Activation of Angiogenesis Under Influence of Red Low Level Laser Radiation

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Abstract: The positive influence of low level laser irradiation (LLLI) on the treatment of vascular disorders, ischemic heart disease, activation of microcirculation, as well as on tissue regeneration and reparation is well known. One of the mechanisms of positive effects of LLLI can be connected with activation of angiogenesis due to direct or indirect influence on stem and endothelial progenitor cells. These cells are responding to the influence of vascular endothelial growth factor (VEGF) and playing a key role in angiogenesis, tissue regeneration and reparation. The aim of the current study is to investigate red low level laser light influence on angiogenesis *in vitro*, and to compare it influence with effects of VEGF application.

Thoracic aortal rings from Sabra rats were used in the study. Samples of group 1 served as control, group 2 samples were incubated with VEGF, group 3 samples were irradiated with low level laser light (660 nm, 20 mW) for 10 min, and group 4 samples were incubated with VEGF after receiving 10 min laser irradiation. The samples were cultivated for 1 week, after that the cultures were fixed with formalin and stained with gencyan violet solution in ethanol. The stained samples were photographed by a camera connected to the microscope.

In the control group (1) development of new vessels was not detected. In VEGF group (2) the area covered by new vessels was 1,3 ± 0,24 mm^2 and the maximal length of vessels was 0,93 ± 0,11 mm. In LLLI group (3) – 1,9±0,29 mm^2 and 0,75 ± 0,10 mm accordingly. No statistical significant difference was discovered between groups 2 and 3. In VEGF + LLLI group (4) the area covered by new vessels was 6,98 ± 0,88 mm^2 ($p < 0,001$) and the maximal length of vessels was 1,7 ± 0,23 mm ($p < 0,001$). Study shows, that LLLI initiates the process of angiogenesis *in vitro* at the same extent as application of VEGF, as well as significantly strengthens effects of VEGF.

Key words: low level laser irradiation, low level laser therapy, angiogenesis, vascular endothelial growth factor, aortic rings

INTRODUCTION

Currently low level laser therapy (LLLT) is used in the treatment of different pathologies. Several studies have reported positive effects of laser therapy for activation of wound healing, especially in complicated cases. In experiments and clinical studies it was shown that red laser irradiation with type 1 diabetes can protect vessels of different muscles, pancreas, kidneys and other organs from degeneration. Very promising results have been obtained after applying low level laser irradiation (LLLI) for the treatment of

ischemic heart disease and conditions, following myocardial infarction (Zhukov B. *et al*., 1996; Tuner J. *et al*., 1999; Moskvin S. *et al*., 2000; Paleev N. 2001).

The common treatment protocols and newly developed technologies for all above mentioned pathologic conditions include actions to improve blood microcirculation in affected tissues (application of nitric oxide donors and other medication, surgical interventions), to stimulate angiogenesis, to induce reparation and regeneration of the damaged tissues. Recent preclinical and pioneering clinical studies have shown that introduction of bone marrow–derived endothelial and hematopoietic progenitors can restore tissue vascularization after ischemic events in limbs, retina and myocardium and contribute to neoangiogenesis during wound healing. Stem cell induced angiogenesis (a process of new blood vessel development from pre-existing vasculature) can restore long-lasting organ vascularization and function (Asahara T. *et al*., 1997, 1999 a, 1999 b; Kalka C. *et al*., 2000; Schatteman G.C. *et al*., 2000; Crosby J.R. *et al*., 2000; Takahashi T. *et al*., 2000; Rafii S. 2000; Orlic D. *et al*., 2001; Iwaguro H. *et al*., 2002; Luttun A. *et al*., 2002; Rafii S. *et al*., 2002; Kocher A.A. *et al*., 2001; Jackson K.A. *et al*., 2001; Edelberg J.M. *et al*., 2002; Majka S.M. *et al*., 2003).

