Effect of carbon dioxide laser (10600nm) on hydroxyproline in mouse skin

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Abstract: The histological and ultrastructural changes of skin of Wistar rats were compared with 10600nm CO_2 dot laser irradiation and external use of A acid preparation. The effects of dot matrix CO2 laser and Nd: YAG laser (quasi long pulse width 10600nm wavelength) on the expression of matrix metalloproteinase -1 (MMP-1) and matrix metalloproteinase inhibitor -1 (TIMP-1) in skin of natural aging mice were investigated, and the skin tender mechanism was further explored. To explore the long-term efficacy and timeliness of 10600nm CO_2 dot laser and A acid treatment, so as to provide reference for clinical practice. The skin of hair on the back of mice was irradiated with dot CO_2 laser and Nd: YAG laser. They were taken at 2 weeks, 1 month and 3 months after irradiation respectively. The dynamic changes of skin HSP47, HSP70 and TGF - β 1 were detected by immunohistochemistry, and the difference of two laser irradiation effects was compared. 45 female Wistar rats were randomly divided into 9 groups. The back skin was used as the experimental observation area. After the depilation, the observation was divided into four parts by using the cross shaped marking line: the left side of the proximal end was the normal control group (A area). At the proximal end, the left side was the vitamin A acid group (B area). The right side of the tail was the combined treatment group (C area), and the right side of the proximal end was the dot laser group (D area). The C and D regions irradiated 10600 CO_2 dot matrix laser 1 times at the beginning of the experiment. The parameters were: energy 15mJ, energy density 5%, frequency 300Hz. The fig. is square, 10mm * 10mm; after the laser irradiation, the B and C areas begin to wipe the 0.025% dimensional A cream every day for 3 weeks. The positive expression of MMP-1 and TIMP-1 in dot CO_2 laser and Nd: YAG laser irradiation area was most obvious at 2 weeks. The MMP-1 gray value (115.14 + 5.23) and TIMP-1 gray value (104.01 + 3.15) of the dot matrix laser irradiated area were lower than the MMP-1 gray value (121.75 + 4.39) and TIMP-1 gray value (109.26 + 3.88) in the Nd: YAG laser irradiation area. That is, the positive expression of CO_2 laser irradiation area was higher, and the difference was statistically significant ($P < 0.05$).

Keywords: Lattice CO_2 laser, Nd: YAG laser, MMP-1, TIMP-1, HSP47, HSP70, TGF-β1

INTRODUCTION

Aging is one of the most basic laws of natural biology, which is universal, endogenous, progressive and harmful (Ashraf and Sarfraz 2016). It is the gradual degeneration of the body with age (Huang *et al.*, 2016). It is characterized by degeneration of various organs, degeneration of function and decline of adaptability (Zhan *et al.*, 2016) Laser cosmetic therapy has been widely applied in clinic for its treatment of wrinkles, chloasma and light damage by selective photothermal therapy. The rapid development of laser medicine has made it a good assistant for dermatologists (Li and Lu, 2016). The number of laser rejuvenation patients is increasing year by year, and its application is becoming more and more popular. In physiology, senescence begins with fertilized eggs and goes along with the human body until the end of life from pathology (Chuang *et al.*, 2016), aging is the result of injury and infection, malnutrition, immune response decline, stress and strain, and bad habits. People still do not know enough about the molecular biological mechanism of laser induced skin (Hoseini *et al.,* 2016). A comparative study of histology and heat shock protein (HSPs) was carried out after dot CO_2 laser irradiation on human skin. HSPs were involved in remodeling of the dermis. Although there are many researches on the mechanism of laser therapy, many studies are limited to histomorphology, and little is known about the molecular mechanism of laser rejuvenation (Chen *et al.,* 2016). Therefore, the mechanism of treatment should be further studied. Some scholars have suggested that heat shock protein, transforming growth factor and matrix metalloproteinase have time dependent changes, which can promote the synthesis of collagen fibers to reach the skin rejuvenation effect. It has been studied that matrix metalloproteinase -1 (MMP-1) and matrix metalloproteinase inhibitor -1 (TIMP-1) are related to the metabolism of collagen fibers, and their content changes are related to the deposition of collagen.

