Helium–Neon Laser Irradiation Stimulates Cell Proliferation through Photostimulatory Effects in Mitochondria

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Previous reports have shown that cellular functions could be influenced by visual light (400–700 nm). Recent evidence indicates that cellular proliferation could be triggered by the interaction of a helium–neon laser (He-Ne laser, 632.8 nm) with the mitochondrial photoacceptor-cytochrome *c* oxidase. Our previous studies demonstrated that He-Ne irradiation induced an increase in cell proliferation, but not migration, in the melanoma cell line A2058 cell. The aim of this study was to investigate the underlying mechanisms involved in photostimulatory effects induced by an He-Ne laser. Using the A2058 cell as a model for cell proliferation, the photobiologic effects induced by an He-Ne laser were studied. He-Ne irradiation immediately induced an increase in mitochondrial membrane potential ($\Delta\Psi_{mt}$), ATP, and cAMP via enhanced cytochrome *c* oxidase activity and promoted phosphorylation of Jun N-terminal kinase (JNK)/activator protein-1 (AP-1) expressions. He-Ne irradiation-induced A2058 cell proliferation was significantly abrogated by the addition of $\Delta\Psi_{mt}$ and JNK inhibitors. Moreover, treatment with an He-Ne laser resulted in delayed effects on IL-8 and transforming growth factor-β1 release from A2058 cells. These results suggest that He-Ne irradiation elicits photostimulatory effects in mitochondria processes, which involve JNK/AP-1 activation and enhanced growth factor release, and ultimately lead to A2058 cell proliferation.

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INTRODUCTION

Previous reports have shown that cellular functions could be influenced by visual light (400–700 nm) irradiation; however, the mechanisms by which visible light induces its biologic effects remain to be established. A low-energy laser has been found to modulate various biological processes in cell cultures and animal models (Karu, 1999; Oron *et al.*, 2001). At the cellular level, low-energy laser irradiation can generate significant biological effects including cellular proliferation, collagen synthesis, and release of growth factors from cells (Sommer *et al.*, 2001). Low-energy lasers produce biologic alterations due to their direct irradiation effects but not thermal effect (Krippner, 1980). In this system, the temperature elevation in the irradiated tissues is limited to less than 0.1–0.5°C (Basford, 1989; Babapour *et al.*, 1995), and its output power is in the range of mWatts (Walsh, 1997). Photochemical processes are based on the excitation of the electronic state of the atom or molecule by visible light irradiation (Letokhov, 1983). Other proposed mechanisms of biostimulation suggest that irradiation may affect cell functions by modulating the production of growth factors (Yu *et al.*, 1994). The mechanism of biostimulation by a low-energy laser may occur at the mitochondrial level (Passarella *et al.*, 1984; Pastore *et al.*, 2000). It has been reported that a helium–neon (He-Ne, 632.8 nm) laser stimulates a number of biological processes, including cell growth and proliferation (Smol'yaninova *et al.*, 1991; Marra *et al.*, 1997). In addition, an He-Ne laser irradiation can affect both bioenergetics and biogenesis of mammalian mitochondria (Salet *et al.*, 1979; Marra *et al.*, 1994; Passarella *et al.*, 1994).

A2058 cells are derived from metastastic human melanoma cells. Our previous study demonstrated that migration and proliferation of human melanocytes were significantly enhanced by He-Ne laser irradiation (Yu *et al.*, 2003). Owing to the limited lifespan of melanocytes on a culture system, the melanoma cell line A2058 was used for further investigation since it shared similar characteristic with melanocytes after He-Ne irradiation. More specifically, He-Ne laser irradiation also resulted in A2058 cell proliferation. Because only proliferation, but not migration, was stimulated by He-Ne laser irradiation, A2058 cells provide a unique model for

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Abbreviations: AP-1, activator protein-1; CCCP, carbonyl cyanide m-chlorophenyl-hydrazone; He-Ne laser, helium–neon laser; JNK, Jun N-terminal kinase; $\Delta\Psi_{mt}$, mitochondrial membrane potential; TGF-β1, transforming growth factor beta 1

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studying the biochemical and molecular events that occur in cells after visual light irradiation. Mitochondria might be a special target of He-Ne laser light (Karu *et al.*, 1984) since it contains most of the cellular chromophores (Salet *et al.*, 1987). One hypothesis explaining the mechanism of photostimulation is that the energy is absorbed by intracellular chromophores and is converted into metabolic energy, that is, the respiratory chain (Belkin *et al.*, 1988; Conlan *et al.*, 1996; Karu, 1999), which produces changes in the redox status in both mitochondria and cytoplasm. Activations of the electron transport chain result in an increase in the electrical potential across the mitochondrial membrane ($\Delta\Psi_{mt}$), an increase in the ATP pool, and finally the activation of nucleic acid synthesis (Yu *et al.*, 1997). Cytochrome *c* oxidase was reported as a mitochondrial photoacceptor upon which the He-Ne laser exerts its effect (Pastore *et al.*, 2000). In addition, it has been reported that He-Ne laser light can stimulate cell proliferation accompanied with an increased cytokine/growth factor release (Yu *et al.*, 1996; Khanna *et al.*, 1999). The aim of this study was to investigate the mechanisms involved in photostimulatory effects induced by He-Ne laser irradiation on A2058 cells.

RESULTS

He-Ne laser irradiation stimulated A2058 cell proliferation

The effect of He-Ne laser irradiation on A2058 cell growth was determined by direct cell counting (Figure 1). The cell number of A2058 treated with dosages of $\geqslant 1.0\,J/cm^2$ after 3 days was significantly higher than that of the control group ($P<0.05$). Moreover, cell proliferation activity induced by an He-Ne laser was examined using the CellTiter cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) (MTS) assay). The results of MTS assay also revealed that cell proliferation activity was significantly higher in the laser-irradiated group than in the control group on day 3 (data not shown). Additionally, the temperature of A2058 treated with dosages of 0, 0.5, 1.0, and $2.0\,J/cm^2$ He-Ne laser irradiation was 23.1 ± 0.07, 23.2 ± 0.14, 23.5 ± 0.07, and $23.6\pm0^{\circ}C$, respectively. These results were consistent with previous reports indicating that low-energy laser irradiation induced minimal temperature elevations. It also confirms that biologic alternations are due to the direct irradiation effect, but not to the thermal effect (Basford, 1989; Babapour *et al.*, 1995).

