power irradiation is indicated by gray shading in Table I. Cytotoxic effect of laser irradiation was slightly enhanced during prolonged incubation time (24, 48 or 72 h), indicating that the process of the cytotoxicity induction by CO₂ laser irradiation in HPLF cells was slightly slower than that observed for HPC cells (Figure 3).

Little or no hormetic effect was found at the lower irradiation dose range (0.5–5 W). The cell number was slightly, but significantly ($p < 0.01$) increased, using dispatch mode of CW [2–3 W (48 and 72 h)], SP1 [3–5 W (48 and 72 hours)] and SP2 [2–5 W (48 and 72 h)] (Figure 3). The maximum hormetic response was 24.70%. The mean of the maximum hormetic response achieved with the use of either

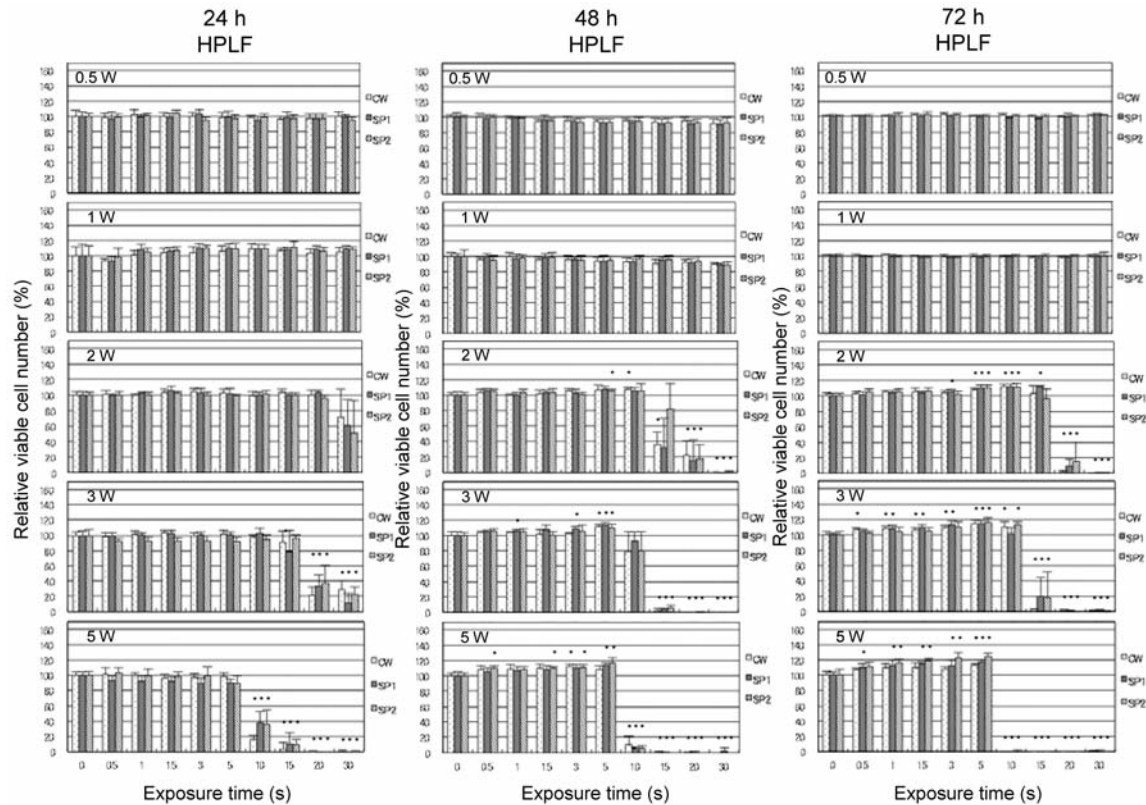


Figure 3. HPLF cells were irradiated for 0 (control), 0.5, 1, 1.5, 3, 5, 10, 15, 20 or 30 sec at 0.5, 1, 2, 3 or 5 W with dispatch mode (CW, SP1, SP2), and then cultured for a further 48 hours in DMEM/10% FBS to determine the viable cell number. Each value represents the mean \pm S.D. of 12 samples. * $p < 0.01$.

CW, SP1 or SP2 dispatch mode was 11.75, 13.61 and 17.68%, respectively, yielding the mean value of 14.35% (Table II).

Discussion

The present study demonstrated that CO₂ laser irradiation induced a very low level of hormesis (maximum: 24.79 and 14.35, respectively) in both HPC and HPLF cells (Table II). The hormetic response was found within the delivered energy density of 7.98-79.77 J/cm², and significant cytotoxicity emerged at deliveries over 132.96 J/cm² (Table I). This is consistent with our previous finding that the hormetic response induced by CO₂ laser irradiation in HGF cells was observed within a narrow range of irradiation doses (15.95-79.77 J/cm²), slightly lower than that of cytotoxic doses (106.37-398.87 J/cm²) (17). This suggests that the hormetic response may be the common survival mechanism by which cells escape from radiation-induced injury.

