

# Changes in Cell Biology under Low-Level Laser Therapy

Subjects: Biophysics

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In clinical practice, low-level laser therapy (LLLT) was introduced by E. Master in the second half of the 1960s. Since that time, this type of radiation has been successfully used in cardiology, hematology, dermatology, surgery, orthopedics, and other clinical specialties. Two main LLLT features seem to have the strongest impact on cell biology. First is the wavelength. Wavelengths from 600 nm up to 1070 nm have the greatest impact on the promotion of cell proliferation. This phenomenon is probably related to the absorption or interference of light beyond this range. Light with shorter wavelengths is strongly absorbed by hemoglobin, while longer wavelengths are absorbed by water. The second important factor is energy density. In general, lower energy density ( $0.05 \text{ J/cm}^2$ – $10 \text{ J/cm}^2$ ) promotes cell proliferation, while higher energy density (above  $50 \text{ J/cm}^2$ ) enhances apoptotic processes. The mutual transition of both phenomena has a continuous nature. This biphasic response is also known as the “Arndt–Schulz law”.

Keywords: low-level laser therapy (LLLT) ; cell metabolism ; cell biology ; photobiomodulation

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## 1. ATP Synthesis

Mitochondria are dynamic organelles that play a critical role in energetic metabolism and intracellular signaling <sup>[1]</sup>. The process of oxidative phosphorylation leads to the formation of high-energy ATP (adenosine triphosphate). When energy is consumed in metabolic processes, it converts to either adenosine diphosphate (ADP) or adenosine monophosphate (AMP). ATP synthesis in cells occurs primarily in mitochondria, which transport high-energy electrons from substrates through a series of protein complexes called the electron transfer chain (ETC). One of the most important proteins in this pathway is cytochrome c—a hemoprotein that acts as an electron transporter in the respiratory chain. It is capable of oxidation and reduction reactions; however, it does not directly bind oxygen <sup>[2]</sup>. It transfers electrons between Complexes III and IV. In addition, it also plays an important role in apoptosis <sup>[3]</sup>.

Low-level laser therapy irradiation of cell cultures has been shown to result in an immediate and long-lasting increase in ATP production <sup>[4]</sup> and in the upregulation of mitochondrial function with a therapeutic window for wavelengths from 650 nm to 1200 nm <sup>[5]</sup>. This effect is closely related to the influence of laser irradiation on the aforementioned mitochondrial cytochrome c oxidase (Cox) <sup>[6]</sup>. The stimulated ATP synthesis is caused by the increased activity of Cox. Cox is a phosphorylated enzyme controlled mainly by allosteric ATP inhibition, depending on the ATP/ADP ratio. However, there is another, non-“classical” control pathway via increased mitochondrial membrane potential ( $\Delta\Psi_m$ ), and the production <sup>[7]</sup> of ROS and low-level laser therapy (LLLT) has a major impact on this control pathway <sup>[3]</sup>. It is postulated that the increased ATP synthesis after laser irradiation is directly involved in biological effects, including: improvement in the function and recovery of rat hearts stored in the cold <sup>[8]</sup>, increase in the resistance of muscles to fatigue during intense physical exercise <sup>[9][10]</sup>, healing of burn wounds <sup>[11]</sup>, improvement in depressive behaviors <sup>[12]</sup>, improvement in tissue regeneration and collagen synthesis <sup>[13]</sup>, anti-inflammatory effects <sup>[14]</sup>, and many others. It is noteworthy that the biologically beneficial effect of increased ATP synthesis after LLLT irradiation depends not only on the intracellular ATP amount but is also associated with increased amounts of extracellular ATP <sup>[15]</sup>. Evidence suggests that the effect of increased ATP synthesis is closely related to the irradiation parameters and that an inappropriate choice of laser light properties may trigger the opposite effect <sup>[16]</sup>. This biphasic response follows the “Arndt–Schulz law” and was demonstrated in the previous study <sup>[17]</sup>.

