**INTRODUCTION**

In recent years a considerable amount of research has been devoted to understanding the angiogenesis process and the problem of healing chronic wounds. Despite increasing knowledge about the wound healing process, its complexity and diversity of pathologic mechanisms continues to cause considerable medical problems.

Contemporary medicine had little influence upon the biological phenomenon of wound healing and the angiogenesis connected with it. Thanks to the development of molecular biology techniques, it has been confirmed that the cell healing process occurs as a consequence of a dynamic cooperation between many factors at the cell level. The introduction of therapeutic standards in hard-to-heal-wounds has cut down the time of therapeutic treatment and provided an opportunity to improve quality of life, hence the potential to explore effective methods that accelerate the healing of damaged cells. In addition to surgical and pharmacological treatment, modern dressings and compressotherapy can be included to compliment methods supporting wound healing. There is a great interest in the protein receptors connected to growth factors and transgenic therapy, aimed at supplying to the damaged cell, the DNA that codes for the optimal chemical environment (1).

**PHOTOTHERAPY WITH LOW-LEVEL LASER INFLUENCES THE PROLIFERATION OF ENDOTHELIAL CELLS AND VASCULAR ENDOTHELIAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-BETA SECRETION**

The healing process and the angiogenesis associated with it, is a very important but currently poorly understood area. Low level laser therapy (LLLT) has been reported to modulate the process of tissue repair by stimulation of cellular reaction such as migration, proliferation, apoptosis and cellular differentiation. The aim of this work was to evaluate the influence of laser radiation in the range of visible and infrared light on the proliferation of vascular endothelial cells in vitro and the secretion of angiogenic factors: vascular endothelial growth factor (VEGF)-A and transforming growth factor (TGF)-β. Vascular human endothelial cells (Ecs) were exposed to radiation with laser beam of the wavelengths: 635 nm (1.875 mW/cm²) and 830 nm (3.75 mW/cm²). Depending on the radiation energy density, the experiment was conducted in four groups: I) the control group (no radiation, 0 J/cm²); II) 635 nm - the energy density was 2 J/cm²; III) 635 nm - 4 J/cm²; IV 635 nm - 8 J/cm²; II) 830 nm - the energy density was 2 J/cm²; III) 830 nm - 4 J/cm²; IV) 830 nm - 8 J/cm². The proliferation and concentration of VEGF-A and TGF-β were examined. LLLT with wavelength 635 nm increases endothelial cell proliferation. Significant increase in endothelial cell proliferation and corresponding decrease in VEGF concentration may suggest the role for VEGF in this process. The wavelength of 830 nm was associated with a decrease in TGF-β secretion.

**Key words:** angiogenesis, low level laser therapy, transforming growth factor-β, vascular endothelial growth factor, radiation, extracellular matrix
blood by leukocytes and blood platelets. Its production is activated in the environment of a cell with insufficient amounts of oxygen, which is a characteristic feature of the on-going inflammatory process (5). The expression of the VEGF gene is quickly and reversibly stimulated in hypoxic cells. The transcription of mRNA VEGF is induced by binding of HIF-1 factor (hypoxia inducible factor 1), which stimulates production and enhances VEGF secretion. The process of VEGF secretion can also be stimulated under the influence of interleukin IL-6 and IL-8, endothelins, calcium ions, nitric oxide (NO), and the transforming growth factor beta (TGF-β). VEGF influences cells through its receptors. Two of them, VEGFR-1 and VEGF-2, are located on the endothelial cells of arteries and veins, and the third one - VEGF-3 on lymphatic endothelial cells.

It is believed that VEGF is responsible for the initial stages of angiogenesis, regulating and stimulating the initiation of this process. This factor induces vessel permeability the most (50,000 times more than histamine), causing the efflux of blood plasma to the extracellular space, leading to coagulation of extravasated fibrinogen and organisation of extracellular matrix (ECM). Forming of ECM initiates the migration of endothelial cells and germination of vessels (5). VEGF is an indicator of angiogenesis: its concentration increases in patients with atherosclerosis and cardiac ischemia (6, 7). The increase of expression of this factor was discovered in distal located tissues (foot), especially in the necrosis and ulceration areas. The concentration of VEGF also increases gradually after a myocardial infarction.