He-Ne laser irradiation stimulated an increase in ki-67 labeling

The p53 gene is considered a tumor-suppressor gene (Hutson *et al.*, 1995), whereas the bcl-2 protein promotes survival by inhibiting apoptosis (Fanidi *et al.*, 1992). ki-67 is a monoclonal antibody which can be used to assess the growth fraction of human neoplasms. We found a significant increase in the expression of ki-67 protein level of cells treated with 0, 0.5, 1.0, and $2.0\,J/cm^2$ He-Ne laser of 39.0 ± 3.6, 33.0 ± 3.2, 50.3 ± 0.6, and $57.3\pm2.5\%$, respectively. In contrast, there was no significant inductive effect on the p53 and bcl-2 expression after He-Ne treatment, as compared with the control group (Figure 2). To investigate further the effect of He-Ne treatment on cell apoptosis, Annexin V/PI binding and TUNEL assays were done. Data from flow cytometric analysis showed that no significant apoptotic effect was seen after $1.0\,J/cm^2$ He-Ne treatment as compared with the control group (data not shown). These results confirmed that He-Ne laser irradiation stimulated A2058 cell proliferation, but did not induce apoptotic cell.

He-Ne laser irradiation stimulated a significant increase in cytochrome *c* oxidase activity of A2058 cells

Cytochrome *c* oxidase is the enzyme that catalyzes the final step in the mitochondrial respiratory chain. It has been reported as a mitochondrial photoacceptor upon which the He-Ne laser exerts its effect (Pastore *et al.*, 2000). As shown in Figure 3, He-Ne treatment resulted in a significant increase in cytochrome *c* oxidase activity. Compared with the untreated control group, the cytochrome *c* oxidase activities of A2058 cells were increased by about 70, 140, and 170% after irradiation with 0.5, 1.0, and $2.0\,J/cm^2$ He-Ne laser treatment, respectively.

He-Ne laser irradiation stimulated a significant increase in $\Delta\Psi_{mt}$ of A2058 cells. The mitochondrial membrane potential ($\Delta\Psi_{mt}$) is an important parameter not only for mitochondrial

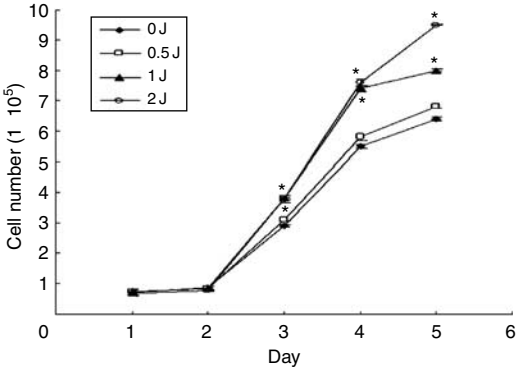

Figure 1. Effect of He-Ne laser irradiation on A2058 cell proliferation. Cells in 24-well plates were irradiated in triplicate with 0, 0.5, 1.0, and $2.0\,J/cm^2$ He-Ne lasers. The number of visible cells was counted by trypsinization and assisted with trypan blue dye exclusion. $^*P<0.05$ as compared with the control.

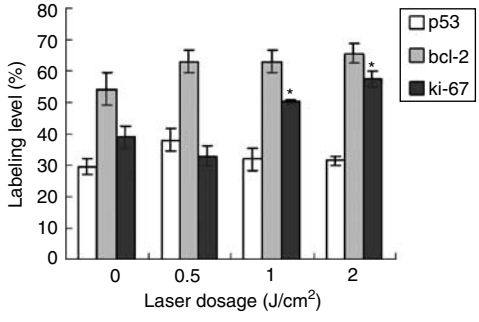

Figure 2. Immunocytochemical staining of p53, bcl-2, and ki-67 proteins of A2058 cells in response to different dosages of He-Ne laser irradiation. Percentage of A2058 cells with or without He-Ne laser irradiation in this study labeling for p53, bcl-2, and ki-67 at different levels, respectively. $^*P<0.05$ as compared with the control.

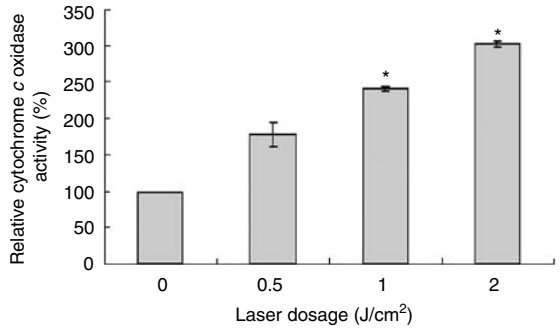

Figure 3. Effect of He-Ne laser irradiation on the cytochrome *c* oxidase activity. The measurement of cytochrome *c* oxidase activity was determined following the experimental protocol described in the Materials and Methods section. The data are expressed as relative levels of cytochrome *c* oxidase activity compared with that of untreated control.

but also of cellular status. To assess the mechanism involved in $\Delta\Psi_{mt}$ perturbation induced by the He-Ne laser, A2058 cells were treated with 0, 0.5, 1.0, and 2.0 J/cm^2 irradiation and analyzed by flow cytometry after $DiOC_6$ dye labeling. The fluorescence intensities after 0, 0.5, 1.0, and 2.0 J/cm^2 He-Ne laser irradiation were 1.3 ± 0.1, 3.0 ± 1.2, 28.4 ± 0.8, and 34.5 ± 3.5%, respectively. A representative histogram is shown in Figure 4a. It is obvious that cells treated with irradiance higher than 0.5 J/cm^2 exhibited a marked increase in cellular uptake of the fluorochrome. In addition, to confirm whether enhanced $\Delta\Psi_{mt}$ is due to He-Ne laser irradiation, cells were treated with the mitochondria-specific ionophore carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (30 μM). CCCP significantly abrogated the increased $\Delta\Psi_{mt}$ of A2058 cells irradiated with 2.0 J/cm^2 He-Ne laser (Figure 4b).

