박사학위논문

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상기자의 치의학박사 학위논문을 인준함

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Contents

A comparative study of blue and red light irradiation on PMA-treated HaCaT cell line on ROS scavenging effect

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(Directed by Professor : Choi, Hong Ran)

Intracellular reactive oxygen species (ROS) produced in a various pathologic state was known to intermediate many cellular response such as inflammation. Recently, low level light irradiation by HeNe laser used in many clinical field could improve inflammatory state by scavenging intracellular ROS through photo - detachment / dissociation process. The purpose of this study is to investigate the differential effects of blue and red light irradiation on ROS scavenging effects.

Immortalized human oral keratinocyte HaCat cells were used. Phorbol 12-myristate 13-acetate (PMA) was treated for inflammation. Red (635nm) and blue (470nm) light irradiation was carried out. To asses the intracellular ROS by light irradiation, confocal microscopic and flow cytometric assay with DCF fluorescence for total ROS and ESR spectrometry of DMPO-O_2^- for superoxide anion were caried out. And microarray was performed for mRNA expression level.

Released intracellular total ROS in PMA treated HaCat cell lines was

dissociated efficiently by red light irradiation, while blue light irradiation did not. Rather, blue light irradiation increased ROS formation. For superoxide anion generated the first synthetic form of ROS, red light irradiation reduced its amount but blue light irradiation did not. In the mRNA expression in line with cyclooxygenase (COX) pathway, prostagrandin endoperoxide synthase 1 (PTGS 1), prostagrandin endoperoxide synthase 2 (PTGS 2) and phospholipase A_2 (PLA_2) were increased by both light irradiation and they were decreased as time flows. And genes associated with ROS releasing, mRNA expressions of tumor necrosis factor receptor (TNFR) and interleukin 1beta (IL1B) were increased by 1 hour red light irradiation but did not by blue light irradiation.

As a result, red and blue light irradiation showed different response in affecting the level of ROS. These findings indicate that red light rather than blue light is more useful for anti-inflammation in clinical field.

Introduction

The skin is exposed to exogenous and endogenous pro-oxidant agents leading to the harmful generation of ROS.[16,17] The cellular production of oxygen radicals has been implicated in a wide variety of biological processes including host defense, regulation of cell apoptosis, modulation of cell signalling and the generation of pathological conditions.[1-9] A variety of reactive oxygen species (ROS) are able to cause direct damage to proteins, DNA carbohydrates and lipids.[10] In physiologic condition, however, generated ROS are scavenged by various natural skin anti-oxidants such as keratinocyte catalase (CAT), superoxide dismutase (SOD), glutahione peroxidase (GP) and ascorbic acid.[18]

Keratinocyte inflammation has been shown to be crucial for tissue fibrosis and chemical carcinogenesis.[11-15] In view of oral mucosa, by activating a number of signalling pathways by chemotherapy and radiation, ROS and their damaged DNA precipitate the start of the biologic process that resulted in mucosal injury.[19]

Recently, many reports have suggested that a specific wavelength of light irradiation could be an alternative anti-inflammatory tool for wound healing in clinical fields.[20.21] Many physiological clinical results of low-level laser treatment (LLLT) for pain reduction, anti-inflammation, and acceleration of wound healing have been reported.[23-25] In our previous study, red light (635nm) irradiation inhibited PGE_2 synthesis like existing COX inhibitors, and its inhibition of PGE_2 synthesis, unlike indomethacin and ibuprofen, was due to decrease of ROS levels.[22]

Blue light (470nm) has been widely used in clinical dental area to initiate polymerization of resins and cements used for restoration of the teeth. Several studies have reported that blue light disrupts cellular processes such as mitosis, mitochondrial function, or DNA integrity, and that light-induced

reactive oxygen species (ROS) probably mediate these effects.[26-28] Though many trials have been approached in clinic area, however, there's no distinctive criteria or differential effects for clinical application by using different wavelength of light irradiation.

The purpose of this study was to investigate the ability of ROS scavenging by blue and red light irradiation and explored the gene expression using by miroarray. In this study, for inflammatory state in oral mucosa, Phorbol 12-myristate 13-acetate (PMA) was treated in immortalized human oral keratinocyte cell line. Blue and red light was irradiated to compare ROS scavenging effects and differentially expressed genes.

