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RESEARCH ARTICLE

EFFECT OF LOW-LEVEL LASER IRRADIATION (LLLI) ON EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND INDUCIBLE NITRIC OXIDE SYNTHASE (INOS) IN RATS MODEL OF MYOCARDIAL INFARCTION

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ABSTRACT

Objective: To observe the effect and significance of low-level laser irradiation (LLLI) on expression of vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) in rat model of myocardium infarction (RMMI).

Method: 110 SD rats were randomly divided into Sham, control and treatment group. Control and treatment group established rat model of myocardial infarction (RMMI) by ligation of left anterior descending artery while Sham group performed only threading around the same site of ligation. After 3 weeks of RMMI, irradiation was done at infarct region in the treatment group by using low level laser diode (635nm, 6mW, 125s, 0.96J / cm^2), while the control group and the sham group were irradiating without putting the power on. 4 to 6 rats were euthanized at 1h, 24h, 48h, 72h and 7d after LLLI. The irradiated myocardial tissue were excised to measure the expression of VEGF and iNOS by using Western blot method.

Results: After the LLLI treatment, VEGF expression continued to rise at 1h-48h in the treatment group, peak expression compared with the control group (2.27 ± 0.22:1.46 ± 0.19,P<0.05) there was a statistically significant difference. iNOS expression started to rise after exposure at 1h in the treatment group, peaked at 48h, compared with the control group (1.90 ± 0.18:1.4 ± 0.08,P<0.05) there was a statistically significant difference. 1 week after the 2nd thoracic irradiation, compared control group with the treatment group; left ventricular ejection fraction (LVEF) (%) (39.83 ± 1.64vs47.62 ± 2.75,P<0.05), left ventricular fractional shortening rates (LVFS) (%) (18.03 ± 1.25vs24.15 ± 2.53,P<0.05), there was a statistically significant difference; 1 week after LLLI, a new capillary density compared between the control group and the treatment group (69.50 ±14.50)/mm2 vs. (111.50 ± 9.00)/mm2,P<0.05, there was a statistically significant difference.

Conclusion: LLLI can significantly increase expression of VEGF and iNOS in infarcted myocardium which can promote angiogenesis to improve the microcirculation of infarct zone and improve the cardiac function.

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INTRODUCTION

Myocardial infarction (MI) is a manifestation of coronary artery disease where coronary blood supply drastically reduced or interrupted. The corresponding myocardial tissue leads to serious and prolonged acute myocardial ischemia and induces acute necrosis. It is lethal and emergency cardiovascular disease which is one of leading causes of disability and death in clinical practice (Fox *et al*., 2007).

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Ventricular remodeling after myocardial infarction refers to neuro-hormonal and genetic regulatory mechanisms activated by inflammatory cytokines causing changes in quality of cell morphology and functions of myocardial cells (Pfeffer and Braunwald, 1990; Rouleau *et al*., 1993). Remodeling the structure and function of the heart in accordance with a certain pattern causing abnormalities of cardiac morphology, blood flow (hemodynamic), progressive expansion of left ventricle, gradually reduced contractility, eventually leading to heart failure (HF) and death. Hence heart failure is an unfavorable consequence of ventricular remodeling after myocardial infarction (Sun, 2009).

Various ways of opposing VR and improving cardiac function after MI have been studied and developed. It was not long after the discovery of the first lasers (the ruby laser in 1960 and the helium-neon (HeNe) laser in 1961) that they began to be used in medical applications. In 1967, Endre Mester in Hungary noticed the ability of the HeNe laser to increase hair growth (Mester *et al*., 1971) and stimulate wound healing in mice (Mester *et al*., 1967), and, shortly afterward, he began to use lasers to treat patients with nonhealing skin ulcers (Mester *et al*., 1972). Since those early days, the use of low-power lasers (as opposed to high-power lasers that can destroy tissue by a photothermal effect) has steadily increased in diverse areas of medical practice that require healing, prevention of tissue death, pain relief, reduction of inflammation, and regenerative medicine. In recent years, the development of light-emitting diodes (LEDs) as alternative light sources for LLLT has added to the confusion. These devices produce light with wavelengths similar to those of lasers, but they have broader output peaks (ie, they are less monochromatic) and lack the coherence that is a particular feature of laser light. LEDs have the advantage of being significantly less expensive than laser diodes (by a factor of approximately 100 on a milliwatt basis), and the LLLT community is engaged in a vigorous ongoing debate about their respective benefits. So, Low-energy laser irradiation (low-level laser irradiation, LLLI) is a sophisticated, safe and effective physical therapy. Yang, (8) research shows that LLLI can increase left ventricular wall thickness and improves ventricular remodeling after myocardial infarction in rat. In our study, we explore the effect of LLLI on expression of vascular endothelial growth factor in myocardial tissue (VEGF) and inducible nitric oxide synthase (iNOS) addressing the effects on myocardial microcirculation, and the protective effect of myocardial tissue after MI.

