저작자표시-비영리-변경금지 2.0 대한민국

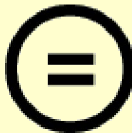
이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.


변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

[UCI]I804:21031-200000292713

Thesis for the Degree of Master of Engineering

Anti-tumor effect of low power laser irradiation on gastroenterological cancer

by

Ye Tian

Interdisciplinary Program of

Biomedical, Mechanical & Electrical Engineering

The Graduate School

Pukyong National University

February 2020

Anti-tumor effect of low power laser irradiation on gastroenterological cancer
(저출력 레이저의 위장암에 대한 항종양 작용)

Advisor: Prof. Hyun Wook Kang

by

Ye Tian

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Engineering

in Interdisciplinary Program of Biomedical, Mechanical & Electrical Engineering, The Graduate School,

Pukyong National University

February 2020

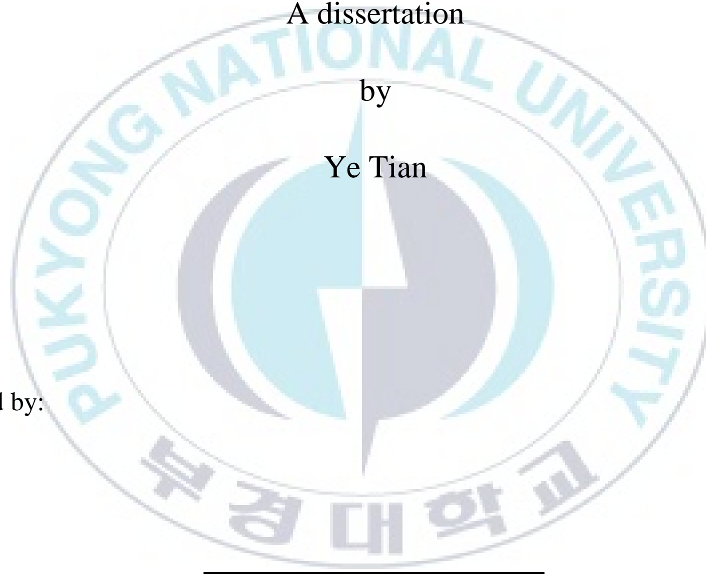
PUKYONG NATIONAL UNIVERSITY
부경대학교

Anti-tumor effect of low power laser irradiation on gastroenterological cancer

A dissertation

by

Ye Tian



Approved by:

____

(Chairman: Yongwook Lee Ph. D)

____________________ ____________________

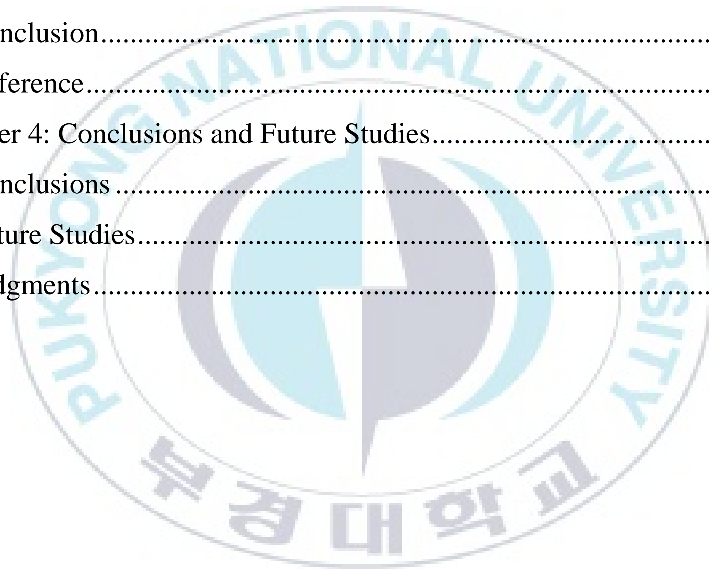
(Member: Hyoung Shin Lee M. D) (Member: Hyun Wook Kang Ph. D)

February 2020

CONTENTS

List of Figures ... iii
Abbreviations ... v
Abstract ... vi
1. Chapter 1: Introduction ... 1
1.1. Motivation ... 1
1.2. Thesis contribution ... 3
1.3. Reference ... 4
2. Chapter 2: Low power laser irradiation for gastroenterological cancer cells treatment ... 6
2.1. Abstract ... 6
2.2. Introduction ... 6
2.3. Materials and Methods ... 9
2.3.1. Chemicals ... 9
2.3.2. Cell culture ... 9
2.3.3. LPLI ... 10
2.3.4. Cell viability by MTT assay ... 11
2.3.5. Statistical analysis ... 12
2.4. Results ... 12
2.5. Results ... 16
2.6. Conclusion ... 18
2.7. Reference ... 18
3. Chapter 3: *In vitro* investigation of anti-tumor effects of low power laser irradiation ... 22
3.1. Abstract ... 22
3.2. Introduction ... 22
3.3. Materials and Methods ... 25
3.3.1. Live and dead cells fluorescence staining ... 25

3.3.2. Cell migration assay 26
3.3.3. Cell apoptosis analysis 27
3.3.4. Statistical analysis 28
3.4. Results 28
3.4.1. FDA/DAPI/PI staining 28
3.4.2. In vitro cell migration assay 30
3.4.3. Cell apoptosis analysis 32
3.5. Results 34
3.6. Conclusion 36
3.7. Reference 36
4. Chapter 4: Conclusions and Future Studies 40
4.1. Conclusions 40
4.2. Future Studies 40
Acknowledgments 42



List of Figures

Figure 1. Hepatocellular carcinoma incidence rate, by sex 2

Figure 2. Age-adjusted colorectal cancer incidence and death rates and Healthy People 2020 (HP 2020) targets 3

Figure 3. Experimental setup for in vitro 532-nm laser irradiation on gastroenterological cancer cells. 11

Figure 4. Cell viability after MTT assay experiment: Squamous cell carcinoma VX2 cells were seeded in 24-well plate overnight and exposed to LPLI at fluences of 5-600 J/cm^2 and water bath heating for 2 hours. Solid triangles represent the medium temperature (℃) after LPLI (*$p < 0.01$ vs. control). 13

Figure 5. Cell viability after MTT assay experiment: Murine colon carcinoma CT26 cells were seeded in 24-well plate overnight and exposed to LPLI at fluences of 10-600 J/cm^2 and water bath heating for 2 hours. Solid triangles represent the medium temperature (℃) after LPLI (*$p < 0.01$ vs. control). 15

Figure 6. Cell viability after MTT assay experiment: Effect of high-fluence LPLI on human skin fibroblast WS1 cells viability were examined. 16

Figure 7. LPLI stimulates the production of mitochondrial reactive oxygen species (ROS) leading to the release of various inflammatory factors in cancer cells.. 25

Figure 8. Fluorescence microscopy analysis of VX2 cell death at various fluences: Nuclei were stained with FDA (green), DAPI (blue), and dead cells were stained with PI (red): (a) control, (b) 100 J/cm^2, (c) 300 J/cm^2, and (d) 600 J/cm^2 (Magnification = 40× and scale bar = 20 μm). 29

Figure 9. Fluorescence microscopy analysis of CT26 cell death at various fluences: Nuclei were stained with FDA (green), DAPI (blue), and dead cells were stained with PI (red): (a) control, (b) 100 J/cm^2, (c) 300 J/cm^2, and (d) 600 J/cm^2 (Magnification = 40× and scale bar = 20 μm). 30

Figure 10. Wound healing assay of VX2 cells: (a) the images of wound variations at 0, 12, 24 and 48 hours after laser treatment (Magnification = 100× and scale bar = 20 μm) and (b) wound size variations as function of time (*$p < 0.05$ vs. control). 31

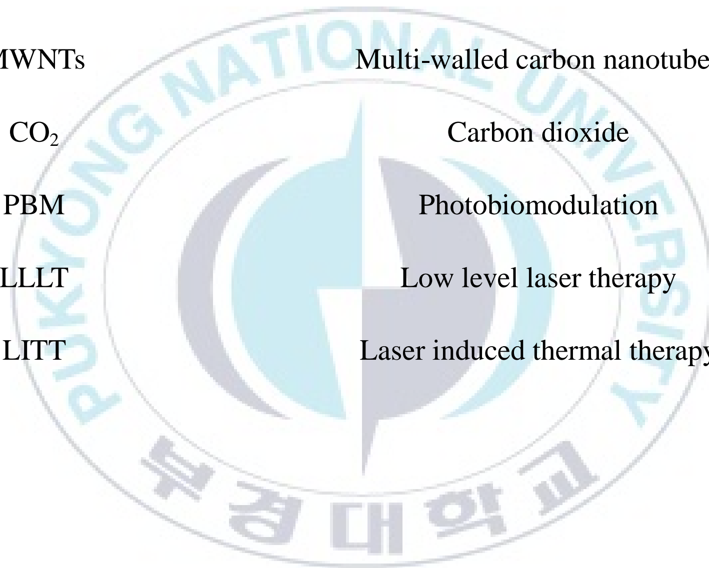
Figure 11. Wound healing assay of CT26 cells: (a) the images of wound variations at 0, 12, 24 and 48 hours after laser treatment (Magnification = 100× and scale bar = 20 μm) and (b) wound size variations as function of time (*p <

0.05 vs. control) 32

Figure 12. (a) Effect of LPLI on apoptosis in VX2 and CT26 cells: (a) Squamous cell carcinoma VX2 and murine colon carcinoma CT26 cells were exposed to LPLI at fluences of 0, 100, 300, or 600 J/cm^2 and then incubated for 4 hours. The cells were harvested for Annexin V/PI staining and were analyzed with a FACS flow cytometer. (b) The percentages of early apoptotic cells (AnnexinV+/PI-) and late apoptotic/necrotic cells (AnnexinV+/PI+) in LPLI-treated cells were evaluated 33

Abbreviations

Abbreviation	Full detail
Laser	Light Amplification by Stimulated Emission of Radiation
LPLI	Low power laser irradiation
HCC	Primary hepatocellular carcinoma
CRC	Colorectal cancer
MWNTs	Multi-walled carbon nanotubes
CO_2	Carbon dioxide
PBM	Photobiomodulation
LLLT	Low level laser therapy
LITT	Laser induced thermal therapy



Anti-tumor effect of low power laser irradiation on gastroenterological cancer

Ye Tian

Interdisciplinary Program of Biomedical, Mechanical & Electrical Engineering, The Graduate School,
Pukyong National University

Abstract

Primary hepatocellular carcinoma (HCC) and colorectal cancer (CRC) are two of the most common clinical malignancies along with high morbidity and mortality. As low-power laser irradiation (LPLI) can induce cytotoxicity or cell apoptosis on several types of hyperplasia, LPLI may be a potential alternative treatment for gastroenterological cancers. The current in vitro study focused on LPLI-induced apoptosis and mechanism after 532-nm laser irradiation on two different carcinoma cells. Squamous cell carcinoma (VX2) and murine colon carcinoma (CT26) cells were cultured for the feasibility LPLI testing. The applied fluence varied from 0 to 600 J/cm^2. MTT analysis, fluorescence imaging, wound healing assay, and cell apoptosis tests were performed 24 hr post-irradiation to monitor cellular responses. The current results demonstrated a dose-dependent stimulatory effect of LPLI on the cell viability, migration and apoptosis of VX2 and CT26 cells. The therapeutic fluence of 600 J/cm^2 induced statistically significant inhibition in the cell viability. Both the wound healing assay and the cell apoptosis tests confirmed that LPLI with high fluences could inhibit cell migration as well as induce cell apoptosis. The current findings demonstrate that LPLI might be a potential treatment for the carcinoma cells. Further studies will be performed

to evaluate the feasibility of LPLI in in vivo tumor models.