Material & Method

Primary cell culture and chemicals

Immortalized human oral keratinocyte HaCat cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) supplemented with Antibiotic Antimycotic Solution (GibcoBRL, UK) and 10% heat-inactivated fetal bovine serum (Hyclone, Canada) in humidified 5% CO_2/95% air at 37 ℃. Cells were cultured at a density of $1x10^5$cells/ml in 10cm sized culture dish according to the condition of analysis and 12 ug/mL of Phorbol 12-myristate 13-acetate (PMA, SIGMA, USA) was treated for 3 hours at 24 hour after plating.

Light source and irradiation

After PMA treatment, light irradiation was performed in a 5% CO_2 humidified chamber at 37°C by manufactured LED irradiation tool kit (Biophoton, Korea). The source of light for irradiation was a continuous-wave LED (U-JIN LED, Korea) emitting at a wavelength of 635 and 470nm and the manufactured energy density was 5 mW/cm^2 on the sample surface.

Enzyme-linked immunoassay for PGE_2

At 24 hour after irradiation, the amount of released PGE_2 was measured in the supernatants using a commercially available enzyme immunoassay kit (R&D system, USA) according to the manufacturer's protocol. The absorbance for PGE_2 was measured at 586 nm by using a colorimetric microplate reader (Bio Tek, USA).

Flow cytometer and laser scanning confocal microscope analysis for detection of ROS formation

The reactive oxygen species (ROS) were assayed by using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma, USA). DCF-DA enters cells passively, where it is enzymatically deacetylated by esterases to become the nonfluorescent 2,7-dihydrodichlorofluorescein (DCF-H). Meanwhile, the oxidizing molecules convert DCF-H to the highly fluorescent DCF.

To measure the intracellular ROS level, cells were detached using a trypsin-EDTA solution after irradiation immediately, and then incubated with 10uM of DCF-DA for 20 min. After incubation, the ROS level was analyzed by flow cytometery (Beckman Coulter, USA) using 485 nm of excitation and 530 nm of emission filters.

To visualize intracellular ROS, DCF fluorescence was mornitored by confocal microscope (Carl Zeiss, German). HaCat cell line grown on cover slips were incubated with 10 uM of DCF-DA for 20 min. After the cells were washed with phosphate-buffered saline containing 10 nM of glucose, DCF fluorescence intensity was monitored using a confocal microscope , set at the excitation and emission wavelengths of 488, 525respectively.

Electron spin resonance(ESR) spin trapping determination of superoxide anion(O_2^-)

O_2^- was detected by ESR spectrometry using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Dojindo, Japan) as a spin trap [24]. In brief, after experiment immediately, 1.5 μl of DMSO, 10μl of sample ($1x10^5$ cells in 0.1M phosphate buffer (pH 7.4)), 40 μl of 1M DMPO were placed in a test tube and mixed. In the reaction mixture, to eliminate the effect of the hydroxyl radical, DMSO was added as a hydroxyl radical scavenger. The mixture was transferred to glass capillaries (50 μl, Paul Marienfeld GmbH & Co. KG, Germany) and the DMPO-O_2^- spin adduct was quantified. The measurement conditions for ESR (JEOL, Japan) were as follows: field sweep, 317-337mT; field modulation frequency, 100KHz; field

modulation width, 0.4mT; amplitude, 300; sweep time, 30s time constant, 0.01s; microwave frequency, 9.166GHz; microwave power, 10mW. Signal intensities were evaluated from the peak height of the first signal of the DMPO- O_2^- spin adduct.

Total RNA isolation

At 6 hour after irraditation, the total RNA was isolated using Trizol® Reagent (Invitrogen, USA) according to the manufacturer's instruction. Extract RNA was dissolved in DEPC water, analyzed microarray.

Analysis of mRNA expression by microarray chip.

Analysis of Illumina microarray was performed by Illumina Bead Station 500X manual (Microgen Co. Korea). Biotynylated cRNA was synthesized by Illumina Amplification Kit (Ambion Inc., USA), and purified by RNA easy kit (Qiagen, USA). After the sample was hybridized on the Sentrix HumanRef-8 Expression BeadChip (Illumina, Inc., USA), the scanning was performed by confocal laser scanner. Acquired scanning image was analyzed by BeadStudio Program (Microgen, Korea)

Statistical Data Analysis

Among the total 40,000, only the probes of detection p-value <0.05 which were 17351 (Non-treat), 17596 (PMA vs Con), 17158 (PMA) analyzed. Fold changes and adjusted FDR p-value were calculated by comparison with the each sample. The genes were categorized by physiological, functional standard using panther Classification system (http://www.pantherdb.org). Statistical analysis was performed by Arrayassist® (Stratagene, USA).

