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Effect of CO_2 Laser Irradiation on Hormesis Induction in Cultured Oral Cells

KENSUKE IWASAKA[1], KAYOKO TOMITA[1], YUMI OZAWA[1], TADASHI KATAYAMA[1] and HIROSHI SAKAGAMI[2]

Divisions of [1]Operative Dentistry and [2]Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan

Abstract. *Background: Many drugs (including toxicants) and radiation therapy have been reported to exert bi-phasic hormetic effects on cultured cells, but only when both the concentration and treatment time were optimal. Most previous studies have been carried out with multiple laser modalities other than CO_2 laser, and there has been no comparison of the hormetic response between normal and tumor cells. We investigated here whether CO_2 laser treatment induces hormesis in human gingival fibroblast (HGF) and oral squamous cell carcinoma (HSC-2) cells. Materials and Methods: 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: CO_2 laser irradiation stimulated cell growth at low and inhibited it at high irradiation power. Among three dispatch modes, super pulse (SP)2 most effectively induced growth stimulation in HGF, at an irradiation dose slightly lower than that which induced cytotoxicity. Higher irradiation doses were comparably cytotoxic against both normal (HGF) and tumor (HSC-2) cells, reaching a plateau of cytotoxicity within 24 hours. Conclusion: Since both the range and magnitude of hormetic response in HGF cells were very narrow and small, it is crucial to establish the optimal conditions for hormesis induction for clinical application in dentistry.*

Various laser modalities have been used in the treatment of cutaneous (1), periodontal (2, 3) and oral (4) disorders. However, as compared with Er:YAG laser therapy, there is insufficient evidence for safety and effects to support the clinical application of CO_2, Nd:YAG, or diode laser (5, 6). Many drugs (including toxicants) and radiation therapy have been reported to show growth-stimulatory effects at lower concentrations and growth-inhibitory effects at higher doses, and this dose-dependent bi-phasic effect is known as 'hormesis' (7, 8). Similarly, high level laser treatment (HLLT) induced cell membrane and DNA damage (9), whereas low level laser treatment (LLLT) stimulated the growth of cells and wound healing (10-12). It has been reported that hormesis is detectable only under optimal conditions (such as the dose of the test compounds and the duration of treatment) (7). On the other hand, we found that sodium fluoride (13), three herbal extracts (14), gefitinib (15) and 2-aminotropone derivatives (16), at wide ranges of concentrations, did not induce any clear-cut hormetic effects in cultured normal human oral cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] and oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4). These negative results with oral cells suggest that the extent of hormetic induction may depend on the type of cell used. Furthermore, most previous *in vitro* studies have been carried out with laser modalities other than CO_2 laser. Based on this, in the present study, whether CO_2 laser irradiation can induce hormesis in both normal (HGF) and tumor (HSC-2) cells was investigated using wide ranges of irradiation doses and incubation times.

Materials and Methods

Cell culture. HGF cells were established from the first premolar extracted tooth in the lower jaw and periodontal tissues of twelve-year-old girl, according to the guideline of the Intramural Board of Ethics Committee (no. A0808) (17). HGF cells had an *in vitro* life span [cumulative cell population doubling level (PDL)] of 47 (17). HGF (8-12 PDL) and HSC-2 (Riken Cell Bank, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), under a humidified 5% CO_2 atmosphere.

Correspondence to: Kensuke Iwasaka, Division of Operative Dentistry, Department of Restorative and Biomaterials Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 492855511 ext. 381, 429; Fax: +81 492855171, e-mail: iwasaka@dent.meikai.ac.jp/sakagami@dent.meikai.ac.jp

Key Words: CO_2 laser irradiation, cytotoxicity, gingival fibroblast, oral squamous cell carcinoma, hormesis.