PUKYONG NATIONAL UNIVERSITY
부경대학교

저출력 레이저의 위장암에 대한 항종양 작용

Ye Tian

부경대학교 대학원 의생명기계전기융합공학협동과정

요 약

원발성 간세포 암종 (HCC)와 결장 직장암 (CRC)은 높은 이환율과 사망률을 가진 가장 흔한 임상 악성 종양 중 하나이다. 저전력 레이저 조사 (LPLI)는 여러 유형의 과형성에서 세포 독성 또는 자연사를 유도함으로써, 위암에 대한 잠재적 대안 치료법이 될 수 있다. 따라서, 본 연구는 2가지 상이한 암종 세포에 532 nm 레이저를 조사하여 LPLI에 의해 유도된 세포 사멸 및 기전에 초점을 맞추었다. 편평 상피 세포 암종 (VX2)와 결장암종 (CT26) 세포를 배양하여 실험을 진행하였다. 레이저는 0에서 600 J/cm^2 사이의 조건으로 조사되었다. 세포 반응을 모니터링 하기 위해 레이저 조사 후 24시간에 MTT 분석, 형광 이미지 측정, 상처 치유 및 세포 사멸을 평가하였으며 이를 통해 VX2와 CT26 세포의 생존도, 이동 및 자연사에 대한 LPLI의 선량 의존적 자극 효과를 입증하였다. 600 J/cm^{2}의 치료적 선량은 세포 생존율에서 통계적으로 유의한 억제를 유도하였다. 또한, 상처 치유 분석과 세포 사멸 시험을 통해 높은 선량의 LPLI가 세포 이동을 억제할 뿐만 아니라 세포 사멸을 유도할 수 있음을 확인하였다. 본 연구는 LPLI가 암종 세포 치료의 잠재적 대안이 될 수 있음을 입증하였으며, 나아가 생체 내 종양 모델에서 LPLI의 가능성과 유효성을 평가하기 위해 추가 연구가 수행 될 것이다.

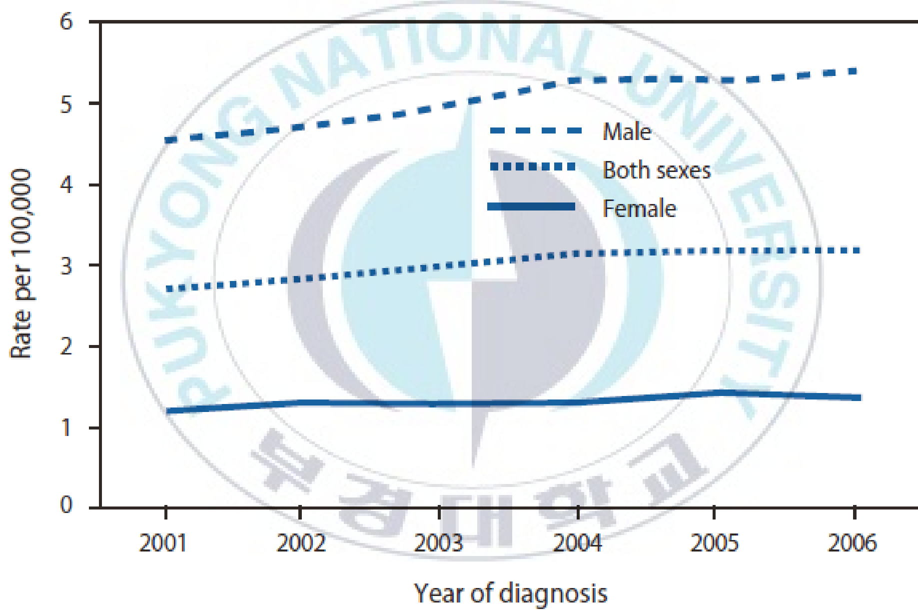
1. Chapter 1: Introduction

1.1. Motivation

As the second global leading cause of cancer deaths, liver cancer has long been a widely concerned issue. Primary hepatocellular carcinoma (HCC), a major form of liver cancer, is currently known to be attributed to chronic HBV and HCV infection, alcoholism, autoimmune hepatitis, diabetes, obesity, and a variety of metabolic diseases [1]. The incidence of this cancer is almost equivalent to its mortality rate [2]. According to data [3] from the Centers for Disease Control, the incidence of HCC in the United States increased year by year from 2001 to 2006 (Figure 1). In 2012, the number of the diagnosed HCC cases worldwide was estimated to be 700,000 [4]. In addition, colorectal cancer (CRC) is one of the most common malignant tumors in the digestive system [5], and the second leading cause of cancer mortality among cancers that affect both men and women [6] (Figure 2). The disease usually begins as a polyp (benign tumor), and the polyps eventually become cancerous over time [7]. The risk of colorectal cancer is influenced by genetic and lifestyle factors. Studies of family clustering have shown that about 20%–30% of colon cancer cases have a potentially definable genetic cause, also it is estimated that 3%–5% of colon cancer occur in genetically defined high-risk colon cancer family syndromes [8]. Therefore, the prevention and treatment of CRC has become a hotspot in cancer control research. As the existing postoperative chemotherapy drugs are not effective, it is of great clinical significance to study and find the new alternative treatment schemes.

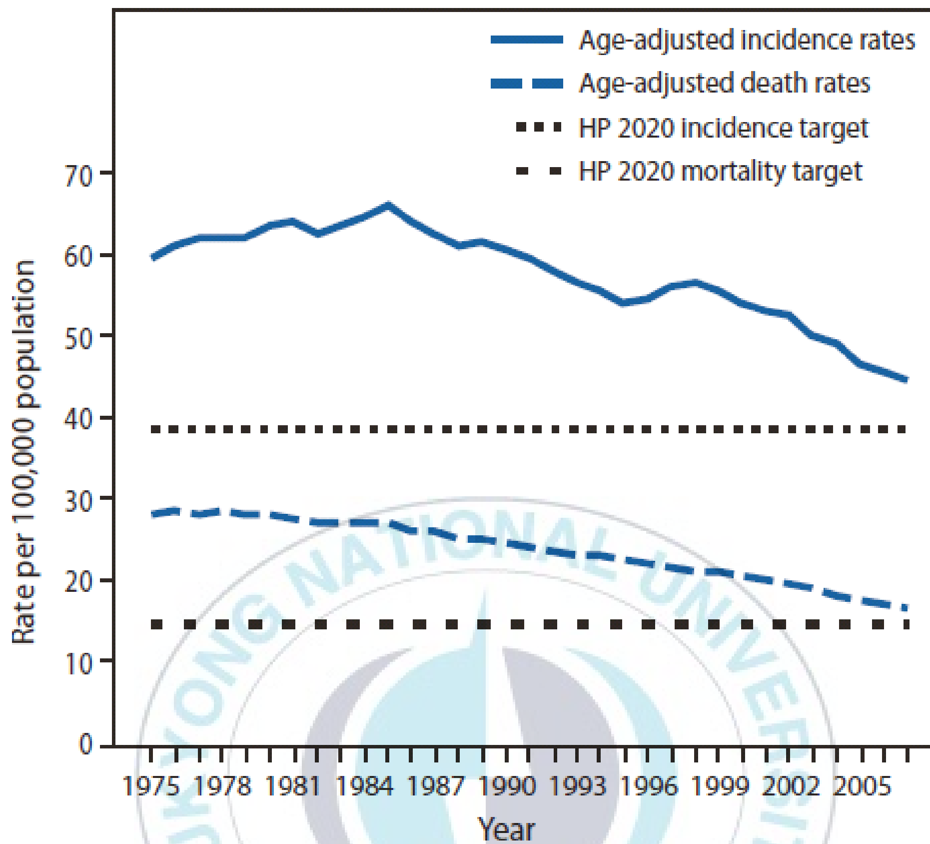
In recent years, the rabbit epidermoid VX2 tumor, which was derived from a

virus-induced skin papilloma, has been used as a model system for studying liver cancer because of similarities of its blood supply to that of human liver tumors [9, 10]. Furthermore, a previous study reported that both VX2 and human HCC model cell line (HepG2) share quite a few common features, which also supports the rationality of VX2 cells as a model of liver cancer [11]. On the other hand, CT26 from colon carcinoma in mice was often used as a colon cancer model in vitro due to its rich blood supplies and rapid growth, which is similar to human colon carcinoma in pathological and imaging characteristics [2].



[Figure adopted from Ref. (Hepatocellular Carcinoma --- United States, 2001--2006, Morbidity and Mortality Weekly Report, May 7, 2010 / 59(17);517-520)]

Figure 1. Hepatocellular carcinoma incidence rate, by sex



[Figure adopted from Ref. (Vital Signs: Colorectal Cancer Screening, Incidence, and Mortality --- United States, 2002--2010, Morbidity and Mortality Weekly Report, July 8, 2011 / 60(26);884-889)]

Figure 2. Age-adjusted colorectal cancer incidence and death rates and Healthy People 2020 (HP 2020) targets

1.2. Thesis contribution

The aim of this current study was to elucidate the induction of apoptosis and its mechanism after 532-nm diode-pumped solid-state (DPSS) laser irradiation of squamous cell carcinoma (VX2) and murine colon carcinoma (CT26) cells. In **Chapter 2**, we constructed in vitro cell models of colon cancer and liver cancer,

and investigated the effects of different laser fluences on the cell viability of the two types of cells. In order to exclude the influence of temperature, the temperature of each well was monitored after LPLI treatment. In **Chapter 3**, fluorescence staining, wound healing assay, and flow cytometry were performed 24 hours post-irradiation to monitor cellular responses. we quantitatively compared the effects of different fluences of laser on cell migration, apoptosis, and necrosis. Finally, **Chapter 4** summarized the work, put forward conclusions and future directions for development.

