

Modifying effect of intravenous laser therapy on the protein expression of arginase and epidermal growth factor receptor in type 2 diabetic patients

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Received: 11 December 2015 / Accepted: 24 June 2016 / Published online: 12 July 2016
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Abstract The epidermal growth factor receptor (EGFR) signaling pathway may be involved in cell activation and may influence the neuronal microenvironment, microglia activation, and production of proinflammatory cytokines. Arginase and nitric oxide synthase (NOS) both use L-arginine as a common substrate. Decreasing the arginase expression may increase L-arginine consumption by NOS and increase nitric oxide (NO) synthesis. Intravenous laser blood irradiation (ILBI) is an effective systemic treatment for different pathologies including diabetes mellitus. Previous studies have shown that low-level laser therapy can have an effect on the release of certain cytokines and growth factors. The aim of this study was to evaluate the effects of ILBI on the expression of arginase and epidermal growth factor receptor in type 2 diabetic patients. We used 630 nm red laser light, 1.5 mW, continuous mode, intravenously for 30 min in 13 type 2 diabetic patients and compared their

blood samples using the flow cytometry technique, before and after ILBI. The difference between the percentage of cells before and after therapy was analyzed using repeated-measures ANOVA, and the relationship between EGFR and arginase expression in blood and tissue was evaluated by calculating the Pearson correlation coefficient. We found a significant decrease in the expression of both arginase- and EGFR-positive cells after laser therapy ($P < 0.01$). In conclusion, laser therapy may have a beneficial effect for diabetic patients via decreasing arginase expression and activation of the NOS/NO pathway which increases NO production and vasodilation, and decreasing EGFR expression which may reduce neuroinflammation and its secondary damages.

Keywords Diabetes · Laser therapy · Flow cytometry · Arginase · Epidermal growth factor receptor

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Introduction

Metabolic diseases including diabetes and cardiovascular diseases are one of the main public health problems [1], and atherosclerosis is a hallmark in the development of myocardial infarction, stroke, and other cardiovascular disorders [1]. Micro- and macrovascular angiopathy is one of the main complications in long-term-duration diabetes mellitus [2]. Since 40 years ago, low-level laser therapy (LLLT) or laser photobiomodulation has been used in experimental and clinical fields as an adjuvant therapy. LLLT can stimulate or inhibit disturbed biological functions and normalize them. LLLT has been reported to accelerate wound healing, collagen production, and modulation of the immune system. It has been used for various medical conditions including wound healing, dermatological disorders, pain, inflammation, neurologic damage, blood disorders, and musculoskeletal complications and can

modulate chronic stress including stress due to chronic disease [3–7]. This technique is non-thermal and non-cytotoxic, using continuous or pulsed red or near-infrared light (600–1100 nm) with output power of 1–500 mW [8]. One of the methods for laser irradiation is intravenous or intravascular laser blood irradiation (ILBI) which has been used in Russia, China, Germany, and Iran since more than 20 years ago [7, 9–12]. This is a safe and cost-benefit treatment with systemic effects. In this method, laser light directly irradiates blood via a sterile disposable catheter. Two millimeters of the fiber optic enters the vein and irradiates the circulating blood. This procedure is just like serum injection. As ILBI can produce systemic effects, it can be effective in pathologies like diabetes which has generalized complications. Many functions in diabetic patients are impaired and laser irradiation may affect these impairments [9, 10, 13].

Laser light can activate microcirculation, angiogenesis, and regeneration. It can regulate the expression of growth factors including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) [14–16]. It seems that laser light can increase production of arginine and nitric oxide [4] and affects several signaling pathways [17]. He-Ne laser can increase the endothelial NO synthase gene and protein expression and NO secretion in humans [18].

Most studies highlight the role of nitric oxide on endothelial dysfunction as the main mechanism in developing cardiovascular disease [19]. Nitric oxide plays a key role in decreasing vascular tone [2]. Hyper production of free radicals decreases the production of nitric oxide and is suggested to be responsible for endothelial dysfunction in diabetes [20]. L-Arginine is an amino acid which participates in the wound-healing process. This semi-essential amino acid is a common substrate for arginase enzyme and nitric oxide synthase (NOS) [21]. It can be metabolized by one of these two pathways, arginase or nitric oxide synthase. If L-arginine is metabolized by the NOS enzyme, the result is nitric oxide (NO) and L-citroline formation. NO is a free radical which is involved in endothelium-dependent relaxation of vascular smooth muscle [22]. L-Arginine can be metabolized by arginase too. Arginase is a manganese-metallo-enzyme that hydrolyzes L-arginine to urea and L-ornithine [23]. Arginase uses the same substrate for the known NOS isoforms: endothelial NOS, neuronal NOS, and inducible NOS [24]. Increasing the arginase expression may increase L-arginine consumption and convert it to L-ornithine and urea; thus, available L-arginine for NO synthesis by NOS reduces. Several studies show increasing activity of arginase in diabetes mellitus type 2 and hypertension, which proposes the critical role of this enzyme in the pathogenesis of cardiovascular disorders [25]. Studies showed that NOS/NO has an important role in many VEGF-induced functions. VEGF induces the release of NO from vascular endothelial cells [26, 27]. A balance in the NOS/L-arginine/arginase axis is necessary for maintenance of NO homeostatic levels. There