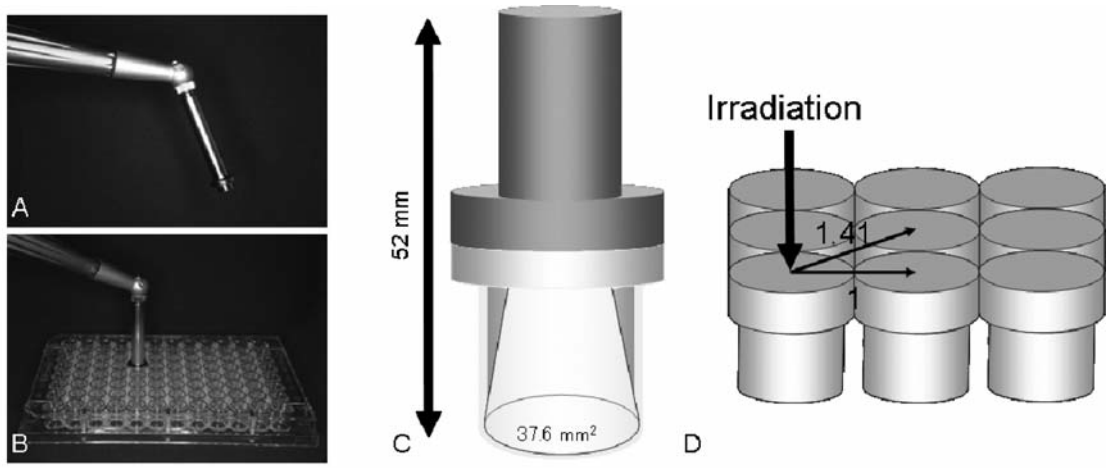

Figure 1. *Experimental procedure of laser irradiation on the cells. A: Tip of CO_2 laser (Opelaser PRO LA12). B: Application of laser tip to cells grown on 96-microwell plate. C: Range of irradiation area. D: The distance between one well to the next well was defined as 1 unit.*