1.3. Reference

[1] Yang J D , Roberts L R . Hepatocellular carcinoma: a global view[J]. NATURE REVIEWS GASTROENTEROLOGY & HEPATOLOGY, 2010, 7(8):448-458.

[2] Ni Y , Wang H , Chen F , et al. Tumor models and specific contrast agents for small animal imaging in oncology[J]. Methods, 2009, 48(2):0-138.

[3] Hepatocellular Carcinoma --- United States, 2001--2006. Morbidity and Mortality Weekly Report. May 7, 2010 / 59(17);517-520.

[4] Ferlay J , Soerjomataram I , Dikshit R , et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012[J]. International Journal of Cancer, 2015, 136(5):E359-E386.

[5] T. Stokbroekx, A. de Boer, R. M. Verdaasdonk, M. E. Vuylsteke, and S. R. Mordon, Lasers Med. Sci. 29, 501-7 (2014).

[6] Vital Signs: Colorectal Cancer Screening, Incidence, and Mortality --- United States, 2002--2010, Morbidity and Mortality Weekly Report, July 8, 2011 / 60(26);884-889.

[7] Bosman, Frank T. (2014). "Chapter Chapter 5.5: Colorectal Cancer". In Stewart, Bernard W.; Wild, Christopher P. World Cancer Report. the International Agency for Research on Cancer, World Health Organization. pp. 392–402. ISBN 978-92-832-0443-5.

[8] Grady W M . Genetic testing for high-risk colon cancer patients[J]. Gastroenterology, 2003, 124(6):1574-1594.

[9] Pauser S , Wagner S , Lippmann M , et al. Evaluation of efficient chemoembolization mixtures by magnetic resonance imaging therapy monitoring: An experimental study on the VX2 tumor in the rabbit liver[J]. Cancer Research, 1996, 56(8):1863-1867.

[10] Geschwind J F H , Artemov D , Abraham S , et al. Chemoembolization of Liver Tumor in a Rabbit Model: Assessment of Tumor Cell Death with Diffusion-Weighted MR Imaging and Histologic Analysis[J]. Journal of vascular and interventional radiology : JVIR, 2000, 11(10):1245-1255.

[11] Nass N , Streit S , Wybranski C , et al. Validation of VX2 as a Hepatocellular Carcinoma Model: Comparison of the Molecular Reaction of VX2 and HepG2 Tumor Cells to Sorafenib In Vitro.[J]. Anticancer Research, 2017, 37(1):87-93.

2. Chapter 2: Low power laser irradiation for gastroenterological cancer cells treatment

2.1. Abstract

Low power laser irradiation has been shown to regulate a variety of biological processes, including cell viability, cell proliferation, cell migration and apoptosis. The current study investigated the effect of LPLI with different fluences at 532 nm on the growth of two types of gastroenterological cancer cells in vitro. After 24 hours of laser irradiation, cell activity was measured by MTT assay. Meanwhile, in order to eliminated the influence of photo-thermal effect, the temperature of medium was monitored during the experiment. The experimental results showed that high fluence (300-600 J/cm^2) LPLI significantly inhibited cell viability, and the inhibitory effect of LPLI on cell viability mainly depends on cell type and laser fluence. Furthermore, detection results of the temperature (< 42 ℃) also showed that the cellular responses were associated with non-thermal processes.

2.2. Introduction

Low-power laser irradiation (LPLI), which is also referred to low-level laser

therapy (LLLT), usually irradiates at 1 to 1000 mW/cm^2 [1]. This form of laser therapy also known as photobiomodulation (PBM), primarily uses light to promote tissue repair, reduce inflammation, and produce analgesia, with a low-power light source (laser or LED) [2]. Due to the low power (usually less than 1 W depending on the target tissue) there is no significant temperature rise in the treated tissue, therefore, the basic structure of the tissue will not be significantly changed after treatment [3]. Studies have shown that various biological processes, including cell viability, cell proliferation, cell migration and cell apoptosis, can be regulated by LPLI [4]. Some reports suggest that LPLI interfere with cell cycle development and induce cytotoxicity or cell apoptosis in some types of hyperplasia, revealing that it is a potential alternative cancer treatment [5]. A 632 nm laser can induce significant cell growth with fluences ranging from 3 to 15 J/cm^2, whereas 50 J/cm^2 of laser irradiation inhibited the viability of HeLa cells [6]. LPLI at 808 nm with fluences of 18, 32 and 54 J/cm^2 reduced tumor proliferation in human-derived glioblastoma A-172 cells in a dose-dependent stimulatory manner [7]. With respect to the cytotoxicity of laser induced thermal therapy (LITT) on tumor treatments, LITT can not only reduce the growth of tumor cells but induce the death of glioma cells isolated from the

brains of rats [8]. Furthermore, LPLI with high fluences (≥ 60 J/cm^2) induced apoptosis of ASTC-a-1 cells in human lung adenocarcinoma [9, 10]. However, a study conducted by Frigo L et al. (2009) showed that LPLI with 1050 J/cm^2 at 660 nm promoted melanoma tumor growth in vivo [11]. These studies showed that these two additional variables (power density and fluence) could affect cellular response to laser irradiation.

LPLI can regulate various processes in different biological systems [12-14]. Passarella et al. [15] reported that compared to non-irradiated mitochondria, laser irradiation could generate additional electrochemical potential and increase the synthesis of Adenosine Triphosphate (ATP) in mitochondria. A recent research [16] showed that the release of platelet-derived growth factor (PDGF) and TGF-β induced by low-energy laser biological stimulation increased the proliferation of fibroblasts. In addition, Yu et al.[16] found that the cytokines released by the irradiated fibroblasts were significantly different from those released by the nonirradiated fibroblasts. The data from these studies concluded that the influences of LPLI on cell apoptosis and proliferation mainly rely on the laser fluences and cell types. Nevertheless, despite various studies, the direct effect of LPLI on VX2 and CT26 cells in culture has been poorly explored.

Therefore, the aim of this current study was to elucidate the induction of apoptosis and its mechanism after 532-nm diode-pumped solid-state (DPSS) laser irradiation of squamous cell carcinoma (VX2) and murine colon carcinoma (CT26) cells.

2.3. Materials and Methods

2.3.1. Chemicals

Dimethyl sulfoxide (DMSO) was purchased from Samchun Pure Chemical Co., Ltd. (Pyeongteak-si, Gyeongi-do, Korea). All cells were cultured in medium including Dulbecco's modified Eagle's medium (DMEM). Dulbecco's modified Eagle's medium/F12 (DMEM-F12) were obtained from Cellgro (Mediatech, Massachusetts, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma-Aldrich. Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco. All the chemicals were used directly in experiments without further purifications.

2.3.2. Cell culture

Squamous cell carcinoma (VX2) cell was purchased from the Korea cell line Bank (Seoul, Korea). The cells were grown in DMEM-F12, which was

supplemented with 10% FBS and 1% penicillin (100 units/ml)-streptomycin (100 mg/ml) at 37 ℃ in an atmosphere that humidified 5% CO_2

Murine colon carcinoma, metastatic CT26 cell was purchased from the Korea cell line Bank (Seoul, Korea). The cells were grown in DMEM containing 10% FBS and penicillin (100 units/ml)-streptomycin (100 mg/ml), at 37 ℃ in an atmosphere that humidified 5% CO_2.

2.3.3. LPLI

A 1-W 532-nm diode-pumped solid-state (DPSS, Changchun New Industries Optoelectronics Technology Co, Ltd, Changchun, China) laser was employed to transmit laser light through a 600-μm optical fiber in the continuous wave (cw) mode for cell treatments (Figure 3). The average power at the distal end of the optical fiber was measured by using a power meter (Handheld Laser Power & Energy Meter NOVA II, Ophir, Israel) before testing. The laser irradiation experiments were conducted in triplicate at room temperature. Before the laser irradiation, the plastic cap was removed from 24-well plates. The cells were irradiated at the equivalent power density of 1 W/cm^2 for 100, 150, 200, 300, and 600 seconds. The corresponding fluences were 100, 150, 200, 300, and 600 J/cm^2, respectively.

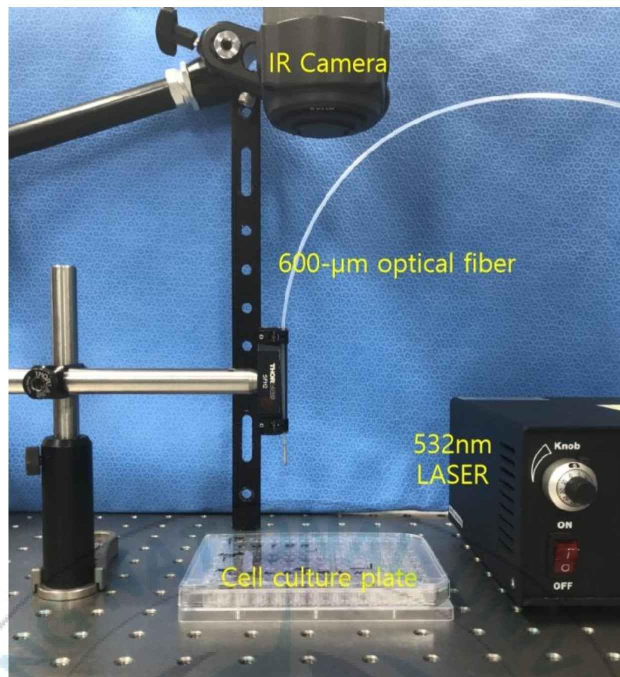


Figure 3. Experimental setup for in vitro 532-nm laser irradiation on gastroenterological cancer cells.

2.3.4. Cell viability by MTT assay

Cell viability was determined by using MTT assay that is based on the conversion of MTT into formazan crystals by living cells. The cells were inoculated on a 24-well plate (5×10^4 cells/well) under standard conditions (37 ℃, 5% CO_2 atmosphere). The cells were irradiated with laser and then incubated for 24 hours. For detection of cell viability, MTT reagent was added to the LPLI-treated cells, and the cells were cultured in a humidified atmosphere for 3-4 hours. An increase in staining quantity can directly reflect the number of

viable cells. The viability assays were based on the manufacturer's instructions. The cell viability after 532 nm laser irradiation was detected by MTT assay. The MTT assay solution was obtained by dissolving 50 mg MTT powder in a 50 mL PBS solution, and the mixed solution was filtered. After the cell medium was removed, 300 μl MTT solution was added to each well, and the cells were incubated for 3-4 hours at 37 ℃ and 5% CO_2. Then, the MTT solution was removed, and 300 μl DMSO was added to each well. The absorbance of the solution at 540 nm was measured after pipetting and shaking. In order to exclude the influence of temperature, the temperature of each well was monitored after LPLI treatment.