Can it be possible that laser therapy utilizes some of the aforementioned mechanisms of treatment? Laser therapy employs several treatment mechanisms. Recent studies showed that red laser light irradiation in vitro and in vivo can increase production of nitric oxide (NO) (Klebanov G.E. *et al*., 1998; Klebanov G.E. *et al*., 2000; Maegawa Y. *et al*., 2000) with subsequent up-regulation of guanylate cyclase activity and increased synthesis of cyclic GMP in platelets and smooth muscle cells (Brill G.E. *et al*., 1997). This mechanism can explain the laser induced vasodilation, inhibition of blood platelet aggregation, and some other effects on cellular and organismal levels (Brill A.G. *et al*., 2000; Yaakobi T. *et al*., 2001; Mirsky N. *et al*., 2002). NO can modulate the production and secretion of several cytokines and other mediators, which can then modulate production of vascular endothelial growth factor (VEGF), mobilization and homing of stem cells (Jozkowicz A. *et al*., 2004).

VEGF is one of the most important growth and survival factors for endothelium (Ferrara N., 1999). VEGF induces angiogenesis and endothelial cell proliferation and it plays an important role in regulating vasculogenesis and vascular permeability (Houck K. *et al.,* 1992). In addition, VEGF causes vasodilation, partly through stimulation of nitric oxide synthase in endothelial cells. VEGF can stimulate cell migration and inhibit apoptosis (Dvorak H.F. *et al*., 1999).

Faster recovery of patients with vascular pathology and after myocardial infarction after LLLT can be a result of activation of angiogenesis, mediated by VEGF as well as stem cells or endothelial progenitor cells mobilization and homing.

The aim of the current study is to investigate the influence of red low level laser light on angiogenesis in vitro, and to compare with effects of VEGF application, as well as to study laser modification of VEGF effect.

MATERIALS AND METHODS

Thoracic aorta was isolated from Sabra rats (weight 160-190 g, Harlan Laboratories Ltd., Israel) and immediately placed into warm medium Bio-MPM. Fibroadipose tissue around the vessel was accurately removed by fine microdissecting forceps and scissors. The aorta was sliced to rings 1 mm wide. The rings were than washed 3 times in sterile warm Bio-MPM medium containing 1% glutamin, penicillin, streptomycin, and nystatin.

Samples of group 1 served as control, group 2 samples were incubated with VEGF (25 ng/ml), group 3 samples were irradiated with low level laser (660 nm, 20 mW) in a drop (10 μl) of the medium during 10 min, and group 4 samples were incubated with VEGF after 10 min laser irradiation. Then all samples were embedded in collagen gel

obtained from rat tail tendons. The collagen solution was prepared by mixing 7 parts of collagen with 1 part of medium MEM x 10 and 2 parts of 0.3 M $NaHCO_3$. The ring-containing collagen gel was prepared in 24-well tissue culture plates (Costar, Massachusets) in quadruplicates and Bio-MPM (500 µl) supplemented with 1% glutamin, 100 U/ml penicillin and 100 µg/ml streptomycin was added. The plates were maintained for 1 week (37°C, 8% CO_2, humidified atmosphere) and the medium was changed each two days. After this time period, the cultures were fixed with 4% formalin during 24h and stained with 0.02% gencyan violet solution in ethanol (Sigma, Israel). The stained samples were photographed by the camera Olympus connected to the microscope (Olympus CK40, Japan; objective x2). The images of the same experiment were taken at the equal zoom. Measurement of area covered with blood vessels (in mm^2) and maximal length of new formed vessels (in mm) were performed using the software Image View.

RESULTS

Studies show, that in control (group 1) without VEGF or red laser light influence no angiogenesis was recorded (Fig. 1). As expected, VEGF application (group 2) significantly activated angiogenesis: the area covered by new vessels was 1,3±0,24 mm^2 and the maximal length of vessels was 0,93±0,11 mm (Fig. 2). After influence of laser light (group 3) stimulation of angiogenesis was also quite clear: the area covered by new vessels was 1,9±0,29 mm^2 and the maximal length of vessels was 0,75±0,10 mm (Fig. 2). No statistical significant difference was seen between influence of VEGF and laser light ($p>0,2$). So, laser light can stimulate the process of angiogenesis at the same level as application of VEGF, the most potent stimulator of angiogenesis (Tab. 1).