The present study demonstrated for the first time that HPCs showed the highest hormetic response among oral cells that we have investigated here and in our previous paper

(17). Combined with our previous data (17), we found that the strength of the hormetic response observed during 24-72 hours' incubation was in the order of HPCs (24.79%) > HPLFs (14.35%) > HSC-2 (10.86%) > HGFs (8.76%). The higher hormetic response of HPC cells may reflect their potential for differentiation into dentin. Among three dispatch modes, SP1 was more effective for hormesis induction in HPC cells (31.69%), as compared with CW (22.30%) and SP2 (20.38%) (Table II).

Both fibroblast lines (HPLF and HGF) showed a similar time-course of hormesis response, with response increasing with the incubation time [10.23% (24 h) \rightarrow 14.19% (48 h) \rightarrow 18.61% (72 h) in HPLFs; 5.70% (24 h) \rightarrow 9.78% (48 h) \rightarrow 10.81% (72 h) in HGFs]. Among three dispatch modes, SP2 was more effective in inducing the hormesis in both HPLF and HGF cells (17.68 and 10.71%) than SP1 (13.61 and 6.62%) and CW (11.75 and 8.95%, respectively).

It has been reported that low level laser treatment stimulated the growth of HGFs (19-23), HPCs (24) and HPLFs (25). Many possible mechanisms for this growth stimulation have been proposed: the induction of heat shock-proteins (10), production of collagen (26) and prostaglandin E₂ via cyclooxy-

Table I. Energy density (J/cm^2) as a function of irradiation time (0-30 s) and irradiation power (0.5-5 W).

Irradiation power (W)		Energy density (J/cm ²)								
		Irradiation time (s)								
		0.5	1	1.5	3	5	10	15	20	30
HPC										
0.5	CW	0.66	1.33	1.99	3.99	6.65	13.30	19.94	26.59	39.89
	SP1									
	SP2									
1	CW	1.33	2.66	3.99	7.98	13.30	26.59	39.89	53.18	79.77
	SP1									
	SP2									
2	CW	2.66	5.32	7.98	15.95	26.59	53.18	79.77	106.37	159.55
	SP1									
	SP2									
3	CW	3.99	7.98	11.97	23.93	39.89	79.77	119.66	159.55	239.32
	SP1									
	SP2									
5	CW	6.65	13.30	19.94	39.89	66.48	132.96	199.44	265.91	398.87
	SP1									
	SP2									
HPLF										
0.5	CW	0.66	1.33	1.99	3.99	6.65	13.30	19.94	26.59	39.89
	SP1									
	SP2									
1	CW	1.33	2.66	3.99	7.98	13.30	26.59	39.89	53.18	79.77
	SP1									
	SP2									
2	CW	2.66	5.32	7.98	15.95	26.59	53.18	79.77	106.37	159.55
	SP1									
	SP2									
3	CW	3.99	7.98	11.97	23.93	39.89	79.77	119.66	159.55	239.32
	SP1									
	SP2									
5	CW	6.65	13.30	19.94	39.89	66.48	132.96	199.44	265.91	398.87
	SP1									
	SP2									

Black and gray shaded areas represent the ranges where growth stimulatory and inhibitory effects of CO₂ laser irradiation were observed, respectively.

Table II. *Hormetic induction in normal oral cells by CO₂ laser irradiation. The maximum hormetic response was determined as the highest value of hormetic response observed during 24-72 hours' incubation of HPC (Figure 2), HPLF (Figure 3), HGF (Figure 3 in ref. 17) and HSC-2 cells (Figure 4 in ref. 17).*

		Maximum hormetic response (%)			Mean
		24 h	48 h	72 h	
HPC	CW	20.93	27.54	18.44	22.30
	SP1	22.60	50.00	22.47	31.69
	SP2	28.35	17.92	14.88	20.38
	Mean	23.96	31.82	18.60	24.79
HPLF	CW	9.18	11.60	14.47	11.75
	SP1	10.27	13.89	16.67	13.61
	SP2	11.25	17.09	24.70	17.68
	Mean	10.23	14.19	18.61	14.35
HGF	CW	10.11	5.46	11.29	8.95
	SP1	3.55	8.67	7.65	6.62
	SP2	3.43	15.21	13.49	10.71
	Mean	5.70	9.78	10.81	8.76
HSC-2	CW	16.80	17.60	4.50	12.97
	SP1	15.90	9.00	2.80	9.23
	SP2	21.20	4.90	5.00	10.37
	Mean	17.97	10.50	4.10	10.86

genase-2 expression (23), reduction of cyclic AMP production (27), and activation of PI3K/AKT (28) and MAPK/ERK (23) pathways, and modification of cytokine gene expression (29). It remains to be investigated whether hormesis induction by low level of CO₂ laser irradiation in HPC and HPLF cells is mediated *via* these changes of intracellular events or not.

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