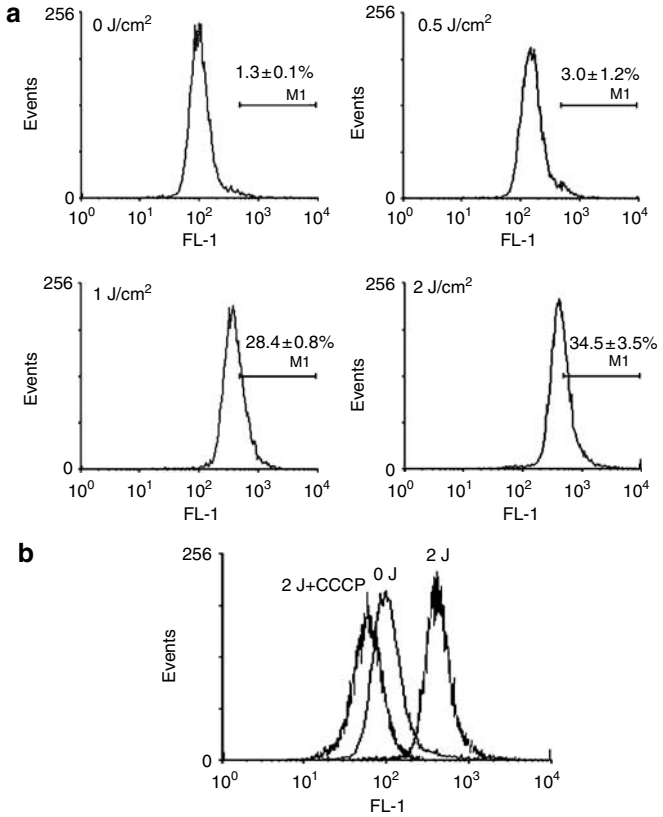

Figure 4. Effect of He-Ne laser irradiation on the mitochondrial membrane potential ($\Delta\Psi$mt). (**a**) A2058 cells were treated with different dosages of He-Ne laser irradiation, then stained with $DiOC_6$ dye and analyzed immediately by flow cytometry. The number M1 indicates the percentage with increased $\Delta\Psi_{mt}$. (**b**) As a control, $\Delta\Psi_{mt}$ was measured in the presence of CCCP, an uncoupling agent that reduces $\Delta\Psi_{mt}$.

He-Ne laser irradiation increased intracellular ATP content in A2058 cells

ATP is the central parameter of cellular energetics, metabolic regulation, and cellular signaling; therefore, determination of intracellular ATP is worthwhile in the characterization of cellular physiology. Intracellular ATP was measured by continuously monitoring ATP production by firefly luciferase luminescence. As demonstrated in Figure 5a, cells treated with irradiance higher than 0.5 J/cm^2 exhibited significantly increased intracellular ATP content in A2058 cells. Compared with that of the untreated control, ATP formation significantly increased in A2058 cells exposed to 1.0 ($P<0.01$) and 2.0 J/cm^2 ($P<0.05$) irradiation, respectively. Additionally, to confirm whether increased ATP is due to enhanced $\Delta\Psi_{mt}$ after He-Ne laser irradiation, cells were treated with 30 μM CCCP before 1.0 J/cm^2 He-Ne laser treatment. Our results showed that CCCP had a significant effect on the reduction of ATP content compared with the group treated with He-Ne laser irradiation alone (Figure 5b).

He-Ne laser irradiation increased intracellular cAMP level in A2058 cells

cAMP, a molecule derived from ATP, is important in many biological processes. Because the cAMP system has been demonstrated to control the biosynthesis of DNA and RNA, the present work also investigated the role of cAMP in the stimulative effect of He-Ne laser on A2058 cells. Compared with that of the untreated control, the intracellular cAMP level of A2058 cells increased about 40, 160, and 120% after 0.5, 1.0, and 2.0 J/cm^2 He-Ne laser treatment, respectively. It is obvious that cAMP formation by A2058 cells treated with irradiance higher than 0.5 J/cm^2 significantly increased as shown in Figure 6a. Moreover, to elucidate whether an increase in cAMP level might be due to the increase in cellular ATP synthesis, cells were pretreated with ATP (1×10^{-4} M) before 0 and 1.0 J/cm^2 He-Ne laser irradiation. Our results showed that pretreatment with ATP followed by a 1.0 J/cm^2 He–Ne laser increased the cAMP level more than either He-Ne laser irradiation alone or treatment with exogenous ATP alone (Figure 6b). CCCP also significantly abrogated the increased cAMP level of A2058 cells irradiated with 1.0 J/cm^2 He-Ne laser (data not shown).

cAMP analog significantly enhanced the phosphorylation of JNK

cAMP has been shown to activate *c-jun*, a component of AP-1, in PC12 cells (Wu *et al.*, 1989). Since Jun N-terminal kinase (JNK) is known to be the upstream kinase of AP-1, we investigated whether the increased JNK phosphorylation is due to increased cAMP after laser irradiation. Cells were

