Effects of Erythrosine With/out Nano-TiO_2 Dioxide-Mediated Photodynamic Therapy On HGF-1 and HOK Cells By PrestoBlue® Viability Assay

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ABSTRACT

The photodynamic therapy (PDT) is an alternative treatment for localized target tissue (e.g. tumors, cancers, etc.) that mainly results in target cell death in oxidation mechanism in producing reactive oxygen species (ROS). Erythrosine dye, not only is known as a standard disclosing agent in Dentistry, with its photosensitizer (PS) property, it also has been used as an antimicrobial PS. However, the cell viability of normal human oral cells of Erythrosine dye tested with Nano-titanium dioxide (Nano-TiO_2) which is widely utilized as a photo-catalyst in order to enhance a photodynamic reaction still has been unknown.

Objectives: To study the effects of Erythrosine with/out Nano-TiO_2 as a photosensitizer on viability and morphology of normal human gingival fibroblast cell line (ATCC® CRL-2014, HGF-1) and Primary Human Oral Keratinocytes; HOK (ATCC® PCS200-014™) primary cells.

Methods: Eleven test groups of each HGF-1 and HOK were tested by Erythrosine as a photosensitizer in different concentration namely, Erythrosine (Ery) at 0 µM, 55 µM, 110 µM, 220 µM, and 440 µM in the presence and/or absence of 1% w/w Nano-TiO_2 with blue light, Nano-TiO_2 alone, and control groups (positive control = DMEM, abd negative control = H_2O_2). With LED-dental curing light (BA Optima 10, 420-480 nm, 16-19 J/cm^2) for 1 min, at 1, 6, or 24 hrs, cell viability assays using PrestoBlue® (Invitrogen, Life Technologies GmbH, Darmstadt Germany) with Varioskan™ at excitation/emission wavelength 560/590 nm. Cell morphology was investigated by SEM (S3000N, Hitashi, Japan).

Results: The difference between HGF-1 and HOK cell viability in Erythrosine-Nano-titamium dioxide-mediated photodynamic therapy was not statistically significant (p-value < 0.05) among the groups at all-time point, except for Erythrosine 440 µM that significantly reduced cell viability observed at 24 hrs. by utilizing one-way ANOVA and post hoc test. Furthermore, some morphology changes were observed between test and control groups by SEM.

Conclusions: Erythrosine with Nano-TiO_2, a novel PDT photosensitizer in the range of the present study exerted no toxicity on HGF-1 and HOK cells.

Key words: Photodynamic therapy, Erythrosine, Blue LED light, Fibroblast, Human oral keratinocytes.

Introduction

The PDT is an alternative/adjunctive treatment for some localized oral lesions, consisting of 3 components; photosensitizing agent (PS), an appropriate wavelength light, and oxygen (in the target living tissue/cell). The PS undergoes a transition from a low-energy 'ground state' to a higher energy 'triplet state', where reaction between triplet-state photosensitizer and biomolecules produces free radicals and radical ions, or reactive oxygen species (singlet oxygen), producing oxidation mechanism to plasma membranes and DNA results in cell death in the target tissue. ROS production due to PDT (very short life-time (0.05 ms.) [1,2,3]causes a highly elevated oxidative stress in the cell.[3,4] The two most well-characterized cell death mechanisms following PDT are apoptosis and primary necrosis[6,7,8]. Necrosis usually predominates when using high dose PDT, whereas apoptosis is more often seen with comparatively lower PDT doses. Apoptotic pathways (which involves the activation of various cellular factors Caspase 2, 8, 9 and 10) and executioner caspases (Caspase 3, and 7)[9]) usually involve mitochondria, which is frequently regarded as the central processing organelle for apoptosis[8,9] Therefore, mitochondrial damage is often essential for the induction of apoptosis within cells following PDT[8].

Photosensitizers used in PDT are classified into 5 groups; Phenothiazine dyes, Phthalocyanine dyes, Chlorine, Porphyrins, Monoterpene, and Xanthenes groups. Although PS such as Foscan (chlorine) and 5-aminolaevulinic acid (porphyrin) [10] provide effective results in some previous studies, yet it has some limitations[11,12] for clinical sustainability and legislative assessments that significantly delay the translation of many such photosensitizers into the clinic.