Skin photoaging refers to skin aging or accelerated aging due to long-term sunlight exposure. Long term and excessive ultraviolet radiation affect various cellular components of the skin and activates macrophages and mast cells infiltrating around the vessels (Scheiblhofer *et al*., 2017). Inflammatory mediators and cells can cause the release of tissue lysis, such as elastase and collagenase, and then slowly dissolve the extra cellular

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matrix components and aggravate the inflammatory response of the dermal tissue. Compared with traditional skin rejuvenation drugs, vitamin A acid and new exfoliative dot laser technology and the combined application of the two methods on skin changes of Wistar rats (Perino *et al*., 2015). Histological changes and ultrastructural changes of transmission electron microscope in different treatment groups were observed and compared. Using natural aging mice, the back depilatory skin of the back was irradiated by dot matrix CO_2 laser and Nd: YAG laser. The expression level of HSP47, HSP70 and TGF - β 1 were observed and the relationship between the three were analyzed and the mechanism of skin beauty was discussed. In this experiment, the changes of MMP-1 and TIMP-1 in skin tissues of natural aging mice irradiated by dot matrix CO_2 laser and Nd: YAG laser were compared, and the interaction and influence were analyzed.

Guided by the theory of focal photothermal effect proposed by MANSTEIN in 2004, lattice laser is safely applied to the treatment of skin diseases such as photoaging (Zhao and Ashraf, 2016). There are annular tissue coagulation zones or thermal damage zones around each MTZ, which are normal tissues without damage. Epidermal cells can be quickly repaired by migration of surrounding cells. When Bae *et al* (2015) studied the molecular mechanism of photoaging, it was found that skin photoaging was mainly caused by changes in extracellular matrix (ECM) and collagen (Luo *et al*., 2016). MMPs are involved in the degradation of extracellular substances and play an important role in the degradation and remodeling of ECM under various physiological conditions. Since 2015, non-contact fractional laser has promoted hair follicle regeneration, and the evaluation theory of irradiation parameters and tissue response has been studied (Qin *et al*., 2016). In 2017, the theory of *in vivo* imaging of retinal hypoxia using low x-4 dependent fluorescence in a laser-induced retinal vein occlusion (RVO) mouse model was proposed (Juhua *et al.*, 2016). However, in 2018, the protective immunity theory of mice and rats induced by subcutaneous injection of a small amount of influenza H7N9 isolate vaccine was also proposed by relevant scholars (Xun *et al*., 2015).

MATERIALS AND METHODS

Twenty-four clean female Kunming mice, weighing (45±7) g, aged 11-12 months, were provided by the Experimental Animal Center of Hunan Traditional Chinese Medicine College. The contrast between the local skin and the non operated local skin after the operation of carbon dioxide lattice laser (10600nm) was compared to the one week, two weeks, four weeks, six weeks, eight weeks, ten weeks and twelve weeks respectively. The 2 * 2cm focal spot area of the mouse is 12 x 12 dot matrix density 10mJ energy, and two 2 x 2 of the focal spots are used to facilitate the late skin detection, and the operation completes the local redness and swelling for about 3 days for 7 days, and the collagen fiber is mainly containing collagen. Amino acids are glycine, proline and hydroxyproline. The mice were anesthetized with 10% chloral hydrate 4m L / kg, and the dorsal skin was divided into three areas by the posterior line of the middle line of the spinal cord and the 1/2 transverse line of the back. The whole left side is the control area, without any treatment; the right head area is the dot matrix CO_2 laser (10600nm) irradiation area (called the dot matrix area), the tail region is the Nd: YAG laser (the quasi long pulse width 1064nm wavelength) irradiation area (short for the Nd: YAG region). The skin of the irradiated area was routinely sterilized by 1% new bromo disinfectant. The parameters of dot matrix CO_2 laser are: 50m J/cm2, square spot (4 mm * 4 mm), pulse width 2ms, density 100 /cm2, and no overlap between spots. Nd:YAG laser irradiation parameters: energy 14.1m J/cm2, circular spot (3.0mm), pulse width 0.3ms, spot overlap 1 /3, irradiated two times. The two groups of irradiated areas were about 2 x 2cm2. 24 mice were irradiated by laser for 4 times, 10d at each interval. 8 mice were collected from second weeks, 1 months and 3 months respectively. The hair samples from the dorsal lattice area, Nd: YAG area and control area were produced by paraffin sections and analyzed by MMP-1 and TIMP-1 immunohistochemical staining.