## 2. Retrograde Mitochondrial Signaling

Because of the paramount role of the nucleus compared to other organelles, including mitochondria, the mechanisms of anterograde regulation and communication have been well studied and described for decades. However, retrograde regulatory mechanisms constitute the subject of more recent, intensive studies. In this reverse information transmission

pathway, changes in the functional state of the organelle affect cell activity via changes in gene expression. Retrograde regulation affects various cellular activities, including the processes of growth and development, aging, the maintenance of homeostasis, and the control of appropriate metabolic processes [18]. Mitochondrial retrograde signaling is initially defined by the change in mitochondrial membrane potential ( $\Delta\Psi_m$ ), and later, other changes occur—particularly in other secondary elements of mitochondrial retrograde signaling (ROS, pH, nitric oxide—NO) [19]. The increase in the mitochondrial membrane potential ( $\Delta\Psi_m$ ) after LLLT is among the best-demonstrated influences of laser light on cell function. This effect is also observed due to other mentioned mitochondrial retrograde signaling mechanisms such as ROS, pH, and NO [1]. It has been proven that this mechanism is responsible for the pro-proliferative effect of LLLT. Low-level laser therapy causes the phosphorylation of tyrosine kinase receptors (TPKR) due to the abovementioned changes in retrograde mitochondrial signaling, a stimulatory effect on the MAPK/ERK kinase signaling pathway, the activity of which leads to increased cell proliferation [20]. LLLT stimulates proliferation not only via this signaling pathway but also by retrograde mitochondrial signaling. Increased membrane potential is involved in the regulation of melanoma cell proliferation induced by  $\Delta\Psi_m$ /ATP/cAMP/JNK/AP-1 [21]. ROSs are one of the elements of mitochondrial retrograde signaling that directly affect proliferation. There is no doubt that the production of ROS is stimulated by LLLT and that they act as important secondary messengers regulating the activity of various protein kinases. The Src kinases are a known target of ROS [22] and play a critical role in regulating fundamental cellular processes, including cell proliferation, migration, and commitment. LLLT has been shown to induce bio-stimulatory effects by activating the Src tyrosine kinase by increasing the ROS level [23]. The production of ROS triggered by LLLT also leads to an activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B) [24], which modulates the expression of target genes involved in cell growth, survival, and death. An example of a prolonged cell growth effect could be observed in the chondrocyte population [25], which may be associated with accelerated bone healing after LLLT.

### **3. Nitric Oxide**

Nitric oxide (NO) and its role as a cellular transmitter has been the subject of numerous studies [26]. It has been shown to promote angiogenesis [27], inextricably linked with tissue and cell growth. Nitric oxide modulates inflammatory processes and the immune response of cells. This effect is achieved through multiple mechanisms which affect cell signaling systems such as cGMP and G-protein, but also the cAMP, JAK/STAT, or MAPK-dependent signal transduction pathways. NO may also modulate transcription factors' activity and the expression of other mediators of inflammation [28][29]. Nitric oxide is known to have a twofold nature regarding a tumor's biology and might simultaneously have pro-oncogenic and anticancer properties. The multidirectional nature of this signal molecule under different conditions can be associated with the effect of time and concentration which affects different phenotypes' expression [30]. There is no doubt that low-energy laser irradiation increases the production of NO in the in vitro models [31], which is also observed in the in vivo experimental models [32][33], including in humans [34]. The exact mechanism of this phenomenon has not been fully understood. However, it is suggested that several different processes are involved. As previously mentioned, LLLT has a strong activating influence on the mitochondrial respiration chain, and, through the cytochrome C oxidase, LLLT increases the production of NO. The molecular basis of this phenomenon is founded on competition between O<sub>2</sub> and NO for the active center in the enzymes of the mitochondrial respiratory chain [35]. LLLT, by stimulating the activity of the cytochrome C oxidase complex, increases the release of NO from the active sites of the enzyme. This phenomenon, occurring after laser irradiation, is referred to as the "NO hypothesis" [36]. This is not the only pathway by which LLLT increases NO production. Another potential mechanism is the influence of low-level laser therapy on the induction, expression, and activation of nitric oxide synthesis. The literature suggests that activation may be contributed by the kinase pathway PI3K/eNOS [37]. However, it seems that the mechanism responsible for increased NO synthesis under the influence of LLLT is far more complicated. Clinical trials carried out in human models showed that intravenous LLLT significantly decreases the expression of arginase and EGFR. The downregulation of arginase and the increase in the metabolism of L-arginine by NOS, which induces NO production, may be one of the mechanisms of the vasodilatation and acceleration of wound healing that occurs after laser therapy [38]. On the other hand, Lindgård A. et al. [39] postulate that laser irradiation resulted in elevated levels of NO but had no effect on the iNOS or eNOS activity. They indicate that irradiation at 634 nm releases NO, possibly from a preformed store, and additionally reduces the production of intracellular ROS. Confirmation of this theory may be the study by Mittermayr R. et al [40]. The researchers suggest that laser-irradiation-induced NO release from NO-Hb complexes may be a novel concept and may explain the abovementioned [17] reduction in the aggregative potential of human platelets after blood irradiation.