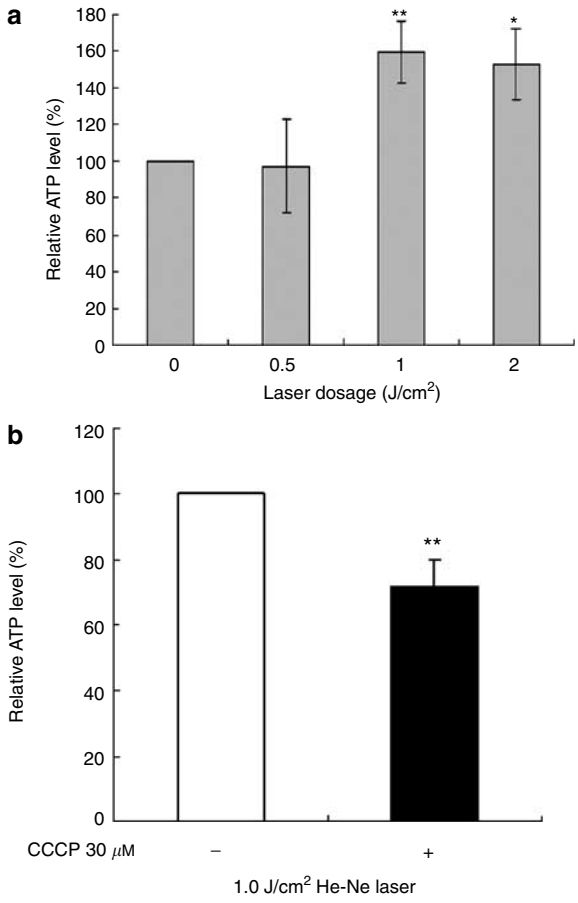

Figure 5. Effect of He-Ne laser irradiation on the intracellular ATP levels of the A2058 cells. (**a**) A2058 cells were irradiated with 0, 0.5, 1.0, and 2.0 J/cm^2 of an He-Ne laser. Relative ATP levels were calculated as the percentage of the dose 0 J/cm^2 level. (**b**) The relative ATP content of A2058 cells was obtained after 1.0 J/cm^2 He-Ne laser irradiation with or without 30 μM CCCP. *$P<0.05$, **$P<0.01$ as compared with the control.

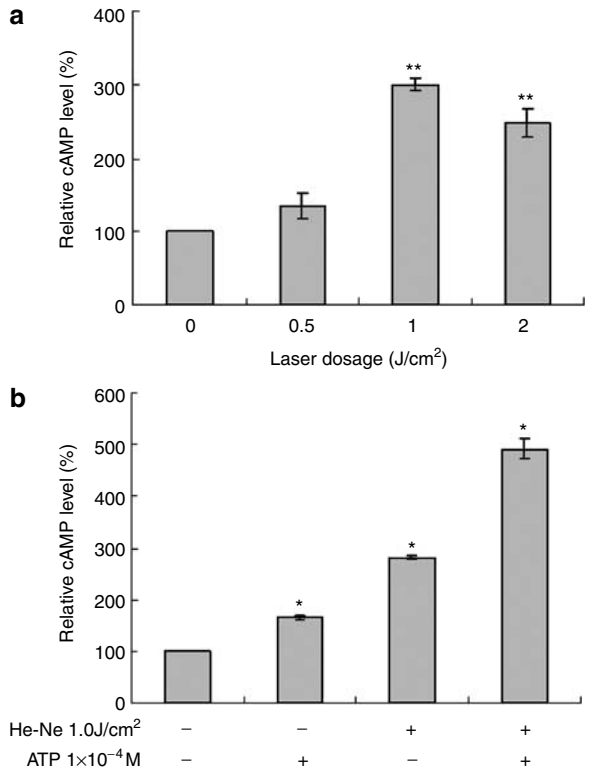

Figure 6. Effect of He-Ne laser irradiation on the intracellular cAMP levels of the A2058 cells. (**a**) The change of intracellular cAMP levels was determined using the experimental protocol. Relative cAMP levels were calculated as the percentage of the dose 0 J/cm^2 level. (**b**) The relative cAMP content of A2058 cells was obtained after 0 and 1.0 J/cm^2 He-Ne laser irradiation with or without ATP (1×10^{-4} M). *$P<0.05$, **$P<0.01$ as compared with the control.

pretreated with a membrane-permeable form of cAMP, 8-bromo-cAMP (0.3 mM) before 1.0 J/cm^2 He-Ne laser irradiation. Our results showed that 8-bromo-cAMP stimulated phosphorylation of JNK–mitogen-activated protein kinase (Figure 7).

He-Ne laser irradiation induced the activation of JNK/AP-1 pathway

It is known that JNK causes concomitant activation of its substrates (c-Jun, ATF2, p53) and plays a key role in the cellular response to extracellular stimuli. The expressions of JNK activity were determined using immunoblotting assay. Compared with the untreated control group, the JNK phosphorylation of A2058 cells increased after He-Ne laser irradiation (Figure 8a). For quantification analysis, the relative ratio of JNK-P to actin was determined, and the increase of JNK-P expression after He-Ne laser irradiation was more than twofold as compared with the 0 J/cm^2 irradiated group. A representative blot is shown in Figure 8b. On the other hand, no significant effect was found on the expressions of p38 phosphorylation after He-Ne laser treatment (data not shown). JNK is known to be an upstream kinase of AP-1 and has been shown to directly phosphorylate proteins of the AP-1 complex; therefore, the role of AP-1 in He-Ne laser-induced cell proliferation was also investigated. As demonstrated in Figure 9a, cells treated with irradiance higher than 0.5 J/cm^2 exhibited significantly increased AP-1 activity in A2058 cells. Compared with that of the untreated control, AP-1 activity was increased in A2058 cells exposed to 1.0 ($P<0.05$), and 2.0 J/cm^2 ($P<0.01$) He-Ne laser irradiation, respectively. To further address the effect of SP600125 on AP-1 activity, the transfected A2058 cells were pretreated with SP600125 followed by 1.0 J/cm^2 He-Ne laser irradiation. Compared with that of the He-Ne laser-treated control group, the relative expression of AP-1 activity showed a significant change in the presence of SP600125 (Figure 9b).

He-Ne laser irradiation induced a significant increase in IL-8 and TGF-β1 release from A2058

IL-8 and transforming growth factor (TGF)-β1 are overexpressed in advanced human melanoma. IL-8 has been shown to act as an autocrine growth factor in melanoma, and it has been reported to enhance tumor growth (Luca *et al.*, 1997). TGF-β1, the most abundant isoform of the TGF-β

family, is elevated in the plasma of melanoma patients, especially those with metastatic lesions (Krasagakis *et al.*, 1998). The assay for IL-8 and TGF-β1 release from cells was performed 24 hours after He-Ne laser treatment. As shown in Table 1, under 0, 0.5, 1.0, and 2.0 J/cm^2 He-Ne laser irradiation, the stimulatory effect of IL-8 and TGF-β releases in A2058 cells was dose-responsive, that is, 7,800±0, 10,000±707.1, 11,350±495.0, and 13,600±1414.2 pg/ml in IL-8 release and 1,904±15.8, 2,038.4±324.7, 2,217.6±79.2, and 2,212±51.5 pg/ml in TGF-β1 release, respectively. The stimulatory effect of He-Ne laser treatment was dose-dependent. In addition, to address

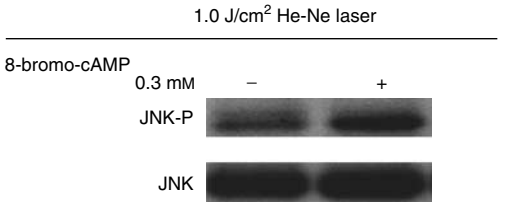

Figure 7. cAMP analog significantly enhanced the phosphorylation of JNK. Cells were untreated or incubated with 0.3 mM 8-bromo-cAMP for 60 minutes before 1.0 J/cm^2 He-Ne laser irradiation, and JNK phosphorylation was detected. $^{**}P<0.01$ as compared with the control.