MATERIALS AND METHODS

Animals

110 adult female SD rats, weight 220g~250g, provided by the laboratory animal Center of Henan province, all animal experiments were approved by Committee on animal, the second affiliated Hospital of Zhengzhou University.

Main reagents and instrument

VEGF polyclonal antibody (rabbit anti-rat) (United States Proteintech company); iNOS polyclonal antibody (rabbit anti-rat) (United States Proteintech company); biological signal collection and analysis system BL-420S type (Chengdu Thai au technology limited); small animal breathing machine HX-100E type (Chengdu Thai au technology limited); echocardiography machine Sonos 5500 type (Netherlands Philips company) ; Laser treatment TY-1 type (instrument technology limited Beijing Branch), nucleic acid-protein Analyzer BioPhotometer plus type (Germany Eppendorf); Image J 1.49v professional image analysis software (United States national institutes of health)

The grouping method

SD rats were randomly divided into three groups. Sham operation group (30), control group (40) and the treatment group (40). The control and treatment groups prepared MI model by ligation of the left anterior descending artery however sham group had only threading around the coronary artery of the same ligation site. Three weeks after the MI model preparation, sham operation group, control group, the treatment group again underwent thoracotomy for the second time to treat LLLI.

Preparation of rats MI model (RMMI)

Anesthesia of healthy female SD rats was done by intraperitoneal injection of 10% chloral hydrate solution (300 g/Kg), rats were breathing smoothly after anesthesia and were made to lie supine on the small animal operating table with head and all the limbs tied in the supporting nails. Preoperative intramuscular injection of antibiotic was given 30 minutes before the procedure (penicillin sodium 200000U). A venous cannula of 18-gauge (3mm) was used as the intubation tube and fixed on the operating table with tapes. Small animal ventilator was set to the respiratory ratio of 1:1, respiratory rate 50 beats / min, tidal volume 3ml / 100g. About 1cm to the left from the mid sternal line, an incision of length 2-3cm was made parallel to the ribs in the 4th intercostals space where the thrill of heart beat was most obvious. After dissecting the tissues, two eyelid retractors were used to retract the ribs and keep the intercostals spaces opened widely proving enough space to expose the heart. Using two small sharp ophthalmic forceps, the apical pericardium was torn open gently with care. Gently press the eyelid retractors on both chest wall of the rat making the heart to expose fully out of the thoracic cavity.

After the heart was fully exposed, cotton swab was applied on the right lower part of heart, lungs and immobilize the apex by a gentle pressure. 0.5ml lidocaine sodium was sprayed on the surface of the heart to prevent arrhythmia during the procedure. At meeting point of left auricle and cone of pulmonary trunk, about 3-4mm away from the aortic root, a needle of 1 ~ 2mm, 6-0 prolene thread is passed with depth of 1.5mm and span of 3mm around the LAD. After the needle was passed, the pressure on both chest walls was relaxed immediately and waits. Ligation of thread was performed after the heart beat became regular and rhythmic. Decreased left ventricular wall motions, color changes to white, ST segment elevation in electrocardiogram were observed after ligation and Myocardial Infarction model was successfully established. After closing the chest, rats were kept back to the rearing area in room temperature 26□ into the cage providing adequate food.

