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ORIGINAL ARTICLE

Assessment of the effect of laser irradiations at different wavelengths (660, 810, 980, and 1064 nm) on autophagy in a rat model of mucositis

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Abstract It is known that high-dose radiation has an effect on tissue healing, but tissue healing does not occur when low dose radiation is applied. To clarify this issue, we compare the treatment success of low dose radiation with programmed cell death mechanisms on wounded tissue. In this study, we aimed to investigate the interactions of low and high-dose radiation using an autophagic mechanism. We included 35 adult Wistar-Albino rats in this study. All animals were injected with 100 mg/kg of 5-fluorouracil (5-FU) on the first day and 65 mg/kg of 5-FU on the third day. The tips of 18-gauge needles were used to develop a superficial scratching on the left cheek pouch mucosa by dragging in a linear movement on third and fifth days. After mucositis formation was clinically detected, animals were divided into five groups (n=7). Different wavelengths of laser irradiations (1064 nm, Fidelis Plus, Fotona, Slovenia; 980 nm, FOX laser, A.R.C., Germany; 810 nm, Fotona XD, Fotona, Slovenia; 660 nm, HELBO, Medizintechnik GmbH, Wels, Austria) were performed on four groups once daily for 4 days. The laser irradiation was not performed on the control group. To get the tissue from the left cheek at the end of fourth day from all animals, oval excisional biopsy was performed. Molecular analysis assessments of pathological and normal tissue taken were performed. For this purpose, the expression analysis of autophagy genes was performed. The results were evaluated by normalization and statistics analysis. We found that *Ulk1*, *Beclin1*, and *Atg5* expression levels were increased in the rats when the Nd:YAG laser was applied. This increase showed that a 1064-nm laser is needed to activate the autophagic mechanism. However, in the diode applications, we found that *Beclin1*, *Atg10*, *Atg5*, and *Atg7* expressions numerically decreased. *Atg5* is responsible for the elongation of autophagosome. *Becn1* is a control gene in the control mechanism of autophagy. The reduction of the expression of these genes leads us to think that it may depend on the effect of drug (5-FU) used to form model. Expressions of therapeutic genes increase to ensure hemostasis, but in our study, expressions were found to decrease. More detailed studies are needed.

Keywords Mucositis · Autophagy · *Atg* family · LLLT

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Introduction

Programmed cell death plays an important role in development and disease [1]. There are three main types of programmed cell death: apoptosis, autophagy, and necrosis. Apoptosis works on the two basic pathways called extrinsic and

intrinsic; when there is a stress factor in a cell, apoptosis starts. When apoptosis starts, the cell digests itself via activation of proapoptotic genes in the *Bcl2* gene family [2]. Unlike apoptosis, necrosis is a caspase-independent type of cell death. The most typical morphological feature of necrosis is the swelling of the cell and the emptying out of its contents. When necrosis occurs in the cells, inflammation is seen in that region [3]. The last type of programmed cell death is autophagy, a physiologic phenomenon. Autophagic pathways are responsible for the digestion of durable proteins and cytoplasmic structures to provide energy, but this phenomenon is a targeted demolition rather than fragmentation [4]. Sometimes, autophagy can cause the cells to die on caspase-independent pathways in conditions in which apoptosis cannot function due to the disruption of apoptotic components [5]. This dual role of autophagy [6] still is not clear [7], but it is known that this mechanism is controlled by more than 30 Atg (autophay-related protein) genes [8].

Laser therapy is a method of treatment that is frequently referenced. Although there are many studies on this approach, it is still very difficult to understand the mechanism of action of lasers.

A study aiming to shed light on the molecular mechanism of autophagy was performed by Lihuan et al. [9]. They showed that 1-h photodynamic therapy with chlorophyllin f, which increases susceptibility to photodynamic therapy, increases the expression of *Beclin1* [9], which regularly forms in autophagosome and in the fusion of autolysosome [10].

Due to the fact that the effects of laser applications on programmed cell death mechanisms are still a mystery, explanation of the data is as difficult as an explanation of autophagy.

In this study, we aimed to throw light on the interaction of lasers with molecular mechanisms by investigating the effects of curative radiotherapy on autophagic mechanism in a rat model of mucositis. For this purpose, we selected five different genes (*Atg5*, *Atg7*, *Atg10*, *Becn1*, and *Ulk1)* which play a role in the autophagic mechanism [11], and we observed the effect of laser irradiations at different wavelengths (660, 810, 980, and 1064 nm) on the expression of these genes.