Results

PGE_2 release and ROS detection

Relative level of PGE_2 release by light irradiation in PMA treated HaCat cell line was shown in the Fig.1. PMA treatment in HaCat cell line increase PGE_2 level to 105%. And 635nm irradiation decreased PGE_2 level, while 470nm irradiation increased PGE_2 as the time flows.

DCF fluorescence indicating intracellular total ROS monitored by confocal microscope was highly increased by PMA treatment (Fig. 2.). Decreased intensity of DCF fluorescence was shown as 635nm irradiated, and their intensity was weaker as time flows. However, with 470nm irradiation, intensity of DCF fluorescence stronger as the time flows.

The quantitative DCF fluorescence by flow cytometry increase to 6.09% in PMA treated HaCaT cell line (Fig. 3.). With 635nm irradiation, amount of DCF fluorescence was decreased 4.39% to 3.08% by irradiation time progression. For 470nm irradiation, amount of total ROS in 30 min irradiation was higher than PMA treated group, and decreased to 5.34% in 60 min irradiation.

Superoxide anion which was generated the first synthetic form of ROS was measured by ESR spectrometry of DMPO-O_2^-(Fig. 4.). Signal intensity which were evaluated from the peak height of the first signal of the DMPO-O_2^- spin adduct was increased to 1163 by PMA treatment, and then decreased to 911 gradually by irradiation time progressed in 635nm irradiation group. The value of peak height in 470nm irradiation, its value was higher than PMA-treated group. And its value was slightly decreased as time flows.

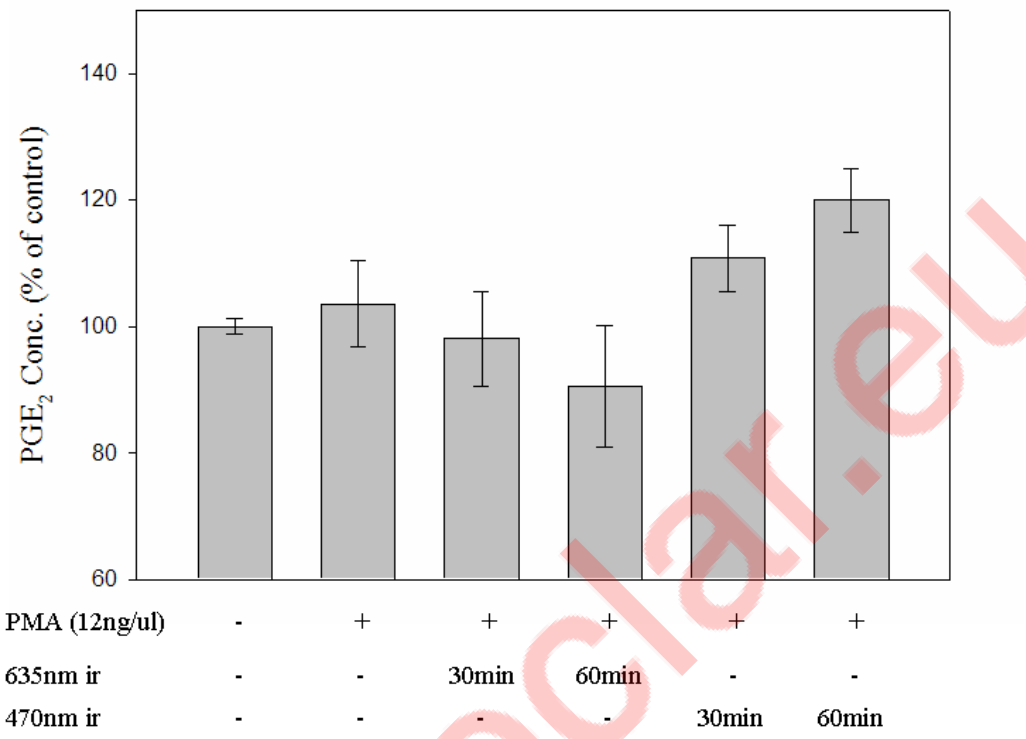


Figure 1. Relative level of PGE_2 release by light irradiation in PMA treated HaCat cell line. The vertical bars indicate the means ± SD (n = 3).