The second important proangiogenic factor is TGF-β. This protein is generated by various types of cells and organs, i.e. macrophages, platelets and kidney cells. TGF-β is a multifunctional factor with very different influence on the growth and differentiation of cells. The main effect of TGF-β is the increase in accumulation of ECM, including collagen of type I. TGF-β causes a decrease in production of degradation proteases and increase in production of ECM elements and the level of protease inhibitors. During the growth and differentiation of cells it has however two functions; in vivo it indirectly stimulates angiogenesis whilst in vitro it is an endothelial cell growth inhibitor (1, 8) but in low concentrations it has stimulating influence on the proliferation (9). TGF-β can cause cells to go into the pathway of apoptosis. During signal transmission with TGF-β, there are two types of receptors involved: TβRI, TβRII and the Smad proteins. Receptors belonging to serine/threonine kinases regulate the intracellular Smad pathway. The Smad proteins are the molecular family that conduct the signal from receptors of the cell membrane to the nucleus, where they regulate transcription by interaction with proteins bonding with DNA. Disorders in TGF-β expression have been observed in many pathological processes, including tumors (8).

Low level laser therapy is an increasingly popular method used in the treatment of hard-to-heal wounds. Scientific evidence (10-12) confirms that it is safe for patients, however, the basis of its biological action is still not fully known.

Laser radiation by laser phototherapy can influence individual cells only through substances that absorb it. Such cell organelles are mitochondria, which supply energy to cells. They have a series of enzymes that take part in the redox reactions of the respiratory chain which are the principal mechanism by which metabolic reactions take place in a cell. One of the light (red and infrared range) absorbents are cytochrome c oxidase and superoxide dismutase - NADH. They contain active centres which are photoinitiated, changing their level of oxidation. As a consequence, the electrons in a molecule flow out. Information on that process seems to confirm that photoinitiated reactions of electron transfer are able to start the synthesis and conformational changes of proteins and lead to increased synthesis of DNA and RNA (13).

The activation of electron transfer in the respiratory chain affects the shape of the electrochemical trans-coat proton gradient in mitochondria. Not only is the redox of the mitochondrial and cytoplasm changed, but also of the whole cell. The activation of coat enzyme that is Na+-K+ ATPase controls the CAMP level (cyclic adenosine monophosphate) in a cell, which can be responsible for the increase in cell proliferation (13, 14).

The aim of this work was to evaluate the influence of low level laser therapy (LLLT), using visible red and infra-red on the growth of vessel endothelial cells and on release of angiogenic factors (VEGF-A and TGF-β).

**MATERIAL AND METHODS**

The approval of the Bioethics Commission of the NCU Collegium Medicum in Bydgoszcz was obtained, No KB/312/2007, date: 11.05.2007.

**Human umbilical vein endothelial cells (HUVEC) isolation and culture**

Endothelial cells (Ecs) were derived from human umbilical veins by using collagenase according to the method described by Strober (15). Cells were placed in growth medium consisting of medium M199, fetal bovine serum (FBS) - 20% v/v and penicillin 100 IU/ml (Gibco™) with addition of 50 µg/ml of endothelial cell growth supplement (ECGS - Biomedical Technologies Inc. USA) and heparin. Before and after laser irradiation the cells were kept in the incubator at 37°C. The temperature of the incubator was kept at 37°C. After a few passages and placing the cells in 6-well culture plates, the experiment was conducted. Ecs were plated at a density of 7.5×10⁵ per 1 cm². Endothelial cells came from three independent isolations.

