Lasers Med Sci (2013) 28:693–700
DOI 10.1007/s10103-012-1127-y

ORIGINAL ARTICLE

High-power helium–neon laser irradiation inhibits the growth of traumatic scars in vitro and in vivo

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Received: 7 September 2011 / Accepted: 24 May 2012 / Published online: 8 June 2012

Abstract This study explored the inhibitory effect of the high-power helium–neon (He–Ne) laser on the growth of scars post trauma. For the in vitro study, human wound fibroblasts were exposed to the high-power He–Ne laser for 30 min, once per day with different power densities (10, 50, 100, and 150 mW/cm^2). After 3 days of repeated irradiation with the He–Ne laser, fibroblast proliferation and collagen synthesis were evaluated. For in vivo evaluation, a wounded animal model of hypertrophic scar formation was established. At postoperative day 21, the high-power He–Ne laser irradiation (output power 120 mW, 6 mm in diameter, 30 min each session, every other day) was performed on 20 scars. At postoperative day 35, the hydroxyproline content, apoptosis rate, PCNA protein expression and FADD mRNA level were assessed. The in vitro study showed that the irradiation group that received the power densities of 100 and 150 mW/cm^2 showed decreases in the cell proliferation index, increases in the percentage of cells in the G0/G1 phase, and decreases in collagen synthesis and type I procollagen gene expression. In the in vivo animal studies, regions exposed to He–Ne irradiation showed a significant decrease in scar thickness as well as decreases in hydroxyproline levels and PCNA protein expression. Results from the in vitro and in vivo studies suggest that repeated irradiation with a He–Ne laser at certain power densities inhibits fibroblast proliferation and collagen synthesis, thereby inhibits the growth of hypertrophic scars.

Keywords Helium-neon (He–Ne) · Laser · Scar · Collagen · Fibroblast · Growth

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Introduction

Hypertrophic scar is a common post-traumatic complication seen especially in burns. Abnormal scarring has numerous ramifications, ranging from changes in appearance and normal body function to disfigurement and the loss of the ability to engage in normal activities of daily living, which can lead to physiological and psychological damages [1]. Various therapeutic regimens have been used for the management of hypertrophic scars, such as surgical removal, cryotherapy, corticosteroid, pressure and radiation therapy. Unfortunately, most of these treatments often were of little benefit or had side effects that were nearly as severe as the original scar [2–4].

Since the 1980s, laser therapy has been used to treat scars, with vaporization ablative lasers and CO_2 canonization lasers being the most commonly used forms of laser treatment. However, scar recurrence or even worsening of the wound inevitably occurs because of the formation of a new wound surface [3, 4]. Low-intensity lasers (LIL), such as He–Ne lasers, do not cause local tissue damage, even when applied at a high power for a longer duration of time. The wavelength of the He–Ne laser is consistent with the absorption wavelength of fibroblasts (630 nm) [5] and can penetrate human skin [6]. Numerous studies have reported data concerning the activating and stimulatory effects of He–Ne laser irradiation [7–9]. The biological effects of the He–Ne laser have been widely applied in the clinical treatment of refractory wounds and chronic ulcers [10]. Many studies have examined the inhibitory effects of the He–Ne laser on fibroblast growth. Breugel et al. [7] reported that He–Ne laser irradiation with 58.08 and 55.87 J inhibited fibroblast proliferation. Gross et al. [11] using a He–Ne laser with 3.81~45.44 J showed that repeated irradiation inhibits the growth of kidney epithelial fibroblasts. Our previous study reported that repeated irradiation with the high-power He–Ne laser (57.6 J) for three and five times inhibits fibroblast growth in culture [12], suggesting that the high-power He–Ne laser can be used in the prevention and treatment of hypertrophic scars.

The purpose of this study was to investigate the effect of high-power He–Ne laser irradiation on scar formation through both in vitro and in vivo investigations. In vitro, we examined the relationship between the power density of the He–Ne laser, scar fibroblast growth and collagen. In vivo, we explored the inhibitory effects of high-power He–Ne laser irradiation on hypertrophic scars by establishing a specific animal wound model to provide experimental evidence for its clinical applicability with respect to the prevention and treatment of hypertrophic scars.

