Effects of laser irradiation on arthritic histopathology and heat shock protein 70 expression in C57 black mice with osteoarthritis

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Objective: To study the effects of three different laser treatments (650 nm alone, 10.6 µm alone and combined laser of 650 nm and 10.6 µm) on experimental osteoarthritis of the knees in C57 black mice.

Methods: Sixty C57 black mice were divided randomly into 6 groups. Ten mice were assigned to a normal control group (no intervention) and the other 5 groups were subjected to a forced running regime to induce osteoarthritis. One group was set as the model control group. The other 4 groups were given 90 s of a 650 nm laser, 90 s of a 10.6 µm laser, 90 s of a combined laser, or a sham treatment on acupoint Dubi (ST35) of the rear left leg 3 times per week for 4 weeks. The modified Mankin score was used to evaluate the degree of cartilage degradation.

Results: Mankin scores of the model control group and the sham control group were significantly higher than that of the normal control group (P<0.01). Mankin score of the combined laser group was significantly lower than that of the model control group (P<0.01). Compared with the normal control group, there was a significant induction of HSP70 in the articular chondrocytes of the combined laser group.

Conclusion: The articular cartilage induced in C57 black mice improved significantly after combined laser treatment of 650 nm and 10.6 µm lasers. This effect may be related to the induction of HSP70 in the articular chondrocytes. The two different lasers appear to have a synergistic effect.

Keywords: osteoarthritis, knee; laser acupuncture; Mankin score; HSP70 heat-shock proteins; mice

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Osteoarthritis (OA) of the knee is very common in middle-aged and elderly people, and knee pain and dysfunction can seriously affect the patients’ quality of life[1,2]. Western medicine typically uses non-steroidal anti-inflammatory drugs (NSAIDs) to control the pain associated with OA of the knee. However, NSAIDs have some adverse effects on gastrointestinal tract, and often cannot be tolerated[3]. Therefore, it is necessary to develop alternative methods to reduce the pain associated with OA. Acupuncture is commonly used to treat OA, but there are some adverse effects associated with needle insertion, such as pain, cross-infection and fainting. Moxibustion, an adjunct of acupuncture treatment in traditional Chinese medicine (TCM), has been widely used to treat various disorders. The main adverse effect of moxibustion is eye and nose irritation due to the moxibustion smoke.

The previous preliminary study conducted by the authors indicated that human acupoints and traditional indirect moxibustion both have an infrared wavelength of 10 μm. This sympathetic vibration is the biophysical base of the therapeutic function of traditional indirect moxibustion[4]. As the wavelength of the CO₂ laser is exactly 10.6 μm, it is hypothesized that it may share a sympathetic vibration with human body tissues. The previous clinical study showed that the combined use of 10.6 μm and 650 nm lasers was effective for treating knee OA[5]. However, it did not show which laser played the most important role.

There are numerous animal models of knee OA. OA is induced by a variety of methods such as knee immobilization, surgery, or intra-articular injection[6]. Silberberg et al[7] first created a C57 black mice OA model in 1960 and since then many other researchers also used this model for study of OA[8,9]. In C57 black mice, OA occurs spontaneously without any intervention because the articular cartilage degenerates progressively with age due to the unique characteristics of articular cartilage in the black mice[10]. Moreover, exercise can accelerate the process of cartilage degeneration. Wang et al[11] found that after treadmill training the incidence of OA was 66.7% for C57 black mice in the 12th week and was 88.3% in the 20th week. Further, in the aging group, the incidence of OA was 0% for C57 black mice in the 12th week and was 16.7% in the 20th week. The manifestations of OA in this kind of mice are similar to those observed in human patients in clinical practice. Therefore, the strain of C57 black mice is an ideal animal model of OA.

In the present study, the therapeutic effects on knee OA of a 650 nm laser, a 10.6 μm laser, and the both lasers combined on C57 black mice were compared.

1 Materials and methods

1.1 Animal preparation A total of 62 16-week-old C57 black mice of clean grade (31 males, 31 females, body weight 20 to 25 g) were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine. The mice were placed in different cages by gender. Ten mice were randomly selected as the normal control and received no interventions. The remaining 52 mice served as experimental models as previously described[11]. These mice received one week of adaptive training on an electric treadmill, and then they were trained once per day. They were driven to run and maintain their movement continuously at a speed of 5 m/min for 50 m once per day for 6 consecutive weeks.