**Laser irradiation and measurement of growth factors**

A semiconductor-based (GaAlAs) laser (Roithner Lasertechnik GmbH, Austria) was used to generate visible laser beam with the wavelength of 635 nm and the wavelength of 830 nm in the infrared. At the cell-layer level, the power density measured using a laser power meter (Gentec, Model SOLO2 R2, Canada) was 1.875 mW/cm² for 635 nm and 3.75 mW/cm² for 830 nm. The power was constant in all experiments. The distance between the laser source and the surface of application was 10 centimeters, application was carried through an optical fiber, the irradiated area was 80 cm². In this paper the authors have used the optoelectronic set for controlled, reproducible exposure of electromagnetic radiation of biological structures in the spectral band of tissue transmission window 600–1000 nm (16).

The groups were: I) the control group (no radiation, 0 J/cm²; II) 635 nm - the energy density was 2 J/cm² (1066 s); III) 635 nm - 4 J/cm² (2132 s); IV) 635 nm - 8 J/cm² (4264 s); II) 830 nm - the energy density was 2 J/cm² (533 s); III) 830 nm - 4 J/cm² (1066 s); IV) 830 nm - 8 J/cm² (2132 s). All experimental groups, including the control group, were exposed to the same environmental and stress conditions like temperature and humidity. After irradiation all cultures were incubated in aforementioned culture media. The cells were incubated in the standard air/C0₂ incubator for 6 days, with two radiations on the day No 2 and 4 with one day-break. Then the supernatant from the used material was centrifuged and levels of VEGF-A and TGF-β1 antigen were measured by enzyme immunoassay using...
the ELISA kits of Bender MedSystems Company according to manufacturer’s instructions. Absorbance was measured at 450 nm using a Multiskan EX of Thermolabsystems plate reader and analyzed using Ascent 2.6 software. Concentrations of VEGF-A and TGF-β1 were quantified using standard absorbance curves. For evaluation of Ecs proliferation, after 6 days incubating cells were harvested using 500 µl of trypsin and 1100 µl of media. The cell-growth curve was determined by direct cell counting with the assistance of dye exclusion staining. The trypsinized cell suspension was mixed with the equal volume of 0.4% trypan blue. The viable cells were counted on a hemocytometer chamber.

Statistical evaluation

Statistical analysis was carried out with the use of Statistica 8.0 computer application. A one-way ANOVA was used for parametrical analysis (proliferation) and a Kruskal-Wallis test was used for nonparametrical comparisons (other parameters). The results of proliferation were presented as relative values in reference to the control group, which was not subjected to radiation. The optical density of the control group was considered as 100% of viability. Statistical significance was defined as p<0.05.

RESULTS

The use of laser radiation wavelength of 635 nm (1.875 mW/cm²) was associated with a statistically significant increase in proliferation of endothelial cells (Table 1). The energy density of 2, 4, and 8 J/cm² brought significant increases in proliferation, with the most optimal energy density of 4 J/cm² (p=0.0007). The use of laser radiation wavelength of 830 nm (3.75 mW/cm²) with energy density of 2, 4, and 8 J/cm² resulted in increase in cell proliferation compared with the nonradiated group but the results were not statistically significant (p=0.05) (Table 2).

Furthermore laser radiation with the wavelength of 635 nm lead to a non-significant decrease in the secretion of angiogenic VEGF-A levels (p=0.0886) (Table 1). The wavelength of 830 nm was associated with a small increase in VEGF-A secretion, the largest at a energy density of 2 J/cm² (48.33 pg/10⁵ cells) (Table 2). The results were, however, not statistically significant.

In addition laser radiation at wavelength of 635 nm was associated with the greatest level of change in TGF-β secretion with the best level at energy density of 4 J/cm² (4.50 ng/10⁵ cells), but due to the high variability of this parameter, the differences between results for the individual doses were not statistically significant (p=0.6546) (Table 1). The wavelength 830 nm caused a decrease in TGF-β secretion, the largest at a density of 8 J/cm², but the results were not statistically significant (Table 2).

DISCUSSION

In recent years there has been a continued increase in the number of publications focussing upon cell healing processes (17-19), and attempting to explain the complex mechanisms of angiogenic process (20). A number of in vivo studies regarding delayed wound healing, for example in diabetes, describe TGF-β as an enhancer of the healing process (1, 9). Animal research has shown that TGF-β can accelerate the wound healing process by stimulating fibroblasts to increase the production of collagen. During empirical research on mice it has been shown that inhibition of TGF-β synthesis is the focus of many inflammatory processes in various organs, leading ultimately to delayed wound healing.