2.3.5. Statistical analysis

All experiments were executed and repeated at least three times, and data were reported as mean ± SD. The data were statistically analyzed by using the non-parametric Mann-Whitney test, and $p < 0.05$ was considered significant.

2.4. Results

Anti-tumor effects of LPLI on cancer cells was verified by cell viability tests in vitro. The cell viability of the irradiated VX2 and CT26 cells was measured 24

hours post-irradiation. Death of the VX2 and CT26 cells was induced by low-power laser irradiation in a dose-dependent stimulatory manner. For VX2 cells, after 24-hour incubation, the cell viability decreased significantly as laser fluences increased (Figure 4).

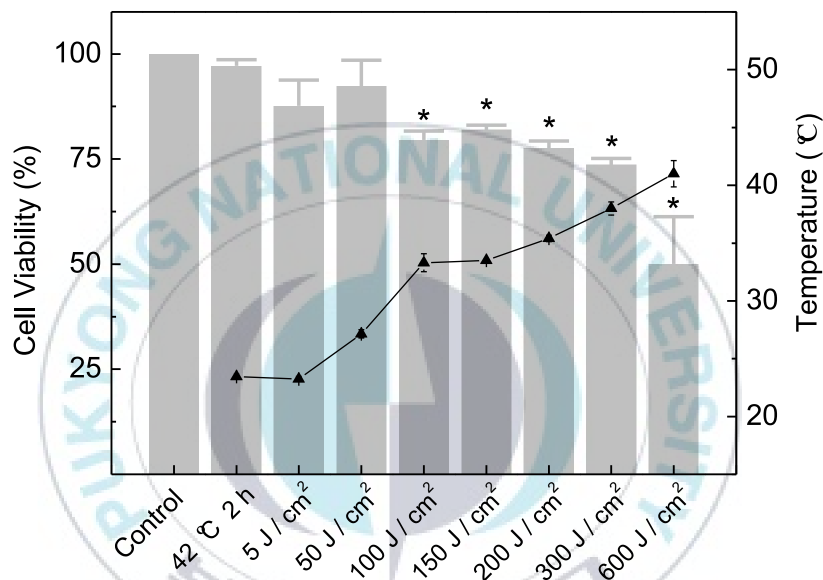
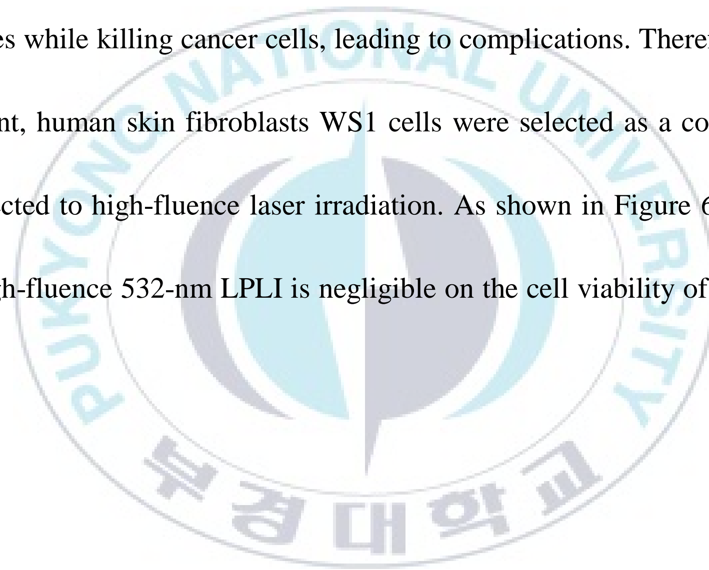


Figure 4. Cell viability after MTT assay experiment: Squamous cell carcinoma VX2 cells were seeded in 24-well plate overnight and exposed to LPLI at fluences of 5-600 J/cm^2 and water bath heating for 2 hours. Solid triangles represent the medium temperature (℃) after LPLI (*p < 0.01 vs. control).

For the CT26 cells, the cell viability did not changed initially as the fluences increased, but after 200 J/cm^2, the cell viability declined rapidly (Figure 5). During the tests, the temperature of the medium was measured and found to be

below 42 ℃. It should be noted that the effect of temperatures between 36 ℃ and 42 ℃ for 2-hour incubation on the tumor cell viability is negligible [17]. To confirm non-thermal effect during LPLI, both the cells lines were also heated in a 42 ℃ water bath (Figure 4 and 5). The results showed that the cellular responses were associated with non-thermal processes. In addition, previous studies [18, 19] reported that laser irradiation therapy may damage normal cells and tissues while killing cancer cells, leading to complications. Therefore, in this experiment, human skin fibroblasts WS1 cells were selected as a control group and subjected to high-fluence laser irradiation. As shown in Figure 6, the effect of the high-fluence 532-nm LPLI is negligible on the cell viability of the normal cells.



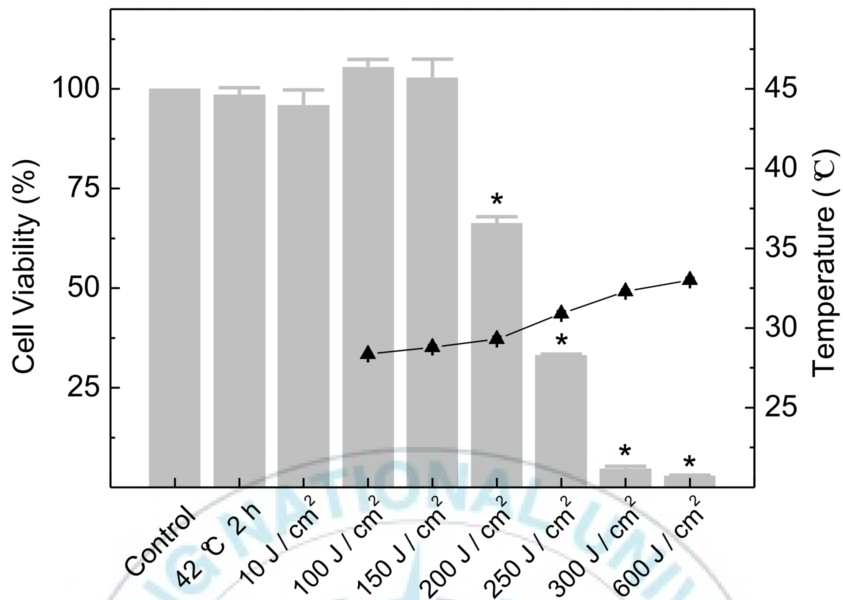


Figure 5. Cell viability after MTT assay experiment: Murine colon carcinoma CT26 cells were seeded in 24-well plate overnight and exposed to LPLI at fluences of 10-600 J/cm^2 and water bath heating for 2 hours. Solid triangles represent the medium temperature (℃) after LPLI (*$p < 0.01$ vs. control).

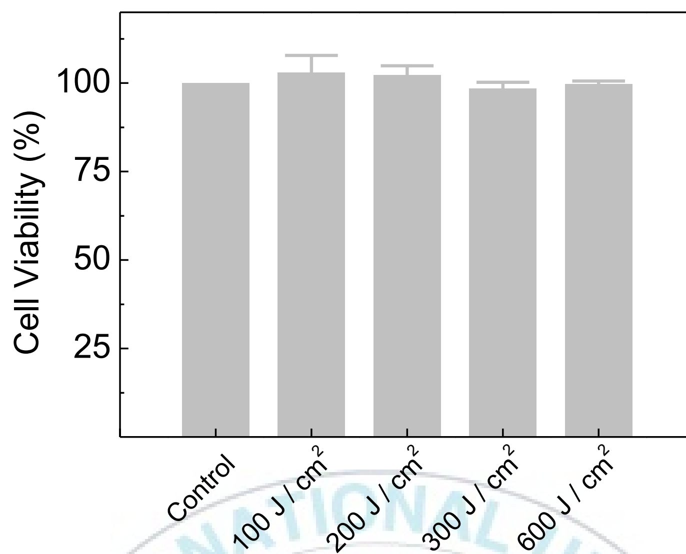


Figure 6. Cell viability after MTT assay experiment: Effect of high-fluence LPLI on human skin fibroblast WS1 cells viability were examined.

2.5. Results

LPLI has been widely researched due to its ability to induce inhibition tumor cells, which makes it a potential cancer treatment. Effect of LPLI on cell proliferation and death mainly depends on cell type and laser fluence. However, the direct effect of LPLI on squamous cell carcinoma and murine colon carcinoma cells in culture remains unexplored. In this study, the differences in the cell viability between untreated cells (control) and LPLI-treated cancer cells were evaluated by using MTT analysis. As shown in Figure 4, LPLI at 532 nm with a fluence of 600 J/cm^2 exerted a strong cytotoxicity, inhibiting the viability of VX2

cells. However, for CT26 cells, a significant inhibitory effect was generated at a much lower fluence of 200 J/cm^2, which implicates that LPLI has different effects on different cell lines. In addition, some studies showed mechanism of laser thermal inhibition of tumor [20, 21]. For instance, Fisher JW (2010) reported that a combination of laser irradiation and multi-walled carbon nanotubes (MWNTs) elevated the temperature of the media and reduced the size of the tumor in human prostate cancer and murine renal carcinoma cells [21, 22].

In the current study, all the cells were subjected to laser irradiation at room temperature (26 ℃), and the medium temperature was monitored after the laser irradiation (0–600 J/cm^2). The temperature monitoring confirmed that after the irradiation, the temperature of the media slightly increased to 42 ℃ (Figure 4 and 5). Moreover, the cells in a 42 ℃ water bath experienced negligible thermal responses, indicating that the anti-tumor effects of LPLI in this study was associated with a non-thermal process. In order to verify whether LPLI may have an effect on normal cells while killing cancer cells, human skin fibroblasts were also selected for high-fluence laser treatment in the experiment. The results (Figure 6) showed minimal effect of LPLI on the WS1 cell viability.

2.6. Conclusion

Above all, this phase of study confirmed that high fluence LPLI significantly inhibited the growth of gastroenterological cancer cells in vitro. The 600 J/cm^2 and 200 J/cm^2 532 nm laser irradiation reduced the viability of VX2 and CT26 cells, respectively, indicating that LPLI has different effects on different cell lines. In addition, the temperature monitoring results also exclude the influence of laser thermal effect. The next phase will continue to explore the specific anti-tumor mechanism of LPLI on gastroenterological cancer cells in vitro.