Screening MI model

Three weeks after prepared MI model, echocardiographies of rat heart were performed. All rats in each group were taken after 3 weeks of myocardial infarction, anesthesia by intra-peritoneal injection of 10% chloral hydrate (300 mg / kg). After stable anesthesia, fixed supine position and remove body hair in the middle of chest. Echocardiography used Philips Sonos 5500 ultrasound system, transducer frequency 12 MHz. The criteria for the standard model of myocardial infarction includes: 1. A left ventricular ejection fraction (LVEF) <60%; 2.

Left ventricle fraction shortening (LVFS) <30%; 3. Ultrasound practitioner judgment, appearance of one or more contractile abnormalities found in the anterior wall. At least two of the above is required to select the MI model.

Low-Level Laser irradiation (LLLI) process

Rats with qualified echocardiography MI screening were treated with Low energy laser irradiation. (Figure 1) Thoracotomy was performed again as described previously to expose the heart by giving incision between the 5th and 6th ribs to reduce the risk of bleeding in the healed area of last incision. We used a gallium-arsenic (GaAs) laser diode, with 600 quartz optical fiber, continuous wavelength of 635nm and an adjustable maximum power output of 20mW. The output power was set at 6mW continuously with uninterrupted wave mode.

The optical fiber tip, in all experiments, was placed 15mm above the surface of heart to allow the laser beam diameter of 10mm. The power density on the myocardium was 7.64mW/cm^2. Thus, the laser beam could spread over most of lateral wall of left ventricle including the infarct myocardial area. The irradiation lasted for 125seconds constantly. Apparently, the energy density to the myocardium was 0.96J/cm^2. All the rats in 3 groups were treated with LLLI however the rats in the sham and the control group underwent the same surgical procedure and laser irradiation, but without switching on the laser power supply. After the irradiation was complete, rats were observed for postoperative management and recovery as in the first time surgery.

Specimen collection

At 1,24,48,72 hours and 7d after the second surgery, i.e.post LLLI treatment, rats were give intraperitoneal injection of 10% chloral hydrate solution. Intubation and mechanical ventilator were connected as before. Heart was harvested quickly after median sternotomy and kept in the physiological saline solution at 4□. Wash and rinse the heart with same solution twice until there is any residual blood. The infarct tissue zone of ventricle was clipped by an ophthalmic scissor, put into a test tube and was frozen then kept into an ultra rapid cryogenic refrigerator at - 80 □.

Determination of VEGF and iNOS expression

Collected tissues are determined the expression of VEGF and iNOS using Western blot method. Using image analysis software ImageJ 1.49v gray scale analysis with the target protein to an internal reference with gray bar represents the ratio of the relative content of protein, the same sample was repeated at least 3 times.

Evaluation of left ventricular function in rats

l weeks after LLLI treatment, the 2nd time echocardiography was performed to evaluate left ventricular function of control and treatment group rats (n = 4 ~ 6 / group). The equipment used in the inspection process, and the evaluation method were same as in the 1st echocardiographic screening for evaluating MI model.

Determination of newborn capillary density

One week after LLLI treatment, the rats (4-6 / group) treatment group and the control group were sacrificed, removed the heart and collected the infarcted tissue by immunohistochemistry within the unit area of CD31 positive expressing cells are used to calculate the density of new blood capillaries

Statistical Methods

Statistical analysis were done using SPSS19.0 software (IBM, USA), data were expressed as mean ± standard deviation (`x ± s),the groups were compared using ANOVA, mean pairwise compared using LSD test, $P < 0.05$ was considered statistically significant.

RESULTS

Myocardial infarction in rats

69 rats (86.3%) were remaining 3 weeks after the MI model in control group and the treatment group. There were eight rats (72.7 %) died due to heart failure after 1 week of MI model, After ultrasound screening, a total of 53, (66.3%) were selected as standard MI model in the control group(26), the treatment group (27)

The expression of VEGF in myocardial tissue after myocardial infarction

After LLLI processing, VEGF expression in sham group and the control group continued to increase at 1h, 24h and showed to decline at 48h,72h and 1week. In the treatment group, VEGF expression elevated at 1h, 24h, and 48h and declined at 72h, 1 week continued to decline. VEGF expression in treatment group was significantly higher than the control group at 1h,24h and 48h, and the difference was statistically significant ($P < 0.05$, Figure 1). At 72h and1 week, expression of VEGF treatment group although still higher than the control group, the difference was not statistically significant ($P > 0.05$), Table 1.