Not only malignancies of head and neck, brain, lung, prostate, ovaries, skin, oesophagus[7,13,14,15], and oral cancer can be treated by PDT, it can also be used to kill bacteria. *Staphylococcus aureus* has been studied in using photosensitizers such as Haematoporphyrin,[16] Phthalocyanine,[17] 5-Aminolaevulinic acid, Photofrin[18], and Erythrosine.[19]

In recent days, Erythrosine dye used in dentistry has been studied and resulted a promising photosensitizer. The Three major advantages of erythrosine over certain other photosensitizers are – (1) it is not toxic to the host, (2) it is approved by the FDA for usage in food/food products, and (3) it has already been approved for use in dentistry, thereby making its applicable into clinics easier[20,21]. The potential of Erythrosine to undergo photochemical reactions and generate singlet oxygen has been known in the past[22] and has been used as a PS of biofilms of *Streptococcus mutans* for the PDT-based cell killing of these oral infectious bacteria[21]. Since Erythrosine is a hydrophilic dye[23], the efficacy of PDT is related to the cellular uptake kinetics of the photosensitizer and its subcellular localization characteristics [23,24] which are the primary target for PDT. Erythrosine is likely to accumulate preferentially in lysosomes and mitochondria[25]. These localization characteristics may help in determining the mechanism of cell death. The previous studies showed PDT with Erythrosine activated by green light (532 +/- 10 nm, 90 mW, an energy of 16.2 J, a time of 3 min, a fluency rate of 237 mW.cm^{-2} or 42.63 J/cm^{-}2) could kill *C. Albicans & C. dubliniensis* if at least 390 µM was required for a statistically significant reduction in CFU/mL [27]

Erythrosine (400 μM, 1 mins application time) activated by green LED (3 mins exposure time 14.34 J/cm^{-2}) significantly reduced the amount of yeast[26]. In vitro: PDT on recovered yeasts PDT reduced *C. albicans* adherence to buccal epithelial cells by 35% without damaging adjacent tissues [27].

In dentistry, LEDs are used as bleaching tools that do not damage oral tissues. Lately, the study Blue LED (λ 420-480 μM, 250 mW/cm^2, 15 mins application time, 1 minute exposure time) Erytrosine with Nano-TiO_2 gel; (adding carbomer 3%) has demonstrated that the Erythrosine combined with Nano-TiO_2 concentration 440 μM is the most effective concentrations to kill *Candida Albicans* biofilms, yet the Erythrosine concentration 220 μM was found to generate the highest amount of singlet oxygen[28] **,** However the concentrations of Erythrosine that killed *Candida Albicans* biofilms have not been confirmed about its cytotoxicity to oral tissue.

To make the PDT practical, this study used the light-emitting diodes (LEDs), suggested as alternative light sources to lasers for their wider emission bands, smaller size, light weight and cost, greater flexibility in treatment irradiation time and easy operation.[29,30] Our aim was to examine the cytotoxicity of our established Erythrosine with blue light photodynamic therapy method in HGF-1 and HOK cells, and subsequently observed how the photosensitizer damages the cellular components by using Scanning Electron Microscopy (SEM) also had been observed.

2. Objectives of the study

To study the effects of Erythrosine with/out Nano-TiO_2 as a photosensitizer on cell viability, and morphology of HGF-1, and HOK cells after PDT.

3. Materials and methods

Sample characterization

Eleven tested groups of different photosensitizers were studied: Erythrosine (Ery) at 0 μM, 55 μM, 110 μM, 220μM, and 440μM in the presence and or absence of 1% w/w Nano-TiO_2 + blue light, Nano-TiO_2 alone (1% w/w by weight of Erythrosine 440 μM), and control groups (+ve control = DMEM, -ve control = H_2O_2) to both of normal human gingival fibroblast cell lines (ATCC® CRL-2014, HGF-1) and normal primary human oral keratinocytes; HOK (ATCC® PCS200-014™).