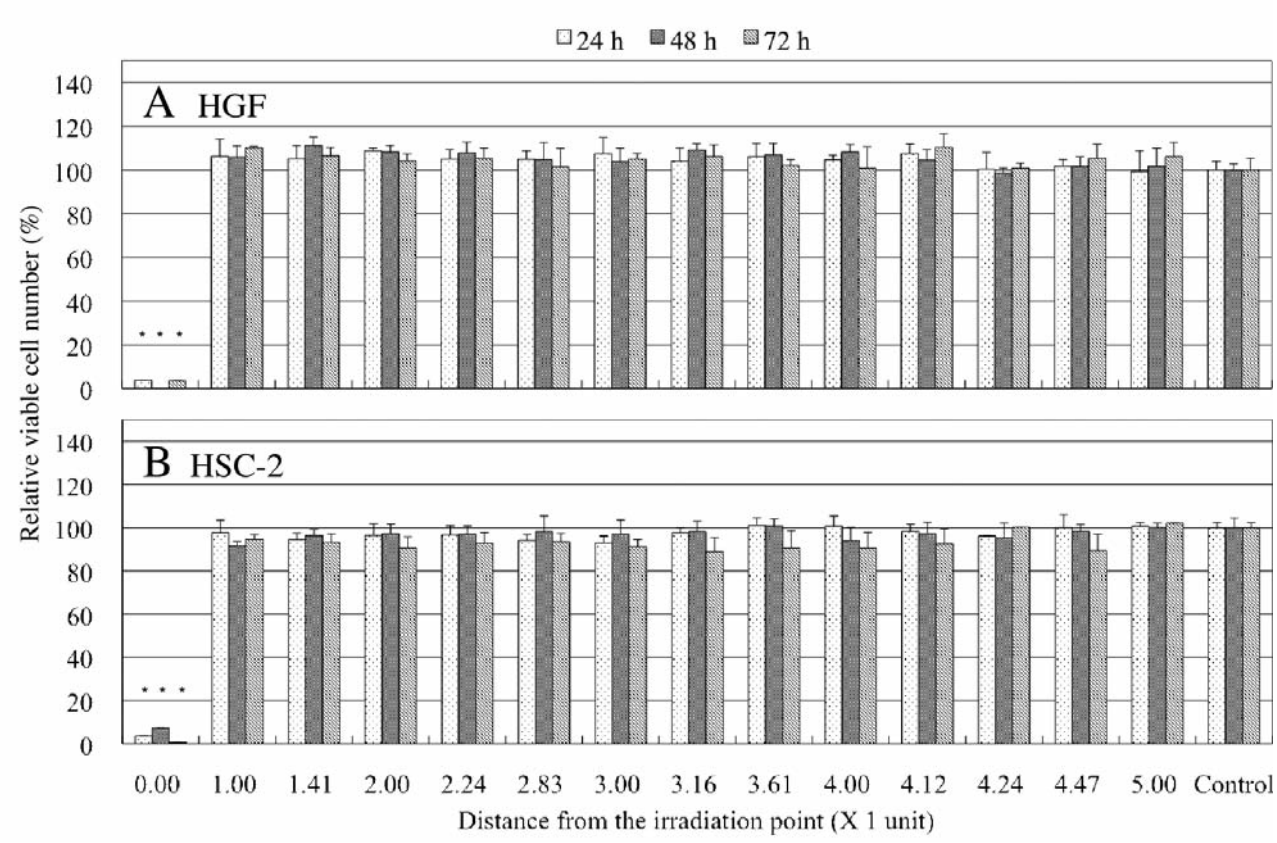

Figure 2. *Ripple effect of laser irradiation on the viability of the cells grown on the adjacent wells. HGF (A) and HSC-2 (B) cells were inoculated on a 96-microwell plate, and incubated for 48 hours to achieve complete attachment. A CO_2 laser tip (Opelaser PRO LA12) was set just 52 mm above the cell surface, and the cells were exposed to CO_2 laser irradiation (5 W, 30 s) without removing the medium (100 μl). After medium change, the cells were further incubated for 24, 48 or 72 hours, and the number of viable cells (expressed as a percentage of unirradiated 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. *$p<0.01$.*