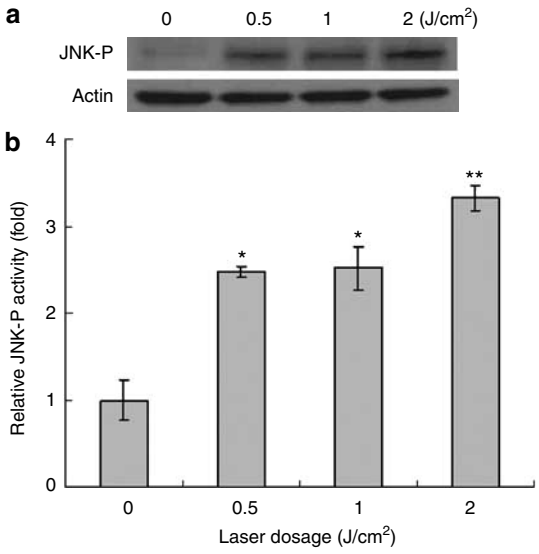

Figure 8. Immunoblot analysis showed the effect of He-Ne laser irradiation on phosphorylation of JNK. (**a**) After exposure to different dosages of laser, cell lysates were collected and immunoblotted with specific antibodies as indicated. For the internal control, the same amounts of protein extract were also probed with antibody against actin. (**b**) The pattern of JNK phosphorylation by A2058 cells showed a dose-responsive effect produced by He-Ne laser irradiation. $^{*}P<0.05$, $^{**}P<0.01$ as compared with the control.

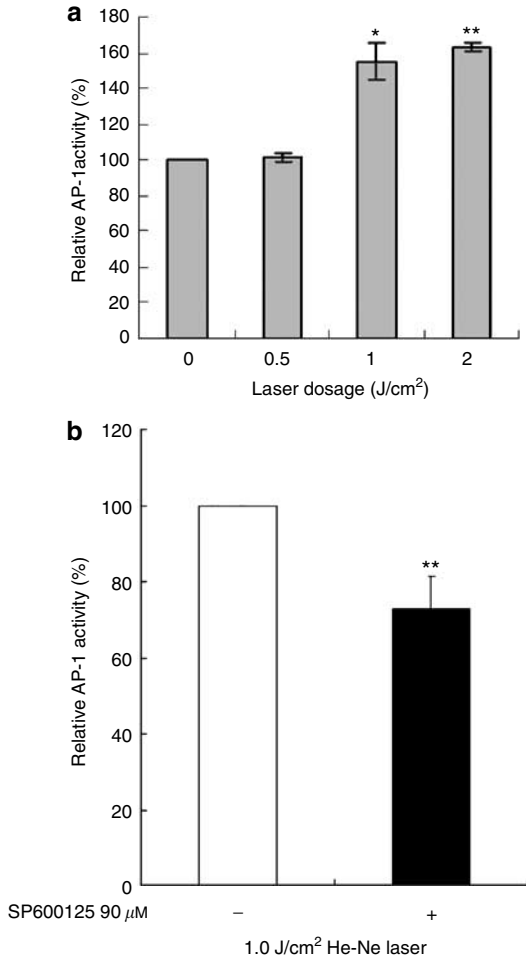

Figure 9. Effect of He-Ne laser irradiation on AP-1 activity. (**a**) A2058 cells were co-transfected with pAP1-luc and pSV-βgal reporter plasmids as described in the Materials and Methods section. The transfected cells were exposed to different dosages of an He-Ne laser and the cell lysates were collected. Luciferase activity was determined and then normalized with β-galactosidase activity. (**b**) The transfected A2058 cells were with or without 90 μM SP600125 before 1.0 J/cm^2 He-Ne laser treatment. $^{*}P<0.05$, $^{**}P<0.01$ as compared with the control.

Table 1. Concentrations of cytokines in A2058 supernatant after He–Ne laser irradiation

	Laser dosage (J/cm^2)			
Cytokine (pg/ml)	**0**	**0.5**	**1.0**	**2.0**
IL-8	7,800±0	10,000±707.1*	11,350±495.0**	13,600±1,414.2*
TGF-β1	1,904±15.8	2,038.4±324.7	2,217.6±79.2*	2,212±51.5*

TGF-β1, transforming growth factor beta 1.
The data are expressed as mean±SD.
$^{*}P<0.05$, $^{**}P<0.01$ as compared with the control.

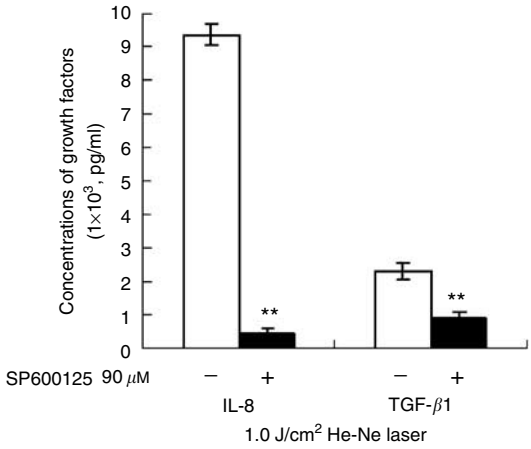

Figure 10. Effect of JNK inhibitor on IL-8 and TGF-β1 release after He-Ne laser treatment. A2058 cells were treated or untreated with 90 μM SP600125 before 1.0 J/cm^2 He-Ne laser treatment. The concentrations of growth factors in the culture supernatants derived from A2058 cells after 24 hours irradiated were measured. $^{**}P<0.01$ as compared with the control.

the effect of JNK inhibitor (SP600125) on growth factors release, cells were treated with SP600125 before 1.0 J/cm^2 He-Ne laser treatment. Our results showed that SP600125 significantly reduced IL-8 and TGF-β1 release (Figure 10).

