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Effect of low level laser irradiation on *VEGF* gene expression in cultured endothelial cells

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ABSTRACT

Background. Endothelial cells play a crucial role in the angiogenesis which is initiated by vascular endothelial growth factor (VEGF). Low level laser therapy (LLLT) stimulates repair processes which are based on the formation of new blood vessels.

Aim. The aim of this study was to evaluate the impact of LLLT on *VEGF* gene expression in endothelial cells cultured *in vitro*.

Material and methods. Freshly isolated endothelial cells from the human umbilical vein endothelial cells (HUVEC) line were used in the study. The cells were irradiated with a semiconductor laser emitting visible laser radiation at the wavelength of 630 nm and the power of 30 mW, and radiation at the wavelength of 808 nm and the power of 60 mW in the infrared range. The study was performed with cell cultures subjected to four different procedures: I — control cells (not subjected to irradiation); II — cells subjected to an energy dose of 2 J/cm^2; III — cells subjected to an energy dose of 4 J/cm^2; and IV — cells subjected to an energy dose of 8 J/cm^2. The cells were cultured for six days, and exposed to irradiation twice. The next step was to evaluate the *VEGF* gene expression by applying real-time PCR.

Results. By using low power laser irradiation, we obtained a statistically significant increase in *VEGF* gene expression, particularly at doses of 2 and 8 J/cm^2 in the wavelength range of 630 nm. The wavelength of 808 nm had a similar effect on increases in gene expression. However, the differences were statistically non-significant when compared to the control cells.

Conclusions. This study has shown that low-power laser radiation in the visible light spectrum (630 nm) results in *de novo* formation of *VEGF-A* in the endothelial cells in culture.

Key words: low level laser therapy, angiogenesis, *VEGF* gene

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Introduction

Vascular endothelial cells (EC) play an extremely important role in the body, participating in the maintenance of vessel homeostasis. They are the first line of contact with other cells and substances found in the blood. These cells react quickly to stimuli and can easily be activated by various pathological factors related to diabetes, hypertension and inflammation. Such knowledge leads to great clinical importance. Experiments with the endothelium may be carried out in a non-invasive manner by the use of *in vitro* culture. One of the basic endothelial cells models is the human umbilical vein endothelial cells (HUVEC) line. Endothelial cells synthesise and release numerous factors affecting vasomotoric properties, haemostasis, inflammation, and angiogenesis. The violation of endothelial integrity, e.g. in wound healing, triggers the repair process associated with the expression of growth factors [1, 2].

One of the most important of these is vascular endothelial growth factor (VEGF) which induces the formation of new blood vessels in the complex process of angiogenesis. This process consists of events that occur sequentially and simultaneously at several levels. Hypoxia

is the main stimulus of angiogenesis. In response to hypoxia, transcription factor HIF-1 activates numerous genes [3, 4], including the *VEGF* gene, stimulating the proliferation and chemotaxis of endothelial cells.

Numerous *in vivo* studies have shown that the use of low level laser therapy (LLLT) has positive effects such as pain reduction, suppression of inflammation and tissue healing acceleration. This kind of treatment is safe for the patient, and its effectiveness has been confirmed by several scientific reports [5–7]. However, the origin of its biological effects is still not fully understood.

Low level laser radiation can affect individual cells only through the substances which absorb that radiation. Mitochondria are such cell organelle, containing a number of enzymes involved in the redox reactions of the respiratory chain. The energy required for the life of every cell is stored in mitochondria. Studies confirm that the photo-induced reactions of electron transfer can initiate synthesis and conformational changes of proteins. They can also increase DNA and RNA synthesis [8].

Studies performed in rats [9] after tongue surgery show that LLLT has a positive effect on wound healing. Another study by Reddy et al. [10] suggested that LLLT accelerates wound healing in rats with induced diabetes by accelerating the production of collagen and the promotion of the overall stability of the connective tissue.

Neovascularisation of the retina is an important feature of diabetic retinopathy. Vascular endothelial growth factor plays a crucial role in microvascular complications. Prevention of the overexpression of *VEGF* gene or blocking its pathological action is extremely important for the treatment of diabetic retinopathy [11].

LLLT's effect on the endothelium and, in particular on the expression of genes coding for the factors associated with angiogenesis, is not fully understood and requires further study. The aim of this study was to evaluate the impact of LLLT on *VEGF* gene expression in cultured endothelial cells.

Material and methods

The study involved the use of freshly isolated HUVEC. The endothelial cells were isolated under sterile conditions in a laminar chamber through the collagenase digestion of the umbilical vein according to the method described by Jaffe et al. [12]. Derived cells were placed in a culture medium (M199) supplemented with foetal bovine serum (FBS) 20% v/v, 100 IU/ml of penicillin (Gibco reagents) and growth factors: 50 μg/ml of endothelial growth factor (ECGS, Biomedical Technologies Inc. USA), and heparin. After several passages, the cell line was obtained. The Petri dishes with the cells were stored in a humidified incubator with 5% CO_2 content.