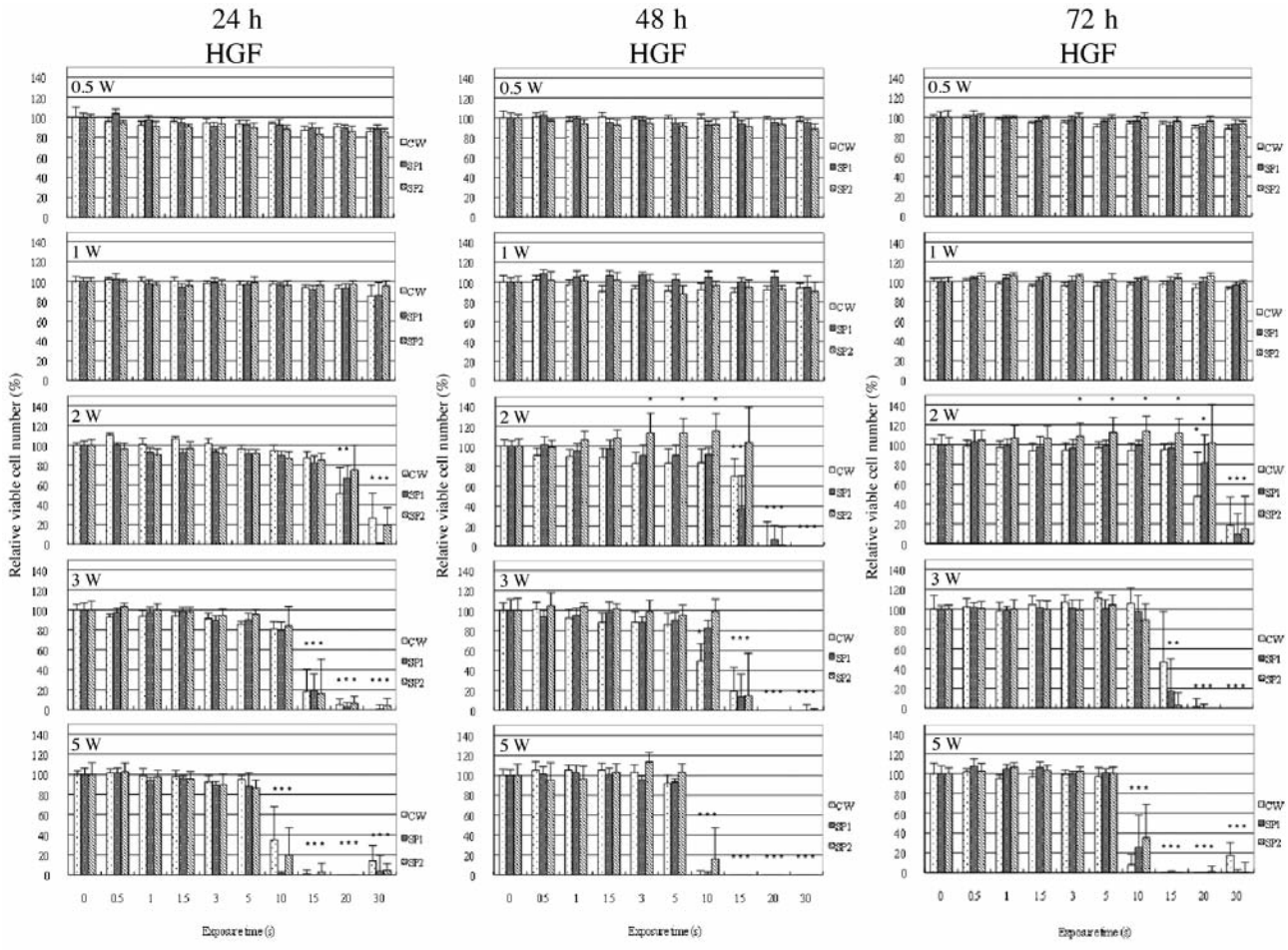

Figure 3. *HGF cells were irradiated for 0 (control), or different durations at different powers and with different dispatch modes, and then cultured for a further 48 hours in DMEM/10% FBS to determine the viable cell number. Each value represents the mean±S.D. of 12 samples. *p<0.01.*

Assay for cytotoxicity of laser irradiation. Cells (1.5×10^3) were inoculated on a 96-microwell plate (37.6 mm^2) (Becton Dickinson, Franklin Lakes, NJ, USA), and incubated for 48 h to allow complete attachment. The medium was changed for fresh medium, and the cells were placed at 52 mm from the CO_2 laser source (Opelaser PRO LA12, Yoshida, Tokyo, Japan) (Figure 1C). The cells in each well were evenly irradiated with a specially manufactured tip (Figure 1A, B), 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) (Figure 1). After the medium was changed, the cells were incubated for 24, 48 or 72 h, and the viable cell number was then determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. In brief, cells were incubated for a further 4 h with 0.2 mg/ml MTT reagent in DMEM/10% FBS at 37˚C, and dissolved with 0.1 ml of dismethyl sulfoxide (DMSO) to determine the absorbance at 540 nm with a plate reader.

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 CO_2 laser irradiation. As a preliminary test, we first confirmed that there was no ripple effect, *i.e.*, the irradiation of individual wells did not affect adjacent cells in the same plate. When HGF (upper panel) and HSC-2 (lower panel) cells were exposed to laser irradiation (5 W, 30 s) at 52 mm distance, almost all of the cells under the irradiation point died after incubation for 48 h in regular culture medium (Figure 2). On the other hand, the cells that had been inoculated in the wells adjacent to those exposed to laser irradiation all survived, judging from the comparable cell growth compared with that of control (unirradiated) cells (Figure 2). This finding made it possible for us to perform sequential CO_2 laser irradiation, without damaging the viability of the cells grown on the adjacent wells.