Preparation of Erythrosine

Photosensitizers in this study were prepared by dissolving Erythrosine powder (Sigma-Aldrich, St. Louis, MO, USA) in distilled water into solution, 0.22 μm pore syringe filter sterilization. Erythrosine concentration 440 μM was the stock solutions which was then diluted into a series of twofold dilutions of 220 μM, 110 μM, and 55 μM. 1% w/w Nano-TiO_2 (Sigma-Aldrich, St. Louis, MO, USA) was suspended into the solution, vibrated, and mixed equally in colloid. H_2O_2 3% or 1 M were prepared for negative control.

Cell cultures

HGF-1 cells were plated in DMEM containing 10% fetal bovine serum (Invitrogen/ Gibco, Carlsbad, CA) and Antibiotic-antimycotic (all from Gibco™, USA). After 24 hour-incubation of HOK, special dermal cell basal

medium (ATCC.PCS-200-030) and Keratinocyte Growth Kit (ATCC.PCS-200-040) were utilized. For HGF-1 DMEM - Dulbecco's Modified Eagle Medium (Invitrogen/ Gibco, Carlsbad, CA) was used. The medium was changed according to the ATCC guideline. When both cells reached 80% confluence, subcultures were performed. The media were changed every 3 days for HGF-1, and 2 days for HOK. Cultures were maintained at 37˚C, 5% CO_2, 95% humidity. Primary HOK were used at passage 4-7. Both cells then were seeded 10^4 cells/cm^2 in 96-well plates standard condition (37˚C, 5% CO_2, and 95% humidity) for 24 hrs. Then the PDT was started.

Photodynamic therapy

Photodynamic therapy was performed in a dark room. A 20 µl of photosensitizer (Ery) in each group, pipetted into each tested wells (A, D, and H vertically also 2, 4, 6, 8 horizontally) 15 minutes pre-irradiation time was set in order to let the solution endocytosed into cells. Test groups were irradiated with LED-dental curing-light (BA Optima 10, 420-480 nm, 16-19 J/cm^{-2}) for 1 min in the dark box (Fig. 1). Observations were performed at 1, 6 and 24 hrs.

Fig.1

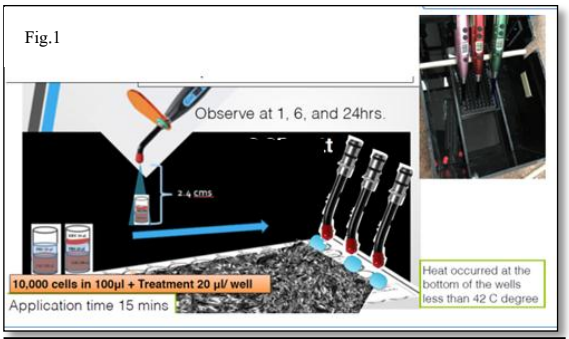


Fig. 1
Setting for photodynamic reaction, the dark box was set as irradiation environment. with 3 dental LED curing machines.

Prestoblue viability assay.

Cell viability assays then were performed by PrestoBlue® (Invitrogen, Life Technologies GmbH, Darmstadt Germany). Each well was incubated 30 mins with PrestoBlue solution, then the analysis of absorbance were accomplished by Varioskan™ at excitation/emission wavelength 560/590 nm. All cells in this assay were cultured in serum free DMEM medium. The control was the mean absorbance from untreated cells and the background was the cell culture media without cells.

Scanning Electron Microscope (SEM)

In order to analyze the surface and morphology of the cells after the PDT, the cell culture (5×10^5 cells/mL) were seeded on a cover slip in 6-well plates. The medium were removed from cells in all tested groups, then fixed with a 4% glutaraldehyde for 45 mins and dehydrated in a graded acetone solution series with Ethanol 30%, 50%, 60%, 70%, 80%, 90% and 100% for 2 mins in each concentration. hexamethyl disilazane (HMDS) was used in the final drying stage by soaking the samples in pure HMDS at room temperature for 30 mins After deposition of a thin gold layer, the cell spreading on the samples were examined by SEM 1000x and 3000x (S3000N, Hitachi, Japan).