After LLLI treatment, the regulation of iNOS expression elevated in each group from 1h until 48h then declined. Regulatory iNOS expression in treatment group at 1, 24,48h were significantly higher than the control group, and the difference was statistically significant ($P < 0.05$, Fig. 1) At 72h and1 week time point, the expression of iNOS in treatment group were better than the control group but was not statistically significant ($P > 0.05$), table 2.

Evaluation of Left Ventricular Function

Comparison of treatment group before and one week after LLLI treatment: LVEF (%) (39.37 ± 1.35 vs 47.62 ± 2.75, $P < 0.05$), LVFS (%) (19.23 ± 3.12 vs. 24.15 ± 2.53, $P < 0.05$); differences were statistically significant; comparison of the control group and the treatment group after one week of LLLI treatment: LVEF (%) (39.83 ± 1.64 vs. 47.62 ± 2.75, $P < 0.05$), LVFS (%) (18.03 ± 1.25vs 24.15 ± 2.53, $P < 0.05$), the differences were statistically significant.

Table 1. VEGF expression at variuos time points after LLLI treatment (time point in each group n = 4 ~ 6)

Group	1h	24h	48h	72h	1week
Sham	1.36±0.13 (n=6)	1.40±0.10 (n=6)	1.34±0.03 (n=6)	1.33±0.14 (n=6)	1.26±0.11 (n=6)
Control	1.37±0.20 (n=6)	1.56±0.25 (n=6)	1.46±0.19 (n=4)	1.42±0.15 (n=6)	1.28±0.05 (n=4)
Treatment	1.74±0.14* (n=5)	2.11±0.18* (n=5)	2.27±0.22* (n=6)	1.48±0.09 (n=6)	1.30±0.08 (n=5)

Note: Compared with the control group, * P <0.05

4.3 iNOS expression in myocardial tissue after infarction:

Table 2. iNOS expression at each time point after LLLI treatment (time point in each group n = 4 ~ 6)

Group	1h	24h	48h	72h	1week
Sham	1.08±0.09 (n=6)	1.18±0.05 (n=6)	1.35±0.10 (n=6)	1.30±0.06 (n=6)	1.19±0.07 (n=6)
Control	1.20±0.04 (n=6)	1.26±0.06 (n=6)	1.40±0.08 (n=4)	1.30±0.08 (n=6)	1.20±0.05 (n=4)
Treatment	1.40±0.13* (n=5)	1.59±0.09* (n=5)	1.90±0.18* (n=6)	1.31±0.05 (n=6)	1.24±0.02 (n=5)