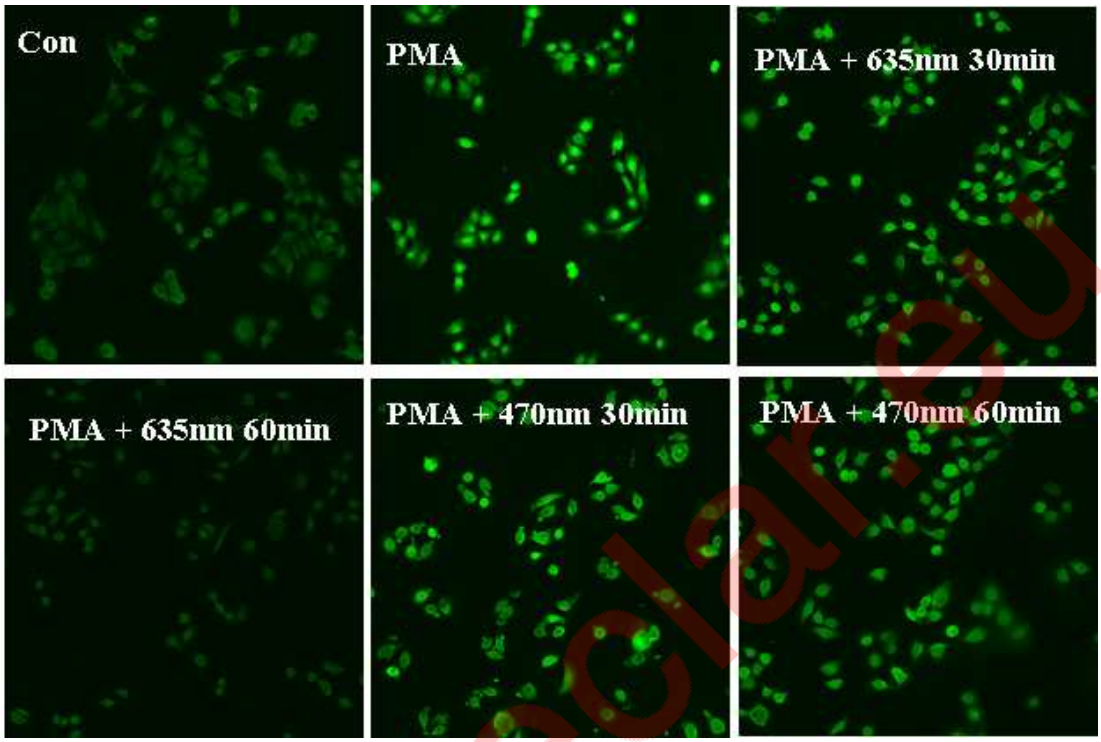


Figure 2. Confocal microscopic assay of DCF fluorescence by light irradiation in PMA treated HaCat cell line. (All magnification is x 200)

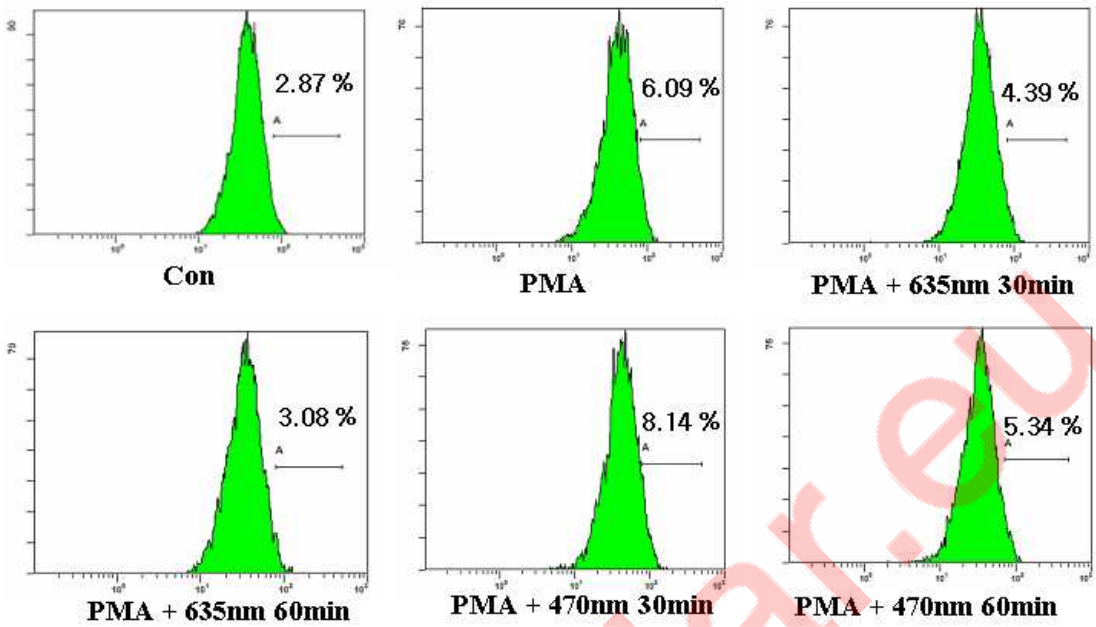


Figure 3. Flow cytometric analysis of DCF florescence by light irradiation in PMA treatd HaCat cell line. (X axis represent DCF fluorescence, Y means cell number)

