

The Effectiveness of a Portable Fluorescence Spectrophotometer for
Early Detection of Oral Cancer

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Title: The Effectiveness of a Portable Fluorescence Spectrophotometer for Early Detection of Oral Cancer

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Abstract

Purpose: The purpose of this study was to investigate whether photodynamic diagnosis (PDD) using a portable fluorescence spectrophotometer (FC-1) can easily and objectively discriminate between normal and tumor cells at the dental chairside, and to further compare it with PDD that requires speculum examination by focusing on protoporphyrin IX (PPIX).

Methods: Three cell lines (2 human oral squamous cell carcinoma-derived cell lines, HSC-2 and HSC-3 cells, and oral keratinocytes, HOK cells) were cultured. 5-Aminolevulinic acid hydrochloride (5-ALA) and deferoxamine mesylate (DFO) were mixed in Dulbecco's Modified Eagle Medium (DMEM), and the mixture was set to Control (DMEM only) and PDD (5-ALA+DFO) groups. The cells were incubated at room temperature (RT) and 37°C, then the fluorescence was measured. Then, the two conditions were combined with the Control and PDD groups to form the Control/RT, Control/37°C, PDD/RT, and PDD/37°C groups. Additionally, the amount of singlet oxygen ($^1\text{O}_2$) generated by irradiation with 405 nm LED was measured using an electron spin resonance spectroscopy to detect PPIX in the cell supernatant after 24 hours.

Results: In HSC-2 and HSC-3, the fluorescence intensity values

increased significantly at 2 hours between the Control/RT and PDD/RT groups. In addition, there was a significant difference between HSC-2 and HSC-3 compared to HOK. In all cell lines, the fluorescence intensity of the PDD/37°C group were significantly higher than those of the PDD/RT group. The amount of $^1\text{O}_2$ generated by 405 nm LED irradiation was higher in the cell supernatants of all cell lines in the order of Control/RT < Control/37°C < PDD/RT < PDD/37°C group, and HSC-3 in the PDD/37°C group showed a significant increase compared to HOK.

Conclusion: It is suggested that PDD using a portable fluorescence spectrophotometer can clearly distinguish between normal cells and tumor cells in *in vitro* studies using cell lines at 2 hours under 37°C, and it can detect not only intracellular PPIX, but also extracellular PPIX.

<Keywords>

Photodynamic diagnosis, Portable fluorescence spectrophotometer, Oral cancer, Protoporphyrin IX

INTRODUCTION

Cancer of the oral cavity and pharyngeal region causes approximately 8,000 deaths per year in Japan, and the number of deaths has tripled over the past 30 years [1]. As Japan becomes a super-aged society, the number is expected to continue to increase. The prognosis for advanced oral cancer has a significant impact on the quality of life of the patient, not only in terms of functions such as chewing and swallowing, but also in terms of aesthetics, such as facial appearance; therefore, early detection is very important. Traditionally, oral cancer has been considered to be an area that can be seen directly and is easy to detect. However, the detection and diagnosis of oral cancer are relatively easy for the general dental clinician if the disease is advanced, but it is difficult to diagnose oral mucosal diseases, including oral potentially malignant disorders, in the early stages, because the clinical pictures of early-stage oral cancer and oral mucosal diseases are very similar [2]. Visual inspection and digital palpation are mainly effective in detecting oral cancer [3], and various other examinations are also performed [4] [5] [6] [7] [8]. However, these methods rely on the clinical experience of dentists, and there are still few objective screening methods that do not rely on experience. For the early detection of oral cancer, it is

very important to establish an objective diagnostic method that is less prone to disparities among medical professionals.

Recently, objective diagnostic methods such as fluorescence observation systems [2] [9] [10] and photodynamic diagnosis (PDD) [11] [12] [13] [14] [15] using fluorescence have attracted significant attention for oral cancer diagnosis. The former is based on the autofluorescent properties of healthy organisms, such as cofactors (flavin adenine dinucleotide) in the mucosal epithelium and collagen cross-linking in the stroma, and the latter is based on fluorescence microscopy of the fluorescence of protoporphyrin IX (PPIX), which accumulates in tumor cells. However, we consider even these methods to be semiquantitative, with subjective evaluation of the surgeon or speculum examiner playing a large role. Our laboratory has been conducting research on PDD in order to pursue further objective quantitative diagnosis [14] [15]. In other words, we proposed to take up 5-aminolevulinic acid hydrochloride (5-ALA) in scraped cells and quantify the fluorescence level of PPIX, which is metabolized in the cells, in a fluorescent plate reader for diagnosis. In addition, the objective evaluation by quantifying the fluorescence intensity using a fluorescence plate reader is useful for early detection of oral cancer and may also be applied to mass screening,

since many samples can be measured at once. However, individual examinations at general dental clinics are also essential to reduce the mortality rate through early detection of oral cancer. For this purpose, it is desirable to develop a PDD method that enables simple, rapid, and objective evaluation with examination equipment that can be installed in clinic facilities with limited space.