DISCUSSION

In this study, we provide evidence indicating that low-energy He-Ne laser radiation enhances A2058 melanoma cell proliferation. This novel finding raised our interest and prompted us to elucidate the signaling mechanism of A2058 cell proliferation induced by He-Ne laser irradiation. We hypothesized that this photostimulatory effect involves the absorption of a specific wavelength by the functioning photoacceptor molecule. Cytochrome *c* oxidase is the terminal member of the respiratory chain, coupling the transfer of electrons from cytochrome *c* to molecular oxygen. Cytochrome *c* oxidase may be a mitochondrial photoacceptor when cells are irradiated with monochromatic red to near-infrared radiation. Previous studies indicated that irradiation with an He-Ne laser caused considerable activation of cytochrome *c* oxidase (Fedoseyeva *et al.*, 1988; Pastore *et al.*, 2000). Our results indicate that treating A2058 cells with an He-Ne laser higher than 0.5 J/cm^2 results in a significant increase in cytochrome *c* oxidase activity. One can speculate that this might be a secondary effect due to generation of reactive oxygen species following activation of other subcellular chromophores. Thus, two fluorescent dyes of 2′,7′-dichlorofluorescein diacetate (10 μM) and hydroethidine (10 μM) were used to clarify this issue. The intracellular fluorescence intensity of 2′,7′-dichlorofluorescein (DCF) or hydroethidine is proportional to the amount of reactive oxygen species produced by the cells. Reactive oxygen species was not involved in the photobiologic effect induced by an He-Ne laser (data not shown). As a result, we propose that an He-Ne laser may induce a cascade of reactions connected with alteration in cellular homeostasis molecules in A2058 cells.

Several studies demonstrated that mitochondria are sensitive to visible monochromatic light irradiation (Karu *et al.*, 1995). Mitochondria plays a central role in cellular homeostasis, and its homeostatic center is mitochondrial membrane potential ($\Delta\Psi_{mt}$). Thus, the assessment of $\Delta\Psi_{mt}$ in cells is worth investigating. Our experimental data indicated that A2058 cells treated with dosages of higher than a 0.5 J/cm^2 He-Ne laser exhibited a marked increase in $\Delta\Psi_{mt}$. Fluorescence intensity for the irradiated cases was larger than in non-irradiated cells, indicating an increase in ATP synthesis (Hilf *et al.*, 1986; Karu, 1999). In addition, a mitochondria-specific ionophore CCCP strongly abrogated the enhanced $\Delta\Psi_{mt}$ of A2058 cells irradiated with an He-Ne laser. As a consequence, $\Delta\Psi_{mt}$ may play an important role in the He-Ne laser irradiation-induced biologic effect on A2058 cells.

Previous studies showed that irradiation with an He-Ne laser causes an enhancement of DNA and RNA synthesis (Karu *et al.*, 1984). These effects depend on the available energy, and the mechanisms involved in the absorption of energy in the transduction of radiation into chemical energy. The most available energy in cells is the energetic ATP system. Our data showed that cells treated with irradiance higher than 0.5 J/cm^2 He-Ne laser exhibited significantly increased intracellular ATP content in A2058 cells. Application of CCCP abrogated the enhanced $\Delta\Psi_{mt}$ and ATP production of A2058 cells induced by an He-Ne laser. These results are consistent with our hypothesis that the mechanism of photobiomodulation involves the upregulation of cytochrome *c* oxidase, leading to increased $\Delta\Psi_{mt}$ and ATP content in A2058 cells irradiated with at least a 1.0 J/cm^2 He-Ne laser.

It has long been recognized that cAMP is an important intracellular messenger that is capable of regulating diverse cellular states, such as differentiation, proliferation, and synaptic plasticity. Light irradiation causes cAMP elevation, which in turn stimulates both DNA and RNA synthesis (Karu and Tiphlova, 1987). As a result, cAMP is synthesized from ATP by adenylate cyclase, and cAMP level might be dependent on cellular ATP synthesis and concentration (Zhang *et al.*, 2001). An increased cAMP level in A2058 cells pretreated with ATP after laser irradiation was observed. Our data revealed that dosage higher than 0.5 J/cm^2 He-Ne laser irradiation on A2058 cells caused an increased amount of intracellular cAMP. Other reports have shown that cAMP-elevating agents can stimulate mitogen-activated protein kinase activation; the activated mitogen-activated protein kinase translocated into the nucleus and induced transcription factor AP-1 activity (Hunt *et al.*, 1994; Englaro *et al.*, 1995; Pomerance *et al.*, 2000). Our results also showed that increased cAMP significantly enhanced the phosphorylation of JNK after He-Ne laser irradiation.

Low-energy laser irradiation has been found to activate the mitogen-activated protein kinase (Shefer *et al.*, 2002). JNK is activated in response to growth factor signaling and is phosphorylated primarily in response to cellular stress

(Ichihashi *et al.*, 2003). JNK is known to be an upstream kinase of AP-1, which promotes expression of genes involved in cell survival, proliferation, and angiogenesis (Briata *et al.*, 1993). Our study showed that JNK/AP-1 signaling pathways were activated in response to He-Ne laser irradiation. The increase of JNK-P expression after He-Ne laser irradiation was more than twofold as compared with the 0 J/cm^2 irradiated group. A significant increase in AP-1 activity of A2058 cells treated with irradiance equal or greater than a 1.0 J/cm^2 He-Ne laser was also observed. To clarify if JNK/AP-1 activity is induced by He-Ne irradiation, A2058 cells were treated with the JNK inhibitor SP600125 before He-Ne irradiation. The inhibitory effect of SP600125 on JNK phosphorylation as well as AP-1 activity were confirmed.

