An Animal Study of the Effects on p16 and PCNA Expression of Repeated Treatment With High-Energy Laser and Intense Pulsed Light Exposure

Henry H.L. Chan, MD, FRCP,1,2* C.H. Yang,1 Joseph C.K. Leung, PhD,1 W.I. Wei, MS, FRCS,3 and K.N. Lai, MD, DSc, FRCP1
1Department of Medicine, University of Hong Kong, Hong Kong
2Department of Medicine and Therapeutic, Chinese University of Hong Kong, Hong Kong
3Department of Surgery, University of Hong Kong, Hong Kong

Background and Objective: Non-ablative skin rejuvenation treatments that involve the use of laser/light sources together with cooling devices have gained much popularity in recent years due to the lack of down time that is associated with them. One important but neglected issue is long-term safety. Does the repeated use of non-ablative skin rejuvenation lead to photoaging? Are we creating another sun-bed phenomenon? Recently, we performed an in vitro study to examine the effect of sub-lethal QS 755 nm lasers on the expression of p16INK4a on melanoma cell lines, and found that sub-lethal laser damage could increase DNA damage, which led to an increase in p16 expression. Our objective was to assess the cutaneous effect of repeated exposure to high-energy lasers and intense pulsed light sources on male Institute of Cancer Research (ICR) mice.

Study Design/Materials and Methods: Twenty-eight male ICR mice were divided into four groups. Other than the control group, all groups received either laser (585 nm pulsed dye laser or 1,320 nm Nd:YAG laser) or intense pulsed light (IPL) treatment. All four groups were anesthetized with a mixture of Hypnorm/Dormicum before treatment. The animals were irradiated twice a week for 6 months. Signs of toxicity such as mortality and weight loss were checked once a week. Skin tumor formation was evidenced by lesions of greater than 1 mm in diameter that persisted for 2 weeks. At the end of the 6 months, the expression of proliferating cell nuclear antigen (PCNA) and p16 in the mouse skin was determined by immunohistochemical staining and immunoblotting using specific monoclonal antibodies for mouse PCNA and p16. The results were expressed as mean ± standard error of the mean (SEM). Statistical difference was assessed by multiple ANOVA. A P-value of <0.05 was considered to be significant.

Results: At the end of the 6 months, none of the animals had developed any signs of toxicity such as mortality or weight loss. There was no evidence of tumor formation. There were significant elevations of p16 and PCNA in all treated groups as compared to the control group (ANOVA P<0.05). This particularly applied to the group that was treated with the 1,320 nm Nd:YAG laser.

Conclusion: The repeated use of high-energy laser and intense pulsed light source did not cause any toxicity in mice. The changes in p16 and PCNA imply that further studies are necessary to consider the implications of repeated exposure to longer wavelength radiation in human skin. Lasers Surg. Med. 39:8–13, 2007. © 2006 Wiley-Liss, Inc.

Key words: non-ablative; PCNA; p16

INTRODUCTION

Non-ablative skin rejuvenation involves the use of a laser/light source together with a cooling device, and in doing so improves the features of photoaging including lentigines, telangiectasia, pore size, skin texture, wrinkles, and skin laxity with minimal down time [1–7]. The use of a cooling device protects the epidermis, and in doing so allows a high-energy laser/intense pulse light (IPL) source (with a wavelength ranging from 570 to 1,340 nm) to be delivered to the dermis. A wide range of lasers or light sources can be used for non-ablative skin rejuvenation, including visible green–yellow (532 nm Nd:YAG, 585 nm or 595 nm pulsed dye laser), near infra-red and infra-red lasers (1,064 nm Nd:YAG, 1,320 nm Nd:YAG, 1,450 nm diode, 1,540 nm Erbium glass), and IPL sources [1–7].

Green and yellow lasers/light sources target the epidermal pigment and papillary dermal vessels. Injury to the papillary dermal vessels not only allows effective treatment of facial telangiectasia, but also leads to the subsequent healing process and new collagen formation [1,2]. Near infra-red and infra-red lasers/light sources together with skin cooling target the water content in the dermis, and their photothermal effect, produced as a result of the laser-tissue interaction, causes a rise in the dermal...
temperature [3–7]. The consequences are collagen tightening, increased fibroblastic activity, and increased collagen production.