Statistical analysis

The Shapiro–Wilk test was use as test of normality in cell viability assay. (2) For normality curve result; ANOVA (p value < 0.05) with Tukey test multiple comparison. Kruskal–Wallis Test. and Mann Whitney U test with

Bonferroni adjustment will be performed if abnormality results were obtained to compare differences between groups. (3) Descriptive analysis to cell morphology by SEM 1,000x and 3,000x.

4. Results

The present study showed that HGF-1 cell viability is not significantly different among groups observed at 1 hr (p value < 0.05) (Fig.2). However, the cell viability was reduced after 6 hrs of observation (Fig.3) at Ery conc. 440 and 440 µM + Nano TiO_2 (p value < 0.05).

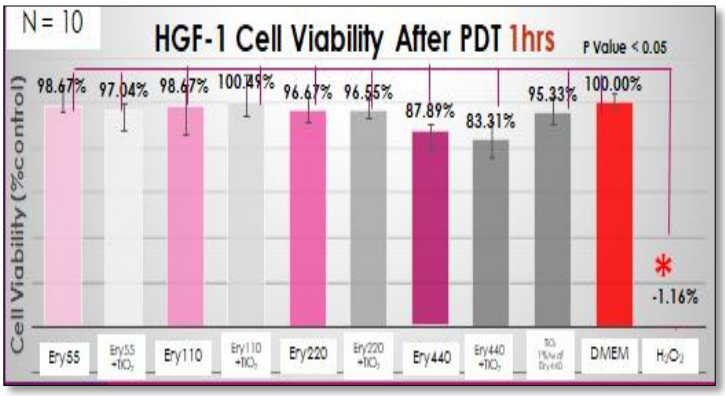


Fig. 2 Comparison of percentage HGF-1 cell viability among Ery groups (conc. 55-440µM) with or without 1% w/w Nano-TiO_2 in PDT after 1 hr (positive control was DMEM group, negative control was H_2O_2) * = significant different from control (DMEM) at p value < 0.05).

After 24 hours of observation, Ery conc. 440 µM, Ery conc.440 µM + Nano-TiO_2, and Ery conc. 220 µM +TiO_2 (Fig. 4) significantly reduced HGF-1 cell viability. Adding Nano-TiO_2 made no significant difference in HGF-1 cell viability (Fig.5). Ery conc. 440 µM + Nano-TiO_2 reduced HGF-1 cell viability number approximately 25% after 24 hours of observation.

Fig. 3
Comparison of percentage HGF-1 cell viability among Ery groups (conc. 55-440 µM) with or without 1% w/w Nano-TiO_2 in PDT after 6 hrs. (positive control was DMEM group and negative control was H_2O_2) * = significantly different from control (DMEM) at p value < 0.05 H_2O_2) * = significantly different from control (DMEM) at p value < 0.05)