Materials and methods

Ethical statement

The experimental protocol applied in the study was confirmed by the Institutional Animal Care and Ethics Committee of the Gaziantep University, Gaziantep, Turkey (12.2010-01).

The animals

In the study, 35 five-month-old male Wistar albino rats with a weight of 250–300-g body mass were used. Before the study, all animals were kept in the laboratory to orientate them to the laboratory conditions—12-h light and 12-h dark cycle, 22–24 °C temperature, and appropriate humidity—for at least four days.

Mucosa wounding

5-fluorouracil (5-FU), 100 mg/kg on the first day and 65 mg/kg of 5-FU on the third day, was intraperitoneally injected to all animals. Then, we scratched the left cheek pouch mucosa with tip of an 18-gauge needle on the third and fifth days. This technique was repeatedly used to develop an ulcerative mucositis. The animals were anesthetized with 3 mg/kg xylazine hydrochloride (XylazineBio) and 90 mg/kg ketamine hydrochloride (Ketasol) before initiating mucosa wounding protocols. After ulcerative mucositis was clinically detected on the animals' left cheek pouch mucosa, the laser therapy was started.

Irradiation protocol

All animals were randomly divided into five groups (n=7). Laser irradiation at four different wavelengths (660, 810, 980, and 1064 nm) was performed with the laser handpiece kept perpendicular to the mucositis at a distance of 1 cm per point. The laser devices used are summarized in Table 1.

The laser irradiation groups and properties

Group 1

Nd:YAG laser (1064 nm; Fotona Plus, Fotona, Slovenia) was used for treatment of mucositis daily from the 1st to the 4th days (pulsed mode, average power 0.25 W, beam area: 0.28 cm^2, application time 9 s, total energy per session 2.24 J, energy density 8 J/cm^2) (0.25 W×9 s/0.28 cm^2= 8.04 J/cm^2).

Group 2

Diode laser (980 nm; ARC FOX, Germany) was used for treatment of mucositis daily from the 1st to the 4th days (continuous mode, average power 0.1 W, beam area 0.12 cm^2,

Table 1 Laser devices used in the study

Laser wavelength	Type	Manufacturer
1064 nm	Nd:YAG	Fotona, Ljubljana, Slovenia
980 nm	Diode	ARC, FOX, Germany
810 nm	Diode	Fotona, Ljubljana, Slovenia
660 nm	Diode	HELBO Medizintechnik GmbH. Wels, Austria

application time 10 s, total energy per session 0.99 J, energy density 8.3 J/cm^2) (0.1 W×10 s/0.12 cm^2=8.3 J/cm^2).

Group 3

Diode laser (810 nm; Fotona XD-2 diode laser, Fotona, Slovenia) was used for treatment of mucositis daily from the 1st to the 4th days (continuous mode, average power 0.25 W, beam area 0.28 cm^2, application time 9 s, total energy per session 2.24 J, energy density 8 J/cm^2) (0.25 W×9 s/0.28 cm^2=8.04 J/cm^2).

Group 4

Diode laser (660 nm; HELBO Medizintechnik GmbH, Wels, Austria) was used for treatment of mucositis daily from the 1st to the 4th days (continuous mode, average power 0.1 W, beam area 0.75 cm^2, application time 60 s, total energy per session 6 J, energy density 8 J/cm^2) (0.1 W×60 s/0.75 cm^2=8 J/cm^2).

Group 5

Because group 5 was the control group, laser irradiation was not performed.

After finishing the laser treatment, tissue samples were taken in an oval excisional biopsy from the left cheek pouch mucosa and were kept in a nitrogen tank for molecular studies.

Tissue homogenization

After the excisional biopsy, the Qiagen TissueLyser LT machine and QIAzol Lysis Reagent were used to disintegrate and to homogenizate the tissues.

RNA isolation

The protocol of QIAGEN RNeasy Mini Kit (250 Catalog No. 74136) was used to extract RNA from tissue samples.

cDNA synthesis

5 μl mRNA samples were incubated at 65 °C in PCR (RT-PCR). After that, a 7.75-μl complementary DNA (cDNA) solution was added to each mRNA sample, and cDNA synthesis protocol was initiated.

cDNA concentration measurement

The epoch (BioTek) measurement machine was used to measure the concentration of all cDNA samples, and the last concentrations were adjusted 50 ng/μl.

Quantitative real-time polymerase chain reaction

In order to do quantitative real-time polymerase chain reaction (qRT-PCR), we designed the primers are shown in Table 2 by using a NCBI BLAST primer tool. For each sample in qRT-PCR, 10 μl of solution, including master mix (5 μl), forward primer (0.3 μl), reverse primer (0.3 μl), RNase/DNase free water (3.9 μl), and cDNA template (0.5 μl) was prepared, and synthesis steps of qRT-PCR were initiated.