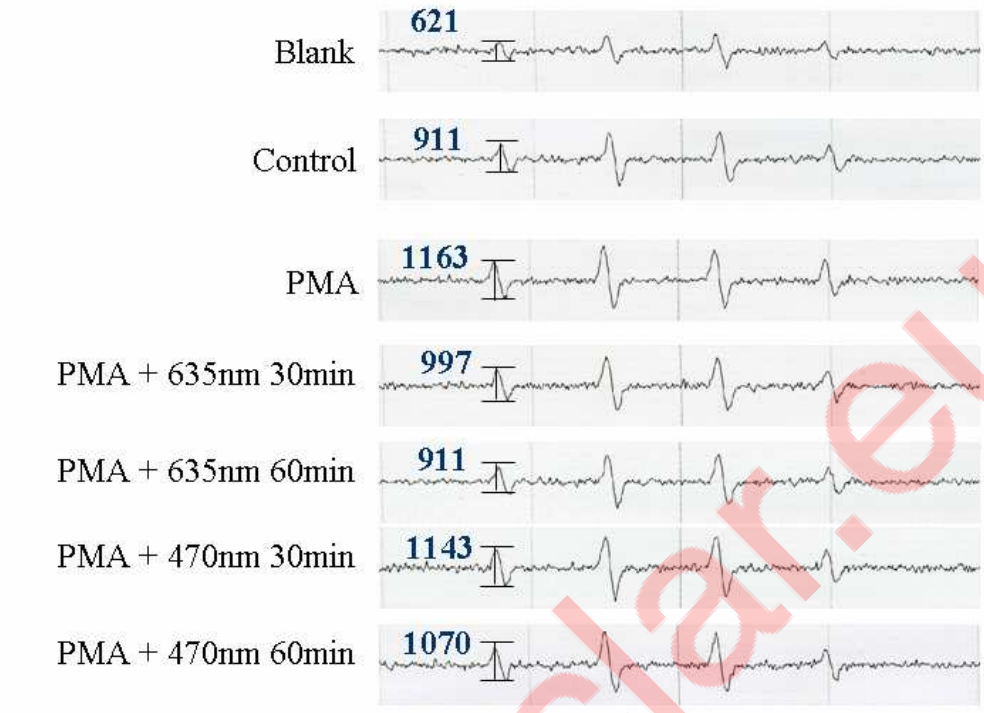


Figure 4. ESR spectrometry of DMPO-O_2^- by light irradiation in PMA treated HaCat cell line. Signal intensities were evaluated from the peak height of the first signal of the DMPO- O_2^- spin adduct.

Microarray analysis

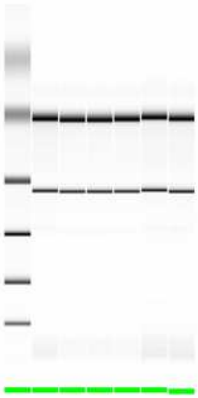
To analyze mRNA expression by 470 and 635nm irradiation in PMA treated HaCat cell line, microarray assay and RT-PCR were performed. Total RNA extracted from each sample was good quality enough to analyze (Fig. 5.).

mRNA expressions in the line with cyclooxygenase pathway, prostagrandin endoperoxide synthase 1 (PTGS 1), prostagrandin endoperoxide synthase 2 (PTGS 2) and phospholipase A_2 (PLA_2) were increased by both light irradiation and they were decreased as time flows (Fig. 6.). Especially, mRNA expression of PLA2 was decreased the most by red light irradiation for 60 min.

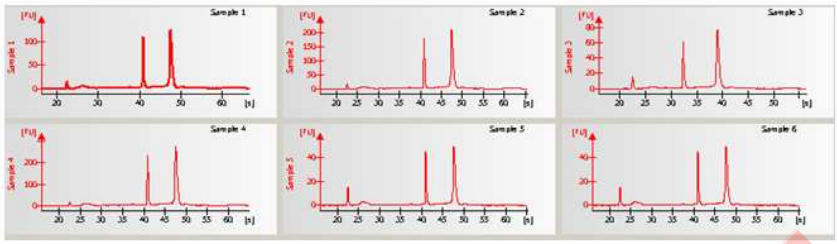
On examining genes associated with ROS releasing, mRNA expression of tumor necrosis factor receptor (TNFR) and interleukin 1beta (IL1B) were in the opposite direction by red light irradiation as time flows. By blue light irradiation, mRNA expression of TNFR was increased, while IL1B was decreased (Fig. 6).