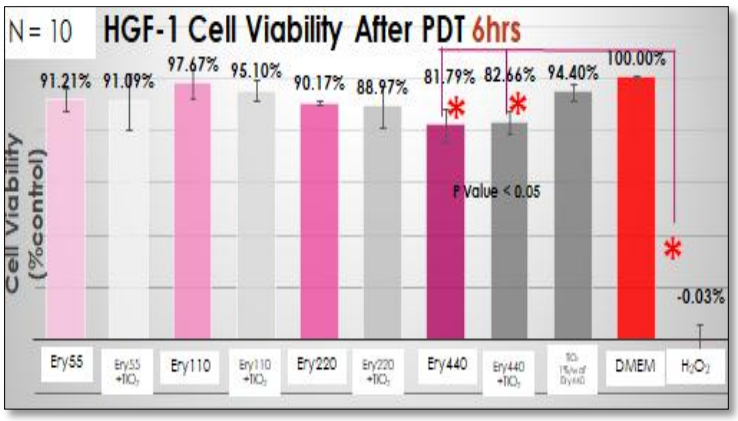
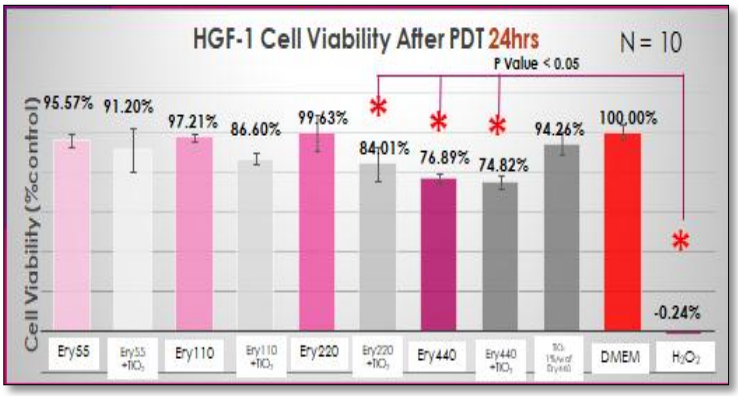


Fig. 4
Comparison of percentage of HGF-1 cell viability among Ery groups (conc. 55-440µM) with or without 1% w/w Nano TiO_2 in PDT after 24 hrs (positive control was DMEM group and negative control was H_2O_2) * = significantly different from control (DMEM) at p value< 0.05)



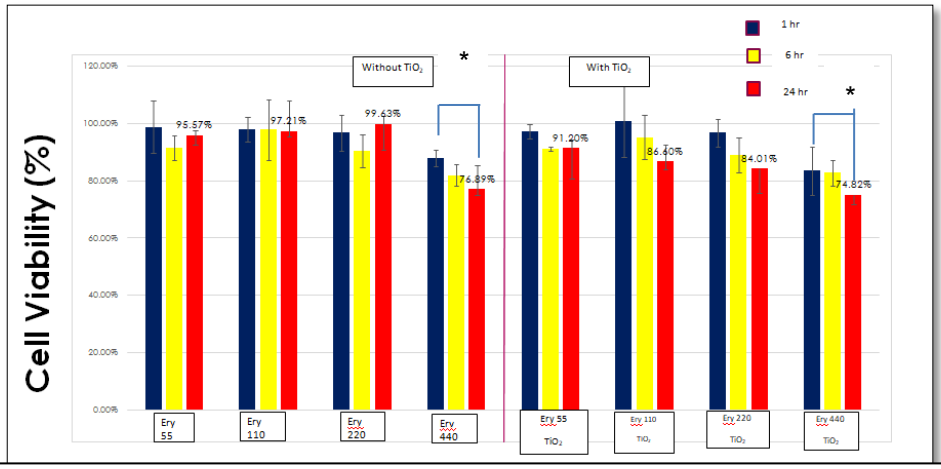
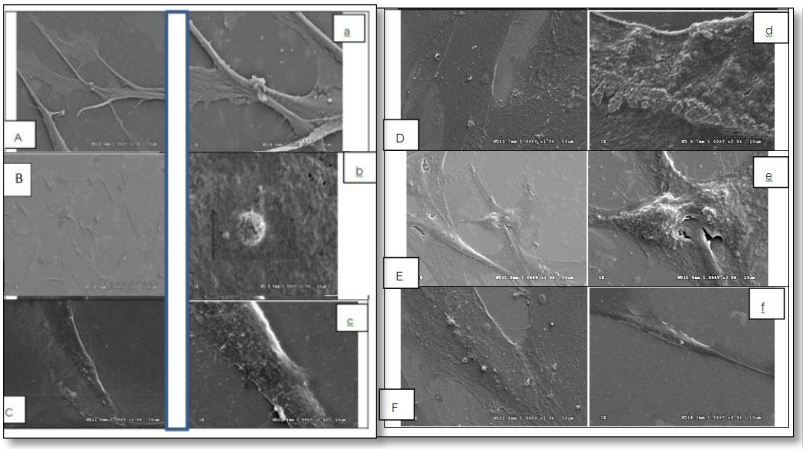