2.7. Reference

[1] Hashmi JT, Huang YY, Osmani BZ, Sharma SK, Naeser MA, Hamblin MR. Role of low-level laser therapy in neurorehabilitation. PM R 2010;2(12 Suppl 2):S292–S305.

[2] Chung H , Dai T , Sharma S K , et al. The Nuts and Bolts of Low-level Laser (Light) Therapy[J]. Annals of Biomedical Engineering, 2012, 40(2):516-533.

[3] Caruso-Davis M K , Guillot T S , Podichetty V K , et al. Efficacy of Low-Level Laser Therapy for Body Contouring and Spot Fat Reduction[J]. Obesity Surgery, 2011, 21(6):722-729.

[4] Rhee Y H , Moon J H , Choi S H , et al. Low-Level Laser Therapy Promoted Aggressive Proliferation and Angiogenesis Through Decreasing of Transforming Growth Factor-β1 and Increasing of Akt/Hypoxia Inducible Factor-1α in Anaplastic Thyroid Cancer[J]. Photomedicine & Laser Surgery, 2016, 34(6).

[5] Chu J, Wu S, Xing D. Survivin mediates self-protection through ROS/cdc25c/CDK1 signaling pathway during tumor cell apoptosis induced by high fluence low-power laser irradiation. Cancer Lett 2010;297(2):207–219.

[6] Zhang J, Xing D, Gao X. Low-power laser irradiation activates Src tyrosine kinase through reactive oxygen species-mediated signaling pathway. J Cell Physiol 2008;217(2):518–528.

[7] Murayama H, Sadakane K, Yamanoha B, Kogure S. Low power 808-nm laser irradiation inhibits cell proliferation of a human-derived glioblastoma cell line in vitro. Lasers Med Sci 2012;27(1):87–93.

[8] Schulze PC, Adams V, Busert C, Bettag M, Kahn T, SchoberR. Effects of laser-induced thermotherapy (LITT) on proliferation and apoptosis of glioma cells in rat brain transplantation tumors. Lasers Surg Med 2002;30(3):227–232.

[9] Wu S, Xing D, Wang F, Chen T, Chen WR. Mechanistic study of apoptosis induced by high-fluence low-power laser irradiation using fluorescence imaging techniques. J Biomed Opt 2007;12(6):064015.

[10] Huang L, Wu S, Xing D. High fluence low-power laser irradiation induces apoptosis via inactivation of Akt/GSK3beta signaling pathway. J Cell Physiol 2011;226(3): 588–601.

[11] Frigo L, Luppi JS, Favero GM, Maria DA, Penna SC, Bjordal JM, Bensadoun RJ, Lopes-Martins RA. The effect of low-level laser irradiation (In-Ga-Al-AsP - 660 nm) on melanoma in vitro and in vivo. BMC Cancer 2009;9:404.

[12] Belkin M , Zaturunsky B , Schwartz M . A critical review of low energy laser bioeffects[J]. Lasers & Light in Ophthalmology, 1988, 2(1):63-71.

[13] Karu T . Photobiology of low-power laser effects.[J]. Health Physics, 1989, 56(5):691-704.

[14] Conlan M J , Rapley J W , Cobb C M . Biostimulation of wound healing by low-energy laser irradiation A review[J]. Journal of clinical periodontology, 1996, 23(5):492-496.

[15] Passarella S , Casamassima E , Molinari S , et al. Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by helium-neon laser.[J]. Febs Letters, 1984, 175(1):95-99.

[16] Yu M , Naim J O , Lanzafame R J . The effects of photo-irradiation on the secretion of TGF and PDGF from fibroblasts in vitro. Lasers Surg. Med. Suppl., 1994 (6): 8.

[17] Giovanella B C , Jr S J , Morgan A C . Selective lethal effect of supranormal temperatures on human neoplastic cells.[J]. Cancer Research, 1976, 36(1):3944-50.

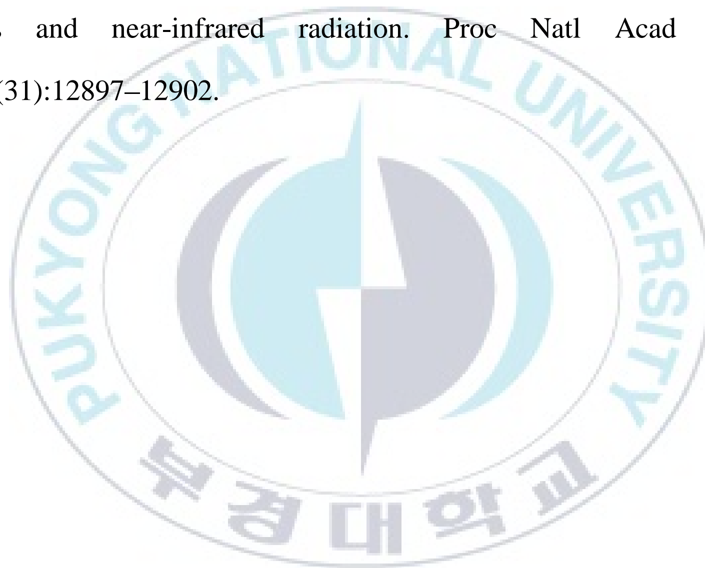
[18] Meirow D, Biederman H, Anderson R A, et al. Toxicity of chemotherapy and radiation on female reproduction.[J]. Clinical Obstetrics & Gynecology, 2010, 53(4):727-39.

[19] Clarke M , Collins R , Darby S , et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials.[J]. Chinese Journal of Breast Disease, 2007, 366(9503):2087-2106.

[20] Shao J, Griffin RJ, Galanzha EI, Kim JW, Koonce N, Webber J, Mustafa T, Biris AS, Nedosekin DA, Zharov VP. Photothermal nanodrugs: potential of TNF-gold nanospheres for cancer theranostics. Sci Rep 2013;3:1293.

[21] Fisher JW, Sarkar S, Buchanan CF, Szot CS, Whitney J, Hatcher HC, Torti SV, Rylander CG, Rylander MN. Photothermal response of human and murine cancer cells to multiwalled carbon nanotubes after laser irradiation. Cancer Res 2010;70(23):9855–9864.

[22] Burke A, Ding X, Singh R, Kraft RA, Levi-Polyachenko N, Rylander MN, Szot C, Buchanan C, Whitney J, Fisher J, Hatcher HC, D'Agostino R, Jr., Kock ND, Ajayan PM, Carroll DL, Akman S, Torti FM, Torti SV. Long-term survival following a single treatment of kidney tumors with multiwalled carbon nanotubes and near-infrared radiation. Proc Natl Acad Sci USA 2009;106(31):12897–12902.



3. Chapter 3: *In vitro* investigation of anti-tumor effects of low power laser irradiation

3.1. Abstract

High fluence LPLI also use low-power light source, but forms overstimulation on cells or tissues through high fluences (usually above 80 J/cm^2), which in turn activates the mitochondrial apoptosis pathway, altering the cell cycle, inhibiting cell proliferation and even causing cell death. Based on the conclusions of the previous chapter, this study aims to investigate LPLI-induced apoptosis and its mechanism after 532-nm high fluence LPLI on two different cancer cells. Fluorescence staining, cell migration assay, and cell apoptosis tests were performed 24 hours post-irradiation to monitor cellular responses. Both wound healing and apoptosis experiments confirmed that high-throughput LPLI could inhibit cell migration and induce apoptosis. The current findings demonstrate that LPLI might be a potential treatment for the carcinoma cells.

3.2. Introduction

In the previous chapter, we mainly introduced the concept and research status of LPLI. Low-power laser irradiation (LPLI) has been shown to regulate a variety

of biological processes [1], including cell proliferation and differentiation [2], cell viability [3], and cell apoptosis [4]. However, current studies have shown that the effect of LPLI on cell proliferation is promoted at relatively low fluence and inhibited at higher fluence [5]. High fluence LPLI also use low-power light source, but forms overstimulation on cells or tissues through high fluences (usually above 80 J/cm^2), stimulates mitochondrial chromophores and the release of various inflammatory factors (Figure 7), which in turn activates the mitochondrial apoptosis pathway, alters the cell cycle, inhibits cell proliferation and even leads cell death [6]. With the advantage of being non-invasive, High fluence LPLI has been widely used in anti-tumor area in recent years. Previous studies have stressed experimental and clinical results supporting the potential anti-tumor effects of high fluence LPLI [7][8]. A phase I trial in patients with advanced neoplasias demonstrated that a first treatment approach using an infrared pulsed laser device was safe for clinical use and improved Karnofsky Performance Status (KPS) and Spitzer Quality of Life Index (QLI) [9]. Also new data have further confirmed that, under certain parameters, high fluence LPLI may indeed be safe for use in cancer patients, despite decades of controversy [10][11]. Anti-tumor activity was observed in 88.23% of patients with a 10-year

of follow-up and 96% compliance of the treatment prescribed [12]. Furthermore, several identified experimental studies [13][14][15] have further documented that LPLI can modulate anti-tumor effects. Surveys such as that conducted by Tanaka (2010) evaluated the non-thermal effects of IR radiation on cancer cells and found that IR light, independent of thermal energy, can kill cancer cells [16]. Wang et al. [17] have showed that low-power laser irradiation may induce apoptosis of human lung adenocarcinoma cells.

Nevertheless, despite various studies have confirmed the anti-tumor effect of LPLI on a variety of different cancers, the direct effect of LPLI on gastroenterological cancer in vitro has been poorly explored. Therefore, in the previous chapter, the anti-tumor effect of LPLI on gastroenterological tumors in vitro were mainly investigated. MTT assay was performed after 24 hours laser irradiation. The current results indicate that high fluence LPLI does significantly inhibit cell activity in two types of gastroenterological cancers. However, the specific effects on cell growth, migration and apoptosis are unclear. Therefore, we will further explore the in vitro anti-tumor mechanism of LPLI in this chapter.

