

Can laser therapy modify the secretion of the tissue-type plasminogen activator and its inhibitor in an endothelial cell culture under hyperglycemia?

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ABSTRACT

Introduction: The effect of low-level laser therapy on the secretion of tissue-type plasminogen activator (t-PA) and its inhibitor (PAI-1) in an endothelial cell (EC) culture under hyperglycemia is the subject of the presented work.

Hyperglycemia associated with diabetes causes vascular EC dysfunction. Low-level laser therapy is a good method to support the pharmacological treatment of diabetes complications.

Materials and methods: We used lasers of 2 wavelengths: 635 nm and 830 nm with dose of 2 J/cm². The experiment was performed *in vitro* in 4 groups of EC: 1 – no glucose in culture medium, no irradiation (control), 2 – glucose, no irradiation, 3 – glucose, laser 635 nm, 4 – glucose, laser 830 nm. After 2 irradiations, cells were counted and the t-PA and PAI-1 antigen (Ag) concentration in the supernatant was measured.

Results: In group 2, we observed a statistically significant lower number of cells ($p < 0.0001$) and a higher concentration of t-PA:Ag and PAI-1:Ag ($p < 0.05$) compared to the control group. However, in groups 3 and 4, the number of cells increased and the concentration of t-PA:Ag and PAI-1:Ag decreased compared to group 2 and nearly reached the values in the control group.

Conclusions: Hyperglycemia affects the fibrinolytic activity of ECs which is manifested by a significant increase in t-PA:Ag and PAI-1:Ag concentrations – recognized markers of endothelial damage. Irradiation of ECs by a low-power laser caused attenuation of the adverse effects of hyperglycemia. A tendency towards a decrease in t-PA:Ag and PAI-1:Ag concentration in the supernatant was observed with a significant increase in the number of cells to values close to control.

Keywords: low-level laser therapy; endothelial cell culture; t-PA; PAI-1; hyperglycemia.

INTRODUCTION

A high glucose level in diabetes mellitus (DM) causes a dysfunction in the vascular endothelium that lines the interior surface of blood vessels. The endothelium plays a key role in maintaining vascular homeostasis via the secretion of active agents involved in many biological processes such as hemostasis, angiogenesis and inflammation [1]. Hyperglycemia induces changes such as an increase of plasminogen activator inhibitor-1 (PAI-1) and endothelin-1 or a decrease in nitric oxide (NO) in the fibrinolytic and vasoactive components. Nitric oxide, by dilating vessels and inhibiting thrombocyte adhesion, is an important factor preventing thrombosis [2, 3]. Under conditions of elevated glucose levels, the weakening of endothelial fibrinolytic activity is also associated with an impairment in the binding of tissue-type plasminogen activator (t-PA) with inactive proenzyme plasminogen, which in turn leads to a decrease in plasmin generation – an active enzyme that is responsible for thrombus degradation [4]. A balance in t-PA and its inhibitor PAI-1 is a requirement for the proper functioning of the hemostatic system. Impaired fibrinolysis under hyperglycemic conditions disturbs this state leading to pro-thrombotic complications [5]. Elevated levels

of t-PA and PAI-1 were observed in diabetic patients with cardiovascular complications and in patients with ischemic heart disease [6, 7]. Plasminogen activator inhibitor-1 is an independent risk factor for the development of diabetes in healthy people as elevated levels were observed in patients with DM and cardiovascular diseases [8]. High levels of t-PA and PAI-1 are considered a predictor of vascular complications [9, 10]. Many studies have shown that hyperglycemia reduces cell proliferation and migration, increases apoptosis and also affects the rate of cell aging [11, 12, 13, 14] which interferes with the wound healing process.

The treatment of diabetes and its vascular complications, such as leg ulcers, are still a huge challenge for modern medicine. A good method for supporting the pharmacological treatment of diabetes complications is low-level laser therapy (LLL) which was developed in the 1960s. During irradiation, photons are absorbed in the mitochondria of the cells, allowing for an improvement in energy metabolism and stabilization of the cell membrane [15, 16]. Low-level laser therapy has a biostimulative effect without a temperature increase, is painless, non-invasive and has no side effects, brings pain relief and affects faster recovery [17].

This work is a continuation of our *in vitro* studies [18] on the effect of hyperglycemia on the vascular endothelial cells (ECs) of the human umbilical vein endothelial cells (HUVECs) line. The aim of the current pioneering research was to evaluate the effect of a low-level laser at 2 wavelengths of 635 nm (visible light) and 830 nm (infrared) on the t-PA and PAI-1 antigen (Ag) concentration in the HUVEC culture supernatant in hyperglycemia.