To make sure that the model was successfully established, two mice were randomly selected for analysis using paraffin-embedded sections. These sections were stained with safranin O and observed under a light microscope. The microscopic image which signifies successful establishment of the model were: decreased density of cartilage cells, abrasion of the cartilage surface, and clustering and hypotrophy of the chondrocytes in some regions[12].

The 50 model mice were randomly divided into 5 groups with 5 males and 5 females in each group. These 5 groups were: model control group, sham control group, combined laser group, 650 nm laser group and 10.6 μm laser group. The mice in the treatment groups (the latter 4) were given treatments after one week of rest.

1.2 Experimental apparatus and materials A self-made combined therapeutic laser instrument (LJ-106/650A, Shanghai University of Traditional Chinese Medicine) was used for all irradiation treatments[15]. This combined laser was composed of a CO₂ laser generator that emitted 10.6 μm (infrared) light via a silver halide optical fiber, with an output of 200 mW and a semiconductor laser generator that emitted 650 nm (red) light via a quartz glass fiber, with an output of 36 mW. Each beam could be transmitted separately or simultaneously through its own optical fiber. The diameter of each laser beam was 0.2 cm.

Heat shock protein (HSP) monoclonal antibodies and the shock protein (SP) immunohistochemical detection kit were obtained from the Huamei Company (Shanghai, China). The glass slides were precut to a size appropriate to the tissue samples. The slides were prepared with acid treatment and high-temperature sterilization.

1.3 Experimental methods The lateral depression of the knee joint of the right rear limb, Dubi (ST35), was selected as the acupoint. First, hair was removed from the skin around the acupoint. There was a fixed plastic tube at the end of the laser probe that was 1 cm in diameter and 2 cm in length. During the experiment, the output end of the laser was kept 2 cm away from the skin. A thick piece of paper with a round 1 mm (diameter) hole was glued to the end of the laser probe. The
laser irradiated the acupoint through this hole. The energy density of the 650 nm laser is 4.13 J/cm², and that of 10.6 μm laser is 22.93 J/cm².

Mice in the normal control group and the model control group received no laser treatment. Mice in the remaining groups were restrained, and the laser probe was aimed at the acupoint Dubi of the right rear limb. The 10.6 μm laser group and the 650 nm laser group received irradiation for 90 s; the combined laser group received both irradiations simultaneously for 90 s. The sham control group mice were restrained and the laser probe was placed at the acupoint Dubi of the right rear limb for 90 s, but the laser was not turned on. Each group received treatment every other day for 30 d. Shanghai University of Traditional Chinese Medicine’s ethical guidelines for the treatment of animals were followed in this experiment.

1.4 Samples and histomorphological examination

The day after the laser treatment, mice were sacrificed and the entire right knee joints, including muscles and other soft tissues, were removed with the joint capsule intact. Knee specimens were fixed and decalcified before they were stained with hematoxylin and eosin (HE). The slides were dehydrated, mounted, and photographed under a light microscope. The morphology of the cartilage, morphology and arrangement of the chondrocytes, position and morphology of the tide mark, staining of the matrix, the joint space, and the morphology of the subchondral bone were observed. Five slides were selected from each group and the degree of cartilage degradation (Minkin score of OA) was calculated. The scoring criteria are shown in Table 1.

1.5 Immunohistochemical staining for HSP70

Following acid treatment and high-temperature sterilization, the appropriately sized glass slides were placed at the bottom of the culture dish. The original generation of chondrocytes and the first and second generations of the passage cells were inoculated. Cells were cultured for 4 to 5 d and the culture was then terminated. The medium was discarded, and the cells were washed three times with phosphate buffered saline (PBS, pH 7.4). After extracting the PBS, the cells were fixed with 95% ethanol for 3 min. The PBS buffer was used to rinse the fixative 5 times (2 min each time), 10% goat serum was added, and the samples were incubated at 4°C overnight. Then, samples were washed with PBS for 5 min, and incubated with 0.3% H₂O₂ for 10 min to eliminate the excess endogenous peroxidase. After that, samples were washed twice with PBS (5 min each time), 10% goat serum was added, and the samples were incubated at 37°C for 20 min. The goat serum was aspirated, and the primary antibody (first antibody working solution of 1: 104) was added. The samples were incubated in a wet box at 37°C for 60 min. The primary antibody was not added to the negative control group. Then, samples were washed with PBS 3 times (5 min each time), the biotinylated secondary antibody was added, and samples were incubated at 37°C for 30 min. Samples were washed with PBS 3 times (5 min each time), the streptavidin enzyme label was added, and the samples were incubated at 37°C for 30 min, and washed with PBS 3 times (5 min each time). Before mounting the samples, they were stained with 3', 3-diaminobenzidine tetrahydrochloride (DAB) for 3 to 5 min, and were then restained with HE for 5 min.