Materials and methods

The inhibitory effect of the He–Ne laser on the fibroblast growth in hypertrophic scars: an in vitro study

Cell culture

Five patients with hypertrophic scars [13] were included in the study. The participants ranged in age from 14 to 38 years old; three were male, and two were female. The scars had been present for an average of 16.7 months (range 8–24 months). Human skin fibroblast primary cultures were derived from scar tissue biopsies [14] and grown in 10 % (*v*/*v*) bovine serum DMEM medium (Gibco BRL, Gaithersburg, MD, USA) in a CO_2 incubator under 100 % humidity at 37 °C. After full diffusion, the cultured cells were trypsinized using 0.25 % (*w*/*w*) trypsin and 0.02 % EDTA-Na_2 (Sigma, Woonsocket, RI, USA). The cells were passaged every 4–5 days. Cell lines 4 to 10 were used for the in vitro experiments.

Approximately 5,000 cells per well were seeded in a 96-well cell culture plate. The cells were seeded in every other row and every other column, yielding a total of five wells per plate for treatments of 10, 50, 100, 150 mW/cm^2 and a control group. The four experimental groups were treated with laser exposure at the corresponding power, whereas the control group was not treated with the laser. A total of 20 culture plates were seeded in the following manner: five plates for cell counting, five plates for cell cycle analysis, five plates for collagen synthesis analysis, and five plates for procollagen gene expression analysis. The seeded cells were incubated in a CO_2 incubator for an additional 24 hours. Prior to laser exposure, the culture medium was removed, and cells were rinsed with Hank's balanced solution. The cells were irradiated by the laser directly, without culture medium. Fresh culture medium was added afterwards.

Laser irradiation

A high-power He–Ne laser (HN-120, Chongqing Aobomedical Corp, Chongqing, China) was used with a wavelength of 632.8 nm. The laser had three output optical fibers. Prior to irradiation, the fiber's position was adjusted such that the exposure spot completely covered the bottom of the well plate (size=0.32 cm^2). The irradiation was vertical. The actual output power was determined by a columnar laser power meter (CLP-1, Beijing Institute of Radiation Medicine, Beijing, China). The output power in this study was 3, 16, 32, and 48 mW, and the corresponding power density was 10, 50, 100, and 150, respectively. The exposure duration was 30 min, once per day. After 3 days of repeated exposure, cells from each group were trypsinized and collected. The Trypan Blue stain was used for cell counting. The cell cycle phase was assessed using flow cytometry. Collagen synthesis and type I procollagen gene expression were assessed using a ^{3}H proline incorporation assay and dot blot assay, respectively.

Cell counting

Trypan Blue stain, 0.1 % (*w*/*w*), was used for cell staining. After random selection of three fields of view, the live and dead cells were counted separately three times, and the average value was used for data analysis.

Cell cycle analysis

The trypsinized cells were collected and fixed in 70 % ethanol then incubated at 4 °C overnight. After the cells were rinsed with PBS and treated with 1 % RNase in Tris–HCl buffer (pH 7.4) for 30 min (37 °C), PI dye was applied

to stain the cell's DNA. FACS420 flow cytometry (BD Biosciences, San Jose, CA, USA) was used to measure the proportion of cells in different phases of the cell cycle. The excitation wavelength was 488 nm.

Cell collagen synthesis assay

Approximately 24 h after the 3 days of repeated laser irradiation, the medium was removed and replaced with 100 μl of serum-free culture medium with 0.5 μCi ^{3}H-proline (China Institute of Atomic Energy, Beijing, China), 50 mg/ml ascorbic acid and 100 μg/ml beta-aminopropionitrile (Sigma, St. Louis, MO, USA). After an additional 24 h of culture, the cells were trypsinized and collected in perchloric-acid-pretreated glass fiber filters and rinsed several times with 5 % (*w*/*w*) trichloroacetic acid and methanol. After drying, the amount of ^{3}H-proline incorporated was measured using scintillation counting with the LKB-1217 liquid scintillation counter (LKB Instruments Inc, Gaithersburg, MD, USA).