### **4. Modulation of Ion Concentrations**

Calcium ions are an important link in intracellular signal transductions. They are involved in many intracellular processes, and changes in their concentration affect the activity and viability of all cell cultures. LLLT has been shown to increase the

permeability of the cell membrane to calcium, leading to an increase in its intracellular level [41][42]. Another mechanism responsible for the increase in calcium levels after LLLT is related to the increased release of  $\text{Ca}^{2+}$  from intracellular stores [43]. An experimental study by Lavi et al. [44] suggests that, within the appropriate radiation range, the increase in the calcium level is directly proportional to the increase in the radiant energy density, measured in  $\text{J}/\text{cm}^2$ , as well as proportional to the increase in reactive oxygen species generation. The researchers also suggest a direct relationship between the intracellular calcium concentration and the levels of ROS. They assume that the mechanism of calcium channel activation is directly related to the production of ROS under the influence of visible electromagnetic radiation. What needs to be emphasized is that laser irradiation increases intracellular calcium levels not only by a ROS-dependent mechanism. Furthermore, LLLT, via the increased production of ATP, may activate multiple subtypes of nucleotide (purinergic) P2 receptors, resulting in the increased level of intracellular calcium levels [45][46]. It is noteworthy that calcium-related signaling pathways include mitochondrial calcium signaling, calcium-sensitive adenylyl cyclase, calcium-sensitive enzymes such as protein kinase C (PKC), calcium-dependent kinase II (CamKII), and extracellular calcium-sensitive receptor (CaSR). Their enhanced activation leads to various changes in the basic activity of the cells and can be summarized as increased metabolism and excitability. This effect affects various cell cultures—myocytes [47], mast cells [48], fibroblasts [49], or reproductive cells [41]. Low-level laser therapy has an impact on the local concentration of sodium and potassium ions. Laser irradiation in a dose-dependent manner alters the ATPase activity of the membrane ion pumps. Depending on the radiation parameters used, an increase or decrease in the  $\text{Na}(+)$ ,  $\text{K}(+)$  ATPase activity is observed [50]. Even though the exact mechanism of this phenomenon has not been fully understood, one study suggests that it may be responsible for the analgesic effect of LLLT [51].

## **5. Growth Factor Release**

### **5.1. Transforming Growth Factor-Beta**

Transforming growth factor-beta ( $\text{TGF-}\beta$ ) is not a single-molecule cytokine but rather a multipotential group of factors consisting of multiple isozymes. Its activation leads to increased activity of a specific  $\text{TGF-}\beta$ -related kinase, which activates a signaling cascade that, in the end, causes changes in regulatory protein levels.  $\text{TGF-}\beta$  also affects the transcription of several target genes that play a role in the differentiation, chemotaxis, proliferation, and activation of many immune cells [52]. This cytokine plays an important role in collagen production by inducing the expression of extracellular matrix components and inhibiting their degradation by inhibiting matrix metalloproteinases (MMPs) [53]. A large body of data suggests that LLLT accelerates bone healing due to the increased proliferation of osteoblasts [54][55]. Enhanced regeneration of connective tissue following laser irradiation is also associated with increased collagen synthesis through activation of the  $\text{TGF-}\beta$ /SMAD pathway [56].