1.6 Statistical analysis SPSS 15.0 software was used for analysis of the data. The Minkin score for OA was used to measure the extent of cartilage degradation. Tests of normality showed the data are not normally distributed, and homogeneity test of variances showed heterogeneity of variances. The data were then expressed as M (Q). Comparisons of data among groups were analyzed by Kruskal-Wallis test. Further comparisons between either two groups were analyzed by Nemenyi test. P<0.05 was considered significant.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Score</th>
<th>Cellularity Score</th>
<th>Matrix staining Score</th>
<th>Tidemark integrity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth surface/normal</td>
<td>0</td>
<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
<tr>
<td>Roughened surface/single crack or area of delamination</td>
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<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
<tr>
<td>Multiple cracks/moderate delamination</td>
<td>2</td>
<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
<tr>
<td>Fragmentation in cartilage or severe delamination</td>
<td>3</td>
<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
<tr>
<td>Loss of fragments</td>
<td>4</td>
<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
<tr>
<td>Complete erosion to tidemark</td>
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<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
<tr>
<td>Erosion beyond tidemark</td>
<td>6</td>
<td>Normal arrangement</td>
<td>0</td>
<td>Normal and intact 0</td>
</tr>
</tbody>
</table>
2 Results

2.1 Histopathological changes of the knee joint
Light microscopy of the knee joints showed varying degrees of damage to the articular cartilage and different numbers of the chondrocytes in the weight-bearing area in mice of the model control group and the normal control group (Figures 1A and 1B). Compared with the articular cartilage, there was less damage to the surrounding cartilage, as indicated by the lighter staining and the presence of blood vessels passing through the tide-mark.

![Figure 1](image)

Figure 1: Histopathological changes of the knee joints of mice in the model control group and normal control group (Light microscopy, ×40)
A: model control group; B: normal control group.

2.2 Mankin score of different groups
Mankin scores of the model control group and the sham control group were significantly higher than that of the normal control group ($P<0.01$). Mankin score of the combined laser group was significantly lower than that of the model control group ($P<0.01$). See Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mankin score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>5</td>
<td>2.0 (1.0)</td>
</tr>
<tr>
<td>Model control</td>
<td>5</td>
<td>5.0 (2.0)**</td>
</tr>
<tr>
<td>Sham control</td>
<td>5</td>
<td>4.0 (2.3)**</td>
</tr>
<tr>
<td>Combined laser</td>
<td>5</td>
<td>3.0 (3.0)ΔΔ</td>
</tr>
<tr>
<td>650 nm laser</td>
<td>5</td>
<td>3.0 (2.3)</td>
</tr>
<tr>
<td>10.5 μm laser</td>
<td>5</td>
<td>3.5 (1.5)</td>
</tr>
</tbody>
</table>

** $P<0.01$, vs normal control group; ΔΔ $P<0.01$, vs model control group.

2.3 HSP70 positive cells in the knee articular cartilage
Figures 2A to 2F present the results of the immunohistochemical staining for HSP70 (light microscopy, ×40). In Figure 2D (combined laser group), there were scattered brown variegated masses with positive staining in the cytoplasm and intercellular space, indicating significant increase of HSP70 expression. The expression of HSP70 in the articular cartilage was also elevated in the normal control group, 10.6 μm laser group, 650 nm laser group, sham control group and the model control group. However, the expression of HSP70 was much greater in the combined laser group.

![Figure 2](image)

Figure 2: HSP70 positive cells in the knee articular cartilage of mice in different groups (Light microscopy, ×40)
A: normal control group; B: model control group; C: sham control group; D: combined laser group; E: 650 nm laser group; F: 10.5 μm laser group. HSP70: heat shock protein 70.

3 Discussion

The Mankin score of the articular cartilage in the model control group was significantly lower than that of the normal control group ($P<0.01$). This is consistent with former reports [11, 12], and indicates that the OA model was successfully established.

Compared with the model control group, articular cartilage degeneration was significantly reduced in the combined laser group ($P<0.01$). However, the articular cartilage was not significantly different between the two single laser groups and the model control group. This indicates that the combined laser treatment is more effective than either of the single laser treatment.