are two main mechanisms for reducing bioactive NO: (1) reduction of its synthesis by NOS and (2) increased oxidative reactivation of NO by reactive oxygen species (ROS) which leads to cardiovascular impairment [28]. Laser irradiation can affect both pathways according to the body condition and balance the axis [29]. Studies show that laser light 350, 420, 470, and 760 nm can affect the turnover of arginine to NO [29].

One of the most important endogenous growth factors in the wound-healing process is epidermal growth factor (EGF) [30]. Signaling through epidermal growth factor receptors is essential for fundamental cellular functions including differentiation, growth, migration, and proliferation. The EGF-related peptides bind to ErbB receptors and induce the formation of homo- and hetero-dimers, which stimulates activation of intrinsic kinase and induces intracellular signaling pathways. In addition, Erbs has an important role in embryogenesis; development of cardiomyocytes; muscle regeneration; development of skin, hair, and eyes; and also cancer [31]. EGF-related peptide binds to the ErbB receptor, activates intrinsic kinase, and stimulates intrinsic signaling pathways [31]. Activation of the tyrosine kinase of the receptor induces signal transduction epidermal growth factor receptor (EGFR) and activates downstream signaling molecules [32].

EGFR has key roles in regulating cell activation. Downregulation of EGFR decreases the EGFR/MAPK cascade and regulates inflammation. EGFR signaling plays roles in several central nervous system disorders and ischemia [33]. Reduction of EGFR expression can decrease microglia activation and IL-1 β and TNF- α production [34].

EGFR overexpression also has been reported in some cancers including breast cancer, small cell lung cancer, and glioblastoma. It seems that an overexpression can induce a transformation of the cell line into malignant phenotype [35, 36].

Our previous studies showed significant effects of LLLT on wound healing, expression of genes involved in wound healing, neuropathy, and metabonomics of the blood in diabetes [7, 9, 10, 15, 37]. In this study, for the first time, we investigate the effects of low-level laser irradiation on the expression of arginase and EGFR after intravenous laser irradiation in diabetic patients.

Materials and methods

After approval of the ethics committee of Tehran University of Medical Sciences, 13 type 2 diabetic patients who were referred to the laser clinic of Milad Hospital and who agreed to participate in our study were selected. After consent was given by the patients, non-fasting venous blood samples were collected in standard 5-ml sodium heparin tubes for flow cytometry study before and after the laser therapy. ILBI was applied intravenously (the laser fiber was inserted into the cubital vein

in the forearm using a catheter, the catheter enters 2–3 cm² into the vein, and 2 mm of the fiber is exposed at the tip of the catheter) with a 1.5-mW, continuous, 630-nm laser therapy apparatus (Mulat, Tecknica Co., Moscow, Russia) for 30 min. The power output was 1.5 mW and the spot size was 0.01 cm². This protocol was designed according to our previous experiments [7]. Another venous blood sample was collected in a standard 5-ml sodium heparin tube 30 min after ILBI. The samples were stored at –20 °C until analysis and thawed prior to use.

Flow cytometry

For cell extraction, tissue of foot debris and lymphocytes from whole blood were, shortly, cultured and incubated with RPMI media (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. Cells were washed twice in KCl 0.075 M and fixed in methanol (Merck KGaA, Darmstadt, Germany).

Staining of the cells was performed by monoclonal anti-arginase liver type, isotype IgG1 (US Biological, Swampscott, MA 01907 USA), and EGFR antibody (528) as a mouse monoclonal IgG2a (Santa Cruz Biotechnology Inc., 69115, Heidelberg, Germany).

The cells were incubated at 4 °C for 25 min and were washed twice by PBS. For the next step, secondary antibody goat anti-mouse IgG2a/R-Pe (AbDSerotec, MorphoSys UK Ltd, Oxford, UK) and antibody anti-mouse IgG1/Cy5 (Rockland, Gilbertsville, PA 19525 USA) were added and incubated for 25 min; then, the cells were washed with PBS. Mouse IgG2a/PE-Cy5 and IgG1/R-Pe (Dakocytomation, Glostrup, Denmark) were used as negative control for the same population.