Table 1. Number of endothelial cells, concentration in supernatant of VEGF and TGF-β after LLLT (wavelength 635 nm) with different energy doses.

<table>
<thead>
<tr>
<th>Energy dose (J/cm²)</th>
<th>%Cells number (% of control), n=8</th>
<th>VEGF concentration (pg/10⁵ cells), n=12</th>
<th>TGF beta concentration (ng/10⁵ cells), n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.00 (10.85)</td>
<td>36.68 (28.60–44.00)</td>
<td>2.90 (1.06–5.55)</td>
</tr>
<tr>
<td>2</td>
<td>120.34 (14.33)</td>
<td>24.68 (20.32–37.57)</td>
<td>3.64 (1.13–5.26)</td>
</tr>
<tr>
<td>4</td>
<td>127.03 (18.08)</td>
<td>23.18 (20.54–28.15)</td>
<td>4.50 (1.27–7.33)</td>
</tr>
<tr>
<td>8</td>
<td>118.18 (7.84)</td>
<td>21.70 (18.97–39.76)</td>
<td>2.58 (0.95–5.58)</td>
</tr>
<tr>
<td>P-values</td>
<td>0.0103 0.0007 0.0253</td>
<td>0.0886 0.6546</td>
<td></td>
</tr>
</tbody>
</table>

Values for cells number are means (standard deviation, S.D.), values for VEGF and TGF beta concentration are medians (percentile 25 - percentile 75), p-values for cells number are only statistically significant in post hoc test.

Table 2. Number of endothelial cells, concentration in supernatant of VEGF and TGF-β after LLLT (wavelength 830 nm) with different energy doses.

<table>
<thead>
<tr>
<th>Energy dose (J/cm²)</th>
<th>%Cells number (% of control), n=8</th>
<th>VEGF concentration (pg/10⁵ cells), n=12</th>
<th>TGF beta concentration (ng/10⁵ cells), n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.00 (11.84)</td>
<td>43.61 (19.30–71.56)</td>
<td>9.16 (5.52–13.18)</td>
</tr>
<tr>
<td>2</td>
<td>108.89 (11.31)</td>
<td>48.33 (21.64–67.16)</td>
<td>6.35 (1.92–8.19)</td>
</tr>
<tr>
<td>4</td>
<td>107.66 (15.61)</td>
<td>43.14 (26.18–63.12)</td>
<td>5.25 (2.32–12.10)</td>
</tr>
<tr>
<td>8</td>
<td>111.55 (17.58)</td>
<td>33.94 (24.83–52.16)</td>
<td>1.61 (0.73–5.04)</td>
</tr>
<tr>
<td>P-values</td>
<td>0.3796 0.8969 0.0886</td>
<td>0.0680 0.0886</td>
<td></td>
</tr>
</tbody>
</table>

Values for cells number are means (standard deviation, S.D.), values for VEGF and TGF-β concentration are medians (percentile 25 - percentile 75).
the death of an animal (21). Human research using the tooth extraction wound model exposed to laser radiation (904 nm, 10 mW, 3 J/cm²) confirmed statistically significant increases in TGF-β concentration compared to wounds not treated by laser therapy (22).

There is also evidence that the laser radiation dose delivered affects the direction of TGF-β performance, particularly in light of in vitro research. Khanna and co-authors (23) examined the influence of laser radiation He-Ne (632.8 nm) on the proliferation and angiogenic factors VEGF and TGF-β secretion by cardiomyocytes. The results of in vitro studies show statistically significant increases in proliferation (p<0.05) at the power level of 5 mW and radiation time of 15 and 20 minutes in comparison with the control group which was not subjected to the laser beam radiation. VEGF and TGF-β genes expression significantly increased (p<0.001 for VEGF, p<0.05 for TGF-β) when exposed to 10, 15 and 20 minutes radiation. Some present study demonstrated the stimulating effect of LLLT on Ecs proliferation, VEGF (24, 25) and TGF-β (8, 22) secretion. Findings from the reviewed studies clearly demonstrate the ability of laser irradiation to modulate gene expression and the release of growth factors and cytokines from cells in culture.