The purpose of this study was to investigate whether PDD using a portable fluorescence spectrophotometer can easily and objectively discriminate between normal and tumor cells at the chairside, and to further compare it with the conventional PDD that requires speculum examination by focusing on PPIX.

MATERIALS & METHODS

1) Cell culture

HSC-2 and HSC-3 cells were obtained from RIKEN (Tokyo, Japan) as human oral squamous cell carcinoma-derived cell lines, and oral keratinocytes (HOK) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) as normal human primary cultured cells. The HSC-2 and HSC-3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin streptomycin (Gibco, St. Louis, MO, USA) in 10-cm diameter dishes. HOK cells were cultured in oral keratinocyte medium (ScienCell Research Laboratories) with 5 ml HOK growth supplement and 5 ml penicillin/streptomycin. The cultures were then maintained in an atmosphere of 5% CO₂, 90% N₂, and 5% O₂ at 37 °C. The culture media were changed every 3 days.

2) Reagents and a portable fluorescence spectrophotometer

5-ALA (MW: 167.6) and deferoxamine mesylate (DFO, MW: 656.8) were purchased from Neopharma Japan (Tokyo, Japan) and Cayman Chemical Company (Ann Arbor, MI, USA), respectively. Following our previous reports [14] [15], 5-ALA was dissolved in DMEM and used as a porphyrin derivative at a final concentration of 2 mM, and

then DFO, which is an iron chelating agent, was added at a final concentration of 5 mM. The 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TMP) was purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were analytical grade.

A portable fluorescence spectrophotometer used FC-1[®] (Tokai Optical Co., Ltd., Aichi, Japan). It is a straightforward configuration that enables simple realization of the principle of fluorescence measurement itself. In brief, the sample in the cell is irradiated with the excitation light ($\lambda=405$ nm), and the emitted fluorescence wavelength ($\lambda = 630$ nm) is detected by the photodetector. The excitation light scattered or reflected in the direction of the photodetector is then passed through the fluorescence filter, and only the fluorescence wavelength is quantified and detected.

3) Fluorescence intensity measurement using a portable fluorescence spectrophotometer

When the HSC-2, HSC-3, and HOK cells reached confluence, the cells were detached with 0.05% trypsin-0.53 mmol/l EDTA · 4Na (Wako, Osaka) solution and passaged in culture dishes. After centrifugation, the cells were adjusted to 5×10^5 cells/ml in a hemocytometer using trypan blue. Then, the experimental groups with 5-ALA+DFO and DMEM only were designated as the PDD and

Control groups, respectively. For 5×10^5 cells / ml of each cell line in the centrifuge tube, 2 ml of each reagent were added, stirred, and stored at room temperature (RT, $22 \pm 0.5^\circ\text{C}$), shielded from light in the atmosphere. The fluorescence intensity was measured for 6 hours using a FC-1 at 2 hours intervals after the start of action. After each measurement, the sample was returned to the centrifuge tube, and the process was repeated. Additionally, the effects of different metabolic environments on fluorescence intensity were investigated.

The experimental groups were placed in an incubator ($37 \pm 0.5^\circ\text{C}$) under the same conditions (37°C condition), and the fluorescence intensity was measured and compared with that obtained under RT condition. In this study, the two conditions were combined with the Control and PDD groups to form the Control/RT, Control/ 37°C , PDD/RT, and PDD/ 37°C groups.

4) Detection of singlet oxygen from excited-PPIX in cell supernatant by 405 nm LED irradiation

The amount of generated singlet oxygen ($^1\text{O}_2$) was measured using an electron spin resonance (ESR) spectroscopy (JES FA-200, JEOL, Tokyo, Japan). The culture supernatants of the Control/RT, Control/ 37°C , PDD/RT, and PDD/ 37°C groups of each cell line 24 hours after the start of metabolism were harvested after

centrifugation. After harvest, the cell supernatant (150 μ l) and 4-oxo-TEMP (150 μ l) were mixed in one well of a 96-well titer plate and immediately irradiated with a 405-nm LED for 60 seconds. Subsequently, the mixture was transferred into a flat quartz cell and then measured using an ESR spectrometer.