The Partec-Denmark flow cytometry apparatus (Sysmex and Partec, Denmark, CyFlow® Cube) and LEICA, DM RXA2-fluorescence microscope (Wetzlar and Mannheim, Germany) were used for cellular assays.

Table 1 Clinical characteristics of 13 treated patients before laser therapy

	Mean	SE
Triglyceride	183.00 mg/dl	29.38
HDL/LDL	0.88 mg/dl	0.26
FBS	176.08 (mg/dl)	20.92
HBA1C	8.41 (mmol/mol)	0.78
BMI	27.85 (kg/m ²)	1.76
H/W	1.05	0.018
Age	60.31 years	2.57
Disease duration	13.85 years	2.01

HDL/LDL high-density lipoproteins/low-density lipoproteins, *FBS* fasting blood sugar, *HBA1C* glycated hemoglobin, *BMI* body mass index, *H/W* waist-hip ratio

Table 2 Percentage of arginase- and EGFR-positive cells in the total cell count of blood samples before and after laser therapy

Marker		Mean	SE
EGFR	Before	4.013	0.69
	After	2.50	0.43
Arginase	Before	4.96	0.70
	After	2.53	0.45

EGFR epidermal growth factor receptor

Results

The number of cells with each marker was expressed and analyzed as percent (Tables 1 and 2). The difference between the percentage of cells before and after laser therapy and the effect of sex and age group were assessed using repeated-measures ANOVA (The age of patients was entered into the equation as a two-level categorized variable: <60 and ≥60 years) (Table 3). The relationship between percentage of EGFR- and arginase-positive cells in blood and tissue was evaluated by calculating the Pearson correlation coefficient (Figs. 1, 2, 3, and 4). All statistical analyses were conducted by SPSS version 22, and an error probability of $P < 0.05$ was regarded as significant.

Two of 13 patients were smokers (20 %). Four of 10 patients had A+ blood type (40 %), four of them had O+ blood type (40 %), just one patient was A–, and one was AB+ (10). The blood type data was not available for other patients. The mean difference between the percentage of positive cells before and after treatment was supposed to be 2. Taking a standard deviation of 2, the power of statistical analysis for a sample size of 13 was calculated using open epi version 3.03a. The power for this sample size was approximately 72 %.

Discussion

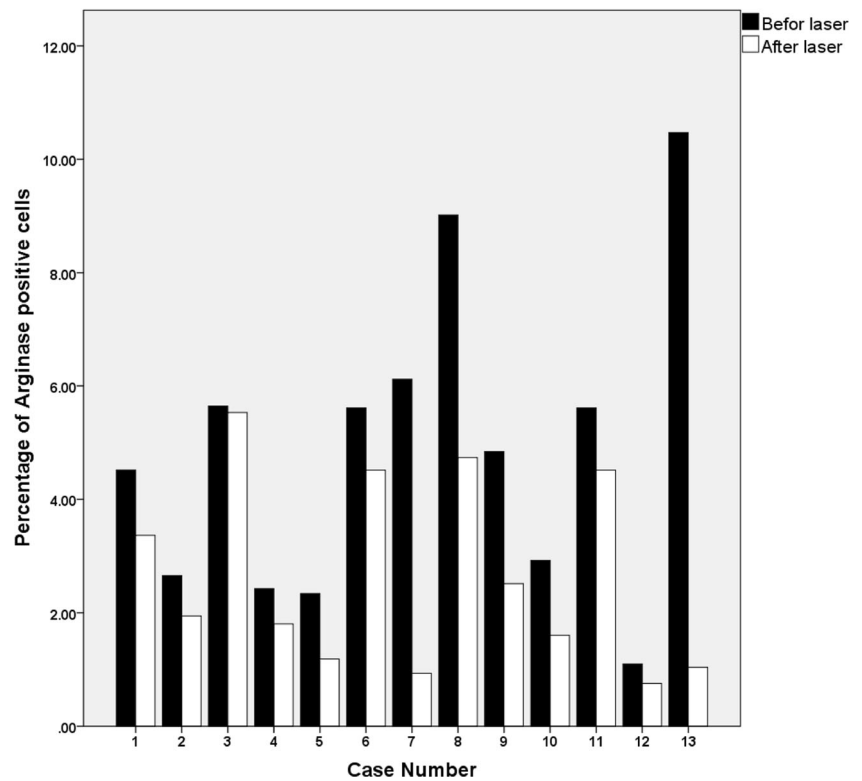
The main finding of this study is that intravenous laser treatment in patients with diabetes mellitus type 2 affects