MATERIALS AND METHODS

Human umbilical vein endothelial cell isolation and culture

Human umbilical vein ECs were isolated and cultured under hyperglycemic conditions as described previously [18]. The main part of the experiment was performed on cells cultured in 6-well plates (seeding density of 7.5×10^3 cells/cm²) and derived from 3 independent isolations.

Characteristics of laser irradiation and the measurement of analyzed parameters

In the experiment we used a semiconductor-based laser in a set for the biological structure irradiation, created by colleagues from the Bialystok University of Technology [19]. Laser irradiation with wavelengths of 635 nm (power density 1.875 mW/cm²) and 830 nm (power density 3.75 mW/cm²) were applied. The exposure dose was 2 J/cm². The irradiation conditions in the study group were identical to those described in our previous experiment [18]. The cells from the 1st group (control) were not exposed to laser irradiation and the culture medium did not contain glucose. The 2nd group of cells was not irradiated either but the medium contained glucose at a level of 30 mM/L. The 3rd and 4th group were cultured in a medium with 30 mM/L glucose and irradiated with wavelengths of 635 nm and 830 nm, respectively.

The levels of t-PA antigen and PAI-1 antigen were determined in the supernatant after 7 days using the ELISA test (eBioscience, Vienna, Austria) according to the manufacturer's instructions. The cells were counted in all wells of the experimental plates by a Burker hemocytometer using trypan blue dye according to Basso et al. [20].

Statistical analysis

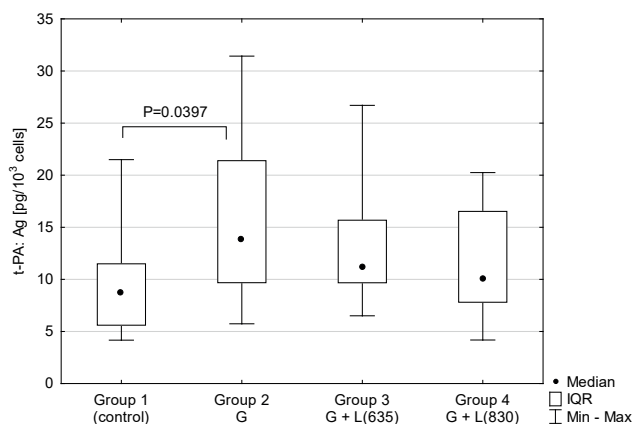
Statistical analysis was performed using Statistica 13.1 (Dell Inc. Tulsa, USA). The one-way ANOVA with *post hoc* test was used. The Fisher test was used for normal distribution variables (cell numbers) and the Kruskal–Wallis test was used for non-normal distribution variables: tissue-type plasminogen activator antigen (t-PA:Ag) and plasminogen activator inhibitor-1 antigen (PAI-1:Ag). Statistical significance was defined as $p < 0.05$. Only statistically significant values were given in the table and figures. The mean \pm standard deviation or median and interquartile range was used for the statistical description.

Ethical considerations

The approval of the Bioethics Commission of the NCU Collegium Medicum in Bydgoszcz was obtained, No KB/135/2009 with the last annex of 19.06.2018.

RESULTS

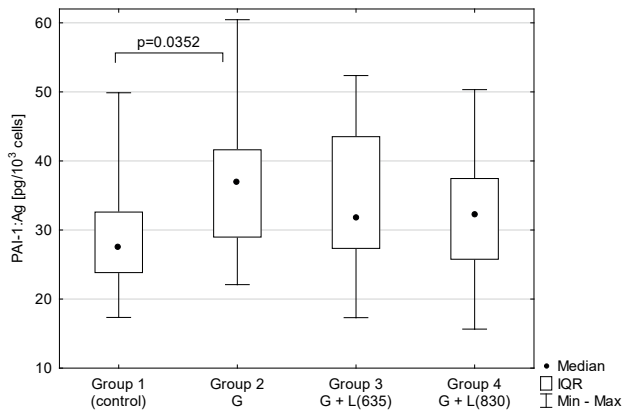
Figure 1 shows the results regarding the effect of low-level laser irradiation on t-PA:Ag concentration in the supernatant of the HUVEC culture. This was cultured with an elevated glucose level in the medium (glucose concentration 30 mM/L). The level of t-PA:Ag in the control group (group 1) without glucose in the medium and not irradiated by the laser was 8.69 pg/10³ cells. In group 2, which contained glucose in the medium and was not irradiated, the concentration of t-PA:Ag was significantly higher (13.86 pg/10³ cells) in comparison to the control group ($p = 0.0397$). In groups 3 and 4, cells were growing in the medium with glucose and irradiated by the laser at a wavelength of 635 nm and 830 nm, respectively. The concentration of t-PA:Ag in group 3 was slightly lower in comparison with group 2, and in group 4 was notably lower. This value amounts to 10.09 pg/10³ cells which is a 37% decrease in relation to group 2. However, these differences were not statistically significant.