Cells were seeded onto 6-well culture plates and specific experiments were performed. Cell seeding density was 7.500/cm^2. HUVEC line cells were derived from three independent isolations.

The irradiation involved the use of a semiconductor laser (GaAlAs) emitting visible laser radiation of the wavelength of 630 nm and 808 nm in the infrared range. Equipment for objective *in vitro* irradiation of cell lines was used in the experiment [13]. Before each irradiation, the power of laser radiation was measured using an energy meter (Gentec, Model SOLO2 R2, Canada). The power of density in all the experiments was constant and amounted to 1.875 mW/cm^2 for 630 nm and 3.75 mW/cm^2 for 808 nm. The cells during irradiation remained in a dark environment, and the distance of the fiberoptic from the irradiated area (80 cm^2) was 10 cm. The study was performed with cell cultures subjected to four different procedures: I — control cells (not subjected to irradiation); II — cells subjected to an energy dose of 2 J/cm^2; III — cells subjected to an energy dose of 4 J/cm^2; and IV — cells subjected to an energy dose of 8 J/cm^2.

The cells were cultured for six days and irradiated twice: on the 2nd and 4th days with a one-day interval between the irradiations. After four days, the medium was poured out and RNA isolation was performed.

Total RNA was extracted from cell cultures with TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA purification was performed by DNase I treatment using TURBO DNA-free™ Kit (Applied Biosystems, Carlsbad, CA, USA). RNA quantity was assessed spectrophotometrically with a Biophotometer (Eppendorf, Hamburg, Germany). Total RNA (2 μg) was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μl, according to the manufacturer's instructions. Real-time PCR reaction was performed with 4 μl of cDNA (20 ng/μl) in 20 μl of reaction mix containing 10 μl of TaqMan® Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA) and 1 μl of TaqMan®Gene Expression Assay (Assay IDs: Hs00900055_m1 for VEGF-A, Hs03929097_g1 for GAPDH) on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Negative control was included. All the reactions were run in triplicate. The gene expression level of *VEGF-A* was determined using the $2^{-\Delta\Delta Ct}$ method with GAPDH as endogenous control.

Statistica StatSoft 10.0 was used for statistical analysis. The Fisher-Snedecor analysis of variance and Tukey's *post hoc* test (with the significance level of $p < 0.05$) were applied to assess the differences

Table 1. Level of *VEGF-A* gene expression of endothelial cells under the influence of LLLT at the wavelength of 630 nm and 808 nm, depending on the dose of irradiation

		Irradiation dose (N = 9)					
		Control	2 J/cm^2	4 J/cm^2	8 J/cm^2	ANOVA	*Post-hoc*
Wave	Stat.	I	II	III	IV	p	p
630 nm	Mean	1.00	1.20	1.13	1.29	0.0008*	0.0064$^{I vs II}$
	SD	0.01	0.05	0.05	0.07		0.0657$^{I vs III}$ 0.0007$^{I vs IV}$
808 nm	Mean	1.00	1.14	1.07	1.13	0.6449	
	SD	0.01	0.05	0.17	0.23		

LLLT — low level laser therapy; VEGF — vascular endothelial growth factor; SD — standard deviation

between the analysed groups. The *VEGF* gene expression levels results are shown in relation to the control, non-irradiated cells according to the $2^{-\Delta\Delta Ct}$ method.

The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Toruń, Poland (no KB 135/2009) on 25 March 2009.

Results

Low level laser radiation at the wavelength of 630 nm and power of 30 mW caused a statistically significant increase of *VEGF* gene expression in vascular endothelial cells compared to the control cells that were not exposed to laser irradiation (Table 1). The highest expression was observed at the energy dose of 8 J/cm^2. This was about 30% higher than in the control cells.

Increased expression of *VEGF-A* gene was also obtained by the use of laser radiation at the wavelength of 808 nm and power of 60 mW at the same doses as in the part applying visible light. However, the differences were statistically non-significant (Table 1).

Discussion

Better understanding of the process of angiogenesis, as well as the possibility of its modulation by the use of laser radiation, can enhance the effect of treatment of diseases connected with the formation of new blood vessels. Contemporary medicine in its therapeutic actions intends to improve and to accelerate the process of wound healing associated with angiogenesis. Due to the development of molecular biology techniques, it has been shown that the tissue healing process results from the dynamic interaction of many factors at the cellular level. Various therapeutic methods affect wound healing, e.g. a non-invasive method using low-energy laser irradiation.