Additionally, LLLT via the  $\text{TGF-}\beta$  signaling pathway can suppress the immune response. These properties of laser irradiation have already been utilized as a part of therapy for chronic kidney disease [57], tendon injury [58], hypothyroidism [59], scars, and keloids [60]. On the other hand, it has been recently shown that the intravascular use of LLLT decreases  $\text{TGF-}\beta 1$  and FGF-2 levels in patients undergoing coronary intervention, which may reduce the neointima formation [61].

### **5.2. Vascular Endothelial Growth Factor (VEGF)**

Vascular endothelial growth factor (VEGF) is a protein that plays a critical role in angiogenesis. It initiates cell migration along with the initial invasion of endothelial cells and the formation of the vascular lumen. Additionally, it promotes the junction process between the new vessel segments and within the existing old ones [62]. VEGF and its activity are directly involved in the pathogenesis of the recovery process from many diseases, such as traumatic injuries to the central nervous system, bone and muscle injuries, rheumatoid arthritis, age-related macular degeneration (AMD), diabetic retinopathy, psoriasis, hair loss, and the whole spectrum of cardiovascular disease [63][64]. There is convincing data on the increased local activity of VEGF after LLLT, which translates into the process of healing and regeneration [65][66]. This effect is probably not permanent and depends on radiation parameters and exposure [67]. Although the side effects after LLLT are hardly observable, still, the safety concerns are not unfounded. Increased activity of VEGF plays an important role in the etiopathogenesis of many tumors, and anti-VEGF therapy is well established in routine clinical practice in oncology or ophthalmology [68]. Increased proliferative potential of the various cells after courses of LLLT via the VEGF pathways implies the necessity of future research, particularly investigating the impact of LLLT on tumor biology.

### **5.3. Hepatocyte Growth Factor (HGF)**

Hepatocyte growth factor (HGF) is a pleiotropic cytokine consisting of an  $\alpha$ -chain and a  $\beta$ -chain. The biological response is triggered by the Met receptor, which affects signaling molecules such as PI3K and MAPK proteins [69]. These proteins have a wide range of activities that affect cell proliferation, mortality, morphogenesis, and the inflammatory process, and

they influence apoptosis [70][71]. Originally, HGF was characterized as a mitogenic protein associated with hepatocytes, but researchers now know that its role extends to many other cells. Its multipotent action leads to many therapeutic applications in various disease models, such as liver cirrhosis, cholestasis, peptic ulcers, acute kidney injury, chronic kidney disease, coronary artery disease and myocardial infarction, emphysema, cerebral ischemia, and skeletal muscle and skin injury [72][73]. LLLT has been shown to enhance the synthesis of HGF and may thus be a supportive therapeutic strategy for the listed diseases [74][75].

#### 5.4. Basic Fibroblast Growth Factor (bFGF)

The basic fibroblast growth factor (bFGF) is responsible for cell growth and survival [76]. It is involved in embryonic development, morphogenesis, tissue repair, tumor growth, and invasion. It is postulated to play a crucial role in wound healing—it acts as a chemoattractant for endothelial cells and fibroblasts [77]. This protein is also involved in granulation tissue formation and re-epithelialization, as well as angiogenesis [78][79]. Due to the multiple roles of bFGF, it plays an important role in the development of numerous diseases, including Paget's disease [80], Alzheimer's disease [81], malignancies and precancerous lesions [82], nerve injury [83], and Crohn's disease [84]. There are many studies suggesting that fibroblast growth factor synthesis is more intense after irradiation of a broad spectrum of wavelengths than after low-level laser therapy [85]. On the other hand, some convincing data suggest an opposite effect [61][86], which most likely depends on many factors, and more studies are required to clarify this issue. A similar dual-response effect after irradiation occurs with insulin-like growth factor I [87][88].