G – glucose in culture medium; L(635) – laser irradiation with a wavelength of 635 nm; L(830) – laser irradiation with a wavelength of 830 nm; IQR – interquartile range; Min – minimum; Max – maximum

FIGURE 1. The antigen concentration of tissue-type plasminogen activator in the supernatant of human umbilical vein endothelial cells line endothelial cell culture depending on hyperglycemia and laser irradiation of different wavelengths

Figure 2 shows the concentration of PAI-1:Ag in analogous groups as in Figure 1. The concentration of PAI-1:Ag in the control group was 27.53 pg/10³ cells and was 34% lower than in group 2 ($p = 0.0352$). Laser irradiation at a wavelength of 635 nm in a hyperglycemic environment (group 3) reduced the PAI-1:Ag concentration to 31.72 pg/10³ cells and a similar effect was obtained for the laser at a wavelength of 830 nm (group 4). These values approximated to values in the control group, however, differences in relation to group 2 were not statistically significant. There were also no differences in the effects of the 2 wavelengths.



G – glucose in culture medium; L(635) – laser irradiation with a wavelength of 635 nm; L(830) – laser irradiation with a wavelength of 830 nm; IQR – interquartile range; Min – minimum; Max – maximum

FIGURE 2. The antigen concentration of plasminogen activator inhibitor-1 in the supernatant of human umbilical vein endothelial cells line endothelial cell culture depending on hyperglycemia and laser irradiation of different wavelengths

Table 1 shows the number of HUVECs was highest in the control group and the lowest number of cells was observed in group 2. The difference was statistically significant ($p < 0.0001$) in comparison with the control group. In group 3, the number of cells was slightly higher compared to group 2 and in group 4 it reached a level closer to the control group. The differences between groups 3–4 were statistically insignificant.

TABLE 1. The number of human umbilical vein endothelial cells (N*105) depending on hyperglycemia and laser irradiation of different wavelengths

Control Group 1	Glucose Group 2	Glucose + Laser 635 nm Group 3	Glucose + Laser 830 nm Group 4	p
6.82 ± 0.96	4.87 ± 0.77	5.35 ± 0.82	6.11 ± 0.71	<0.0001 ^{1 vs. 2} <0.0001 ^{1 vs. 3} <0.0004 ^{2 vs. 4}

The values were described as M ± SD; p – statistical significance according to one-way ANOVA with *post hoc* test.

DISCUSSION

Tissue-type plasminogen activator is the major physiological plasminogen activator. Its concentration depends on the concentration of its inhibitor PAI-1. A significant portion of t-PA circulates in plasma in complexes with PAI-1. The proteins of the fibrinolytic system are also involved in tissue remodeling, angiogenesis and wound healing. Synthesis of t-PA and PAI-1 takes place primarily in the ECs of blood vessels and in the liver and vascular smooth muscle cells [21, 22]. This is why our *in vitro* experimental model is based on ECs of the HUVEC line grown in a medium containing glucose at a concentration imitating the conditions found in the blood of patients with diabetes (30 mM/L). This level of glucose concentration in the culture medium is commonly used in this type of *in vitro* study. An elevated level of blood glucose promotes non-enzymatic glycation of proteins (including activators and inhibitors of the

hemostatic system) and contributes to the release of advanced glycation end products and oxidative stress [23]. Adverse processes then occur in the mitochondrial respiratory chain and there is an increase in the production of free radicals and peroxide anions [1]. Advanced glycation end products bind to specific receptors and activate cells through the proinflammatory nuclear factor (NF-κB) which increases the gene expression of proinflammatory cytokine, growth and hemostasis factors [24].