Type I procollagen gene expression assay

The type I procollagen cDNA plasmid was provided by Dr. Uitto as a gift. Details concerning restrictive enzyme digestion and probe labeling can be found in the reference noted [15]. Twenty-four hours after the 3 days of repeated laser irradiation, the cells were trypsinized and collected. The total cellular RNA was extracted with a one-step procedure. The sample consisted of 10 μl of RNA solution with formamide, formaldehyde, and 20× SSC. The solution was incubated at 68 °C for 15 min to denature the RNA. Then the RNA sample was added to an NC membrane using a point sampler and baked for 3 h at 80 °C in an oven. The sample was then sealed in a plastic bag and added to the pre-hybridization-solution-infiltrated NC membrane at 42 °C overnight. The probe was added at a ratio of 1 μl of probe for 0.5 ml of pre-hybridization solution. The hybridization occurred at 42 °C for 24 h. The membrane was then rinsed using Buffers I and II. The antibody (1:5,000 dilution) was added at 37 °C for 30 min. The membrane was again rinsed using Buffers I and III. The developing reagent was added in the dark. After the appropriate color developed, Buffer IV was added to stop the reaction. A density scan analysis was performed using the thin layer scanner (LS-9000, SHIMADIU Corp, Nakagyoku, Kyoto, Japan) to determine the relative amount of type I procollagen mRNA [16].

The inhibitory effect of the He–Ne laser on the fibroblast growth of hypertrophic scars: an in vivo animal study

Animal wound model

A total of 24 adult Japanese white rabbits were randomly selected by gender and weight, which ranged between 2.0 and 2.5 kg, with an average of 2.35 kg. The wound model was induced as described in previous studies [17]. Briefly, the animals were anesthetized with 3 % pentobarbital sodium (1.5 mg/kg). On the ventral surface of each rabbit ear, four circular areas of full-depth sections of skin, 6 mm in diameter, were removed with a diamond knife. The total number of wounded surfaces was 64. After wounding, the animals were treated with general antibiotics and allowed to eat freely. All animal experiments were performed in accordance with the National Institutes of Health guidelines on animal care and with the approval of the Ethics Committee of Daping Hospital, Third Military Medical University, China.

Laser irradiation protocol

Twenty-one days after the wound creation, the scar thickness, which was most prominent over the wound edge, was measured using a Vernier caliper (Chengdu Measuring & Cutting Tool Corp, Chengdu, China, accuracy=0.02 mm). The measurement was repeated three times for each scar to calculate an average value. Then 20 scars were randomly selected to receive He–Ne laser irradiation (laser group), whereas the remaining 20 scars were considered to be the control group (control group). Prior to irradiation, the optical fiber was adjusted such that the irradiation spot covered the entire wound at a vertical irradiation angle. The HN-120 He–Ne Laser was used. The output power was 120 W (corresponding power density=333 mW/cm^{2}), the irradiation duration was 30 min, and the laser irradiation was performed every other day. Thirty-five days after wounding, the wound tissue was dissected in both the laser and control groups. The wound sample was stored in liquid nitrogen at −196 °C. The scar thickness was measured again prior to the dissection of the wound tissue.

In situ apoptosis assay

The tissue sample stored in liquid nitrogen (−196 °C) was cut into slices (30-μm thickness, AS620, Shandon Corp, Sewickley, PA, USA). Employing the TUNEL technique, we used an in situ cell death detection kit (POD, Boehringer Mannheim Corp, Indianapolis, IN, USA) to identify apoptotic cell bodies, which appeared as a nucleus containing brown granules (TUNEL-positive cells) under light microscopy. Three fields of view were selected randomly to determine the positive cell number under high magnification. The percentage of apoptotic cells is shown as the number of apoptotic cell bodies per 100 cells.

Immunohistochemistry

Based on the streptavidin/peroxidase (SP) method, a PCNA monoclonal antibody (PC-10, Santa Cruz Biotechnology

Inc, Santa Cruz, CA, USA) was diluted at a ratio of 1:100 and incubated with the samples, followed by conventional DAB staining, hematoxylin staining, dehydration, transparency, and sealing. The primary antibody was replaced by PBS for the control. The true-color imaging analysis system (CMIAS007, Chongqing Aobomedical Corp, Chongqing, China) was used for quantitative analysis, and four regions were randomly selected under 20× magnification. The positive cells were selected using a defined threshold, and the average integrated optical density (AIOD) was then measured for each group.