The ESR measurements were conducted under the following conditions: magnetic field, 335 ± 5 mT; modulation width, 0.1 mT; time constant, 0.1 seconds; microwave power, 4.00 ± 0.05 mW; sweep width, 5 mT; sweep time, 2 minutes; and amplitude, 100. Signal intensity was normalized by MnO marker, and the RI value was calculated [16].

5) Statistical analysis

Statistical analyses were performed with an IBM SPSS Statistics ver.22 (IBM, Armonk, NY, USA). The relative fluorescence intensity of HSC-2 and HSC-3 cells against HOK cells in RT, and the RI value obtained by LED irradiation of the cell supernatant of each group were analyzed by Dunnett's *t*-test. The fluorescence intensity obtained from Control/RT and PDD/RT groups, or PDD/RT and PDD/37°C groups were analyzed by Student's *t*-test.

The level of significance was set at 1% for all analyses.

RESULTS

Figure 1 showed the fluorescence intensity values of each cell line in Control/RT and PDD/RT groups. In all cell lines, the fluorescence intensity of Control/RT and PDD/RT groups increased in a 5-ALA metabolism time-dependent manner. In HOK cells, the fluorescence intensity of 5-ALA metabolic initiation was significantly lower in PDD/RT group than in Control/RT group at 2 hours, but was significantly higher at 4 hours ($p < 0.01$). At 6 hours, there was no significant difference between them ($p=0.4$) (Figure 1a). On the other hand, in HSC-2 and HSC-3 cells, the fluorescence intensity in PDD/RT group showed a significant increase compared to that of Control/RT group at all times ($p < 0.01$) (Figure 1 b, c). Furthermore, a comparison among cell lines in the PDD/RT group showed a significant difference in their fluorescence intensities between HOK and oral squamous carcinoma (HSC-2 and HSC-3) in all incubation times ($p < 0.01$) (Figure 1 d).

Figure 2 showed a comparison of the fluorescence intensity obtained from the PDD/RT and PDD/37°C groups of each cell line. HOK showed no significant differences at 2, 4 and 6 hours ($p > 0.01$) (Figure 2 a). In contrast, the fluorescence intensities of HSC-2 and HSC-3 cells increased significantly more in the PDD/37°C group than

in the PDD/RT group ($p < 0.01$) (Figure 2 b, c).

Figure 3 showed the typical ESR-spectra and RI values. The typical ESR spectrum is a 1:1:1 triplet signal characteristic of 4-oxo-TEMPO having a hyperfine splitting constant ($a_N=1.608$ mT), which is produced when 1O_2 reacts with 4-oxo-TEMP. The amplitudes of the spectra increased in the following order; Control/RT < Control/37°C < PDD/RT < PDD/37°C group (Figure 3a). Furthermore, when the RI values of different cell lines were compared for each group, HSC-3 cells showed significantly higher values than HOK cells in the PDD/37°C group ($p < 0.01$) (Figure 3b). No significant difference was observed in the other groups.

DISCUSSION

With the advent of a super-aging society, the importance of prevention, examination, diagnosis, and treatment of cancer and lifestyle-related diseases is increasing. The importance of these issues is also understood in the field of dental medicine, and many reports or protocols have been published on dental caries [17], periodontal disease [18], oral cancer [19], and the relationship between systemic diseases and dental diseases [20] [21] [22] [23]. These results clearly demonstrate that the diversity of examinations and diagnoses has expanded the options for dental treatment, including prevention.

Our laboratory has been investigating a diagnostic method applying PDD that is safe and secure and enables all dentists to detect oral cancer in its early stages [14] [15]. We find that accumulation of PPIX in cells using 5-ALA and DFO, an iron chelator, can clearly distinguish normal cells from tumor cells using a fluorescent plate reader. Furthermore, this method can also be achieved with the number of cells safely scraped from the brush reported by Ikawa et al. [24]. However, the fluorescence plate reader is only suitable for mass examinations, but not for general dentists. In the present study, we investigated whether a portable fluorescence spectrophotometer,

which is considered to be appropriate for general dentists, can distinguish between normal cells and tumor cells in oral tissue.