Table 3 The effect of laser therapy on the percentage of arginase- and EGFR-positive cells in the total cell count of blood samples regarding sex and age factors

Marker	Factor	<i>P</i> value
Arginase	Laser irradiation	0.001*
	Sex	0.037*
	Age	0.006*
	Sex and age interaction	0.007*
EGFR	Laser irradiation	0.009*
	Sex	0.408
	Age	0.149
	Sex and age interaction	0.04*

* $P < 0.05$

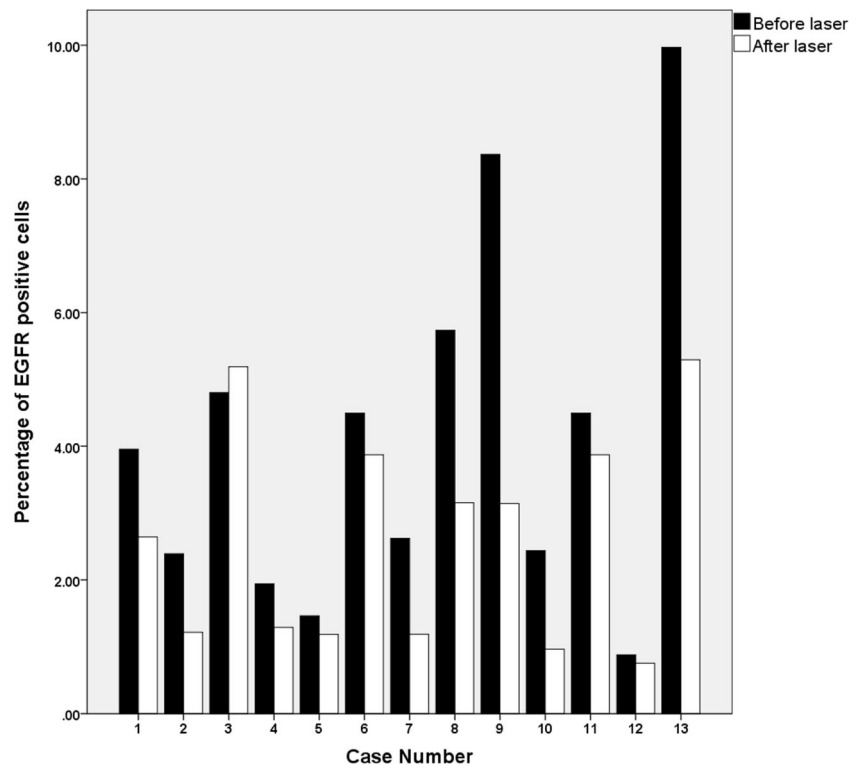
Fig. 1 Percentage of arginase-positive cells before and after laser therapy in 13 studied patients



significantly arginase and EGFR at the level of gene expression and downregulates them, using the flow cytometry technique. Decreasing arginase expression may activate the NOS/NO pathway and induce vasodilatation in patients with macro-

and microangiopathy including patients with diabetes. It is suggested that some diabetic complications including endothelial, cardiovascular, and erectile dysfunction are related to increased arginase gene expression and reduction of NO

Fig. 2 Percentage of EGFR-positive cells before and after laser therapy in 13 studied patients