Hydroxyproline assay in scar tissue

The tissue sample was weighed after it was removed from liquid nitrogen (−196 °C), and it was then placed in hydrolysis tubes. After the addition of 5 ml of hydrochloric acid (6 mol/L), the sample was dried using nitrogen air for 30 min. The tubes were then sealed with a vacuum. After hydrolysis for 22–24 h, the tube was opened and evaporated at 100 °C in a water bath to remove the hydrochloric acid. The hydroxyproline amount was measured using the Amino Acid Analyzer (6300, Beckman Instruments Inc, Fullerton, California, USA).

Statistical analysis

The results are presented as the mean±standard deviation ($x \pm s$). Data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc LSD (least square difference) test with significant set at $P<0.05$.

Results

The effect of He–Ne laser irradiation on scar fibroblast proliferation

The Trypan Blue stain cell-counting assay showed no significant changes in the total cell number using 10 and 50 mW/cm^2 irradiation when compared with the control group. A significant decrease in cell number was observed in the groups irradiated with 100 and 150 mW/cm^2 (P=0.045826 and P=0.038764, respectively).

Cell cycle analysis showed the proliferation index (PI, calculated as [S/(G0/G1+S+G2/M*%)]) of the control group was 50.4 %. No significant difference for PI was found among samples exposed to a 10 mW/cm^2 laser, those exposed to a 50 mW/cm^2 laser and the control group. Cells irradiated with 100 and 150 mW/cm^2 had less PI staining than the control group. The difference was significant (P=0.045386 and P=0.038664, respectively, Fig. 1). Cells exposed to 100 and 150 mW/cm^2 irradiation showed an increase in the fraction of cells in the G0/G1 phase when compared with the control group. The difference was statistically significant (P=0.045625 and P=0.036824, respectively).

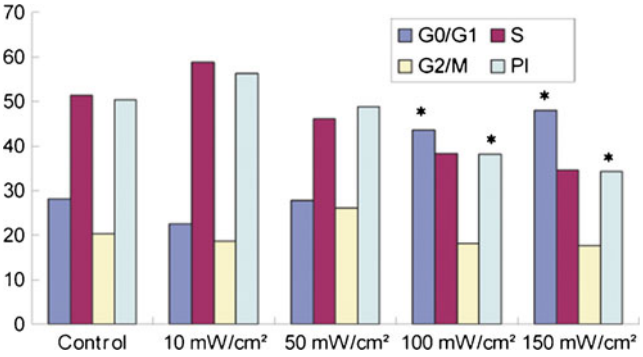

Fig. 1 Relationship between the power density of the He–Ne laser and PI staining in cultured scar fibroblasts. Different from control: *P< 0.05

Effect of He–Ne laser irradiation on collagen synthesis and the type I procollagen gene expression of scar fibroblasts

Figure 2 shows the results of the dot blot hybridization. No significant difference in collagen synthesis and type I procollagen gene expression was found after the cells were irradiated with the 10 and 50 mW/cm^2 lasers. Cells irradiated with the 100 and 150 mW/cm^2 lasers had significantly lower levels of collagen synthesis than the control group (P=0.046588 and P=0.025654, respectively, Fig. 3). They also had lower levels of type I procollagen gene expression than the control group (P=0.048244 and P=0.022032, respectively). The difference was statistically significant. In addition, the group irradiated with 150 mW/cm^2 had lower levels of type I procollagen than the group irradiated with 100 mW/cm^2 (P=0.044652).