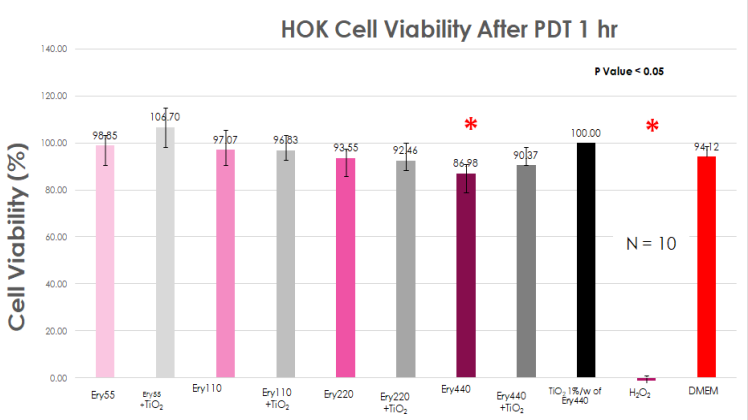
Fig. 5 Comparison of percentage of HGF-1 cell viability among Ery groups (conc.55-440µM) with or without 1% w/w Nano TiO_2 in PDT after 1, 6 and 24 hr (+ve control was DMEM group and -ve control was H_2O_2) * = significantly different from control (DMEM) at $p < 0.05$)



HGF-1 after PDT treated with Ery conc. 440 µM and Ery conc. 220 µM + Nano-TiO_2 µM observed show a significant difference in reducing cell viability after 24 hour-observation (stars on Fig.5).

Fig. 6
SEM of HGF-1 cells; DMEM 24 hr (A,a), Ery conc. 440 µM observed after 1 hr PDT (B,b), Ery conc. 440+ TiO_2 µM observed after 1 hr PDT (C,c), Ery conc. 440 µM observed after 6 hr PDT (D,d), Ery conc. 440+ TiO_2 µM observed after 6 hrs PDT (E,e), Ery conc. 440 µM observed after 24_hr PDT (F), Ery 440+ TiO_2 (f) by SEM 1000x and 3000x (S3000N,

Fig 7
Comparison of percentage of HOK cell viability among erythrosine groups (conc. 55-440µM) with or without 1% w/w Nano TiO_2 in PDT after 1 hr (positive control was DMEM group and negative control was H_2O_2) * = significantly different from control (DMEM) at p value <



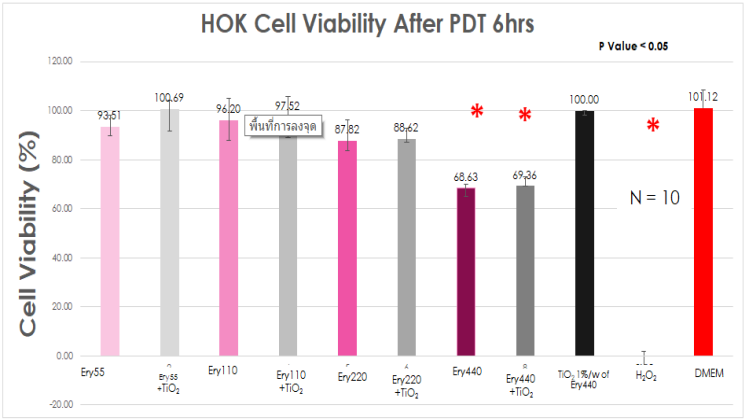


Fig 8
Comparison of percentage of HOK cell viability among Ery groups (conc. 55-440µM) with or without 1% w/w Nano TiO_2 in PDT after 6 hrs (positive control was DMEM group and negative control was H_2O_2) * = significantly different from control (DMEM) at p value <