Histological evidence suggests that non-ablative skin rejuvenation is associated with increased collagen production and shrinkage [8,9]. Trelles et al. studied the use of a 1,320 nm laser on Spanish patients and found histological improvement and fair to significant clinical improvement 4–6 months after twice weekly treatments for a total of 4 weeks (1,320 nm Nd:YAG, 30–35 J/cm², 30 milliseconds dynamic cooling, 40 milliseconds delay, 5 mm spot size, CoolTouch, Laser Aesthetics, Auburn, CA) [10].

One important but usually neglected issue is long-term safety. Does the repeated use of non-ablative skin rejuvenation lead to photoaging? Are we creating another sun-bed phenomenon? [11] Unlike most other forms of laser surgery, after a certain number of treatment sessions, surgery should be terminated if there is either complete improvement or no further improvement. Non-ablative skin rejuvenation can be addictive. Indeed, clinicians in Asia have proposed the daily use of a Q-switched 1,064 nm Nd:YAG laser (Medlite 4 and C6, HOYA, ConBio, Tokyo) for skin rejuvenation [12]. Recently, we saw a patient who was treated by a Hong Kong clinician for full-face skin rejuvenation using a Q-switched 1,064 nm Nd:YAG laser consecutively for 28 days, after which, upon the patient’s request, the chin area was treated for another 10 days, which led to areas of depigmentation on the chin and face (Fig. 1 and Fig. 2).

The cell division cycle is regulated at two restriction points that are located at the G₁/S and G₂/M transitions. At these points, the cell divides or moves into a nonproliferative state. The G₁/S restriction point is regulated by the retinoblastoma gene product (Rb), which acts as a gatekeeper. The retinoblastoma gene product binds to the E2F family of transcription factors and prevents their activities. E2F transcription factors are important in the activation of a wide range of genes, including those that are encoded for thymidine kinases, dihydrofolate reductase, and DNA polymerase alpha. These enzymes are required for the S phase progression of the cell cycle. Hence, the Rb protein indirectly prevents the expression of the S phase genes and inhibits further cell division. Rb activity depends on other regulatory factors that can either inhibit or enhance its function. Cyclin-dependent kinases (CDKs) are catalytic substances that require regulatory subunits known as cyclins to perform their kinase functions. CDK type 4 (CDK4), after binding with cyclin D, can phosphorylate Rb protein and halt its activity. This allows the cell cycle to progress and cell division to occur. The p16 gene competes with cyclin D (CD) for the binding of CD4, and prevents the formation of the CD4/CD complex, which leads to a reduction in the inactivation of the Rb protein. It is therefore an important tumor suppressor. The expression of the p16 locus is unique in the sense that it encodes not one but two genetic products, which arise from different promoters. The p16 gene consists of three exons: E1a, E2, and E3. The alternative transcript also utilizes E2 and E3, but instead of E1a it transcribes E1b, which leads to the production of a slightly smaller protein that is designated as p14ARF (an alternative reading frame).

The p16 gene is an important tumor suppressor, and its mutation is known to be linked to the development of melanoma. Mutation of the p16 gene can be found in approximately 70% of melanoma cell lines [13,14] and in 30%–50% of patients with familial melanoma [15].

Fig. 1. Depigmented macules on the chin after 38 days of consecutive treatment with a QS 1,064 nm Nd:YAG laser for skin rejuvenation (under cross polarized light).

Fig. 2. Depigmented macules on the face after 28 days of consecutive treatment with a QS 1,064 nm Nd:YAG laser for skin rejuvenation (under ultraviolet light).
melanoma cell lines, p16 deletion is more common than mutation. In addition to melanoma, families with known p16 mutation are also at risk of cancers other than melanoma, including pancreatic and breast cancer [16]. Recently, we performed an in vitro study that examined the effect of sub-lethal QS 755 nm laser on the expression of p16INK4a on melanoma cell lines, and found that sub-lethal laser damage could increase DNA damage, which leads to an increase in p16 expression. This effect would be particularly undesirable for patients with p16 mutation [17].

In addition to p16, proliferating cell nuclear antigen (PCNA) is a useful marker that can provide further insight into laser/IPL-tissue interaction. PCNA is expressed in cells with DNA damage that require repair or in cells that are replicating [18,19]. Animal studies that look at the cutaneous expression of these markers, other macroscopic changes such as the development of toxicity (mortality and weight lost), and new tumor formation will be useful in assessing the effect on skin of repeated long term high-energy laser/IPL exposure.