First, the measured fluorescence intensities of the Control/RT and PDD/RT groups for each cell line were compared. In HSC-2 and HSC-3 cells, there were a significant increases in the fluorescence intensity of the PDD/RT group at 2 hours after the start of metabolism. In HOK cells, the PDD/RT group showed a significant increase in the fluorescence intensity at 4 hours after the start of metabolism, but the fluorescence intensity decreased significantly after 2 hours, and there was no significant difference after 6 hours. Furthermore, when comparing HOK cells with HSC-2 and HSC-3 cells in the PDD/RT group, the fluorescence intensity was significantly increased compared to HOK cells at all metabolic times after 2 hours of metabolism. The fluorescence intensity values increased in a metabolic time-dependent manner in the order of HSC-3 > HSC-2 > HOK cells. This is considered to be largely due to the differences in cell differentiation. HSC-2 cells are a highly differentiated cell line, but HSC-3 cells are a poorly differentiated cell line, suggesting that the fluorescence intensity is higher in poorly differentiated cells, which is consistent with the report by Uekusa et al. [25]. On the other hand, it has been reported that the fluorescence intensity is higher in

highly differentiated cell lines, but different opinions [14] [15] have been expressed depending on the cell differentiation, and further studies are needed. Since the fluorescence intensity of HOK cells increases in a time-dependent manner, it is considered to be homeostatically accumulated during the porphyrin metabolism process when 5-ALA is metabolized to PPIX. In addition, the reason for the increase in fluorescence intensity in the PDD/RT group of this study is considered to be that 5-ALA is metabolized to PPIX, and the iron chelator DFO inhibits ferrochelatase and the conversion of PPIX to heme, resulting in an increase in intracellular PPIX and an increase in the fluorescence intensity by excitation [25].

Next, the effect of different metabolic environments, especially temperature, on the fluorescence intensity was investigated. The fluorescence intensity of HSC-2 and HSC-3 cells showed a significantly higher increase in the PDD/37°C group than in the PDD/RT group. It is thought that this is due to enzyme activity and mitochondrial function. Numerous enzymes such as ferrochelatase [26] [27], ALA dehydratase [28], uroporphyrinogen III decarboxylase [28] and porphobilinogen deaminase [28], are thought to be involved in the synthesis of PPIX. These enzymes then function in the cytoplasm or mitochondria. These enzymes have an optimum

temperature, and since all of the above enzymes are active *in vivo*, it is suggested that the optimum temperature for these enzymes is around 37°C. The mitochondria themselves are also active in their metabolic functions at around 37°C. Additionally, regarding the effect of temperature on the accumulation of PPIX in dermatology, Asta et al. [29] reported that lowering the skin temperature to 28-32°C reduced the accumulation of PPIX by 1/3 to 1/2, and that increasing the temperature of the target tissue can enhance the accumulation of PPIX. The present results are similar, suggesting that incubation at 37°C is more clearly distinguishable when measuring fluorescence intensity using a portable fluorescence spectrophotometer.

Finally, PPIX was detected indirectly by irradiating the cell supernatant after 24 hours with a 405 nm LED. In brief, the mechanism of PPIX extracellular efflux by ABCG2, a cell membrane transporter, or by exocytosis has been reported [30]. PPIX has an optical absorption spectrum with five peaks between 400 and 650 nm [31]. Therefore, PPIX changes to a triplet state and transfers energy to nearby oxygen atoms in the ground state to produce $^1\text{O}_2$ by absorbing a photon upon light irradiation. Applying this mechanism, we attempted to detect PPIX indirectly by trapping $^1\text{O}_2$ using ESR,

since the generation of $^1\text{O}_2$ can be inferred to be due to the excited-PPIX. The result showed that HSC-3 cells, which had the highest accumulation of PPIX in the cells had the highest RI value, as well as HSC-2 and HOK cells in that order. Cell lines with a high accumulation of PPIX are thought to have a similarly high extracellular export of PPIX, and a diagnostic method using a portable fluorescence spectrophotometer can detect not only intracellularly accumulated PPIX, but also extracellularly discharged PPIX.