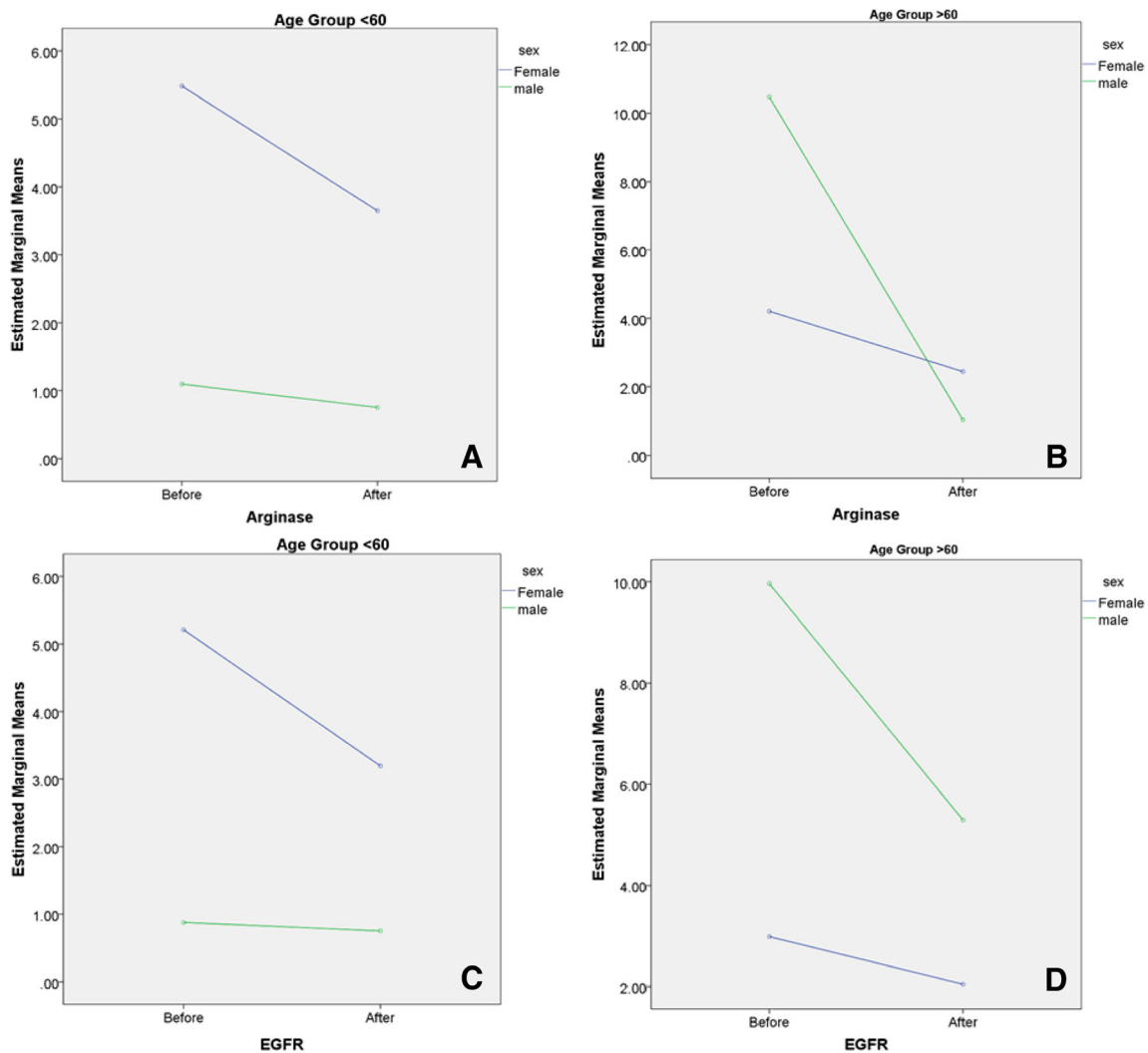


Fig. 3 The effect of sex and age in the reduction of the percentage of positive cells; **a** (left figure) arginase-positive cells before and after laser therapy in male and female patients below 60 years old, **b** (right figure) arginase-positive cells before and after laser therapy in male and female

patients above 60 years old, **c** (left figure) EGFR-positive cells before and after laser therapy in male and female patients below 60 years old, **d** (right figure) EGFR-positive cells before and after laser therapy in male and female patients above 60 years old