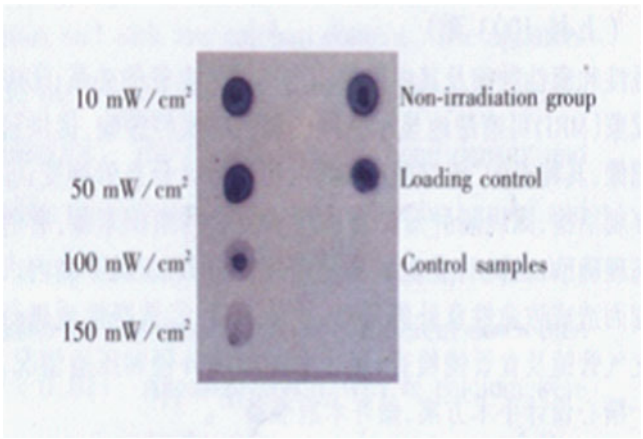

Fig. 2 Blot hybridization of type I procollagen cDNA in cultured scar fibroblasts

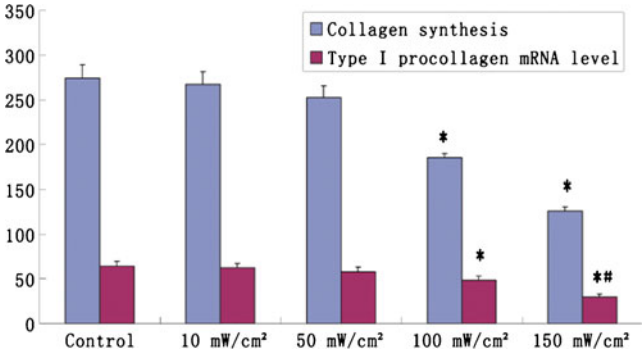

Fig. 3 Effect of the He–Ne laser on collagen synthesis and type I procollagen mRNA levels in cultured scar fibroblasts. Different from control: $^{*}P<0.05$; different from 100 mW/cm^2: $^{\#}P<0.05$

The effect of He–Ne laser irradiation on hypertrophic scars in an animal model

Before laser irradiation (21 days post-wounding), the scar thickness was not significantly different between the two groups ($P=0.837228$). Two weeks after laser irradiation (35 days post-wounding), the scar of the laser group (Fig. 4) was thinner than that of the control group (Fig. 5), and the difference was statistically significant ($P=0.031478$, Fig. 6). Prior to laser irradiation (21 days post-wounding), the levels of ^{3}H-hydroxyproline (ωB/mg g^{-1}) were not significantly different between the two groups. After laser irradiation (35 days post-wounding), the levels of ^{3}H-hydroxyproline were significantly lower in the laser group than in the control group ($P=0.032516$, Fig. 7). Immunohistochemistry showed PCNA protein expression in both the laser and the control groups. Under light microscopy, PCNA protein was found in the nuclei of fibroblasts and vascular cells. The AIOD was 427.78±92.83 in the laser group (Fig. 8) and 950.00±192.75 in the control group (Fig. 9). The difference was statistically significant ($P=0.023720$). TUNEL analysis revealed apoptotic cells in both the laser and control groups. The apoptosis rate in the laser group (Fig. 10) was 25.60 %±2.82 % and 18.00 %±2.48 % in the control group (Fig. 11). This difference was statistically significant ($P=0.034074$).

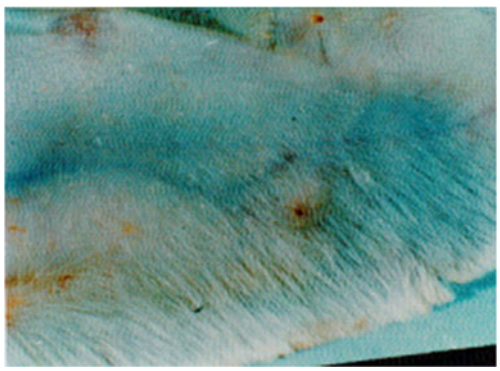

Fig. 4 Morphological observation of hypertrophic scars in rabbits in the laser group

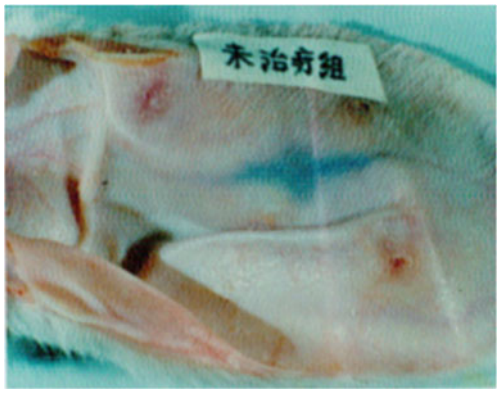

Fig. 5 Morphological observation of hypertrophic scars in rabbits in the control group