In consideration of these factors, objective evaluation by quantifying fluorescence intensity using FC-1 is thought to have a significant advantage over the conventional PDD using a fluorescence microscope. The problems of conventional PDD compared to PDD using a fluorescence spectrophotometer are as follows: 1) subjective evaluation by the naked eye, which is affected by the intensity of fluorescence [32]; 2) the amount of excitation light is not constant, which means that the fluorescence intensity is affected by the focal distance from the microscope and the angle to the optical axis of the surgical field [33]; and 3) must target only PPIX accumulated in the cell. On the other hand, a common problem of the conventional PDD and PDD using a portable fluorescence

spectrophotometer is that the blood on the surgical field surface blocks the excitation light [34]. In addition, this study was an in vitro study using a cell line, and in fact, there are limitations in mentioning the effect of PDD using a portable fluorescence spectrophotometer because of the mixture of normal and tumor cells. In the future, it will be necessary to investigate whether PDD can be diagnosed with cells taken from actual tumor tissues.

CONCLUSION

The results of the present study suggest that PDD using a portable fluorescence spectrophotometer can clearly distinguish between normal cells and tumor cells in *in vitro* studies using cell lines at 2 hours under 37 °C, and it can detect not only intracellular PPIX, but also extracellular PPIX.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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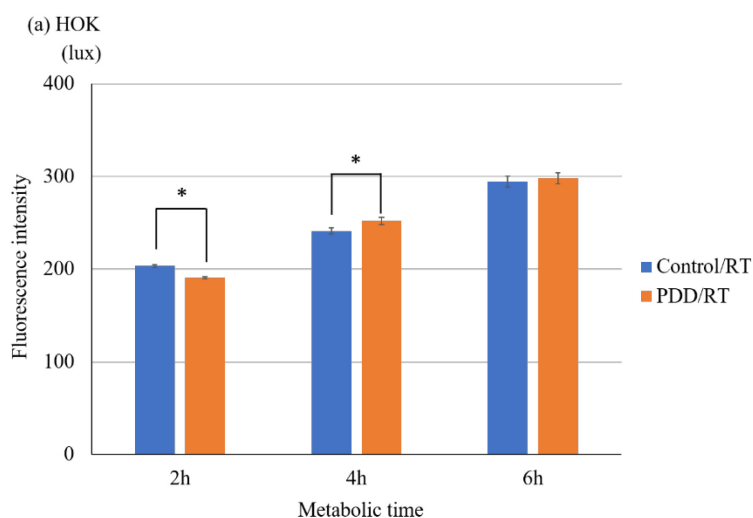
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FIGURES & LEGENDS

Figure 1: Effect of PDD to the fluorescence intensity in different cell lines.

The fluorescence intensities of different cell line in Control/RT and PDD/RT groups. HOK (a), HSC-2 (b), or HSC-3 (c) were incubated for 2, 4 and 6 hours RT with/without PDD (5-ALA+DFO). No PDD treated as a control. Data was analyzed by Student's *t*-test. Then all cell lines were incubated for 2, 4 and 6 hours RT with PDD. Data was analyzed by Dunnett's *t*-test.

In all analyses, the significance level of the P-value was set at 1%. Data are expressed as means \pm SD of multiple experimental replicates (n=4).