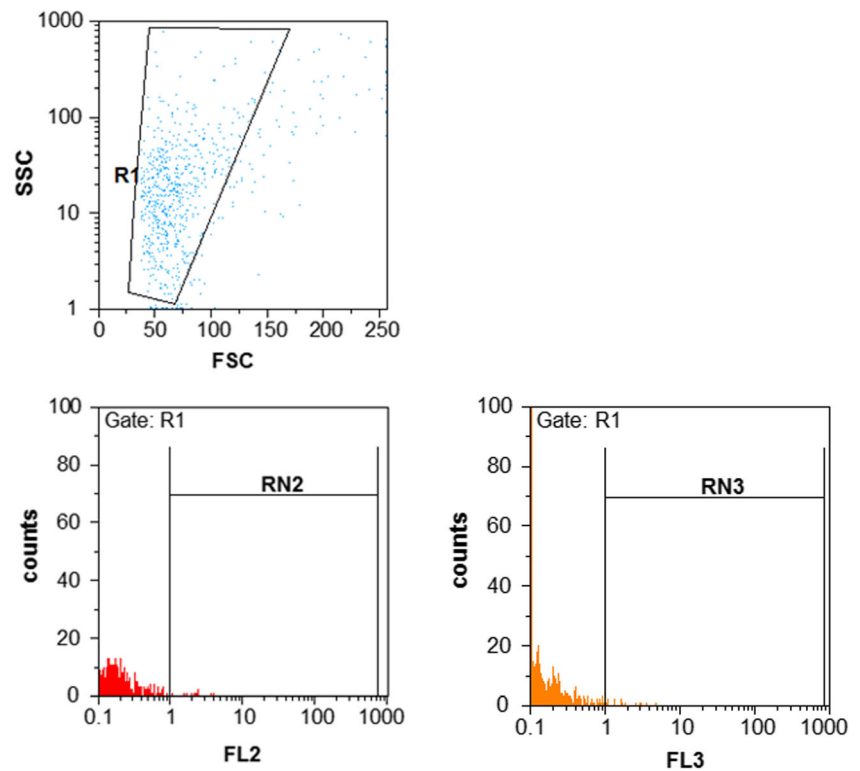
production [38, 39]. Important factors that decrease NO production in these patients are reduction of L-arginine, activity of eNOS [40], and upregulation of arginase. Decreasing EGFR expression may influence the neuronal microenvironment, microglia activation, and production of proinflammatory cytokines [34]. EGFR regulates cell activation and its downregulation regulates inflammation by decreasing the EGFR/MAPK cascade. EGFR signaling plays roles in several central nervous system disorders and ischemia [33]. Our previous study showed that LLLT had a significant effect on diabetic neuropathy, in which this mechanism may be involved [37]. Our study showed that LLLT affects the expression of genes involved in wound healing in diabetic mice. The results showed a significant increase in fibroblast growth factor (FGF) and a downregulation of vascular endothelial growth factor which was not statistically significant, which may be due to an insufficient sample size [15]. Our previous

clinical studies showed that LLLT is an effective therapeutic method for diabetic foot ulcer, neuropathy, and regulating metabolism and blood sugar in these patients [7, 10, 37]. These observations suggest that gene expression regulation is a key mechanism in treating diabetic complications using LLLT.

Our results in this study also showed that the basic level of arginase and EGFR is higher in diabetic women below age 60; however, after age 60, the basic level of both of these markers is higher in men. Although we did not find any reason for this finding in the literature review, the results showed that laser irradiation has a greater effect on the group with the higher basic level of these markers and a more gentle effect on the lower basic levels. It may suggest that laser can modulate cell pathways and bring them to normal functions through a remarkable manner.

Romero et al. reported that arginase I competes with NOS for L-arginine and could cause coronary vascular dysfunction in

Fig. 4 Flow cytometry of the peripheral blood from a patient affected with diabetes before laser therapy. *FL2* the cells conjugated with Pe-cy5 and reflect protein expression of arginase. *FL3* the cells conjugated with R-Pe and reflect protein expression of epidermal growth factor. Particles, 695; *R1*, 572 (82.30 %); *FL2* (arginase), 35 (1.92 %); *FL3* (EGFR), 15 (2.62 %)



diabetic patients [41]. Shemyakin et al. reported that arginase inhibition significantly improved endothelial function in patients with diabetes mellitus type 2 and coronary artery disease [39]. Trinity et al. compared the expression of arginase in cavernosal tissue of healthy people and diabetic patients with

erectile dysfunction. They reported that arginase II gene expression is higher in diabetic patients. They suggested that elevated expression of arginase II in cavernosal tissue of diabetic patients may play a role in erectile dysfunction and other complications related to NO production in diabetic patients [38].

Fig. 5 Flow cytometry of the peripheral blood from a patient affected with diabetes after laser therapy. *FL2* the cells conjugated with Pe-cy5 and reflect protein expression of arginase. *FL3* the cells conjugated with R-Pe and reflect protein expression of epidermal growth factor. Particles, 5001; *R1*, 3118 (62.35 %); *FL2* (arginase), 29 (0.93 %); *FL3* (EGFR), 37 (1.19 %)