Statistical analysis

All the values were calculated as mean±standard deviation by the SPSS for Windows version 22.0 for all evaluated genes. Student *t* test was used to compare the variables of two independent groups. $P<0.05$ was considered to be statistically significant.

Results

Compared to the control group, a statistically significant decrease was found in the expression of *Atg5* of samples to which 810- and 660-nm lasers were applied ($P<0.05$, Fig. 1). *Atg5* expression declined at 980 nm, but this decline was not significant ($P>0.05$, Fig. 1). However, *Atg5* expression significantly increased at 1.064 nm application ($P<0.05$, Fig. 1).

The expressions of *Atg7* and *Atg10* decreased on all wavelengths compared to control groups, but these decreases were not statistically significant ($P>0.05$, Fig. 1).

While the expression of *Beclin1* significantly decreased at the 660-nm laser application compared to the control group ($P<0.05$, Fig. 1), it significantly increased on other all laser wavelengths ($P<0.05$, Fig. 1). Contrary to *Beclin1*, the

Table 2 qRT-PCR: primers

	Forward	Reverse
Atg5	GCCATCAATCGGAAACTCATG	CCCCATCTTCAGGATCAATAGC
Atg7	CTTGGATGTTGGGTTTTGGC	CATGTCAAAAGCACTGAACTCC
Atg10	GCGTCCGAAGTGATTAAATATGAG	GTATTGGATGTTCCTGTTGCG
Beclin1	TTTCAGAGATACCGACTTGTTCC	TCGCCTTTCTCAACCTCTTC
Ulk1	CGCAGTTTCCAGGTGACC	AGGGTGGGGATGGAGATG

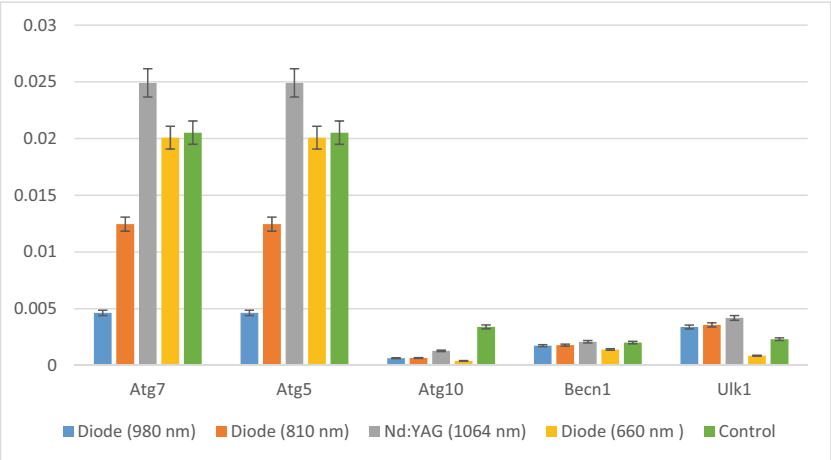

Fig. 1 The changes of gene expressions in laser irradiations on different wavelengths (660, 810, 980, and 1064 nm)

expressions of *Ulk1* significantly increased on all wavelengths, except 660 nm, compared to the control group ($P<0.05$, Fig. 1). There was an insignificant decrease on the 660-nm wavelength ($P>0.05$, Fig. 1).

Discussion

Depending on the state of stress of the cell, autophagy may be an intracellular degradation pathway [12] for nonfunctional organelles as well as a survival mechanism to produce biofuels for cell survival in difficult times, such as hunger. It is also a suicide mechanism for the execution of cells in some conditions in which apoptosis cannot work. However, this complex process is still unclear [13].

The most sensitive proteins to photosensitive drugs are in the Bcl-2 family located on the mitochondria and endoplasmic reticulum (ER) and include anti-apoptotic proteins [14, 15]. If the proteins of Bcl-2 family get damaged due to photodynamic therapy, this damage may start apoptosis or autophagy in the cell [16].

When the autophagic mechanism starts to work, a microtubule-associated protein chain-I (LC3-I) transforms into LC3-II by linking with phosphatidylethanolamine [17]. LC3-II is a biological marker of autophagy in that it plays a role in autophagosome formation [18]. Kessel at al. have shown the transformation of LC3-I into LC3-II in L1210 and DU145 cell cultures when ER damage is caused by photodynamic irradiation [19].