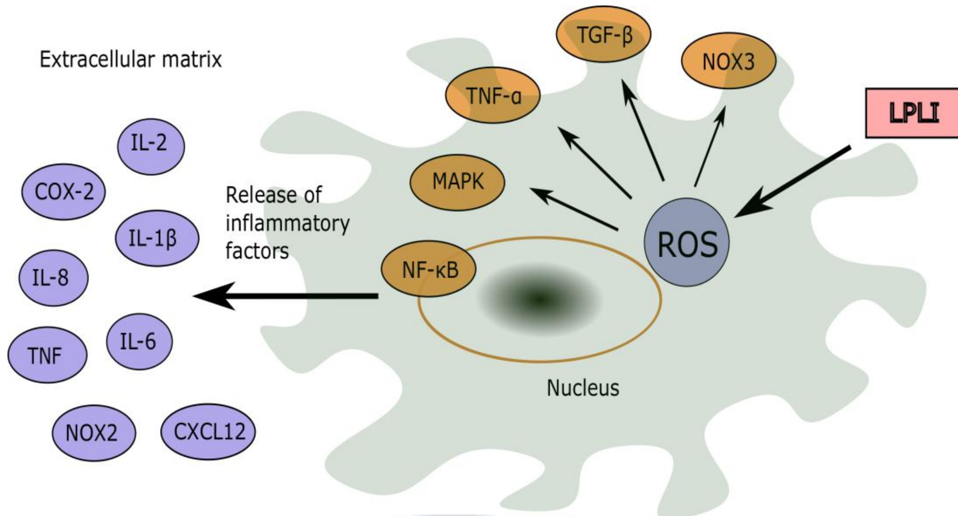


Figure 7. LPLI stimulates the production of mitochondrial reactive oxygen species (ROS) leading to the release of various inflammatory factors in cancer cells.

3.3. Materials and Methods

Flourescein diacetate (FDA), 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and antibodies were obtained from Sigma-Aldrich. All the chemicals were used directly in experiments without further purification. For cells seeding and laser treatment conditions were performed as presented in **Chapter 2**.

3.3.1. Live and dead cells fluorescence staining

VX2 and CT26 cells were inoculated on a 24-well plate (5×10^4 cells/well) under standard conditions (37 ℃, 5% CO_2 atmosphere). The cells were subjected

to laser irradiation. After 24 hours, the LPLI-treated cells were stained with FDA/DAPI/PI fluorescence staining and then observed with a fluorescence microscope to estimate the effect of LPLI on death of tumor cells. FDA stock solution (25 mg/ml dissolved in acetone), DAPI stock solution (1 mg/ml dissolved in acetone) and PI stock solution (1 mg/ml dissolved in distilled water) were stored in the refrigerator of 4 ℃. Then, 7 μl of FDA, 42 μl DAPI and 42 μl PI stock solution were diluted into a 4.2 ml of medium. The staining time was 7–10 minutes. After the staining, the cells were washed three times with 300 μl PBS, and they were then observed under a fluorescence microscope. Bright green or blue cells represented viable cells, while red cells were considered nonviable.

3.3.2. Cell migration assay

To assess effect of LPLI on cell migration, an in vitro wound healing assay was conducted. VX2 and CT26 cells were inoculated into 24-well plates (5×10^4 cells/well) and incubated at 37 ℃ and 5% CO_2 atmosphere for 24 hours. A wound scratch was made to disrupt a confluent cell monolayer. The tumor cells were irradiated by 532-nm light at 1 W/cm^2 for various irradiation times. The cell migration was estimated by applying phase contrast microscopy for a period

of 48 hours, which was sufficient to complete scratch closure. The area devoid of the cells was calculated in pixels at 0, 12, 24 and 48 hours by using Image J (National Institute of Health, Bethesda, MD). The percentage reduction of the scratched area between the first and last time points was calculated.

3.3.3. Cell apoptosis analysis

Annexin V/PI staining analysis was used for further detecting the proportion of early apoptosis cells and late apoptosis/necrosis cells [18]. VX2 and CT26 cells were inoculated into 24 well plates at the density of 4×10^5 cells/well and 3×10^5 cells/well, respectively. Then, the cells were irradiated with 532-nm light and incubated for 4 hours. The cells were harvested with a microcentrifuge tube after incubation and were washed twice with 100 μl cold PBS. Then, the cells were resuspended in a buffer solution (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM Hepes/NaOH) at a concentration of 1×10^6 cells/ml. An Annexin V/PI staining solution was added to the cells. The cells were gently stirred and incubated at room temperature (26 ℃) in the dark environment for 15 minutes. A 400 μl of buffer solution was added to each microcentrifuge tube. The cells were analyzed by using flow cytometry within 1 hour. Each sample was analyzed at least 1×10^4 cells. PI negative, Annexin V positive or both Annexin V and PI positive

percentage cells were used to represent the number of early apoptosis cells and late apoptosis/necrosis cells, respectively. The data were analyzed by the leading platform FlowJo for single-cell flow cytometry analysis.

3.3.4. Statistical analysis

All experiments were executed and repeated at least three times, and data were reported as mean ± SD. The data were statistically analyzed by using the non-parametric Mann-Whitney test, and $p < 0.05$ was considered significant.

3.4. Results

3.4.1. FDA/DAPI/PI staining

The treated VX2 and CT26 cells stained by FDA, DAPI and PI were observed to evaluate anti-tumor effect of LPLI on the tumor cells (Figure 8 and 9) under a fluorescence microscope. The green emission comes from the viable cells nuclei stained by FDA, the blue emission from nuclei stained by DAPI, and the red emission from the nonviable cells stained by PI. Both control (Figure 8a and 9a) and low fluence (Figure 8b and 9b) displayed clear green and blue, and minimal red emissions from the nuclei, indicating that the cells were viable. For VX2 cells, the cells irradiated by a high fluence laser (300–600 J/cm^2) exhibited

a slight increase in the red emission, thus revealing a slight increase in the number of the dead cells (Figure 8c and 8d). In contrast, the CT26 cells treated with a high fluence laser showed a significant increase in cellular death (Figure 9c and 9d), indicating selective anti-tumor effects as a result of the LPLI treatments.

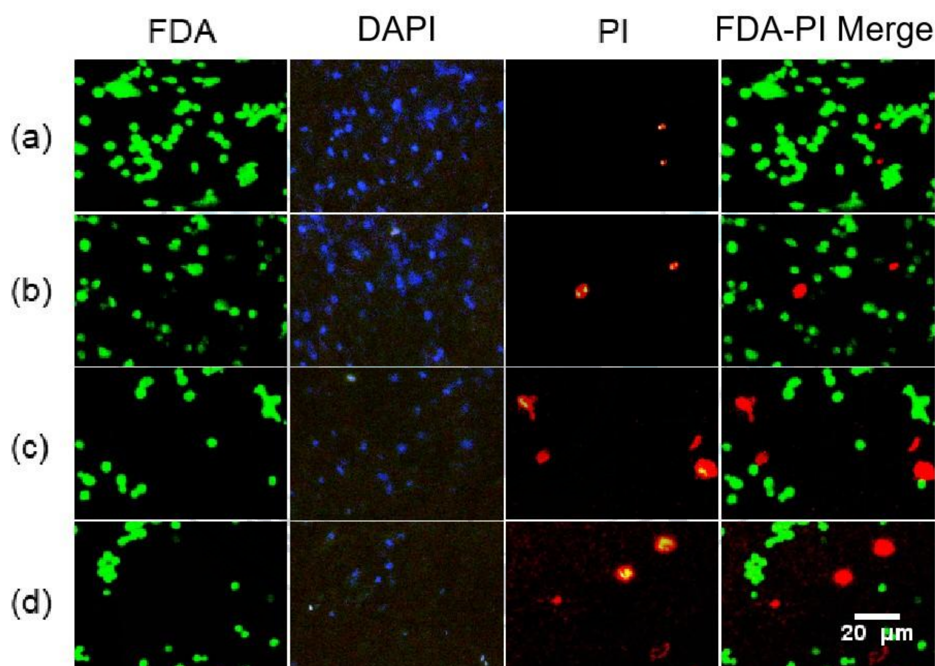


Figure 8. Fluorescence microscopy analysis of VX2 cell death at various fluences: Nuclei were stained with FDA (green), DAPI (blue), and dead cells were stained with PI (red): (a) control, (b) 100 J/cm^2, (c) 300 J/cm^2, and (d) 600 J/cm^2 (Magnification = 40× and scale bar = 20 μm).

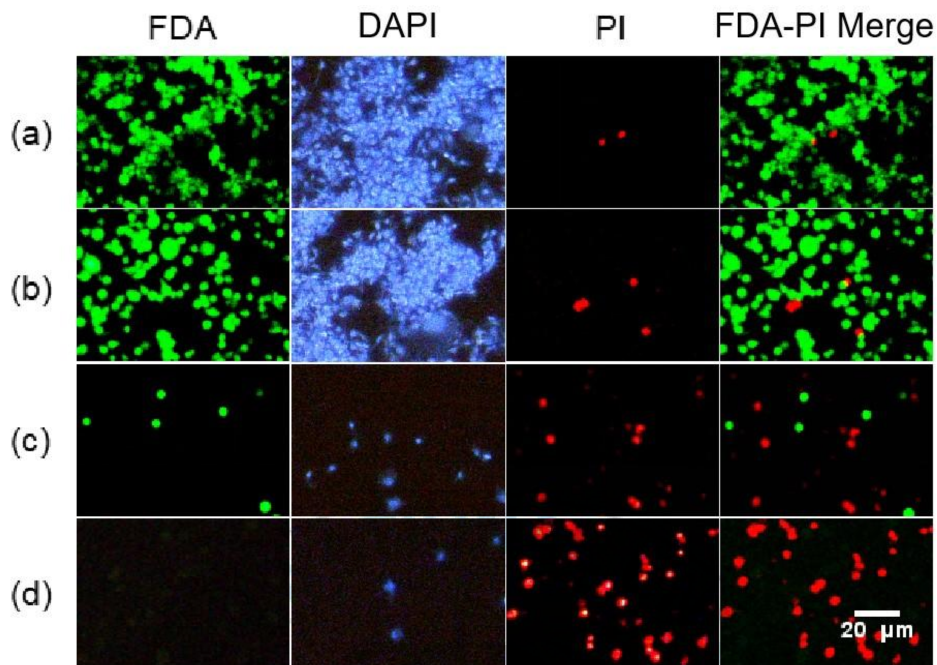


Figure 9. Fluorescence microscopy analysis of CT26 cell death at various fluences: Nuclei were stained with FDA (green), DAPI (blue), and dead cells were stained with PI (red): (a) control, (b) 100 J/cm^2, (c) 300 J/cm^2, and (d) 600 J/cm^2 (Magnification = 40× and scale bar = 20 μm).

3.4.2. In vitro cell migration assay

The correlational analysis results of wound healing assay were presented in Figure 10 and 11. The wound size decreased over time, and the rate of wound healing was used as a measure of cell migration. In a range of laser fluences (100-600 J/cm^2), the migration rate of the cells from the border of the original scratch zone to the inner was decelerated. For VX2 cells, the migration rate was significantly reduced at 600 J/cm^2, while for CT26 cells, the migration rate nearly

stopped at 200 J/cm^2. Figure 10a and 11a present photomicrographs of the cells. Measurements of wound widths indicated that the wound closure was inhibited in an irradiation-dose-dependent manner, compared to non-irradiated control (0 J/cm^2).