The *R* in the formula is laser irradiation parameter, *I* is energy, *Q* is square spot, *D* is pulse width, min is density. Thus, the next formula can be obtained:

$$R_t(p_{1t}, Q_t) = p_{1t} \cdot \min(I_t + Q_t, D_t) - (p_{0t} \cdot Q_t + C_t \cdot AI_t) + R_{t-1} \qquad (1)$$

By the upper (1) available formula (2):

$$I_{t+1} = I_t + Q_t - \min(I_t + Q_t, D_t) = \max(I_t + Q_t - D_t, 0) \qquad (2)$$

The type (2) can be converted into form (3):

$$AI_t = \frac{(I_t + Q_t) + (I_t + Q_t - D_t)}{2} = I_t + Q_t - \frac{D_t}{2} \qquad (3)$$

At the second week, 1 month, and 3 months after irradiation, the skin and the skin of the control area were taken at the same time. The size of the specimen was 5mm* 5mm. Para formaldehyde was fixed, the concentration of ethanol was gradually dehydrated, paraffin embedded and sectioned. The first antibody was Rabbit anti HSP47, HSP70 and TGF - β 1 McAb (1: 150). Biotin labeled two was used as Goat anti rabbit monoclonal antibody, hatched by horseradish enzyme labelled streptomycin, and DAB coloring (Beijing Boosen Biotechnology Co., Ltd.). Positive controls were known as positive slices, and 0.01 mol / L PBS instead of single antibody as negative control. Microscopic examination showed that the positive control showed full color and stopped responding quickly (Safi *et al*., 2015).

Type D was the irradiation site, A was the control site, and B was the size of the specimen. Thus, the next formula

Table 1: Body weight change during the experiment of group A1 rats Unit /g

	0 Weeks	2Week	3Week	4Week
Weight/g	230.5	269.2	289.4	291.3

Table 2: Body weight changes during the experimental period of A2 and group B rats Unit /g

Group	0Weeks	2Weeks	4Weeks	6Weeks	8Weeks
A2	240.5	285.6	351.3	415.6	493.8
B	240.2	289.1	352.1	430.5	486.9

Table 3: Changes of gray value of HSP47 in 2 weeks, January and March after irradiation

Laser zoning		2Weeks	1Month	3Month
Lattice CO_2	Irradiation area	119.5	139.2	142.4
	Control area	195.2	172.1	171.2
Nd: YAG	Irradiation area	143.8	150.5	109.5

Table 4: Variance analysis of gray values of MMP-1 and TIMP-1 at different time points

Biomarker	Control region		Fractional CO_2		Nd: YAG	
	F	P	F	P	F	P
MMP-1	3.26	1.01	43.27	<0.01	71.01	<0.01
TIMP-1	7.21	1.02	55.17	<0.01	60.21	<0.01

Table 5: Changes of HSP70 gray value in 2 weeks, January and March after irradiation

Laser irradiation zoning		2Weeks	1Month	3Month
Lattice CO_2	Irradiation area	110.5	120.2	140.2
	Control area	152.3	157.3	171.9
Nd: YAG	Irradiation area	132.5	135.7	139.8

Table 6: The change of gray value of TGF - β 1 at different time intervals

Lattice partition		2Weeks	1Month	3Month
Lattice CO_2	Irradiation area	113.7	129.7	132.7
	Control area	120.6	145.6	149.1
Nd: YAG	Irradiation area	159.2	130.5	141.2

can be obtained:

$$D_i = a + \sum_{j=1}^{n} b_j p_j + r_i Y + u \qquad (4)$$

By the upper (4) transformation of the available formula (5):

$$D_i = a + \sum_{j=1}^{n} b_j \ln(p_j) + r_i \ln(Y) + u \qquad (5)$$

Using Motic medical image analysis system, the positive cells were quantitatively analyzed, and the gray value of the reaction products was determined. The difference between the 0 grades was black and 255 for white. The difference between the two grades was 256 grades. The darker the positive product, the smaller the gray value. 3 slices of visual field were randomly selected from each slice, and the average gray value of the selected positive substances was obtained.