Discussion

The He–Ne laser inhibits the growth of scar fibroblasts and collagen synthesis

He–Ne and gallium arsenide (GaAs) lasers are the most commonly used LIL in physiotherapy for promoting wound healing and pain modulation [18]. The in vitro biostimulation by LIL is dependent on laser irradiation parameters such as wavelength, laser output power, and energy density [19, 20]. Similar parameters can have different effects on cultured cells. Parameters that effectively improve cell growth can impair protein synthesis [19, 21]. Several parameters have been used to induce cell proliferation in vitro. Most studies have tested different energy densities [19–21]. Our previous study showed that the He–Ne laser can inhibit the growth of scar fibroblasts in culture. The inhibitory effects of the He–Ne laser on cellular growth may be closely related to the energy density of laser irradiation [12].

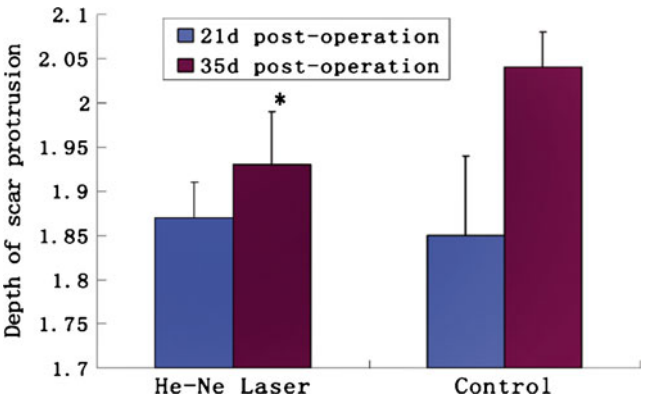

Fig. 6 A comparison of the depth of the scar protrusion between the two groups. Different from control: $^{*}P<0.05$

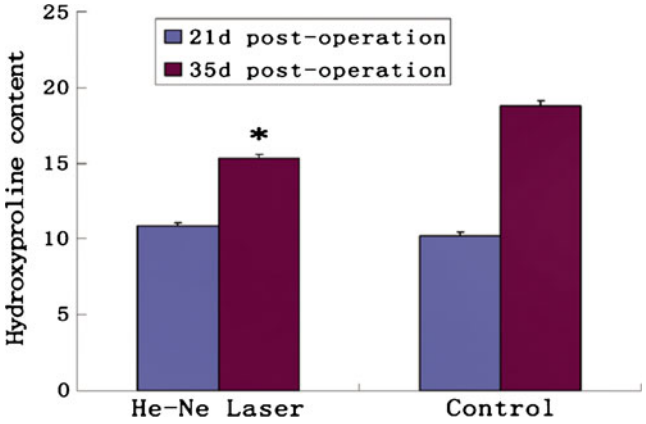

Fig. 7 Hydroxyproline content of scar tissue in the rabbit ear. Different from control: *$P<0.05$

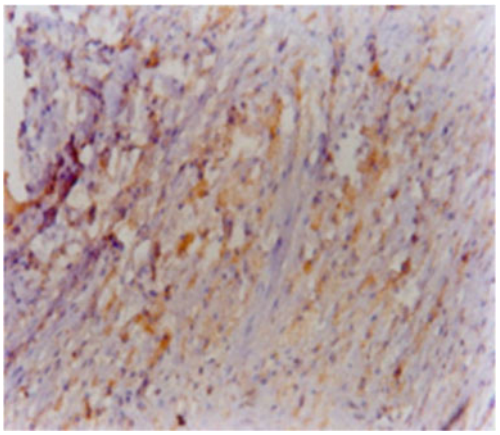

Fig. 9 PCNA protein expression in rabbit scar tissues in the control group (SP ×200)