When cells are over exposed to photodynamic therapy or if apoptotic pathways are damaged and cannot work, it has been reported that autophagy works as a programmed cell death pathway [19–21]. Contrary to this, photodynamic irradiation sometimes cause inactivation of autophagy, as this application causes the photosensitive proteins (including the proteins need to start of autophagy) to collect at hydrophobic regions near the membrane [22]. Apart from these, the cell response may change depending on the organelles targeted by photodynamic therapy [22] or the dose of phototherapy [23]. When low level laser therapy is applied, autophagy plays a protective role by repairing the damage in ER and mitochondria, but as long as the level of phototherapy increases, both apoptosis and autophagy may induce cell death [23]. In ER damage, photodynamic therapy causes Ca^{+2} to be released into cytoplasm [24], and it is thought that the determining factor in the initiation of autophagy is the amount of this Ca^{+} in the cytoplasm [25], but this approach's accuracy has been questioned [26]. Kessel et al. [24] have shown that even if ER is exposed to extensive photodynamic therapy, Ca^{+} released from ER into the cytoplasm was not enough to start apoptosis.

When the tissue is exposed to laser, it may cause an increase in the local tissue's temperature [27], but this increase may be more related to increased microcirculation in the area than laser heating, as Lanzafame et al. [28] showed that the healing effects of low level laser therapy (LLLT) on wound healing are temperature independent in a mice model of pressure ulcers. Besides, laser therapy induces transformation of molecular oxygen into reactive oxygen species (ROS) [29]. Although many studies in the literature have reported that diode lasers speed up the tissue healing process, the effects of laser on autophagic mechanism has not been studied yet [29–32].

The heat stress (41 °C, 30 dk) may cause substantial activation of the autophagic mechanism in rats [33]. Another study, which had rats exercise by using human exercise protocol and had their body temperatures rise above of 39 °C, showed the effect of heat on the autophagic mechanism and that the autophagic mechanism worked [34]. In the cases of long-term stress, high temperatures may change the role of autophagy (lethal or rescuer) depending on intracellular stress [33]. In our study, the expression of autophagic genes on the 1064 nm increased. The autophagy effect of the tissue could be related to the high-energy microsecond pulse of Nd:YAG (1064 nm) which produce different biological effects than a CW laser of the same wavelength and power; so comparisons with CW wavelengths is difficult even with the same average

powers [35]. In addition, for a good benchmark between different laser wavelengths, all the parameters, except for the wavelength, should be same; however, in our study, laser application times, applied energy, and laser options (continuous wave or pulsed) were different in the groups.

Even though heat shock response and autophagy had once been thought of as two different systems that work independently of each other, Dokladny et al. [33] showed the interaction of autophagy with heat exchange. In their in vitro study, Dokladny's team showed that heat shock response regulates autophagy. Moreover, they showed increased LC3 lipidation in the case of HSF-1, as the regulator of heat shock genes [36, 37], is knocked out. Heat shock proteins regulate autophagy not only at high temperatures but also due to starvation [33]. For example, the over expression of HSP70, another heat shock protein, causes inhibition of the autophagic mechanism by depressing LC3-II [33].

When cells or organisms are exposed to stress, such as heat, radiation, heavy metal, ischemia, inflammation, or exercise, resulting in protein denaturation, the heat shock proteins (HSP) act like autophagy. Therefore, the expression of HSP increases to ensure homeostasis [38–42].

ROS accumulated in the cells may lead to cell death via caspase-dependent apoptosis pathways [43]. The studies performed in recent years have shown that when H_2O_2 treatment is applied to human umbilical vein endothelial cells (HUVECs), *Beclin1* and p65 expressions increase [44]. However, when BHA and NAC, which are scavengers of ROS, are added to the medium, autophagy is inhibited by decreasing the expression of p65 and *Beclin1* [44]. If the amount of ROS increases, it has been shown that increased ROS may start autophagic cell death [45, 46]. In addition, Wang et al. [47] reported that a 2-deoxy-D-glucose treatment induces autophagy by causing excessive ROS in the cell via the ROS and AMP protein kinase (AMPK) autophagy pathway. Zeng et al. [44] have shown that the ROS is produced by oxidative stress, especially in the reperfusion phase of ischemia/reperfusion induced autophagy. The effects of ROS on the autophagic mechanism was indicated by Zhang et al. [48], who reported that intracellular ROS may lead cells to apoptosis or autophagy on PI3K/AKT, mTOR/P70S6K, and JNK signal pathways. The changes in the expressions of genes, which control autophagy, depending on cell type [49], may decrease or increase photodynamic irradiation resistance [22]. When the expression of *Atg7* was repressed in the MCF-7 human breast cancer cell line [50], the cells became more resistance to photodynamic therapy [22]. Similarly, it has been shown when low and high level photodynamic therapy are applied to L1210 cells (L1210/*Atg7* is knocked out), the transformation of LC3-I into LC3-II does not occur [23]. The changes in the expression of autophagic genes may increase or decrease the cell resistance not only in photodynamic therapy but also in other irradiations. Repression of *Atg5* in mouse embrionic fibroblasts (MEFs) makes these organisms more sensitive to ultraviolet C radiations [49], and when *Atg5* deficiency in the SK-N-SH neuroblastoma cell line is treated with ab ER stressor, cells become more sensitive to death [51].