STATISTICAL ANALYSIS

The SPSS13.0 software package was used for statistical analysis. The data were described by using X + s, and the data were tested for normality and homogeneity of variance. The normal distribution and homogeneity of variance were satisfied. Paired t test was used to compare the two groups. Variance analysis was used to compare the differences between different time periods, and the normal distribution and homogeneity of variance were not satisfied. The Wilcoxon rank sum test was used to compare the two groups. 22 the P value of comparison is adjusted by bpnferroni method. The test level is =0.05 and $P<0.05$ is statistically significant.

RESULTS

Hydroxyproline (HYP) is one of the amino acids, is a non essential amino acid, is one of the main components of collagen tissue. It is also a special amino acid in collagen, accounting for 13% of the total amino acid content of collagen. Collagen is the most abundant protein in the body, accounting for about 1/3 of the total protein of human body. Hydroxyproline was used in the determination of hydroxyproline in blood and urine. It can understand the situation of collagen catabolism *in vivo*, and can be used as an indicator of connective tissue decomposition. Many diseases can be accompanied by changes in collagen metabolism, resulting in changes in blood, urine and tissue hydroxyproline content. At present, the automatic amino acid analyzer is widely used in clinical tests. There were also colorimetric, gas chromatography, high performance liquid chromatography, capillary electrophoresis and so on. Therefore, hydroxyproline was selected to measure the proliferation of collagen.

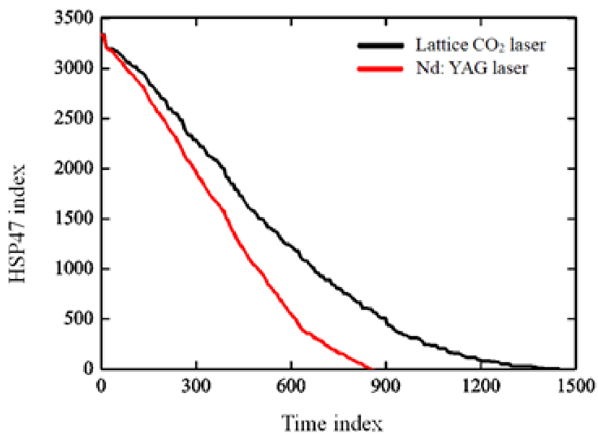

Fig. 1: Expression of HSP47 at different time after dot CO2 laser and Nd: YAG laser irradiation

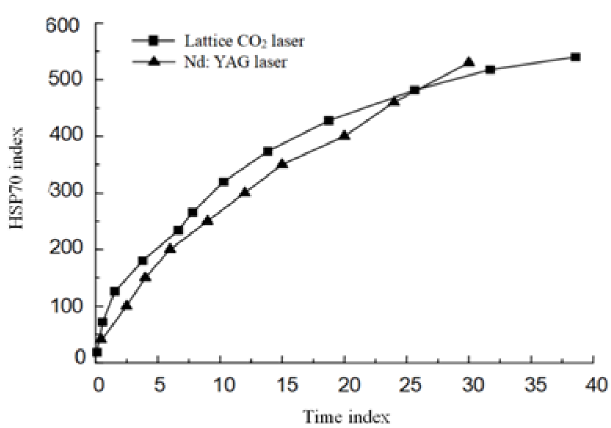

Fig. 2: Expression of HSP70 at different time after dot CO2 laser and Nd: YAG laser irradiation

During the whole experiment, 1 of the experimental group died from general anesthesia, and 15 remained in the A2 group. In the control group, only 1 died and 10 remained, all in second weeks. In group A1, 2, 3, 4 and 2 died at second, third, fourth and 5 weeks respectively. In group A1, activities began to decrease from week 3, poor eating, poor spirits, and no weight gain, and the mortality was higher in the first 4 weeks. After fourth weeks, ultraviolet irradiation was stopped, but the improvement of basal wood was not obvious in rats. The experimental data of group A1 were only taken for the first 4 weeks. As shown in table 1.