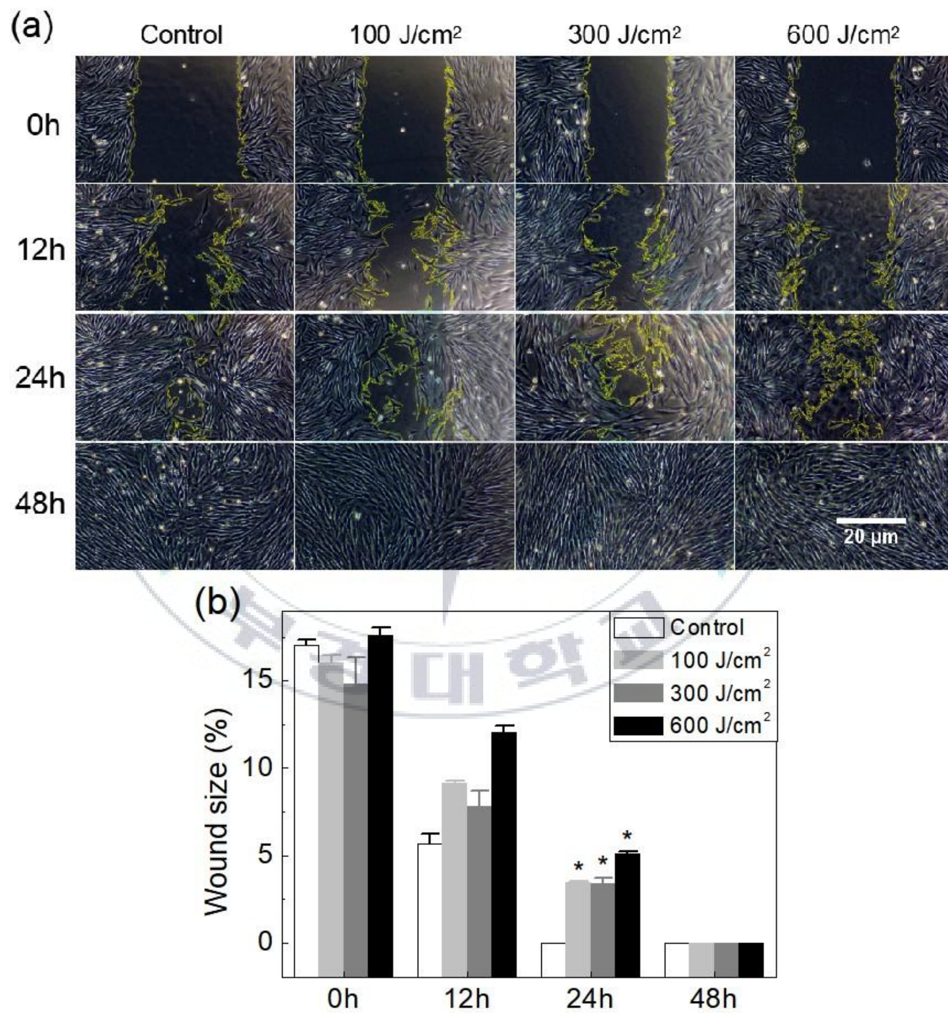


Figure 10. Wound healing assay of VX2 cells: (a) the images of wound variations at 0, 12, 24 and 48 hours after laser treatment (Magnification = 100× and scale bar = 20 μm) and (b) wound size

variations as function of time (*p < 0.05 vs. control).

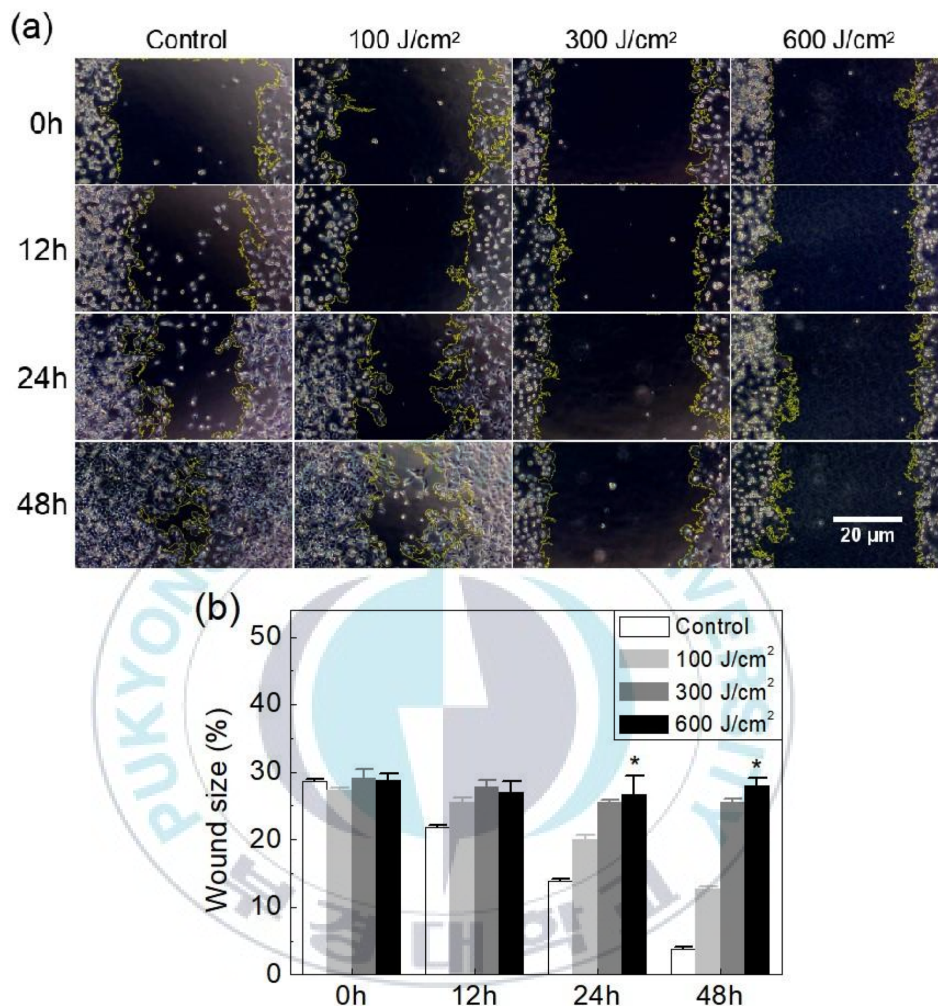


Figure 11. Wound healing assay of CT26 cells: (a) the images of wound variations at 0, 12, 24 and 48 hours after laser treatment (Magnification = 100× and scale bar = 20 μm) and (b) wound size variations as function of time (*$p < 0.05$ vs. control).

3.4.3. Cell apoptosis analysis

As cell apoptosis is an important indicator for cancer therapy, effect of LPLI on

apoptosis induction in VX2 and CT26 cells were evaluated. Experimental results show that high-fluence LPLI increased the number of apoptosis cells in the VX2 cells population. In contrast, for the CT26 cells, LPLI yielded a more significant ability to induce apoptosis at the same fluence (Figure 12a and 12b). The current findings were consistent with those of cell viability assay, suggesting that LPLI is a feasible treatment for carcinoma cells.

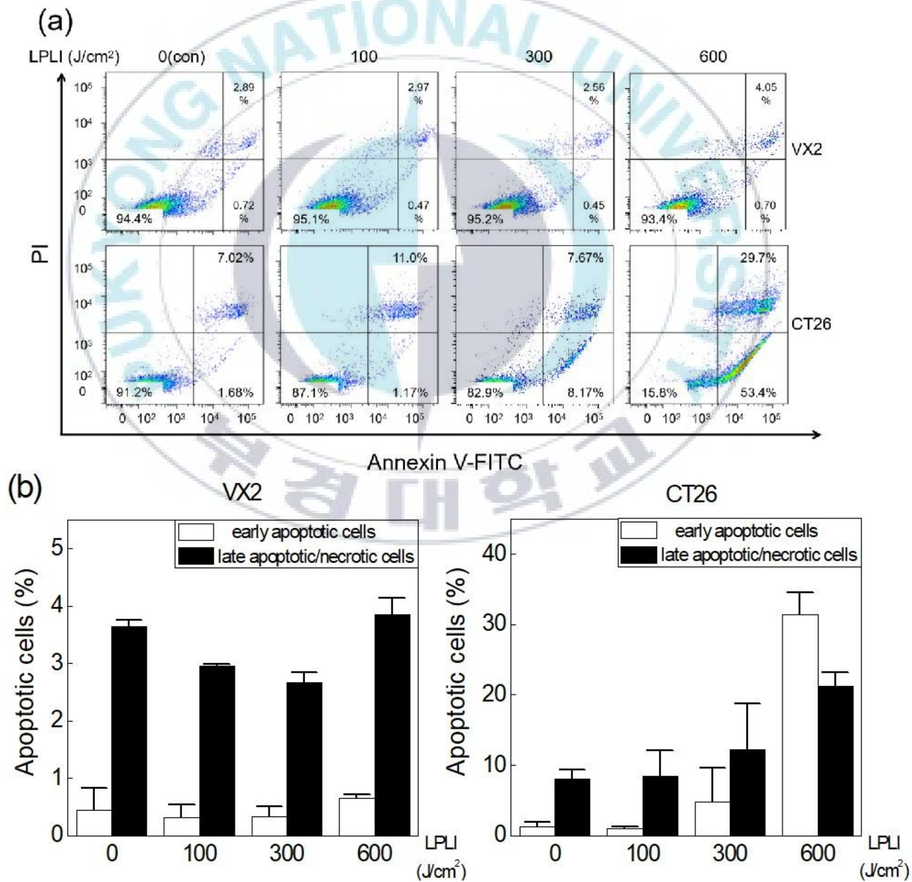


Figure 12. (a) Effect of LPLI on apoptosis in VX2 and CT26 cells: (a) Squamous cell carcinoma

VX2 and murine colon carcinoma CT26 cells were exposed to LPLI at fluences of 0, 100, 300, or 600 J/cm^2 and then incubated for 4 hours. The cells were harvested for Annexin V/PI staining and were analyzed with a FACS flow cytometer. (b) The percentages of early apoptotic cells (AnnexinV+/PI-) and late apoptotic/necrotic cells (AnnexinV+/PI+) in LPLI-treated cells were evaluated

3.5. Results

Cell morphology and the ratio of dead and alive cells after laser irradiation were observed by using fluorescence microscopy following FDA/DAPI/PI staining. As shown in Figure 8 and 9, fluorescence staining images demonstrated that LPLI with high fluences may induce both VX2 and CT26 cell death. However, compared to the VX2 cells, the CT26 cells were more affected by the 532-nm laser irradiation. These results corroborate those of previous studies showing that the cell death induced by laser irradiation mainly depends on the laser fluences and cell type. Furthermore, cell migration is an important feature for spread and metastasis of tumor cells [19, 20]. Thus, the effect of LPLI on the migration of cancer cells was evaluated. It was found that LPLI reduced the migration of the VX2 and the CT26 cells (Figure 10 and 11). For the VX2 cells, the migration rate was significantly reduced at 600 J/cm^2, while for the CT26 cells, the migration rate almost stopped at 200 J/cm^2. The apoptosis results

showed that the high-fluence LPLI induced apoptosis of the VX2 cells. In comparison, LPLI yielded a more significant apoptosis-inducing effect on the CT26 cells at the same fluence. It is speculated that LPLI may trigger mitochondrial oxygen stress by exciting endogenous photoacceptor, thereby inducing mitochondrial apoptosis pathway. However, Further experiments are needed to verify the specific molecular mechanism for apoptosis induction and migration inhibition. The current results of the wound healing assay and apoptosis tests were consistent with those of the previous experiments, suggesting that LPLI be a potential therapy for primary hepatocellular carcinoma and colon carcinoma patients.