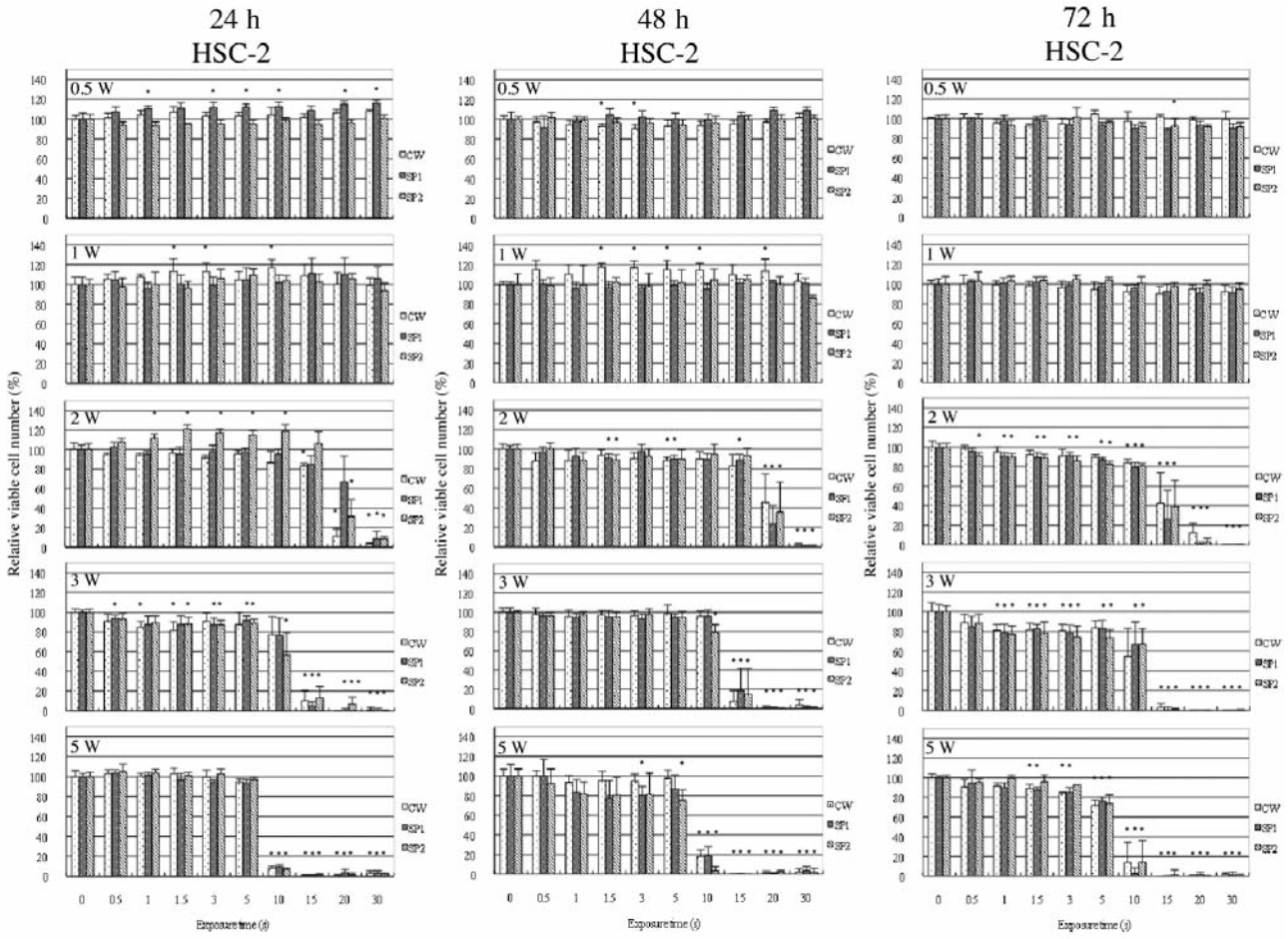

Figure 4. *HSC-2 cells were irradiated for 0 (control), or different durations, at different powers and dispatch modes, and then cultured for a further 48 hours in DMEM/10% FBS to determine the viable cell number. Each value represents the mean±S.D. of 12 samples. *p<0.01.*

Effect on HGF cells. We investigated the effect of irradiation time and power on the viability of HGF cells (Figure 3). Lower power irradiation (0.5-1 W) for up to 30 s did not significantly affect the viability of HGF 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 80%. Cytotoxicity of CO_2 laser irradiation reached nearly a maximum level within 24 h incubation in culture medium (Figure 3).

Slight, but significant ($p<0.01$) growth stimulation (15-20% above that of the control) was observed after incubation for 48-72 h, when dispatch mode of SP2 was selected at 2 W (3-15 s) (Figure 3). However, such a hormetic effect was not observed when dispatch modes of CW and SP1 were used (Figure 3).