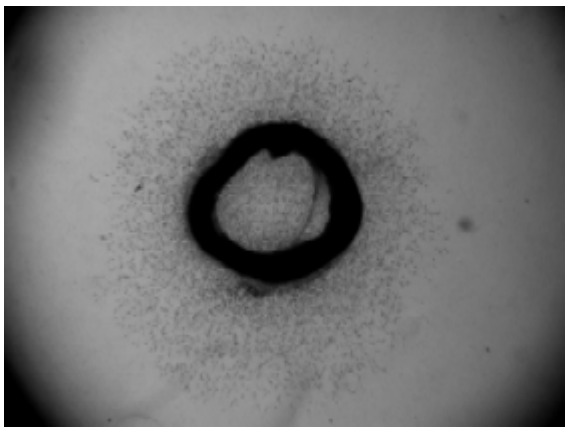

Figure 1
Control (group 1)

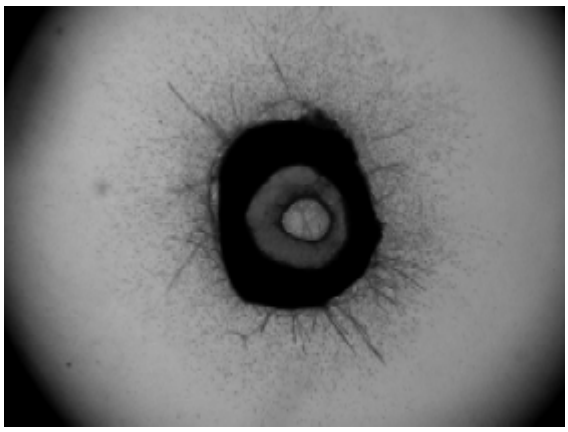

Figure 2
VEGF (group 2)

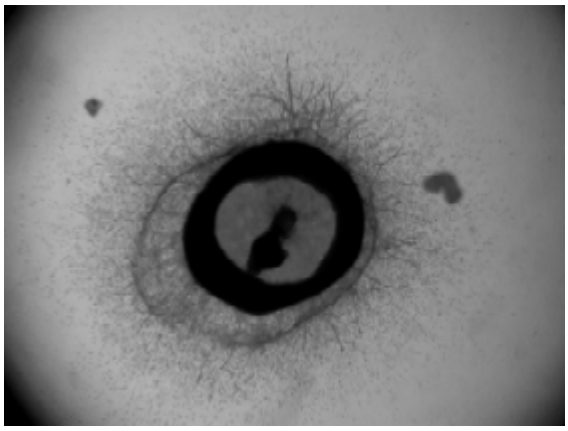

Figure 3
Laser (group 3)

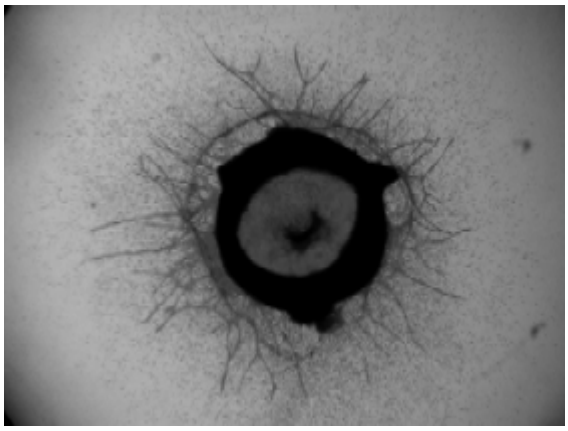

Figure 4
Laser + VEGF (group 4)

The strongest effect was obtained in case of combined influence of laser light and VEGF (group 4). In this case (Fig. 4) the area covered by new vessels was 6,98±0,88 mm^2 ($p < 0,001$) and the maximal length of vessels was 1,7±0,23 mm ($p < 0,001$).

Table 1. The influence of VEGF and low level laser irradiation on angiogenesis

№	Group	Area covered by new blood vessels (mm^2)	P	Maximal length of vessels (mm)	p
1	Control	-		-	
2	VEGF	1,3±0,24		0,93±0,11	
3	Laser	1,9±0,29	$_{2,3}$>0,1	0,75±0,10	$_{2,3}$>0,2
4	Laser + VEGF	6,98±0,88	$_{2,4}$<0,001 $_{3,4}$<0,001	1,7±0,23	$_{2,4}$<0,001 $_{3,4}$<0,001

DISCUSSION

The major findings of the present study are, that low level red laser irradiation launches the process of angiogenesis *in vitro* at the same extent as application of VEGF, as well as significantly strengthens effects of VEGF in vitro.