METHOD

Animals

Twenty-eight male Institute of Cancer Research (ICR) mice were provided by the Laboratory Animal Unit of the University of Hong Kong and were used at 6 weeks of age. The mice were kept at 22 ± 2°C, relative humidity 50 ± 10%, and 12 hours room light per 12 hours dark cycle. The animals were monitored for food and water consumption. Signs of toxicity such as mortality and weight loss were checked for once a week. The 28 mice were divided into 4 groups. Other than the control group, all groups received either laser or IPL treatment. All four groups were anesthetized before treatment by the intraperitoneal injection of a 1:1:6 mixture of Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen Animal Health), Dormicum (midazolam 5 mg/ml, Roche AB), and MilliQ water, giving a final dosage of 6.25 mg Dormicum per kg body weight and 12.5 mg Hypnorm per kg body weight.

Skin Irradiation Procedure

The irradiation of the animals was performed with a pulsed dye laser machine (ScleroPLUS, Candela, Wayland, MA) (585 nm, 1.5 milliseconds, 7 mm spot size 8 J/cm²), with a 1,320 nm Nd:YAG laser (Cooltouch II, Laser Aesthetics, Auburn, CA) (10 mm spot size, 3 passes performed at the test site: 2 pre-cooling passes with a fluence of 20 J/cm² for the first pass and 18 J/cm² for the second pass, and one pass of post-cooling with a fluence of 15 J/cm²). An IPL source (Estelux, Palomar, Burlington, MA) with a green hand-piece (wavelength 500–670 nm and 870–1,400 nm, spot size 12 mm×12 mm, 30 J/cm², 20 millisecond pulse duration) was used. A region of dorsal skin of about 2×4 cm was shaved with an electric clipper and irradiated. The animals were irradiated twice a week for 6 months. At the end of the experiment, skin samples were collected. Portions of the samples were fixed in neutral buffered formalin and embedded with paraffin for immunohistochemical examination. Other portions of skin samples were stored frozen at −70°C for immunoblotting analysis.

Tumor Formation Evaluation

The effects of laser and IPL sources on tumor incidence (the percentage of mice with tumors) and tumor multiplicity (the number of tumors per mouse) were recorded weekly. Skin tumor formation was evidenced by lesion growth to a diameter of greater than 1 mm for 2 weeks [20].

Histological and Immunohistochemical Examination

Four-micrometer thick paraffin-embedded skin sections were deparaffinized with xylene and then rehydrated through a descending gradient of ethanol. The expression of proliferating cell nuclear antigen (PCNA) and p16 in the mouse skin was determined by immunohistochemical staining using specific monoclonal antibodies for mouse PCNA (Dako, Carpinteria, CA) and p16 (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described [21].

Immunoblotting of PCNA and p16

Crude extracts were prepared from 5 mg mouse skin using a solubilization buffer that contained 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, and complete protease inhibitors. The extracts (10 μg) were electrophoresed through 15% SDS–PAGE gel and transferred to PVDF membranes. The membrane was probed with anti-PCNA (1:2,000) or anti-p16 (1:1,000) and detected with ECL plus chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL). The immunoblotting images were scanned on a flatbed scanner and the density of the bands was quantitated using ImageQuant software (Molecular Dynamic, Sunnyvale, CA). The densitometry results were reported as average arbitrary integrated values (units).

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). The data from more than two study groups were analyzed using multivariate ANOVA followed by Bonferroni correction. A P-value of <0.05 was considered to be significant.

RESULTS

At the end of the 6 months, none of the animals had developed any signs of toxicity such as mortality or weight lost. There was no evidence of tumor formation. There were significant elevations of p16 and PCNA in all of the treated groups as compared to the control group (ANOVA P < 0.05) (Figs. 3–6). This particularly applied to the group that was treated with the 1,320 nm Nd:YAG laser. Figure 3 indicates that increased signals of p16 were observed in the skin of the mice that received pulsed dye laser (B), IPL treatment (C), or Nd:YAG (D) as compared with the control mice (A). In Figure 4, increased signals of p16 are shown in the skin of
Fig. 3. Representative immunohistochemical staining (brown) of p16 in mouse skin (magnification 200×).

Fig. 4. Representative immunohistochemical staining (brown) of PCNA in mouse skin (magnification 200×).
mice that received pulsed dye laser (B), IPL treatment (C), or Nd:YAG (D) as compared with the control mice (A).

IPL and laser treatment with Nd:YAG (CT) significantly increased the expression of PCNA. There was no significant increase in PCNA expression after pulsed dye laser (PDL) treatment. The results in Figure 5 represent the mean ± SD of eight animals.