Melanoma cells secrete a variety of growth factors either constitutively or subsequent to induction by other cytokines (Matte *et al.*, 1994). These growth factors/cytokines may act as autocrine growth factors or act in paracrine fashion on the host environment to stimulate growth (Herlyn, 1990). IL-8 is a multifunctional cytokine that can stimulate proliferation of melanoma cells and keratinocytes in both an autocrine and a paracrine fashion (Schadendorf *et al.*, 1993). TGF-β1, the most abundant isoform of the TGF-β family, is elevated in the plasma of melanoma patients, especially those with metastatic lesions (Krasagakis *et al.*, 1998). Our results showed that there was a significant release of IL-8 and TGF-β1 from A2058 cells after treatment with a >0.5 J/cm^2 He-Ne laser.

The data gathered in this study demonstrated that cells irradiated by an He-Ne laser resulted in an increase in cytochrome *c* oxidase, $\Delta\Psi_{mt}$, ATP, and cAMP. In addition, He-Ne laser irradiation also induced mitogen-activated protein kinase/JNK phosphorylation, resulting in an activation of the transcription factor AP-1. These results were obtained immediately after He-Ne laser irradiation. Moreover, He-Ne laser treatment further showed significant stimulatory effects on IL-8, and TGF-β1 release by A2058. Because AP-1 can induce factors/cytokines expression (Wolf *et al.*, 2001), the assay for cytokine release from cells was performed 24 hours after He-Ne laser treatment. Furthermore, the cell growth reached its significant increase in A2058 cells 3 days after He-Ne treatment. As the stimulatory effect of these biomolecules in A2058 cells by He-Ne laser irradiation was time-responsive, we propose that an He-Ne laser stimulates cell proliferation through the mitochondrial pathway, which subsequently activates JNK and increases the transcriptional activities of AP-1. These activations were involved in increased expression of IL-8 and TGF-β1 ultimately leading to cell proliferation (Figure 11). Other pathways may also be involved in this complicated biological phenomenon and further investigation is needed to address this issue. In addition, we describe the effect of He-Ne laser illumination on a single melanoma cell line *in vitro*. Further investigations are needed to elucidate the mechanisms involved in photostimulatory effects induced by He-Ne laser irradiation on normal pigment-producing cells. Our results, nevertheless, indicate that visible light can cause cellular physiologic changes and can have photobiomodulatory effects on cellular proliferation.

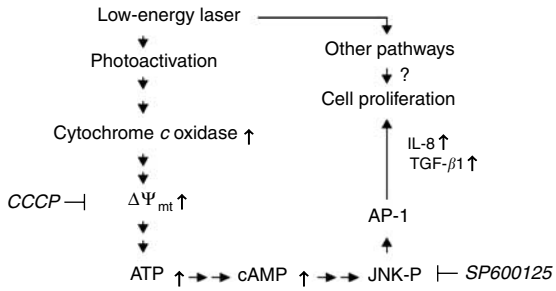

Figure 11. A proposed mechanism for He-Ne laser-induced cell proliferation in the A2058 melanoma cell. He-Ne laser irradiation stimulates A2058 cell proliferation by the mitochondrial pathway and subsequent increases in the transcriptional activities of AP-1, which could be involved in increased expression of IL-8 and TGF-β1 leading to cell proliferation. The locations where various inhibitors (italicized) act are also shown.

MATERIALS AND METHODS

All the described experiments were approved by the Medical Ethical Committee of Kaohsiung Medical University and were conducted according to the Declaration of Helsinki Principles.

Cell culture

Human melanoma cell line A2058, purchased from American Type Culture Collection (Manassas, VA), was maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate (Gibco, Grand Island, NY). Melanoma cells were passaged at confluence after treatment with 5 mM EDTA (Gibco) and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

He-Ne laser irradiation

The method for He-Ne laser irradiation was described in our previous study (Yu *et al.*, 1996). Briefly, an He-Ne laser used (Lasotronic MED-1000, Lasotronic, Zugerstr, Switzerland) has an output of 10 mW with a diverging lens that delivered 7.0 mW (as measured by a power meter, POW-105, Lasotronic) to a platform 17 cm under the lens where the dishes were placed. The cultured cells were rinsed with phosphate-buffered saline (PBS) and then irradiated with 0, 0.5, 1.0, and 2.0 J/cm^2 of He-Ne laser in PBS to minimize the loss of laser energy through absorption by colored culture medium. The calculation formula of designated time for laser treatment is t(s) = energy (J/cm^2) × surface (cm^2)/power (W) (Saliba and Foreman, 1990).

In addition, to clarify whether any biologic alterations are the result of a direct irradiation effect, rather than a thermal event, the laser irradiation-induced temperature changes were measured by a digital thermograph. All irradiation experiments were repeated in triplicate and all dishes within an experiment (including controls) were maintained in PBS at room temperature and atmosphere during the period of experimentation.

Growth curve determination

The effect of He-Ne laser irradiation on A2058 cell proliferation was assessed by counting the number of cells. A2058 cells were seeded on 24-well plates with a density of 4×10^3 cells/well and were incubated overnight. Then cells were irradiated with 0, 0.5, 1.0, and 2.0 J/cm^2 He-Ne laser radiation and incubated for 1–5 days,

respectively. The cells were then washed with PBS and harvested by gentle trypsinization, followed by mixing with an equal volume of trypan blue. The number of living cells was counted with a hematocytometer chamber.

Immunocytochemical staining for p53, bcl-2, and ki-67

To clarify the roles of the p53 tumor suppressor gene, the antiapoptotic protein bcl-2, and the tumor proliferation marker ki-67 in A2058 cells treated with He-Ne irradiation and to correlate its expression with apoptotic and cell proliferation, immunocytochemical studies were carried out for the expression of p53, bcl-2, and ki-67 protein on A2058 cells with and without exposure to He-Ne laser irradiation (48 hours after 0–2 J/cm^2). A2058 cells were seeded on 24-well plates with a density of 4×10^3 cells/well and were incubated overnight. The cells were treated with 0, 0.5, 1.0, and 2.0 J/cm^2 of He-Ne laser irradiation. Immunocytochemistry was performed on all test samples after 48 hours. They were immersed for 30 minutes in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. They were incubated overnight with primary antibodies (mouse anti-p53, mouse anti-bcl-2, and mouse anti-ki-67 at 1:100) at 4°C. Subsequently, slides were incubated with secondary antibodies and streptavidin peroxidase reagent, respectively, at room temperature. In all cases, 3,3-diaminobenzidine was used as chromagen and hematoxylin as counterstaining. One hundred cells for each group were selected to measure the number of positive cells stained by antibodies p53, bcl-2, and ki-67.