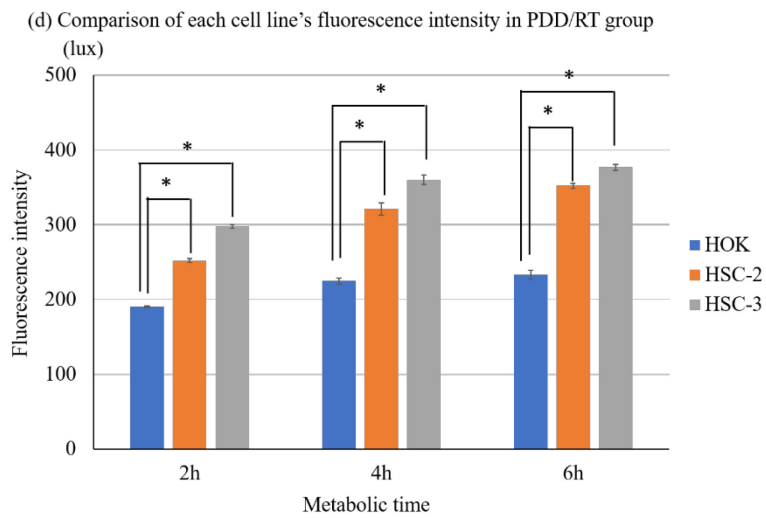
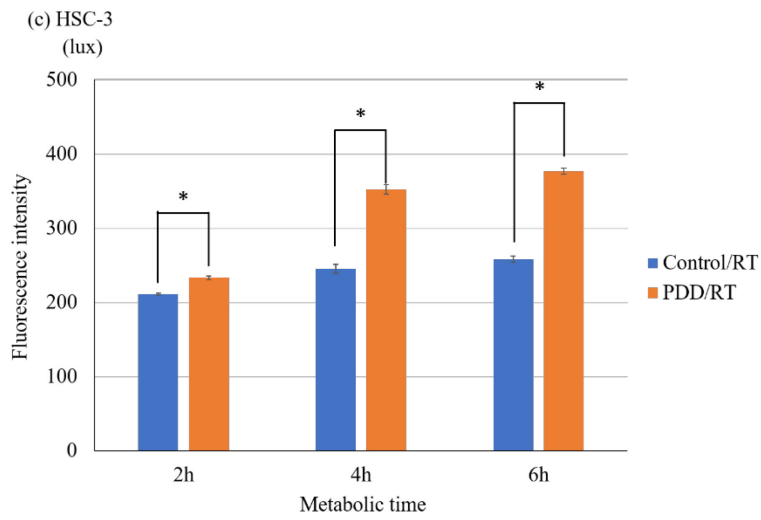
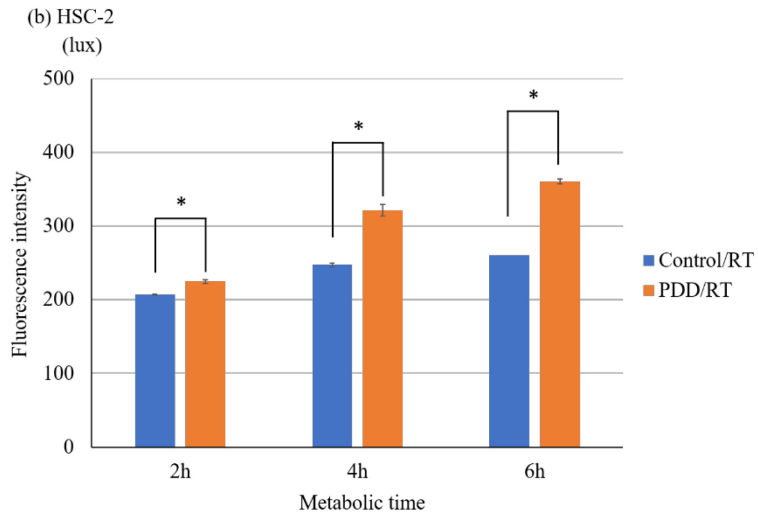
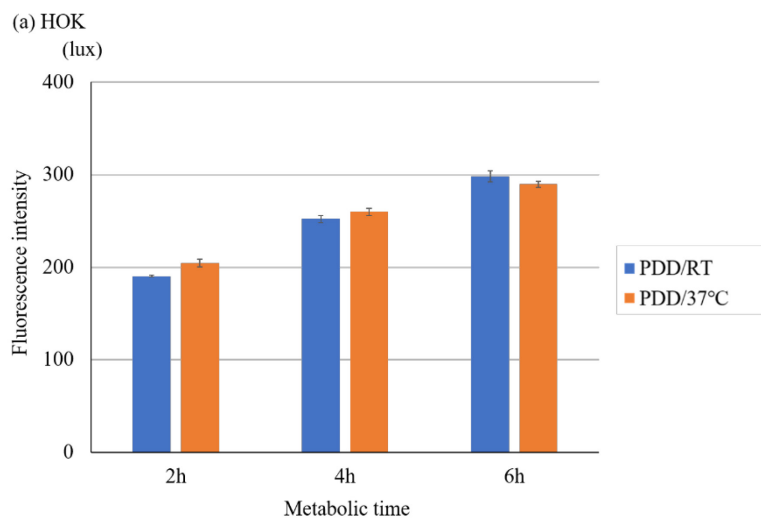


Figure 2: Effect of incubation temperature to the fluorescence intensity in different cell lines. The fluorescence intensities of different cell line in PDD/RT and PDD/37°C groups

HOK (a), HSC-2 (b), or HSC-3 (c) were incubated for 2, 4, and 6 hours RT or 37°C with PDD (5-ALA+DFO). Data was analyzed by Student's *t*-test. The significance level of the P-value was set at 1%. Data are expressed as means \pm SD of multiple experimental replicates (n=4).