IPL treatment, PDL treatment (PDL), and Nd:YAG laser treatment (CT) significantly increased the expression of p16. The results in Figure 6 represent the mean ± SD of eight animals.

**DISCUSSION**

Non-ablative skin rejuvenation by laser and IPL source treatment is a new development in dermatology that has attracted considerable interest in recent years. Non-ablative skin rejuvenation involves the use of a cooling device to protect the epidermis, which allows a high energy laser/IPL source (with wavelengths that range from 570 to 1,340 nm) to be delivered to the dermis [1–7]. Histological evidence has suggested that non-ablative skin rejuvenation is associated with increased collagen production and collagen shrinkage [8,9]. Several mechanisms have been proposed to lead to rejuvenation effects, including damage to the dermal vessels that causes microvascular injury, with the subsequent healing process leading to new collagen formation. The heating of collagen causes shrinkage and subsequent new collagen formation as part of the healing process. The lack of down time that is associated with these procedures has led to their increasing popularity in recent years. Both the industry, and in some cases clinicians, are now actively marketing this therapy to the general public [22]. However, the long-term complications that are associated with the use of these systems are not known. Long-term complications are particularly important because non-ablative skin rejuvenation can be addictive, unlike most other forms of laser surgery, which after a certain number of treatment sessions are terminated due to complete clearing or no further improvement. Despite the lack of clinical data, some clinicians advocate the daily use of Q-switched Nd:YAG laser for skin rejuvenation [12]. Furthermore, in the hands of non-physicians such procedures are performed monthly for long periods on subjects who are in their twenties. This is similar to the use of sun beds. As indicated at the recent American Academy of Dermatology consensus conference on the UVA protection of sunscreen, the action spectrum for dermal photoaging and photocarcinogenesis for melanoma is not well established [23]. Although the role of UVA has been studied, much less is known about the long-term effects of longer wavelength radiation. Hence, we used an animal model to address the potential long-term complications that were associated with the use of non-ablative lasers and IPL for skin rejuvenation. Animal studies are important because it will take decades to determine whether the long-term chronic use of such devices will be harmful to humans.

Our study indicated that there was no sign of toxicity or tumor formation despite twice weekly treatments for 6 months. As the life span of the mouse is about 2 years, our findings are important because they suggest that despite repeated treatment using high-powered lasers and IPL over 25% of the mouse’s life span, there was no effect on the wellbeing of the animal. Nonetheless, that both p16 and PCNA were significantly elevated indicated that DNA damage had occurred. The implication of this is unknown because none of the mice developed tumors or other signs of toxicity. It would be worthwhile to perform a further study using a knockout mouse model. For mice that have both p16 and ARF mutations, tumors such as fibrosarcoma and B-cell lymphoma, but not melanoma, have been detected at an
early stage [24]. However, in a recent study in which mice were mutated specifically in the p16 locus but not in the ARF locus, the application of carcinogen 7,12-dimethylbenzanthracene increased the incidence of melanoma with frequent metastases, and confirmed the importance of p16 in the pathogenesis of melanoma [25]. Further studies that apply high-energy lasers/IPL to mice with specific mutations in the p16 locus but not the ARF locus will be useful in assessing the safety of these devices.

Another interesting observation is that 1,320 nm Nd:YAG is associated with a greater statistically significant degree of PCNA elevation than pulsed dye laser. This is probably due to the difference in the mechanisms of action of the two systems. Pulsed dye laser achieves skin rejuvenation by damage to the microvascular circulation and is more selective than 1,320 nm Nd:YAG, which causes bulk tissue heating. As PCNA expression increases in both DNA damage and cell proliferation, bulk tissue heating can lead to a greater degree of dermal injury, thus causing the higher expression of PCNA.

Finally, it is important to address whether the findings of this study will have any effect on clinical practice. Like those of any animal study, our findings may not be applicable to humans. Nonetheless, the lack of toxicity and tumor formation in this study is at least reassuring. The changes in p16 and PCNA do imply that further studies are necessary to determine the risk of laser injury especially among patients with personal or family history of melanoma and therefore at risk of p16 mutation.

In conclusion, repeated high-energy laser and IPL source treatment did not cause any toxicity in mice. The changes in p16 and PCNA imply that further studies are necessary to ascertain the implications of the repeated exposure of human skin to longer wavelength radiation.

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REFERENCES