According to the study by Breughel et al. [7], power density appears to be more important than total dose in wound healing. Wound healing is dependent on cell proliferation. It would then be important to test different power densities in the studies of cell proliferation in vitro. Azevedo et al. [22] reported that a 660-nm low-intensity GaAlAs diode laser at same energy (2 J/cm^2) can influence differently the cultured human fibroblast growth in function of power density, that is, there was an inverse relationship between power density and cell growth. The results from this study further showed that different power densities have a differential impact on cell proliferation. Laser irradiation using 10 and 50 mW/cm^2 did not result in a change in the cell PI. However, a significant decrease was seen in laser groups treated with the 100 and 150 mW/cm^2 lasers. These results suggest that the inhibitory effect of He–Ne laser irradiation on scar fibroblast proliferation may only occur when specific laser power densities are used. The results from the cell cycle assay showed no significant change in the percentage of cells in the prophase stage (G0/G1), the S phase or the post-phase of DNA synthesis (G2/M) in the irradiation groups using the 10 and 50 mW/cm^2 lasers. Irradiation using 100 and 150 mW/cm^2 resulted in a significant increase in the percentage of cells in the G0/G1 phase and a decrease in the percentage of cells in the S phase, which suggests that the inhibition of scar fibroblast growth may be directly related to cell arrest in the G0/G1 phase.

Use of differing laser power densities resulted in different effects on collagen metabolism in the scar fibroblasts. Wound cells exposed to 10 and 50 mW/cm^2 of irradiation showed no significant change in cellular collagen synthesis. A significant decrease in collagen synthesis has been shown with 100 and 150 mW/cm^2 irradiation. The results suggest that the inhibitory effect of He–Ne laser irradiation on the collagen synthesis of scar fibroblasts may only occur when particular laser power densities are used. There was no significant change in type 1 procollagen expression when using 10 or 50 mW/cm^2 irradiation. However, 100 and 150 mW/cm^2 irradiation resulted in a significant decrease in type 1 procollagen expression. The change in the expression level of type 1 procollagen is consistent with cell collagen synthesis, which indicates that the He–Ne laser

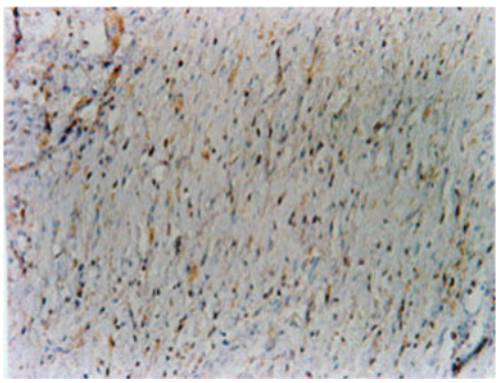

Fig. 8 PCNA protein expression in rabbit scar tissues in the laser group (SP ×200)

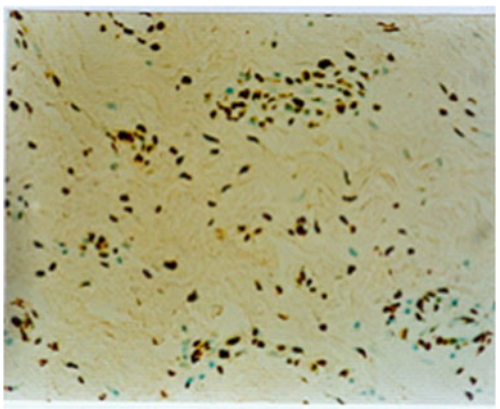

Fig. 10 In situ apoptosis assay in rabbit scar tissues in the laser group (TUNEL ×200)

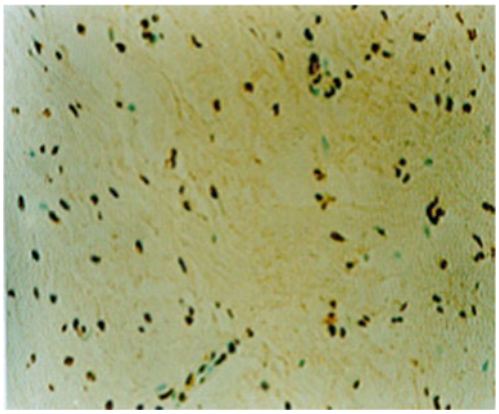

Fig. 11 In situ apoptosis assay in rabbit scar tissues in the control group (TUNEL ×200)

inhibits the collagen synthesis of scar fibroblasts by down-regulating the type 1 procollagen gene.