An in vitro study by Krmpot et al. [52] indicated the effect of LLLT as a kind of phototherapy for the autophagic mechanism. When Krmpot et al. [52] applied a Nd-YVO4 (532 nm) laser to a U251 glioma cell line, they saw an increase in the amount of *Beclin1* protein in the cells, and they emphasized that tissue heat reached 47 °C only for 30 sn; therefore, increased *Beclin1* expression was not related to temperature increases. They thought that the autophagic mechanism might have worked due to an increase in the amount of ROS [19, 20, 53]. In addition, Wu et al. [54] reported that in the irradiation of high-fluence low-power laser, apoptosis is induced directly by mitochondrial ROS generation. Normally, low fluence laser irradiations have stimulatory effects on cell proliferation [55, 56]; however, high fluence low power laser irradiations interfere with cell cycling and inhibit cell proliferations [57, 58]. Consistent with this, Wang et al. [59] reported that when human lung adenocarcinoma cells (ASTC-a-1) are irradiated by He-Ne lasers (632.8 nm, 40 mW) at high fluence (60–120 J/cm^2), caspase-3 is activated.

The studies to date have shown that high power and calorific lasers cause necrotic cell death [60–62], whereas low power laser may cause cell apoptosis [54, 59, 63].

When the literature is reviewed, this study is the first study to show the effect of laser therapy on autophagy. We identified the digestion of intracellular proteins in the case of cytotoxicity, Nd:YAG laser caused. Because the expressions of *Atg5*, *Beclin1*, and *Ulk1* increased in the samples, Nd:YAG was applied. *Atg5* is an E3 ubiquitin ligase, and it is needed to extend autophagosome in autophagy [11]. *Beclin1* is a control gene in the control mechanism of autophagy, and together with class III phosphoinositide 3-kinase (also known as Vps34), it plays a role in the formation of autophagosome [11, 64]. *Ulk1* controls the *Atg9* pool both at the beginning of autophagy and at the concourse phase [65–67]. The expression of *Ulk1* was increased at all wavelengths except 660 nm. Due to the fact that *Ulk1* is responsible for starting autophagy [65–67], this has shown us that there are attempts at autophagy on all wavelengths, except 660 nm; however, on some wavelengths, these attempts were selectively stopped by a mechanism we cannot explain. The decreased expressions of *Atg5*, *Beclin1*, and *Ulk1* with an 810-nm application have shown that the autophagic mechanism did not work on this wavelength. In addition, significant decreases in the expressions of *Beclin1* and *Ulk1* on the 980-nm wavelength and in the expressions of *Atg5* and *Beclin1* on the 660-nm wavelength were found. It is hard to identify autophagy in these wavelengths. We can offer three explanations for decreases in the expressions of autophagic genes at 810, 980, and 660 nm applications.

First, these wavelengths might have blocked the autophagic pathways to protect the cell because it has been reported that these wavelength speed up the tissue healing process [31]. Second, the cell can turn into one of three programmed cell death types (apoptosis, autophagy, and necrosis) in the case of cytotoxicity. Our cells might not have turned into autophagy at these wavelengths (810, 980, and 660 nm), depending on stress intensity and which organelles were targeted by cytotoxicity. Finally, these changes in the expressions of genes may be due to the effect of the drug (5-FU) that was used to form the mucositis model.

Normally, while the expressions of therapeutic genes increase to ensure intracellular homeostasis, decreased therapeutic gene expression to insulate cells from cytotoxicity indicates areas for further study of the interaction mechanism between autophagy and lasers.

Ethical statement The experimental protocol applied in the study was confirmed by the Institutional Animal Care and Ethics Committee of the Gaziantep University, Gaziantep, Turkey (12.2010-01).

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