Effect on HSC-2 cells. We next investigated the effect of irradiation time and power on the viability of HSC-2 cells (Figure 4). Lower power (0.5-1 W) for up to 30 s was not cytotoxic to HSC-2 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 80%. Cytotoxic effect of laser irradiation was slightly elevated during prolonged incubation (24, 48 or 72 h).

A degree of hormetic effect was found at the lower irradiation dose range (0.5, 1 and 2W). The cell number was significantly ($p<0.01$) increased (15-20% above control) using dispatch mode of CW (1 W, 24 and 48 h), SP1 (0.5 W, 24 h) and SP2 (2W, 24 h) (Figure 4).

Discussion

The present data demonstrate that CO_2 laser irradiation bi-phasically modified the growth of both HGF and HSC-2 cells. The growth stimulatory effect (hormetic effect) was observed at lower irradiation power (energy density=1.33-

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

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

Bold and underlined numbers represent growth stimulatory and inhibitory effects, respectively.

79.77 J/cm^2), whereas a growth-inhibitory or cytotoxic effect was observed at HLLT (energy density ≥106.37 J/cm^2) (Table I). The maximum hormetic response (8) was only 20% in both HGF and HSC-2 cells. In HGF cells, such growth-stimulatory effect was observed within a narrow range of irradiation doses (15.95-79.77 J/cm^2), slightly lower than that of the cytotoxic range of doses (106.37-398.87 J/cm^2) and only at an irradiation power of 2 W (Figure 3, upper panel in Table I). This suggests that the hormetic response may be a mechanism by which cells escape from cytotoxicity. It should be noted that the hormetic effect was induced only by SP2, but not by SP1, nor CW dispatch modes (Figure 3), indicating the superiority of the SP2 dispatch mode for clinical application.

On the other hand, the growth-stimulatory effect of CO_2 laser irradiation in HSC-2 cells was rather broadly distributed at lower irradiation doses (1.33-58.18 J/cm^2) (Figure 4, lower panel in Table I). The growth-stimulatory effect was observed after incubation for 24 h (0.5 W with SP1, 1 W with CW, 2W with SP2) and 48 h (1W with CW), but not at 72 h. These data suggest that the optimal irradiation power for hormesis induction by the dispatch mode of CW, SP1 and SP2 is 1, 0.5 and 2 W, respectively. These data suggest that hormesis is more clearly detected in normal oral cells rather than oral squamous cell carcinoma cell lines, in agreement with our previous report (16). It may be possible that high metabolic rate and short cell cycle in transformed cells make them unresponsive to LLLT (12).

It has been reported that LLLT stimulated the growth of HGF (18-22), HPC (23) and HPLF (24). Possible mechanisms for the growth stimulation may include induction of heat-shock proteins (10), production of collagen (25) and prostaglandin E_2 *via* cyclooxygenase expression (22), reduction of cAMP production (26), and activation of PI3K (phosphatidylinositol-3 kinase)/Akt (for enzymes that are members of the serine/threonine-specific protein kinase family) (27) and MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) (23) pathways, and modification of cytokine gene expression (28). The growth-promoting activity of LLLT may serve as the basis for its wound-healing process characterized by more prominent fibroblast proliferation, with young fibroblasts actively producing collagen (25). However, it has been reported that the power density of irradiation influences cell growth in an inversely proportional manner (19). Furthermore, the present study demonstrated that the optimal range of the power density for normal cells is very narrow (Table I), and higher doses of CO_2 laser irradiation induced comparable cytotoxicity in both normal (HGF) and tumor (HSC-2) cells (Figures 3 and 4). Therefore, it is crucial to establish the optimal conditions of hormetic response for clinical application in dentistry.

The present study also demonstrated that both HGF and HSC-2 cells are relatively resistant to hormesis induction by CO2 laser treatment, as is true for other inducers (13-17). This suggests that the extent of hormetic induction may depend on the type of cell, rather than the type of inducer. It remains to be investigated whether the sensitivity of these oral cells to dental materials or drugs is changed by pretreatment with CO_2 laser irradiation.

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