Cytochrome *c* oxidase assay

The expression of cytochrome *c* oxidase was determined based on observation of the decrease in absorbance at 550 nm of ferrocytochrome *c* caused by its oxidation to ferricytochrome *c* by cytochrome *c* oxidase. After indicated laser irradiation, the cells were harvested and lysed immediately with ice-cold radio immunoprecipitation assay buffer and cell extracts were obtained. After centrifugation to remove cell debris, we collected the protein in the supernatants and quantified the protein by the Bradford method (Bio-Rad, Hercules, CA). The measurement of cytochrome *c* oxidase activity was determined according to the protocol provided with the cytochrome *c* oxidase assay kit (Sigma, St Louis, MO).

Assessment of mitochondrial membrane potential

A2058 cells were seeded in 35-mm dishes and allowed to reach exponential growth for 24 hours before treatment. The medium was removed and the adherent cells trypsinized after 0, 0.5, 1.0, and 2.0 J/cm^2 of He-Ne laser irradiation. The cells were pelleted by centrifugation at 400 *g* for 5 minutes and stained in a 100 nM/ml $DiOC_6$ dye (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Each suspension was divided into two 0.5 ml portions, 30 μM CCCP, an uncoupling agent that abolishes the $\Delta\Psi_{mt}$, was added to one portion for 20 minutes at 37°C. Then cells were analyzed immediately for fluorescence (FL-1 detector, filter 530/30 nm band pass) on a FACScan flow cytometer (Elite ESP, Beckman Coulter, Brea, CA). Histograms were analyzed using Windows Multiple Document interface software (WinMDI, Version 2.8).

ATP content bioluminescence assay

The amount of intracellular ATP was determined by bioluminescent assay based on the measurement of the light output of the luciferin–luciferase reaction. The luciferin–luciferase was purchased as a kit from ThermoLabsystems Luminoskan Ascent (Vantaa, Finland). After indicated laser irradiation, total A2058 cell extracts were obtained immediately by lysing methods. After centrifugation to remove cell debris, we collected supernatants for ATP measurement. The amount of ATP was determined by the ATP monitoring kit.

Measurement of intracellular cAMP level

After irradiation with an He-Ne laser (0–2 J/cm^2), total cell extracts from cultured A2058 cells were obtained immediately by lysing methods. After centrifugation to remove cell debris, we collected supernatants for cAMP measurement. The total amount of intracellular cAMP was determined according to the protocol provided with the cAMP immunoassay kit (R&D Systems, Minneapolis, MN).

Protein extraction and A2058 western blotting analysis

Total cell extracts from cultured A2058 cells were obtained by lysing the cells in ice-cold radio immunoprecipitation assay buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 100 μg/ml phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml NaF. After centrifugation at 14,000 g for 30 minutes, protein in the supernatants was quantified by the Bradford method (Bio-Rad). Forty micrograms of protein per lane was applied in 10% SDS-polyacrylamide gel. After electrophoresis, protein was transferred from the gel to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked at room temperature for 1 hour in PBS + 0.1% Tween 20 containing 5% skim milk. After being briefly rinsed with PBS + 0.1% Tween 20, the membrane was incubated with primary antibody at room temperature for 2 hours or at 4°C overnight. Rabbit polyclonal antibodies against JNK (46 kDa), mouse mAb against p-JNK (46 kDa), actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), respectively. For the blocking test, 90 μg/ml of SP600125 (a potent inhibitor for JNK) (Bennet et al., 2001) was added to the media 2 hours before irradiated with 1.0 J/cm^2 He-Ne laser. The membrane was incubated with the corresponding horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 hour. Membranes were washed with PBS + 0.1% Tween 20 four times for 15 minutes and the protein blots were visualized with the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA). The relative amounts of specific proteins were quantified by densitometry scanning of X-ray films, and analyzed by the Eagle Eye Image System (Stratagene, La Jolla, CA).

Reporter transfection and luciferase assay

A2058 cells were plated in 35-mm dishes to 80% confluence. After 1 day of culture, cell transfection was performed according to the Lipofectamine reagent protocol (Life Technologies, Grand Island, NY). In brief, 1 μg of pAP1-Luc plasmid (Stratagene) together with 1 μg of pSV-β-galactosidase plasmid was mixed with 3 μl Lipofectamine reagent to a final volume of 200 μl serum-free Dulbecco's minimal essential medium containing no antibiotics and then was incubated at room temperature for 2 hours to allow transfection complex formation. Dulbecco's minimal essential medium (without antibiotics) (1.3 ml) was added into the reaction tube containing the transfection complexes, mixed, and then transferred to A2058 in the

plates. After 6 hours incubation, 1.5 ml Dulbecco's minimal essential medium containing 10% fetal bovine serum (without antibiotics) was added to the transfected A2058 which were incubated for an additional 12 hours. After the transfected A2058 cells were treated with different dosages of He-Ne laser irradiation, cell lysates were collected immediately using 0.4 ml of lysis buffer (0.1 M KH_2PO_4, pH 7.9, 0.5% Triton X-100, 1 mM dithiothreitol). Luciferase activity (Sambrook and Russell, 2001b) and β-galactosidase activity (Sambrook and Russell, 2001a) were measured by standard protocol. Luciferase activity was normalized with β-galactosidase activity.

Measurement of cytokines in culture supernatant using ELISA

The concentrations of cytokines in the culture supernatants derived from 5×10^5 A2058 cells/ml after 24 hours irradiated with different dosage of He-Ne laser were measured using commercially available ELISA kits, including IL-8 and TGF-β1 (Quantikine, R&D Systems). The results are presented as mean $\pm$ SD.

Statistical analysis

The results are expressed as mean $\pm$ SD and analyzed by using the statistical analysis system (SPSS, SPSS Inc., Chicago, IL). Differences among groups were analyzed by Student's *t*-test. *P*-values <0.05 were considered as significant for all statistical tests.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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