The B animals in group A2 and control group were in good condition and the food intake was normal in each group. As shown in table 2.

Variance analysis of gray values of MMP-1 and TIMP-1 at different time points. It was found that the average gray value of MMP-1 in the skin tissues of the mice and the control area at different time segments had statistical significance ($P<0.05$) by the analysis of variance analysis. The skin tissues of the mice were irradiated with the dot matrix CO2 laser and the Nd: YAG laser. The effect of laser irradiation on the expression of HSP47 in skin is shown by immunohistochemistry. At 2 weeks, the expression of HSP47 increased in the cortex, fibroblasts, vascular endothelial cells and appendages. At 1 month, basal lamina and dermis increased significantly, especially around MTZs. The average gray values of MMP-1 and TIMP-1 in the two groups were the highest at the end of 3 months, and their expression was extremely weak or not expressed. As shown in table 3, table 4 and fig. 1.

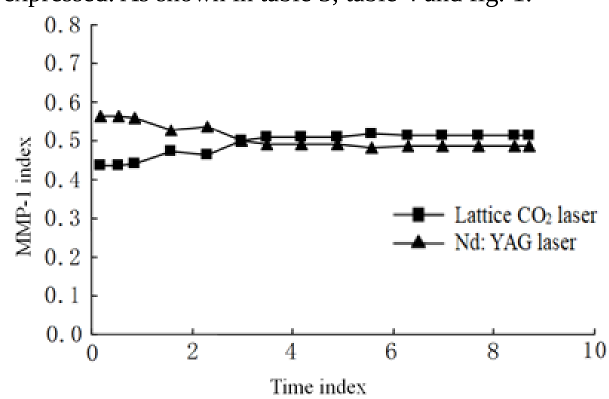

Fig. 3: Expression of MMP-1 at different time after dot CO2 laser and Nd: YAG laser irradiation.

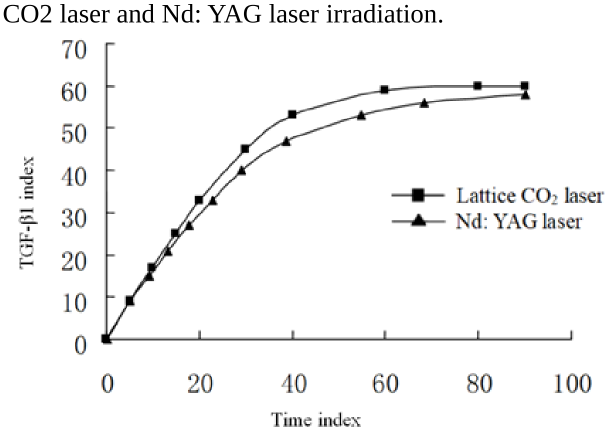

Fig. 4: Changes of TGF- β 1 expression at different time after dot CO_2 laser and Nd: YAG laser irradiation.

The expression of MMP-1 in normal skin is very weak. At 2 weeks, the expression of MMP-1 was mainly seen in the epidermis, but weakly positive in the epidermis, but more positive cells were seen in the dermis. They are mainly endothelial cells and inflammatory cells, and there are more positive granules in dermal interstitium. At 1 month, the expression of full-thickness skin was at a low level. At 3 months, the expression of full-thickness skin was at a very low level. The effect of laser irradiation on skin HSP70 reached 2 weeks after irradiation, and HSP70 expression was obvious around the epidermal spinous cell layer and MTZs. After 1 month, the epidermis and dermis and MTZs were weakly positive, and decreased gradually at 3 months. As shown in table 5 and fig. 2 and 3.