LLLT application increases the proliferation of the cells and induces secretion of growth factors affecting angiogenesis [14–17]. LLLT's effects are associated with absorption of the radiation by cytochrome C oxidase involved in cellular respiration. Literature data suggests that this enzyme is responsible for the absorption and transfer of energy to other intracellular molecules, activating multiple biochemical reactions which affect cellular functions. The results of the studies have shown an increased production of this enzyme after laser irradiation [18, 19]. Other authors have suggested that the stimulation of cell proliferation by LLLT follows the activation of signalling pathways such as the mitogen-activated protein kinase (MAPK) pathway [20, 21]. Studies proving this hypothesis investigate a relationship between stimulation of cell proliferation by LLLT and the activation of phosphatidylinositol 3-kinase (PI3K), which controls a number of intracellular signalling pathways connected to the regulation of gene expression [22]. LLLT improves HUVEC proliferation and migration as well as NO secretion by the activation of the PI3K pathway. LLLT also increases the expression of nitric oxide synthase gene (NOS) [23]. Furthermore, a reduction in NOS expression was observed after the addition of PI3K inhibitor (LY294002). This result indicates that the increased signal activation of the PI3K pathway due to laser radiation may be associated with an increased migration of HUVEC and angiogenesis stimulation.

Initiation of angiogenesis is associated with activation of the *VEGF* gene under the influence of hypoxia inducible factor HIF-1α. This must be transported to the nucleus to be able to take part in the induction of VEGF gene. Importin α and β actively participate in this process [24]. Under the influence of hypoxia, HIF-1α combines with HIF-1β to form an active HIF-1 complex. The mechanism of *VEGF* gene expression is mediated by HIF-1 complex binding to the transcriptional region of a gene [25]. Moreover, the recruitment of transcription factors: P-CREB and P-STAT3 to the

promoter region leads to the initiation of *VEGF* gene transcription [25–27].

The action of LLLT promotes increased cell proliferation, cell metabolism and the expression of various genes (e.g. VEGF) directly involved in the healing processes [5, 14, 15]. LLLT's biomodulation effects have been demonstrated in *in vitro* studies involving different types of cells, such as keratinocytes, fibroblasts, osteoblasts, odontoblasts, cardiomyocytes and endothelial cells [14–16, 28–30]. However, the cellular mechanisms of LLLT's action have not yet been fully explained.

Khanna et al. in their *in vitro* study on cardiomyocytes showed increased cell proliferation and *VEGF* gene expression when exposed to laser irradiation at the wavelength of 632 nm and the power of 5 mW [29]. The study conducted by Basso et al. [28] on keratinocytes treated with LLLT irradiation (780 nm, 40 mW, energy doses 0.5, 1.5, 3.0, 5.0 and 7.0 J/cm^2) identified an increased cell metabolism and proliferation and *VEGF* gene expression, especially at the energy dose of 1.5 J/cm^2.

Our study on the cultured endothelial cells proves an increase in the expression of *VEGF-A* gene under the influence of low-power laser irradiation, particularly at doses of 2 and 8 J/cm^2 at the wavelength range of 630 nm. The irradiation of cells with a laser at the wavelength of 808 nm gives a similar effect of increasing the gene expression. However, the differences are statistically non-significant when compared to the control cells.

Our previous study [31] indicated that laser radiation with similar parameters (in visible and near infrared radiation) results in an increased EC proliferation. Earlier *in vitro* studies by other authors for both the endothelial [30, 32] and other cells [14, 15, 17, 28, 29] are in agreement with our observations. In our other study, we have also demonstrated the presence of VEGF-A in the supernatant obtained from the culture of endothelial cells treated with LLLT [31]. The increased proliferation was accompanied by a reduction in the concentration of VEGF in the supernatant, which can be explained by linking VEGF molecules with its membrane receptor VEGFR-2. This combination activates signalling pathways responsible for EC proliferation. The increased level of expression of VEGF/VEGFR-2 complex may cause increased resistance to EC apoptosis. Autocrine secretion of VEGF is also a defence against stressful conditions such as a lack of serum in the culture medium [33].

The present study showing an increase in *VEGF-A* gene expression exposed to LLLT confirms that the observed EC proliferation is associated with *de novo* production of VEGF-A and its stimulatory effect on cell division. We have also proved that this effect is particularly clear after the application of laser radiation in the visible light range.

Repair processes are complex and involve different tissues. Endothelial cells play an important role in angiogenesis which is a condition for new tissue formation at the lesion site. The process of angiogenesis is initiated by the stimulation of VEGF gene. The above study suggests that the non-invasive technique using a low-energy laser increases the expression of the gene.

Conclusions

This study has shown that low-power laser radiation in the visible range (630 nm) results in *de novo* formation of VEGF-A in cultured HUVEC line endothelial cells.

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