Upstream pathway of PTGS 2 was screened; Hypoxia inducing factor (HIF) and vascular endothelial growth factor (VEGF). By both light irradiation for 30 min, their mRNA expressions were increased. However, with 60 min irradiation, their expressions were decreased except VEGF (Fig. 6)

(a)



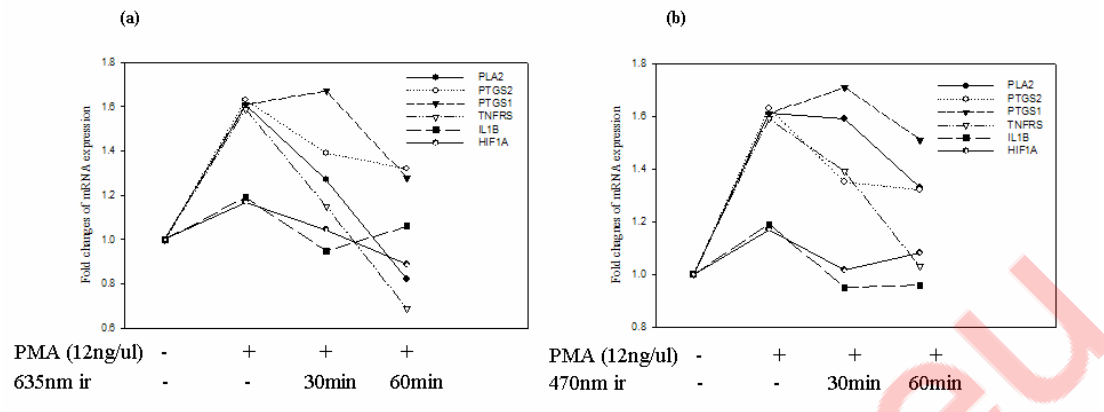
(b)



(c)

Lane	Sample	28S/18S	260/280	260/230	260	ug/ul
1	HaCat with PMA Con	2.1	1.50	1.68	98.51	3.94
2	HaCat with PMA 635nm 30min	1.9	1.93	2.06	65.15	2.61
3	HaCat with PMA 635nm 60min	2.0	1.53	1.72	95.09	3.80
4	HaCat with PMA 470nm 30min	1.9	1.47	1.64	98.61	3.94
5	HaCat with PMA 470nm 60min	1.9	1.56	1.74	94.92	3.80
6	HaCat no-treat Con	2.0	1.68	1.74	88.08	3.52

Figure 5. The plot indicates a result of sample quality check (a). The band's distinction between 28S and 18S using the Bioanalyzer (Agilent) were checked. Heights of each peak show intensity for 28S and 18S (b). Good quality samples' criteria was shown (c).



(c)

SYMBOL	Transcript	con	PMA	p val	R 30	p val	R 60	p val	B 30	p val	B 60	p val
PLA2	NM_005090	1.00	1.610	0	1.274	0.000	-1.29	0.001	1.587	0.000	1.334	0.000
PTGS2	NM_000963	1.00	1.626	0.004	1.395	0.000	1.320	0.001	1.345	0.000	1.324	0.000
PTGS1	NM_000962	1.00	1.606	0.003	1.668	0.003	1.280	0.005	1.710	0.003	1.505	0.003
TNFR	NM_148973	1.00	1.591	0.000	1.152	0.001	-1.55	0.002	1.394	0.000	1.028	0.001
HIF1A	NM_181054	1.00	1.170	0.060	1.046	0.001	-1.17	0.001	1.016	0.001	1.082	0.001
VEGF	NM_001025366	1.00	1.32	0.06	1.05	0.005	-1.12	0.008	1.094	0.004	-1.01	0.006
IL1B	NM_000576	1.00	1.190	0.000	-1.08	0.000	1.060	0.000	-1.08	0.000	-1.07	0.000

Figure 6. Fold changes of mRNA expression associated with intracellular ROS and inflammation by 635 (a) and 470nm(b) irradiation in PMA treated HaCat cell line. (R30: 30min irradiation, R60: 60min irradiation), 470nm (B30: 30min irradiation, B60: 60min irradiation) irradiation in PMA induced HaCat cell line was shown (c).