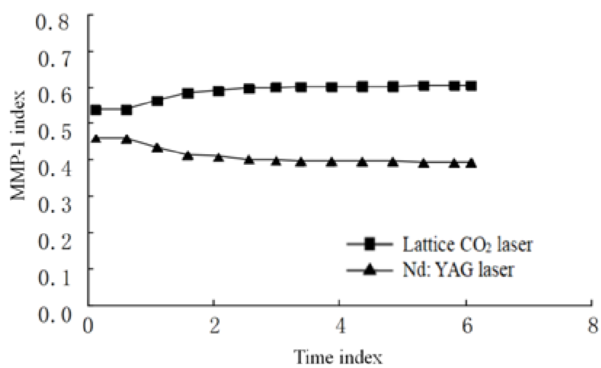

Fig. 5: Expression of TIMP-1 at different time after dot CO_2 laser and Nd: YAG laser irradiation.

No expression of TIMP-1 was found in normal skin. The positive expression of cells in the dermis was more than that of more than that of 2 weeks. At 1 month, the expression of epidermis decreased and the positive expression of dermis was higher. At 3 months, the expression of the epidermis was weaker, while the expression of cells in the dermis decreased, but it was still expressed. The effect of laser irradiation on the expression of TGF- β 1 in normal skin is almost not expressed in normal skin. 2 weeks after laser irradiation, some cells in the basal cells of the epidermis were positive, while the positive expression in the dermis was obvious. The expression of the epidermis was weakened at 1 month. At 2 weeks, positive particles appeared in the epidermis, and positive endothelial cells and fibroblasts appeared in the dermis. At 3 months, the expression of the epidermis was further weakened, while the expression of the dermis was positive, but it was weaker than before. As shown in table 6 and fig. 4 and 5.

DISCUSSION

The negative control expression of the above indexes was negative. HSP47 was lower than that of HSP70, and the expression of HSP47, HSP70 and TGF - β 1 in negative treatment sites was negative. The lattice area at different times is compared with the Nd: YAG area. According to the analysis of variance, it was found that the CO_2 laser and Nd: YAG laser irradiated the mice, and the difference of the average gray value of the MMP-1 in the skin tissues of the control area at different time periods had statistical significance. The lattice area and the long pulse area at different time intervals are compared. According to the analysis of variance, the average gray value of TIMP-1 in the skin tissues of the mice and the control area at different time periods was significantly different from that of the dot matrix CO_2 laser and the Nd: YAG laser. After irradiation with dot CO_2 laser and Nd: YAG laser, HSP47, HSP70 and TGF- β 1 expressed differently in different time periods, showing time dependence. Statistical results showed that both the dot CO_2 laser and the Nd: YAG laser irradiation area was compared with the control area. The expression levels of HSP47, HSP70 and TGF - β 1 were statistically significant. And each index in the dot CO_2 laser group and the Nd: YAG laser group had statistically significant difference.

When studying the molecular mechanism of photoaging, it is found that photoaging is mainly caused by changes in extra cellular matrix (ECM) and collagen. MMPs is involved in extra cellular matter degradation, and plays an important role in the degradation and remodeling of ECM under various physiological conditions. Lattice lasers are guided by the theory of focal photothermal effect and are applied safely to the treatment of skin diseases such as photoaging. A circular tissue zone or a heat damage zone around each MTZ is formed, which is an undamaged normal tissue, and the epidermal cells are rapidly repaired through the migration of the surrounding cells. Light factors are the main exogenous factors that induce the aging of skin. UVB (280nm-320nm) and UVA (320nm-400nm) in sunlight can induce a series of damaging biochemical reactions in tissue cells. A large number of peroxidation free radicals will destroy the transcription and translation of genes, change the differentiation state of cells, and then form an aging damage to the organism. Lattice laser can treat photoaging, but the research of dot matrix laser is mainly focused on the summary of clinical experience. There are not many basic researches on the mechanism of treatment from the aspects of microstructure, cell and molecular level. From the point of view of trauma repair, we treated photoaging rats with dot matrix CO_2 laser, observed the index of photoaging and the changes of TLRs2/TLRs4 and discussed the mechanism of photoaging by dot matrix laser.