The OA model was established by increasing exercise load, which is similar to sports injury-induced OA (blood stasis in TCM). In clinical practice, OA caused by cold or dampness is common.
The treatment of blood stasis focuses on blood-activating and stasis-dissolving effects, and the treatment of cold or dampness focuses on meridian-warming and activating effects. Low power lasers commonly used are 600 to 1 500 nm wavelength lasers in the visible light and near infrared range. These lasers can transverse the skin and make deep penetration into body tissues as “acupuncture”. CO₂ laser could be absorbed in epidermis and cause a rapid and relative higher temperature raise in skin as “moxibustion”. Traditional acupuncture combined with moxibustion is very commonly used in clinical practice, especially in treating OA. The local heat effect produced by CO₂ laser may promote deep penetration and biochemical reaction of red laser. The results suggest that 650 nm and 10. 6 μm lasers act synergistically. Both lasers are necessary to produce the effect which is consistent with the results of the previous study[15].

The production of HSP, also known as SP, is associated with stress, and these proteins play important roles in the maintenance of cell function[19]. In a rheumatoid arthritis mouse model, moxibustion has been shown to promote expression of HSP70 in local tissue[19], suggesting a possible therapeutic pathway of moxibustion. Other studies have found that moxibustion, electro-acupuncture, or electronic moxibustion can induce HSP, and have suggested that HSP may activate immune function[20-22]. One study suggested that the role of acupuncture in disease prevention is related to the induction of HSP[21]. Another study found that the protective effects of moxibustion pretreatment in rats who were subsequently induced with adjuvant arthritis were related to an increase in the HSP70 expression in the hypothalamus[21]. It has also been shown that helium-neon laser (632.8 nm) treatment could enhance the biosynthesis of articular cartilage, and ameliorate arthritic histopathological changes[25].

The results show that HSP70 expression was only significantly elevated in the articular cartilage of the combined laser group which is consistent with the observation that the combined laser group had the least amount of pathological change in articular cartilage. These results are also consistent with several previous studies. For example, one study found that the concentration of HSP in the articular cartilage increased significantly after rats were irradiated with a helium-neon laser and that the protective and restorative effects of this laser were related to the induction of HSP[26]. Another study, which employed gene transfer by electroporation of rat patellar cartilage, found that HSP protected articular cartilage cells from injury[27]. Thus, it is proposed that the therapeutic effects observed from the combined laser might be related to the induction of HSP70. In future studies, we plan to use image analysis to quantitatively analyze HSP expression.

4 Acknowledgements

Statistical analyses were performed under the direction of statistician Ms Hua-ling Song.

5 Competing interests

There are no competing interests exist within this research. The authors have no conflicts of interest to declare in regard to this study.

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激光照射对 C57 黑鼠膝骨关节炎软骨及热休克蛋白 70 的影响

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目的：观察不同波长激光照射 C57 黑鼠的膝骨关节炎软骨对关节软骨软骨损伤的作用及对热休克蛋白 70 的影响。

方法：C57 黑鼠共 60 只分为 6 组，每组 10 只。其中 1 组为正常对照组，其 5 组接受负荷训练。在电动跑道上进行 1 周的适应性训练后，以 5 m/min 的速度连续训练 50 m，并人工驱赶保持其运动状态；每日 1 次，持续 6 周，以引发小鼠关节炎。造模成功后，除模型对照组和假治疗组外，其余 3 组每天分别接受 10.6 μm 激光、650 nm 激光及二者的复合激光分别照射膝骨关节，Mankin 评分评定小鼠的软骨退化情况，免疫组织化学染色检测热休克蛋白 70 在小鼠关节软骨的表达情况。

结果：模型对照组及假治疗组的关节软骨 Mankin 评分高于正常对照组（P<0.01），而激光组的关节软骨 Mankin 评分明显降低于模型对照组（P<0.01）。与正常组比较，激光组、10.6 μm 激光组、650 nm 激光组、假对照组及模型对照组关节软骨的热休克蛋白 70 阳性表达均增强，而激光组的热休克蛋白 70 阳性表达增强较其他组更为明显。

结论：650 nm 及 10.6 μm 和二者的复合激光照射对膝关节软骨退变均有明显治疗效应，激光照射单一激光更为明显，而激光对关节软骨中热休克蛋白 70 阳性表达的增强亦较 650 nm 及 10.6 μm 单一激光明显。激光照射对关节软骨的治疗效应可能与热休克蛋白 70 的表达有关。10.6 μm 与 650 nm 激光具有协同作用。

关键词：骨关节炎，膝；激光针刺；Mankin 评分；HSP70 热休克蛋白；小鼠