Although the VX2 cells have been used to study liver tumor because of similar blood supply to that of human liver tumor, the cells were originally derived from a carcinoma of a domestic rabbit skin wart induced by rabbit papillomavirus [21]. In spite of the favorable outcomes, the inhibitory effect of LPLI on the VX2 cells may not directly evidence that LPLI could be used to treat hepatocellular carcinoma. Additionally, effects of LPLI-induced apoptosis were found to be different between the two types of the cell lines. However, due to the limitations of in vitro cell tests, further in vivo animal experiments are still required to

confirm the current findings. Therefore, the next step of this study is to establish a VX2 model of liver cancer in rabbits and a CT26 model of colon carcinoma in mice, so as to better understand the mechanism of LPLI for gastroenterological tumor treatment.

3.6. Conclusion

The goal of the current in vitro study was to assess effect of LPLI on hepatocellular carcinoma and colon cancer cell models. LPLI with 532 nm at 600 J/cm^2 significantly induced apoptosis in VX2 and CT26 cells. LPLI may be a potential therapy for treating gastroenterological carcinoma. Further studies will be conducted to evaluate the propose treatment in in vivo animal tumor models.

3.7. Reference

[1] Karu T. 1989. Photobiology of low-power laser effect. Health Physiol 56:691–704.

[2] Nadav BD, Gavriella S, Andrey I, Anton W, Uri O, Orna H. 1999. Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. Biochim Biophys Acta 1448:372–380.

[3] Lubart R, Eichler M, Lavi R, Friedman H, Shainberg A. 2005. Low-energy laser irradiation promotes cellular redox activity. Photomed Laser Surg 23:3–9.

[4] Wang F, Chen TS, Xing D, Wang JJ, Wu YX. 2005. Measuring dynamics of caspase-3 activity in living cells using FRET technique during apoptosis induced by high fluence low-power laser irradiation. Lasers Surg Med 36:2–7.

[5] Karu T I. Effects of visible radiation on cultured cells.[J]. Photochemistry & Photobiology, 2010, 52(6):1089-1098.

[6] Chu J , Wu S , Xing D . Survivin mediates self-protection through ROS/cdc25c/CDK1 signaling pathway during tumor cell apoptosis induced by high fluence low-power laser irradiation[J]. Cancer Letters, 2010, 297(2):0-219.

[7] Karu T. Mitochondrial mechanisms of photobiomodulation in context of new data about multiple roles of ATP. Photomed Laser Surg. 2010; 28: 159-160.

[8] Lanzafame RJ. Photobiomodulation and Cancer and Other Musings. Photomed Laser Surg. 2011; 29: 3-4.

[9] Santana-Blank LA, Rodríguez-Santana E, Vargas F, Reyes H, Fernández-Andrade P, Rukos S, et al. Phase I trial of an infrared pulsed laser device in patients with advanced neoplasias. Clin Cancer Res. 2002; 8: 3082-3091.

[10] Myakishev-Rempel M, Stadler I, Brondon P, Axe DR, Friedman M, Nardia FB, et al. A Preliminary Study of the Safety of Red Light Phototherapy of Tissues Harboring Cancer. Photomed Laser Surg. 2012; 30: 551-558.

[11] Santana Blank L, Rodríguez-Santana E, Santana-Rodríguez KE. Concurrence of Emerging Developments in Photobiomodulation and Cancer. Photomedicine & Laser Surgery. 2012; 30: 1-2.

[12] Santana Blank L, Rodríguez-Santana E, Vargas F, Reyes H, Fernández-Andrade P, Rukos S, et al. Phase I trial of an infrared pulsed laser

device in patients with advanced neoplasias. Clin Cancer Res. 2002; 8: 3082-3091.

[13] Tata DB, Waynant RW. Laser Therapy: A review of its mechanism of action and potential medical applications. Laser Photonics Rev. 2011; 5: 1-12.

[14] Tata DB, Fahey M, Mitra K, Anders J, Waynant RW. Near-IR induced suppression of metabolic activity in aggressive cancers. Proc. SPIE. 2007; 6428: 64280E.

[15] Tata DB, Waynant RW. Laser light induced modulations in metabolic activities in human brain cancer. Proc. SPIE. 2008; 6846: 684607.

[16] Tanaka Y, Matsuo K, Yuzuriha S, Yan H, Nakayama J. Non-thermal cytocidal effect of infrared irradiation on cultured cancer cells using specialized device. Cancer Sci. 2010; 101: 1396-1402.

[17] Wang F, Chen TS, Xing D, Wang JJ, Wu YX. Measuring dynamics of caspase-3 activity in living cells using FRET technique during apoptosis induced by high fluence low-power laser irradiation. Lasers Surg Med. 2005; 36: 2-7.

[18] Liang W Z , Liu P F , Fu E , et al. Selective cytotoxic effects of low-power laser irradiation on human oral cancer cells[J]. Lasers in Surgery and Medicine, 2015, 47(9):756-764.

[19] Bravo-Cordero JJ, Hodgson L, Condeelis J. Directed cell invasion and migration during metastasis. Curr Opin Cell Biol 2012;24(2):277–283.

[20] Entschladen F, Drell TLt, Lang K, Joseph J, Zaenker KS. Tumour-cell migration, invasion, and metastasis: Navigation by neurotransmitters. Lancet Oncol 2004;5(4): 254–258.

[21] Aravalli R N , Steer C J , Sahin M B , et al. Stem Cell Origins and Animal Models of Hepatocellular Carcinoma[J]. Digestive Diseases and Sciences, 2010, 55(5):1241-1250.

4. Chapter 4: Conclusions and Future Studies

4.1. Conclusions

In this work, VX2 and CT26 cells were irradiated by 532 nm laser with a series of fluences. And the cells responses were investigated deeply.

High fluence LPLI significantly inhibited the growth of gastroenterological cancer cells in vitro. The 600 J/cm^2 and 200 J/cm^2 532 nm laser irradiation reduced the viability of VX2 and CT26 cells, respectively, indicating that LPLI has different effects on different cell lines.

In addition, the temperature monitoring results also exclude the influence of laser thermal effect. Both the wound healing assay and the cell apoptosis tests confirmed that LPLI with high fluences could inhibit cell migration as well as induce cell apoptosis.

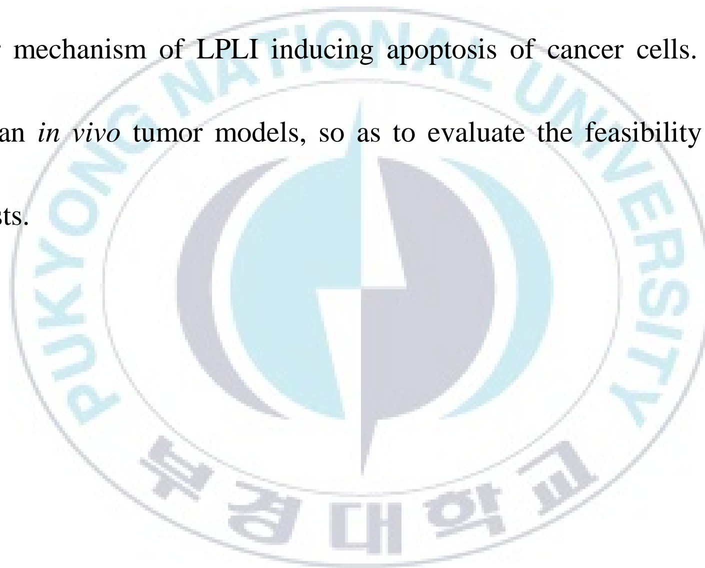
Above all, LPLI with 532 nm at 600 J/cm^2 significantly induced apoptosis in VX2 and CT26 cells. LPLI may be a potential therapy for treating gastroenterological carcinoma.

4.2. Future Studies

Although our work confirmed that 532-nm high fluence LPLI (300-600 J/cm^2)

can induce apoptosis of gastroenterological cancer cells *in vitro*, the specific molecular mechanism is still unknown. Moreover, for an ongoing disease, the complexity of the multicellular environment makes it hard to predict tumor behavior and cell culture studies alone are inadequate to for assessment of tumor responses.

Therefore, for the future studies, it is necessary to further explore the specific molecular mechanism of LPLI inducing apoptosis of cancer cells. Also try to establish an *in vivo* tumor models, so as to evaluate the feasibility of LPLI in animal tests.


PUKYONG NATIONAL UNIVERSITY
부경대학교

Acknowledgments

Foremost, I would like to express my sincere gratitude to my advisor Prof. Hyun Wook Kang for his continuous support of my master study and research, for his patience, encouragement and immense knowledge. His guidance has helped me a lot during the last two years of research and living in South Korea. In addition, he always gives many in-depth discussion about results, and willing to help his students with their research.

I would like to thank Kim Hyejin, Lee Yeachan, Karuoousamy Shanmugapriya, Truong Van Gia, Van Nam Tran, and the other member of Bio-Ablation Lab who as good friends, was always give me best suggestions and kindly mobilization. Special thanks to Prof. Yong wook Lee and Prof. Hyoung Shin Lee, who were willing to participate in my final defense committee.

At last, I'd like to dedicate this work to my parents. Whatever I do, wherever I go, they will always give me encouragement and strength. And thanks to all my relatives for their endless care and support.

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI16C1017).

PUKYONG NATIONAL UNIVERSITY
부경대학교