Discussion

Oral mucosal diseases are extremely prevalent: recurrent aphthous stomatitis (recurrent oral ulceration) affects 20% of the population,[29] and oral lichen planus, up to 4%.[30] These conditions are based on inflammation. And keratinocyte inflammation has been shown to be crucial for tissue fibrosis and chemical carcinogenesis.[11-15] In this study, immortalized oral keratinocyte cell line was used and PMA was treated for inflammation. To verify the inflammation, the level of PGE_2 was assessed and to relieve the inflammation, both red and blue light irradiation was applied. Resultantly, level of PGE_2 was reduced by red light irradiation while increased by blue light irradiation (Fig. 1). This fact indicate that red rather than blue light was useful for anti-inflammation. In our previous study, evoked ROS was reduced by light irradiation,[22] and we speculated that evoked ROS was dependent on specific wavelength of light and different effects by different wavelength of light wound be explored.

In general, the skin is exposed to exogenous and endogenous pro-oxidant agents leading to the harmful generation of ROS.[16,17] And intracellular ROS have been regarded as a critical factor in various kinds of inflammation.[31-32] The strategy of inhibiting evoked ROS is the best way to suppress inflammation. In other words, dissociation or scavenging of ROS by specific wavelength of light is the alternative method against inflammation.

To investigate the intracellular ROS by light irradiation, confocal microscopic and flow cytometric assay for DCF fluorescence and ESR spectrometry of DMPO-O_2^- for superoxide anion which is known to be the first form of ROS was experimented. Intracellular total ROS was increased as PMA treated and its return to decrease was observed by red light irradiation. However, blue light did not reduce the intracelluar total ROS. These facts is in line with the result of PGE_2 release. For ESR spectrometric analysis for

superoxide anion which is known to be the first form of ROS, red light reduced the release of superoxide anion and their release was inversely related with the light dosage (fig. 4). However, blue light irradiation did not reduce the release of superoxide anion. Finally, amounts of intracellular ROS was associated with released PGE_2 and their reducing was effective by red light irradiation rather by blue light irradiation.

Increased ROS, followed by increasing PGE_2 releasing with blue light in this study was coincided with several studies; blue light disrupts cellular processes such as mitosis, mitochondrial function, or DNA integrity, and that light-induced ROS probably mediate these effects.[26-28] That is, blue light irradiation is presumed the cell to be necrotic and/or apoptotic. So it can be used for removing necrotic debris from ulcer boundary. Importantly, dentist extensively using blue light for polymerization of resin and cements, should be aware that blue light can be harmful when the mucosa was exposed exceedingly for long time.

For the microarray analysis, genes associated with COX pathway were mainly examined. The remarkable effects of ROS during inflammation are the oxidative modification of phopholipids through phopholipase A_2 (PLA2) activation within the cell membrane and stimulation of mRNA expression of COX-2.[33,34] mRNA expression of PLA2 was the most decreased by red light irradiation for 60 min. However, blue light irradiation increased the mRNA expression of PLA2. This fact showed that only red light irradiation could reduced the evoked ROS, followed by decreasing mRNA expression of PLA2. In this study, mRNA expression of TNFR was increased by 30 min both lights irradiation, but, for 60 min irradiation, its expression was 3 fold decreased by red light. Shen et al. reported that expressed TNFR was associated with cell apoptosis and ROS was in intermediary role in its signal transduction.[35] Red light irradiation for enough time, in this study, reduced evoked ROS and decreased expression of TNFR led to cell apoptosis.

However, increased expression of TNFR by blue light irradiation was presumed to be opposite result.

A putative pathway was diagrammed (fig. 7) based on the results of microarray in association with COX pathway. And the involvement of both lights irradiation for anti-inflammation was thought to be dissociation of ROS. And further study for the way of ROS dissociation by light irradiation will be needed.

Finally, released ROS in PMA treated HaCat cell lines was dissociated efficiently by red light irradiation in present study. However, blue light irradiation did not reduced the released ROS. As a result, red and blue light irradiation showed different response in affecting the level of ROS. These findings indicate that red light rather than blue light is more useful for anti-inflammation in clinical field.