The x- and y- axes indicate the metabolic time and fluorescence intensity (lux), respectively.



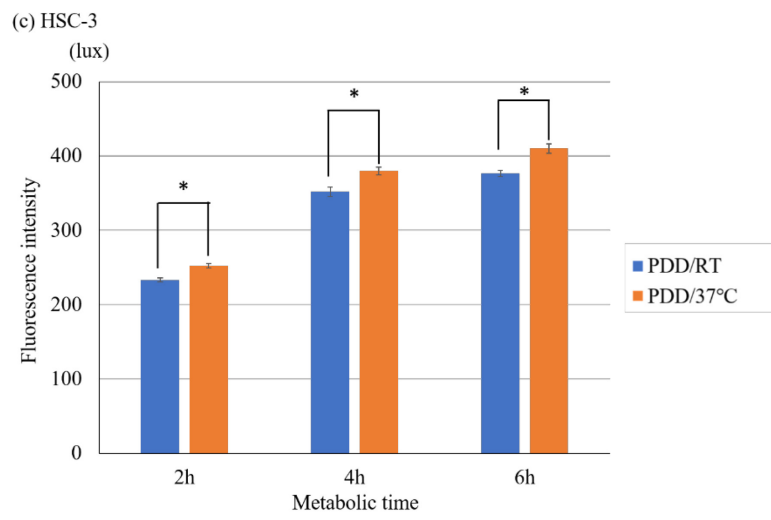
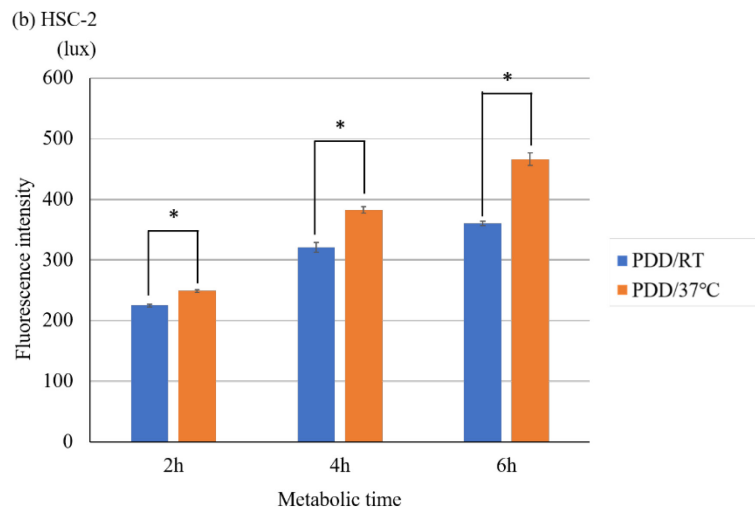
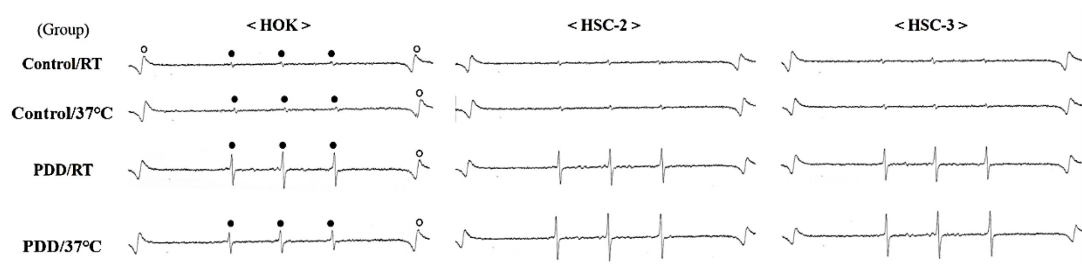


Figure 3: Comparison of the ESR-spectra obtained after 405 nm LED irradiation and the RI-value.

ESR-spectra of HOK, HSC-2, and HSC-3 cell supernatants at 24 hours after 405 nm LED irradiation (A). The white and black circles indicate the Mn^{2+} marker and the nitroxide radical, respectively.

The RI value of different cell line obtained by LED (B) were analyzed by Dunnett's *t*-test, and the significance level of the P-value was set at 1%. Data are expressed as means \pm SD of multiple experimental replicates (n=3).

(A)



(B)