### 6. Activation of Transcription Factors

Laser irradiation also affects cell biology by modulating transcription factors. One of the best-known transcription factors, whose activity is changed by LLLT is nuclear factor-kappa B (NFkB). NFkB regulates many cellular functions (migration, proliferation, inflammatory and stress-induced responses). This protein complex remains in a non-active state, and under the influence of stimulating factors, it turns into the active state [89]. This change occurs without any additional protein synthesis, allowing classification in the “rapid-acting” primary transcription factors category. There are many well-known inducers of NFkB activation, such as tumor necrosis factor-alpha, ROS, bacterial lipopolysaccharides, interleukins, and low-level laser therapy [24][90]. In the case of LLLT stimulation, researchers observe a typical reaction consistent with the Arndt–Schulz law. The appropriate dose of radiation leads to the activation of the enzyme and increases the proliferative and anti-inflammatory potential [91][92]. However, exceeding the radiation dose led to increased oxidative stress and an over-abundant activation of NF-kB [93].

Another transcription factor activity that is modified under the impact of low-energy laser irradiation is the hypoxia-induced factor (HIF). HIF is a small protein associated with a cell's response to hypoxia. In hypoxic conditions, HIF activation led to the upregulation of several genes, such as glycolysis enzymes, which allow ATP synthesis in an oxygen-independent manner. Probably, the therapeutic effect of LLLT is partly achieved by changing the level of HIFs. Furthermore, Gupta et al. [94] suggest that modification of HIF activity (upregulation) under laser light might be involved in enhancing the healing of burn wounds. The same effect of increased activity of HIFs was observed by Cury et al. [95]. They prove that LLLT can promote angiogenesis in ischemic skin flaps. What is interesting is that LLLT does not only increase the activity of HIFs. There is evidence that it can alleviate neuropathic pain and promote functional recovery in rats with chronic constriction injury by decreasing the activity of HIFs [96]. It is noteworthy that LLLT may also have a dark side—some data suggest that LLLT can promote aggressive proliferation in cancer cells by activating the Akt/Hypoxia Inducible Factor-1 $\alpha$  pathway [97].

### 7. Influence on Apoptosis

The mechanism of action of low-level laser therapy is not only related to enhancing cellular metabolism and proliferation, but after application at higher doses, it also shows the ability to induce apoptosis. The exact mechanism of this phenomenon has not been fully understood; however, it might be indirectly related to the production of reactive oxygen species (ROS). On the other hand, laser irradiation activates the  $\beta$ 3 glycogen synthase kinase (GSK3 $\beta$ ), which triggers apoptosis [98]. Interestingly, there is another ROS-related mechanism promoting apoptosis—by the Akt/GSK3 $\beta$  pathway [99]. It is noteworthy that LLLT irradiation can induce both proliferation (low energy density—0.8 J/cm<sup>2</sup>) and apoptosis (higher energy density—60 J/cm<sup>2</sup>) by changing the activity of particular kinases, such as C-kinase [100]. This fact points at the quantitative rather than qualitative nature of promotion of proliferation/apoptosis process after LLLT irradiation. Probably, the direct “effector” through which laser-induced apoptosis is induced is the caspase 3 [101], which is a key mediator of programmed cell death and is involved in the breakdown of many of its essential proteins [102]. Even though the mechanism of apoptosis induced by low-level laser therapy is not fully understood, the majority of researchers agree that the differentiating factor between pro-proliferative and apoptotic effects is the energy provided by the laser.

Interestingly, this process is continuous and mutually permeates—the anti-apoptotic pathways are activated even during irradiation with high energy density. A good representation of this situation is the impact of laser irradiation on the ROS/Src/Stat3 pathway, the activation of which inhibits apoptosis <sup>[98]</sup>. It should be emphasized that in the case of various cell types, the borderline at which there occurs an advantage of apoptotic processes over the pro-proliferative is variable. The specific cell line type, in combination with the recently described <sup>[103]</sup> effective use of fractional descriptions of energy transfer as a tool dedicated to the modification of cell biological functions, can open a completely new field of precise LLLT selected for specific medical indications.

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