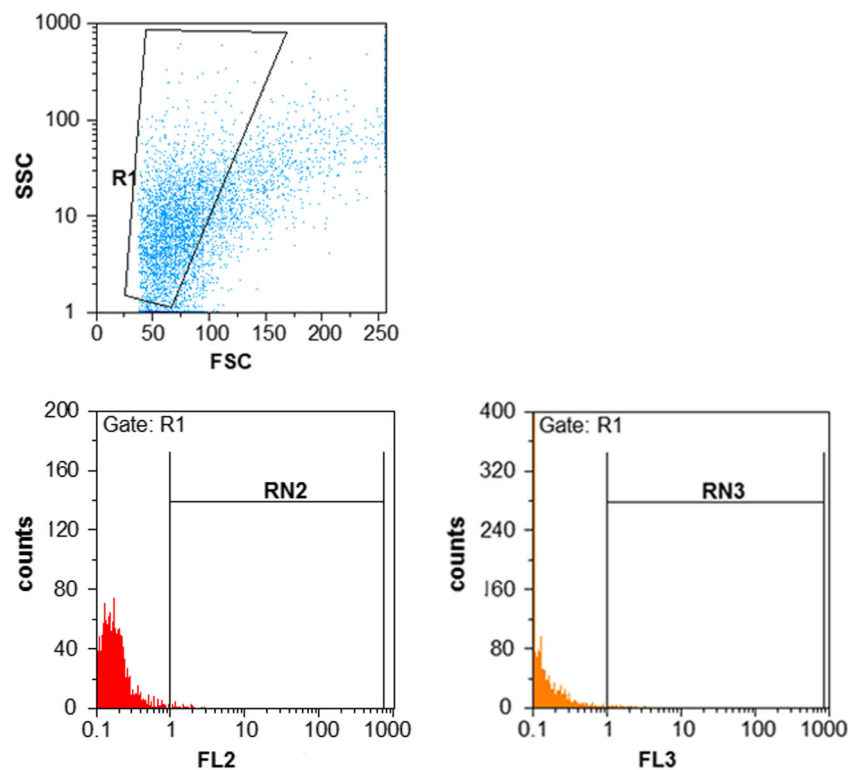
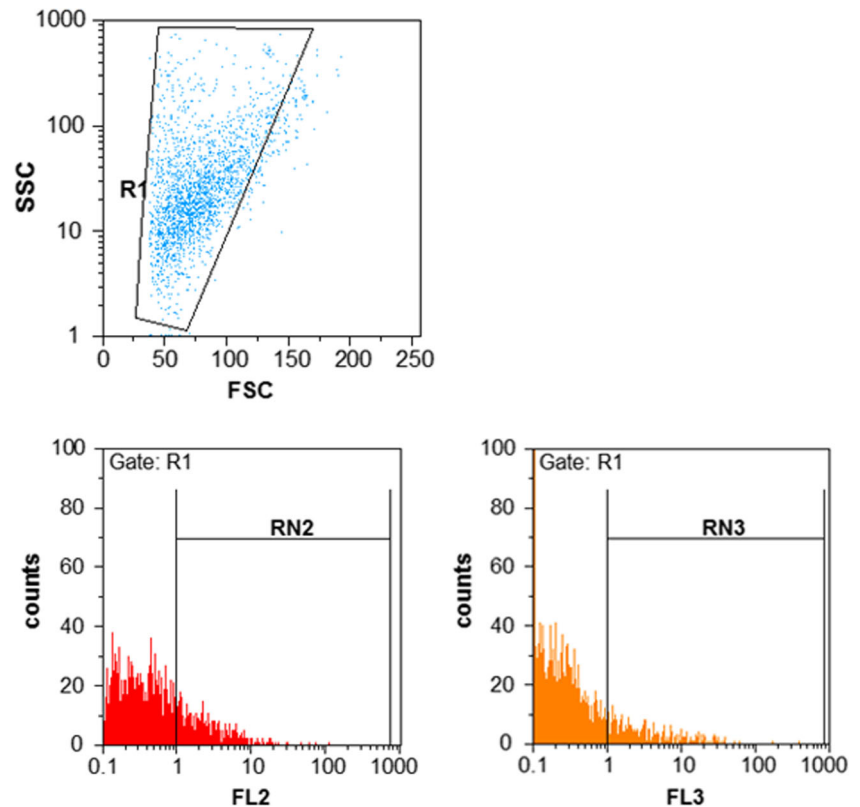


Fig. 6 Flow cytometry of the foot debris from a patient affected with diabetes before laser therapy. *FL2* the cells conjugated with Pe-cy5 and reflect protein expression of arginase. *FL3* the cells conjugated with R-Pe and reflect protein expression of epidermal growth factor. Particles, 2270; *R1*, 2111 (93.00 %); *FL2* (arginase), 348 (16.49 %); *FL3* (EGFR), 471 (22.31 %)



Qu and colleagues showed that EGFR blockade inhibited the EGFR/MAPK cascade, reduced production of cytokines in microglia, and modulated inflammatory response after spinal cord injury (SCI). They reported that inhibition of EGFR phosphorylation decreased the production of TNF- α and IL-1 β in activated microglia [34]. Erschbamer and colleagues reported that in experimental spinal cord injury, inhibition of EGFR improves motor and sensory functions and improves bladder function [33]. Koprivica and colleagues suggested that EGFR inhibition promotes nerve regeneration in optic nerve injury [42]. Liu et al. reported that upregulation of EGFR promotes NOS-2 in astrocytes of the human optic nerve and excessive NO causes pressure damage in the optic nerve [42]. Furthermore, cancer studies showed that EGFR inhibitors may normalize vascular function [43].