Our current results confirm that different wavelength of laser exposure on TGF-β secretion. The wavelength of 635 nm caused, at the ascending dose, a increase in TGF-β concentration in the supernatant, in contrast, the wavelength of 830 nm caused the concentration to decrease to 1.61 ng/10⁵ cells. The different influence laser beam radiation with the wavelength of 635 nm (the highest proliferation increase) and 830 nm (the lowest TGF-β concentration) on the proliferation and angiogenic factors secretion may be caused by different levels of photoreception influences. It seems that in the currently conducted research with the use of laser beam radiation including visible light environment (635 nm) and infrared (830 nm), TGF-β acts like a proliferation inhibitor. Its diminished secretion at an energy density of 8 J/cm² could contribute to the proliferation increase of 112% (Table 2).

The research done on patients with the postoperative backbone degenerative wound showed a lack of statistically significant differences in VEGF concentration in blood serum (26). The study included 20 adult patients after surgical treatment of degenerative changes in the spine. Each patient’s venous blood samples were obtained three times: before the surgery, on day 1 after surgery and between the 5th and 6th day after the operation. The VEGF concentration was assessed using the immunoenzymatic method (ELISA). The control group after the operation. The VEGF concentration was assessed using the ELISA. The control group was not subjected to laser therapy. The current VEGF research in supernatant confirmed that the ascending energy density of laser radiation with the wavelength of 635 nm, and especially the energy density of 8 J/cm² was associated with a decrease in its concentration. The same dose was accompanied by the best expressed proliferation of endothelial cells. Our observation is consistent with the results from other studies (27, 28) who also found an increase in cell proliferation under the influence of laser radiation.

VEGF acts through its receptors located on the endothelial cells: VEGFR1 and VEGFR2, causing the proliferation increase. VEGFR1 and VEGFR2 are known to transduce the full range of VEGFR1 and VEGFR2, causing the proliferation increase. VEGFR1 and VEGFR2 are known to transduce the full range of VEGF responses in endothelial cells, i.e. regulating endothelial survival, proliferation, migration and formation of the vascular tube (29). The lowest level of VEGF at a energy density of 8 J/cm² (λ=635 nm) simultaneously at the statistically significant proliferation level suggests that in such conditions the greatest number of VEGF elements merged with the receptor, which had significant influence on the level of ECs proliferation. Confirming this will need further study. We are currently breeding endothelial cells in order to measure not only the VEGF, but also both receptors (VEGFR1 and VEGFR2) which condition the proliferation action on the breeding cells.

The present study demonstrated changes in VEGF-A and TGF-β concentration after the laser irradiation (635 nm and 830 nm). This study is novel, there are relatively few data available on the effects of LLLT on vascular endothelial cells. There remain a number of questions about molecular mechanism responsible for the function of LLLT on Ecs proliferation and growth factors secretion. More recently, researchers have paid close attention to the signaling pathways involved in the biological effects of LLLT. It has been reported that LLLT could activate the MAPK/ERK (mitogen-activated kinase/extracellular signal-regulated kinases) pathway and promote proliferation of the cells (30). Other studies demonstrated that MAPK signaling cascade regulates VEGF expression and angiogenesis in myocardial microvascular endothelial cells (31, 32).

Further research is needed to describe the influence of low level laser therapy on the proliferation of endothelial cells and the secretion of angiogenic factors. The potential for angiogenesis modulation using LLLT could facilitate its usage in illneses with treatments based on vascularisation.

We conclude that low level laser therapy with the wavelength of 635 nm causes the increase of endothelial cells proliferation; the greatest increase was observed with the use of wavelength of 635 nm and the energy density of 4 J/cm². Significant increase of the endothelial cells proliferation with the significant decrease of VEGF concentration occurred at the same time which may suggests the involvement of VEGF in this process.

Conflict of interests: None declared.

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