The mechanisms associated with the stimulatory effects of He–Ne laser has not been fully elucidated. One possibility may be that the laser energy is absorbed by intracellular chromophores and is converted into metabolic energy, that is, the respiratory chain [23], which produces changes in the redox status in both the mitochondria and cytoplasm. Activations of the electron transport chain result in an increase in the electrical potential across the mitochondrial membrane, an increase in the ATP pool, and finally the activation of nucleic acid synthesis [24]. This study used He–Ne laser power density far greater than reported in the literature, and mitochondrial photoreceptors—cytochrome *C* oxidase is very sensitive to the He–Ne laser [25]. Therefore, we speculate that: higher power density (100 and 150 mW/cm^2) He–Ne laser irradiation induced cell growth inhibition, probably due to the high-power He–Ne laser to change the mitochondrial cytochrome *c* oxidase activity (reduced or enhanced), the lack of mitochondrial ATP synthesis or excessive accumulation, leading to G0/G1 phase cells were arrested, I procollagen gene expression down.

The He–Ne laser inhibits the scarring process in animal models of hypertrophic scarring

The mechanism of hypertrophic scar formation during wound healing is generally considered to involve the abnormal proliferation of fibroblasts and the overproduction of collagen, which leads to excessive collagen deposition in the extracellular matrix [1]. Therefore, the inhibition of cell proliferation and (or) regulation of collagen metabolism represents an important therapeutic approach for hypertrophic scars.

The process of wound healing involves an acute inflammatory phase, elicited by the wounding, followed regeneration, migration, and proliferation of parenchyma and connective tissue cells, remodeling, collagenization, and finally acquisition of wound strength [26]. Large numbers of studies showed positive results of LIL therapy during wound healing, probably by promoting angiogenesis, reducing the inflammatory phase, modulating cytokine expression and inducing collagen synthesis [27–29]. Demidova-Rice et al. [8] reported biphasic dose response between energy density and wound healing, i.e., 2 J/cm^2 has the largest positive effect, 1 and 10 J/cm^2 improve healing to a lesser extent, while 50 J/cm^2 has a negative effect on wound healing. In this in vivo animal study, there was a significant reduction in the thickness of protruding scars 2 weeks after He–Ne laser irradiation when compared with the control group. Moreover, a significant reduction in hydroxyproline content and a significant down-regulation of PCNA protein were also observed in the laser group. The results from animal study suggest that the He–Ne laser inhibits the hypertrophic scar process in an animal wound model by inhibiting the growth of fibroblasts and the synthesis of collagen in scar tissue.

Apoptosis not only is involved in tissue reconstruction during the normal healing process but also is closely related to hypertrophic scar formation and regression [30]. Wounded cells exposed to He–Ne laser irradiation showed higher cell apoptotic rates than the non-irradiated control group, which implies that the induction of apoptosis may be related to the therapeutic effect of the He–Ne laser on hypertrophic scars. He–Ne laser irradiation can transform fibroblasts into myofibroblasts [31, 32]. Myofibroblasts are terminally differentiated cells that disappear during apoptosis [33]. It is still unclear whether myofibroblasts are related to the increase in apoptotic cells in hypertrophic scars exposed to the high-power He–Ne laser irradiation.

Conclusions

Repeated irradiation with the He–Ne laser at certain levels of power density can inhibit the growth of fibroblasts and the synthesis of collagen, thus inhibiting the hypertrophic scar process in an animal wound model. Further studies on the high-power He–Ne laser treatment are needed to determine long-term clinical outcomes with the use of this modality and the therapeutic effects of the laser on human hypertrophic scars.

Acknowledgments The authors are grateful to the Daping Hospital for Third Military Medical University to provide funding for research. We thank our colleagues and students at Daping Hospital of Third Military Medical University who assisted with the intellectual and technical development of this research.

Author disclosure statement The authors declare that there are no competing financial interests.

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