Note: Compared with the control group, * P <0.05

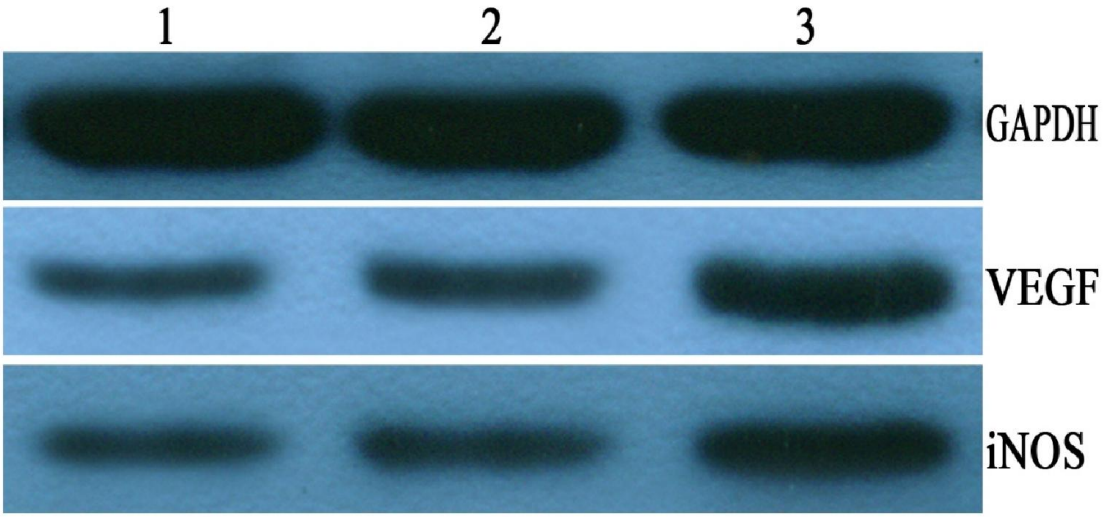

Figure 1. Expression of VEGF, iNOS protein in infarcted myocardial tissue after LLLI treatment in each group at 48h 1: sham group after the LLLI treatment at 48h; 2: control group after LLLI treatment at 48h; 3: treatment group after LLLI treatment at 48h; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; VEGF: vascular endothelial growth factor; iNOS: inducible nitric oxide synthase

Newborn capillary density

One week after treatment of LLLI, comparison of control group and the treatment group; (69.50 ± 14.50) / mm^2 ratio (111.50 ± 9.00) / mm^2, P <0.05, the difference was statistically significant.

DISCUSSION

US Food and Drug Administration (FDA) approved that LLLI is an equipment, safe, easy to operate with no significant harm to the tissues and has been widely accepted clinical practice. Yang *et al*. (2013) found that treatment of myocardial infarction LLLI significantly decreased malondialdehyde (MDA) expression, increase superoxide dismutase (SOD) activity, and effectively improve the ventricular remodeling process. Recently, there has been an increase in its use for the treatment of chronic pain (Walker, 1983; Goldman *et al*., 1980; Iijima *et al*., 1989) and to promote injury repair (Basford, 1986). Vascular reactions during LLLI are also postulated as one of the possible mechanisms responsible for the above-noted clinical effects, because blood flow is an important determinant of wound healing and relief of pain.

For example, Furchgott *et al*. (1961) showed that light in the wavelength range of 310–440 nm from a nonlaser source reduced vascular smooth muscle tone in vitro. Also, Gal *et al*. (1992) showed that low-level laser irradiation reversed histamine-induced spasms in the coronary artery of atherosclerotic microswine. VEGF expression increased by LLLI in Myocardial infarction, VEGF acts on initial phase of microvascularisation only to promote the formation of capillaries, but did not promote the ability to generate arteries (Albrecht-Schgoer *et al*., 2012; Adila *et al*., 2008; Marsano *et al*., 2012). Ardila *et al*. (2008) reported that transplant VEGF protein fiber can improve heart function in rats with myocardial infarction. Ito *et al.* (2011) proved that VEGF promote angiogenesis of endothelial cells by upregulating matrix metalloproteinase and urokinase plasminogen activator. Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy *et al*., 1989; Connolly *et al*., 1989) the chorioallantoic membrane" and the rabbit bone graft model.s! Wang *et al*. (2013) found that VEGF targeted therapy can significantly improve blood supply to the infarct zone of myocardial infarction in rats. Normal myocardial cells do not express or

show low expression of iNOS. Myocardial cells are at the tune of iNOS expression only when myocardial cells are stressed. In studies (Liu *et al*., 2009; Pons *et al*., 2008) showed that iNOS has a protective effect on myocardial tissues and iNOS gene therapy can promote recovery of cardiac function after myocardial infarction. The results of our study show that after the LLLI treatment, expression of VEGF and iNOS in the control group and the treatment group were raised, but increased more significantly in the treatment group and peaked at 48h. LLLI play a role to improve the target tissue, it is no longer statistically significant 72h and 1 week, indicating LLLI not change VEGF, iNOS metabolism kinetics. LLLI by producing "light biostimulation" Improve the target organ, the exact mechanism is not clear. There are views that the LLLI, after treatment of myocardial infarction, increasing the activity of ATP synthesis of antioxidant enzymes in the myocardial cells, which in turn regulate the expression of VEGF (Pons *et al*., 2008). The experimental results show that the LLLI treatment in myocardial infarction increases VEGF, iNOS expression, develops microcirculation and improved cardiac function.

Conclusion

The results of this study show that, LLLI is a safe, low cost, has an obvious effect on increasing VEGF and iNOS expression, improving angiogenesis, microcirculation and heart function after myocardial infarction. But its specific regulatory mechanism remains to be further studied.

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