Several studies suggested the effects of LLLT on gene expression. Ogita and colleagues studied protein expression and

cell proliferation induced by low-level Er:YAG laser irradiation in human gingival fibroblasts by proteomics analysis. They showed that LLLT can promote human gingival fibroblast proliferation and induce protein expression and upregulation of galectin-7 which contributes to cell proliferation [44]. Peplow and colleagues studied the effects of laser irradiation on the release of growth factors, cytokines, and gene expression in animal and human cell cultures in a systemic review article. They concluded that laser therapy can modulate gene expression and the release of growth factors and cytokines in cell culture; however, further clinical studies are needed [45]. Zhang and colleagues used the cDNA microarray technique to evaluate the effects of laser irradiation on the gene expression profile in human fibroblasts. They reported that irradiation of an optimum dose of red light can affect the expression of 111 genes. Most of these genes play roles in enhancement of cell proliferation, directly or indirectly [46]. Conlan and colleagues

Fig. 7 **a** Diabetic foot ulcer since 3 months ago, **b** after 39 sessions of LLLT



showed in a review article that red light laser can enhance basic fibroblast growth factor [47]. In our previous study, we evaluated the effect of laser therapy on the expression of VEGF, PDGF, and FGF in diabetic mice. The results showed that laser therapy can significantly increase FGF expression. It decreased VEGF and increased PDGF but these changes were not statistically significant [15].

Our previous studies indicated the significant effect of LLLT in complications including neuropathy and wound healing and blood sugar regulation in diabetes [7, 9, 10, 15, 37]. Our results showed that using LLLT for treating diabetic foot ulcers was significantly effective (Fig. 5, adopted from reference [10], Figs. 6 and 7) [7].

In another clinical trial, we did a metabonomics study on the serum of the blood of the same patient who participated in the present study (“Modifying Effect of Intravenous Laser Therapy on the Protein Expression of Arginase and Epidermal Growth Factor Receptor in Type 2 Diabetic Patients”). Our results showed that ILBI significantly decreased glucose, glucose 6 phosphate, dehydroascorbic acid, *R*-3-hydroxybutyric acid, *L*-histidine, and *L*-alanine and significantly increased the *L*-arginine level in blood. Blood sugar (BS) in these patients also reduced significantly ($P < 0.05$) [9]. We also compared the effects of blue and red intravenous laser on the blood sugar level in diabetic patients. Both red and blue laser lights decreased the BS level significantly ($P < 0.0001$), but we did not find a significant difference between them [10]. Our clinical trial on the efficacy of LLLT in the neuropathy of diabetic patients clearly demonstrated the significant effect of LLLT on the improvement of nerve conduction velocity in diabetic distal symmetric polyneuropathy [37]. These findings support the therapeutic potential of low-level lasers in diabetic patients.

Our novel findings in this research were as follows: (1) Laser therapy has significant effects on downregulation of arginase and EGFR expression; (2) the basic level of arginase and EGFR is higher in women below age 60; however, after age 60, the basic level of both of these markers is higher in men; (3) as Fig. 4 illustrates, laser irradiation has a greater effect on the group with a higher basic level of these markers and a more gentle effect on lower basic levels. It may suggest that laser can modulate cell pathways and bring them to normal functions through a remarkable manner.

Conclusion

In summary, we report that intravenous laser therapy significantly decreases the expression of arginase and EGFR in diabetic patients. Downregulation of arginase and increasing the metabolism of *L*-arginine by NOS which induces NO production may be one of the mechanisms of vasodilation that occurs after laser therapy. Our results also showed a decrease of EGFR expression which can explain the effects of laser

therapy on the improvement of neuropathy in diabetic patients. We also found a relationship between basic levels of studied markers and the intensity of laser effects that may be explained by regulatory effects of laser therapy. As literature review showed, arginase and EGFR upregulation has a significant role in diabetic complications including endothelial, cardiovascular, and sexual disorders and laser therapy may regulate the expression of these genes. Future studies for measuring clinical effects are needed.

Acknowledgments This work was financially supported by Research Affairs, Tehran University of Medical Sciences. We also thank Behsaz Institute for providing laser apparatus and the staff of the laser clinic and laboratory of Milad Hospital for their support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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