Numerous reports suggest that low level laser irradiation is capable of affecting cellular processes in the absence of significant thermal effect and induce positive therapeutic effects. Laser light helps to treat inflammatory disorders, neurological diseases, diseases of endocrine system, accelerate wound healing, activate haematopoiesis, control pain and in general improve the quality of life. One area of medicine, in which LLLT is applied with promising results, is the treatment of vascular pathologies (ischemic heart disease, atherosclerotic arterial disease, vascular thromboses and so on). Studies have discovered several mechanisms of favorable effect of light on the treatment of patients with myocardial infarction. They include lowering blood coagulation due to influence on blood platelets, increase oxygen supply to damaged tissues via influence on erythrocyte membranes, vasodilating effect due to increase production of nitric oxide. At the same time all above mentioned mechanisms are not enough to explain long-lasting positive effects of laser therapy, such as faster recovery and better function of affected myocardial area after infarction, improvement of blood supply in lower extremities of patients with peripheral arteriosclerosis, faster and cosmetically better wound healing, possibility to stimulate heamatogenesis for better chemotherapy of cancer patients as well as wide range of other positive effects.

Is it possible to find other therapy modalities with such a number of positive effects, connected to tissue repair and regeneration, as well as development of new vessels? Recent progress in biomedical research exhibits growing evidence that application of several growth factors and stem cell therapy can exhibits a wide range of therapeutic effects, mainly connected with faster and better regeneration and reparation of damaged tissues. Shaoheng Zhang and colleagues (2004) studied results of direct injection of bone marrow mononuclear cell suspension into the infarct and peri-infarct regions of rat myocardium. Faster improvement of cardiac function, higher expression of HSP32, HSP70 and VEGF, as well as higher vessel density in the infracted myocardium was found in bone marrow cell transplant group. Stem cells have ability to migrate and reach areas of the most severe tissue damage due to special chemo-attraction mechanisms, so intravenous mode of bone marrow cells transplantation can be applied successfully. Stem cells can induce production of VEGF, the major inducer of angiogenesis and vasculogenesis (Risau W., 1997). Because VEGF is produced in response to hypoxia it describes a physiological mean to ablate the need of nutrients and oxygen by the induction of new blood vessels. In vitro it induced several activities in endothelial cells, which are associated with angiogenesis, such as proliferation, survival and migration.

VEGF is able to attract stem cells from peripheral blood stream into areas of neovasculrization in vivo.

T. Asahara and colleagues (1995) made an interesting observation while they were studying effect of VEGF on re-endothelialization of arteries. They denuded carotid arteries of dogs bilaterally and then delivered VEGF protein locally via a double balloon catheter to one of the carotid arteries. As expected, VEGF markedly increased re-endothelialization of the treated artery. However, researchers also observed that the local delivery of VEGF to one artery improved re-endothelialization of the untreated countralateral artery. Since only small amounts of VEGF were administered, systemic levels of VEGF could not have been appreciably elevated. Researchers considered, that faster re-endothelialization of other artery was the result of activation some precursor endothelial cells as they pass through the region of high VEGF concentration.

Recent studies have discovered, that VEGF, as well as granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and some cytokines have the ability to increase the number of circulating hematopoietic stem cells (HSC) and endothelial progenitor cells (EPCs) by mobilizing them from the bone marrow (Asahara T. *et al*., 1999; Takahsi T. *et al*., 1999). EPCs may then be recruited to areas of neovascularization. EPCs that are capable of contributing to *in vitro* capillary formation can be derived from bone marrow cells (Bhattacharya P *et al*., 2000).

Recent discovery that laser irradiation of different cell types can induce VEGF secretion can be a clue to better understanding of mechanisms of laser therapy. N. Kipshidze *et al*. (2001) demonstrated that LLLI increases production of VEGF by smooth muscle cells (SMC), fibroblasts, and cardiac myocytes and stimulates human endothelial cells (EC) growth in culture. Authors observed that (1) low level laser irradiation of vascular and cardiac cells results in a statistically significant increase of VEGF secretion in culture (1.6-fold for SMC and fibroblasts and 7-fold for cardiomyocytes) and is dose dependent (maximal effect was observed with LLLI irradiance of 0.5 J/cm^2 for SMC, 2.1 J/cm^2 for fibroblasts and 1.05 J/cm^2 for cardiomyocytes). (2) Significant stimulation of endothelial cell growth was obtained with LLLI-treated conditioned medium of SMC (maximal increase was observed with LLLI conditioned medium with irradiance of 1.05 J/cm^2 for SMC and 2.1 J/cm^2 for fibroblasts).