The results of our experiment confirm the adverse effect of hyperglycemia on vascular ECs. It caused a decrease in ECs numbers in comparison to the control (Tab. 1) as well as a simultaneous increase in the concentration of t-PA:Ag and PAI-1:Ag in the supernatant (Fig. 1, 2). The difference between these values was over 30% and statistically significant. Some researchers have observed a decrease in t-PA:Ag levels in patients with diabetes [3], but our research shows that hyperglycemia causes damage to ECs (and a reduction in their numbers, Tab. 1) and at the same time, induces an increased concentration of t-PA:Ag and PAI-1:Ag in the supernatant (Fig. 1, 2), as is confirmed by other researchers [2, 6]. This can be explained by a simultaneous increase in the formation of t-PA-PAI-1 complexes under conditions of high PAI-1:Ag concentration. The research of Pandolfi et al. indicates that hyperglycemia causes an increase in t-PA:Ag levels, but its active form is radically reduced because t-PA:Ag in the complex with PAI-1:Ag is fibrinolytic inactive [25]. Knudsen et al. show that abnormal glucose regulation strengthens fibrinolytic stress and is associated with high levels of t-PA:Ag and PAI-1:Ag in patients after myocardial infarction [8]. For a complete understanding of these processes, further research should be complemented by PAI-1 activity results. The obtained results also suggest the necessity of undertaking immunohistochemistry research on ECs cultures to obtain knowledge about the location of the studied proteins and damage caused by hyperglycemia.

The number of circulating endothelial progenitor cells is significantly reduced in diabetes, as is their proliferation, adhesion and migration [26]. In hyperglycemia, there is also an impairment in angiogenesis and neovascularization. It also induces aging in the vascular cells of mice, which is manifested by an increase in PAI-1, p53 and p21 [27]. This is confirmed by *in vitro* studies on HUVEC. Along with an increase in the number of passages, they proliferate more slowly and show increased expression of PAI-1 [12]. In our study, the number of HUVECs was significantly lower (by 29%) under hyperglycemic conditions when compared to the control group (normal glucose concentration) – Table 1. Considering the high concentration of t-PA:Ag and PAI-1:Ag in the supernatant from the EC culture with glucose (Fig. 1, 2), it is possible to assume that hyperglycemia has led to some of these cells disintegrating and releasing t-PA and PAI-1.

Nowadays, standard treatment methods for diabetic complications such as ulcers and hard-to-heal wounds are often supported by unconventional treatments. Phototherapy using low-power lasers or light-emitting diodes have promising effects. Low-level laser therapy stimulates microcirculation and angiogenesis which accelerates the healing of wounds [28].

In a study conducted by Feitosa et al. on DM patients, the use of a 30 mW laser with a wavelength of 632.8 nm at a dose of 4 J/cm² resulted in a significant reduction in wound size compared to the control group [29]. The mechanism of action of LLLT comprises an absorption of photons by irradiated cells.

The main role in the mitochondrial respiratory chain is played by cytochrome C oxidase. Its concentration increases under the influence of low-level laser irradiation. This enzyme catalyzes the reduction of oxygen for energy metabolism. It increases the transport of electrons in the respiratory chain and increases oxygen consumption within the mitochondria [16]. This process is accompanied by the synthesis of adenosine. Various transcription processes are initiated as well as the activation of signaling pathways [15, 30]. As a consequence, this has an impact on the treatment improvement due to the release of NO and suppression of reactive oxygen species and NF-κβ. Low-level laser therapy also improves cell viability and proliferation which are attenuated by hyperglycemia [11, 13, 31]. In our research, the beneficial effect of laser therapy is manifested by a decrease in the concentration of t-PA:Ag and PAI-1:Ag in the groups of irradiated cells compared to non-irradiated cells growing in the medium containing an elevated glucose level (Fig. 1, 2). In addition, the number of cells growing in hyperglycemic conditions in the groups irradiated by lasers (3–4) was higher than in the non-irradiated group (group 2). A difference of 25% was observed between groups 2 and 4 (p = 0.0004) – Table 1. Differences between the 2 waves are not statistically significant, although slightly more favourable effects were noted for the 830 nm wave (Fig. 1, 2). The research of Houreld and Abrahamse on diabetic-wounded fibroblast cells indicates better cell proliferation under the influence of a 632.8 nm wave than 830 nm [32]. Numerous studies confirm differences in the effects of LLLT at different wavelengths but this mechanism has not yet been explained. This difference may be caused by the activation of ion channels by certain wavelengths. Furthermore, differences in the optimal dose of radiation (J/cm²) for particular wavelengths can also have an impact [33].

CONCLUSIONS

Hyperglycemia caused an increase in the concentration of t-PA:Ag and PAI-1:Ag in the supernatant of an EC culture with a simultaneous decrease in the number of cells, indicating that some of them had been damaged or were apoptotic.

Irradiation of ECs by a low-power laser caused an attenuation of the adverse effects of hyperglycemia. A tendency towards a decrease in the concentration of t-PA:Ag and PAI-1:Ag in the supernatant was observed with a significant increase in the number of cells to values close to the control. There is a need to continue research in this area.

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