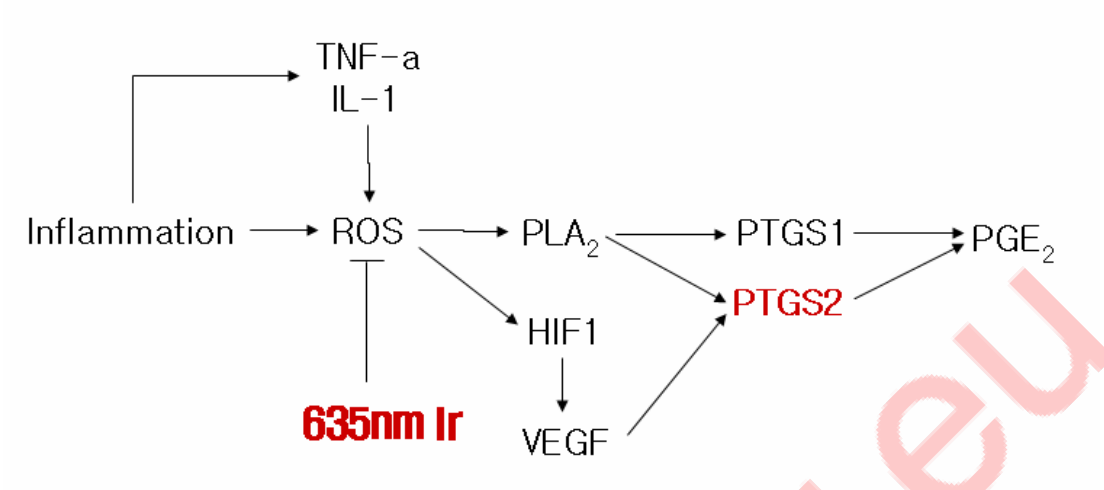


Fig. 7. A diagram shows the putative pathway of ROS and PGE_2 release along with the possible point of involvement of 635nm irradiation.

Conclusion

A comparative study of red and blue light irradiation in PMA treated HaCat cell lines was carried out. Red (635nm) and blue (470nm) light irradiation was carried out. To investigate the intracellular ROS by light irradiation, confocal microscopic and flow cytometric assay for DCF fluorescence for total ROS and ESR spectrometry of DMPO-O_2^- for superoxide anion. And microarray was performed for mRNA expression level.

Released intracellular total ROS in PMA treated HaCat cell lines was dissociated efficiently by red light irradiation, while blue light irradiation did not. Rather, blue light irradiation increased ROS formation. For superoxide anion generated the first synthetic form of ROS, red light irradiation reduced its amount but blue light irradiation did not. In the mRNA expression in line with cyclooxygenase (COX) pathway, prostagrandin endoperoxide synthase 1 (PTGS 1), prostagrandin endoperoxide synthase 2 (PTGS 2) and phospholipase A_2 (PLA_2) were increased by both light irradiation and they were decreased as time flows. And genes associated with ROS releasing, mRNA expressions of tumor necrosis factor receptor (TNFR) and interleukin 1beta (IL1B) were increased by 1 hour red light irradiation but did not by blue light irradiation.

These findings indicate that red light rather than blue light is more useful for anti-inflammation in clinical field.

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PMA 처리한 인간 불멸화 상피세포에서 청색 및 적색 광 조사에 따른 활성산소 제거 능에 대한 비교 연구

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<국문초록>

피부는 외부 및 내부 산화물질에 의해 발생되는 활성산소 (ROS)에 의해 병리적 손상을 입는다. 최근 광 조사를 이용해 이러한 병리적 손상 회복에 대한 많은 보고가 있다. 또한, 치과영역에서 가장 많이 사용되는 청색광에 대한 이해와 활성산소에 대한 보고가 부족하여 본 연구를 시행하였다. 본 연구는 적색 (635nm) 및 청색 (470nm) 광조사가 불멸화 상피세포인 HaCat 세포 주에 미치는 영향을 ROS 제거능에 초점을 맞추어 비교 하였다.

광 조사에 의한 세포내 전체 활성산소량 측정을 위해 DCF 형광물질을 이용하여 공초점 현미경 및 유세포측정기를 사용하였고, 수퍼옥사이드(O_2^-)를 측정하기 위해 DMPO를 이용해 ESR 분광측정기을 이용하였으며, 광 조사 후 발현되는 mRNA 수준을 알기위해 miroarray 분석을 각각 시행하였다.

적색광은 PMA처리 후 증가 된 활성산소를 효과적으로 제거하였으나 청색광은 활성산소의 제거에 효과적이지 못하였다. 적색광은 직접 활성산소 제거를 통하여 항 염증효과를 갖는 것으로 여겨진다.

이상의 결과에서 적색광은 청색광에 비해 임상에서 항염증치료에 이용하는데 더욱 유용할 것으로 사료되었다.