It was also discovered that laser irradiation of T-cells can activate EC proliferation. D. Agaiby *et al*. (2000) reported that laser therapy (820 nm, 5,000 Hz, and 50 mW) stimulates human T-cells to produce factor(s) that can modulate EC proliferation in vitro. EC proliferation increased significantly when incubated with conditioned media collected from resting T-cells exposed to 1.2 and 3.6 J/cm^2. Day 5 conditioned media produced similar patterns of EC proliferation to that of day 3 but at lower magnitude. Conditioned media from 3.6 J/cm^2 laser-treated T-cells induced the maximal EC proliferation in all groups studied. Laser light effect on the lymphocytes is influenced by (1) the amplitude of energy density used for T-cell irradiation, (2) exposing T-cells to both mitogen and laser, and (3) the duration of T-cell incubation in culture.

In accordance with the above mentined studies, A. Schindl with colleages (2003) reported that low level red laser irradiation (660 nm) at specific dose intervals can significantly increase EC proliferation in vitro (HUVEC culture) and might thereby contribute to the increase in angiogenesis and the acceleration of wound healing in vivo. A. Khanna and colleagues (1999) studied effect of LLLI (632 nm) on proliferation of fetal cardiomyocytes in vitro and on the expression of proangiogenic genes, transforming growth factor-beta (TGF-beta), and vascular endothelial growth factor (VEGF). Authors observed a dose-dependent increase in cardiomyocyte proliferation can be obtained with LLLI and a significant increase in VEGF and TGF-beta mRNA expression by cardiomyocytes.

Until recently, VEGF was the only growth factor proven to be specific and critical for blood vessel formation (Ferrara N., 1999; Dvorak H. *et al*., 1999; Eriksson U., 1999). Other long-known factors, such as the fibroblast growth factors (FGFs), had profound effects in various endothelial cell assays (Risau W. 1997), but such factors were also known to be nonspecific. Due to a recent explosion of newly discovered growth factors acting on the vascular endothelium the vascular endothelium-specific growth factors now include five members of the VEGF family, four members of the angiopoietin family, and at least one member of the large ephrin family (Carmeliet P., 2000). A rule that is emerging is that all of these factors must be used in perfect harmony, in a complementary and coordinated manner, to form functional vessels (Gale N. et al. 1999). In addition, many other growth factors that are not vascular endothelium-specific are also required for blood vessel formation, such as members of the platelet-derived growth factor or transforming growth factor-β families, although these factors also have critical roles for many other systems as well (Hellstrom M. *et al*., 1999, Hirschi K., 1999).

We do not have enough data to explain precisely the origin of all angiogenic effects of red laser light in vitro, but it is possible to propose the following hypotheses:

(1) laser light can increase production of NO by EC;

(2) higher levels of NO can increase VEGF production by different cells (fibroblasts, smooth muscle cells);

(3) higher levels of VEGF can increase expression and activity of different matrix metalloproteinases (MMP-9, MMP-2);

(4) MMP-9 is essential for the mobilization of EC induced by growth factors and cytokines; higher levels of MMP-9 can then partially degraded extracellular matrix and make easier endothelial cell migration and angiogenesis.

(5) in addition to effects on EC, laser light can activate fibroblasts, macrophages, cardiomyocytes, smooth muscle cells, other cells, which then can release several cytokines and growth factors (IL-6, IL-8, TGF-β), which can later contribute in angiogenesis and tissue reparation.

Summarizing our research data and information from various reports, it is clear, that red laser light can induce angiogenesis in vitro and in vivo. Moreover, laser light can enhance angiogenic effects of VEGF. More studies are required to determine the most important mechanisms employed in vitro and in vivo. These data may have significant importance leading to the establishment of new methods for myocardial photoangiogenesis and photoregeneration as well as in vitro proliferation of cardiac myocytes, and have value for the development of new treatment methods based on better and faster tissue regeneration and reparation.

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