Ultraviolet irradiation can increase the expression of MMP-1, then initiate the cleavage of type I and type III collagen fibers, and further degrade by MMP-2 and MMP-9. TIMP is a group of secreted glycoproteins that block ECM degradation and inhibit MMP activity. It participates in the establishment and maintenance of tissue structure. Indirect effects depend on ECM signal transduction, and TIMP-1 preferentially suppresses MMP-1. The efficacy of laser rejuvenation has been confirmed clinically. However, the molecular biological changes related to dermal remodeling caused by laser irradiation are not very clear (Sarfraz *et al*., 2016). The pathological changes showed that the keratinocytes in the epidermis were arranged in disorder, the volume of fibroblasts in the dermis was smaller, the number was reduced, and the elastic fibers and the collagen fibers changed. The content of mature collagen decreased, the number of Langerhans cells and melanocytes decreased, and the appendages of sebaceous glands and sweat glands atrophied. The appearance of photoaging skin is often manifested as loose skin, deep and deep wrinkles, nodules, leather appearance, increased pigment spots and capillary dilatation (Iftakhar *et al*., 2016). The appearance of the original geometric fig. is obviously changed or disappeared, the skin color is often gray yellow and can occur all kinds of benign, precancerous or malignant tumors. In the process of wound healing, MMP-1 plays a key role in the initiation of collagen degradation, and its action is influenced by many factors. Among them, TIMP-1 is the most important inhibitor of MMP-1 and forms a complex with MMP-1 at 1: 1, which inhibits the activity of the enzyme.

The study on the role of HSP47, HSP70 and TGF - β 1 in the healing process of skin thermal injury caused by lattice laser has not been reported so far (Huang *et al*., 2015). The results showed that HSP47, HSP70 and TGF - β 1 had time dependent changes after natural aging mice were irradiated by dot CO_2 laser and Nd: YAG laser. Then, collagen fibers and elastic fibers were synthesized and secreted, and their changes were obviously consistent with skin remodeling. The stimulation of dermis can be treated with traditional vitamin A acid or new dot matrix laser. Although there are many researches on these two treatments, the comparative study between them has not been reported. Therefore, the most direct way to judge the effect of photoaging is the change of the naked eye. In the first part, we have successfully prepared photoaging rat models, all of which show typical symptoms of photoaging. The expression of MMP-1 and TIMP-1 is very weak in normal skin. In this study, MMP-1 and TIMP-1 immunohistochemical staining of skin tissue of aged Kunming mice after laser irradiation was carried out. The average gray value of positive cells was analyzed by Motic medical image analysis system (Hu *et al*., 2015). The higher the average gray value, the weaker the expression of antigen. The lower the average gray value, the stronger the antigen expression.

CONCLUSION

The expression of MMP-1 and TIMP-1 in dot CO_2 laser was stronger than that in Nd: YAG laser irradiation area, indicating that the irradiation effects of the two groups were different. The wavelength of the dot matrix CO_2 laser is 10 600nm, which belongs to the exfoliative laser, while the wavelength of the long pulse width of 1 064nm Nd: YAG laser is 1 064nm, which belongs to the non stripping laser, and the effect has significant difference. That is, dot CO_2 laser with longer pulse width of 1 064nm Nd: YAG laser has stronger effect on remodeling of dermis, which provides a reliable scientific theoretical basis for clinical treatment (Daolei *et al*., 2015). In this experiment, by comparing the HSP47, HSP70 and TGF - β 1 between the lattice laser group and the Nd: YAG laser group, it was found that the positive expression in the lattice laser group was significantly higher than that of the Nd: YAG laser group. Combined with another experiment, the synthesis of type I and type III collagen fibers and fibroblasts was obviously increased, suggesting that the improvement effect of lattice CO_2 laser was more obvious than that of Nd: YAG laser. Both dot CO_2 laser and Nd: YAG laser irradiation can promote the expression of MMP-1 and TIMP-1 in skin of natural aging mice to a certain extent. It suggested that the two were directly involved in dermal remodeling. The dot CO_2 laser group was more effective than the Nd: YAG laser group. It should be pointed out that the research object is natural aging mice, although the skin and human skin are most closely related to the structure and function of human skin, but there are some differences after all. Therefore, it is necessary to further verify the results in future clinical work.

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