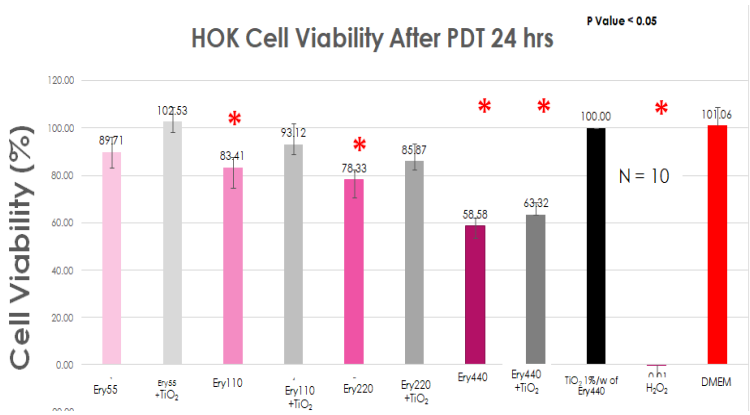


Fig 9
Comparison of percentage of HOK cell viability among Ery groups (conc.55-440µM) with or without 1% w/w Nano TiO_2 in PDT after 24 hr (positive control was DMEM group and negative control was H_2O_2) * = significantly different from control (DMEM) at p value < 0.05

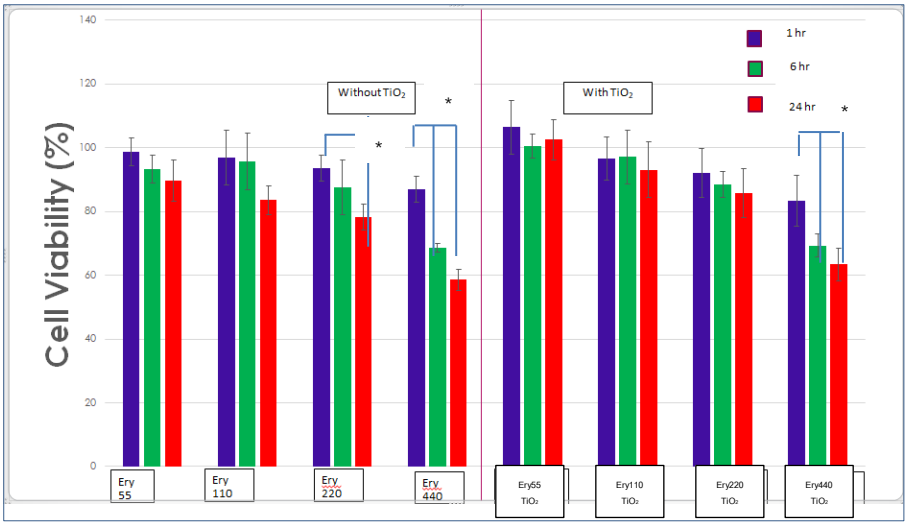


Fig. 10
Comparison of percentage of HOK cell viability among Ery groups (conc.55-440µM) with or without 1% w/w Nano TiO_2 in PDT after 1, 6 and 24 hr (+ve control was DMEM, and -ve control was H_2O_2) * = significantly different from control (DMEM) at p value < 0.05)

HOK viability results showed significant difference (p value < 0.05) in HOK viability observed at 1 hr by PrestoBlue® between DMEM group (positive control) and Ery conc.440 µM. (Fig.7) After 6 hrs Ery conc. 440 µM and Ery conc. 440 µM + Nano TiO_2 showed significantly less cell viability than positive control (Fig.8). HOK viability after 24 hrs PDT demonstrated between group DMEM and Ery conc. 110 µM, 220 µM, 440 µM, 440 µM + Nano-TiO_2 caused significantly lesser cell viability (Fig. 9). Adding Nano-TiO_2 made no significant difference in cell viability (Fig.10). However, HOK cells viability was significantly reduced after PDT with Ery conc. 220 µM observed between 1 and 24 hrs. Both Ery conc. 440 µM, and Ery conc. 440 TiO_2 µM significantly reduced the cell viability observed at all times (stars on Fig.10). HOK cells seem to have a higher sensitivity to the effect of PDT than HGF-1 according to cell viability test (Fig. 5 and Fig.10).

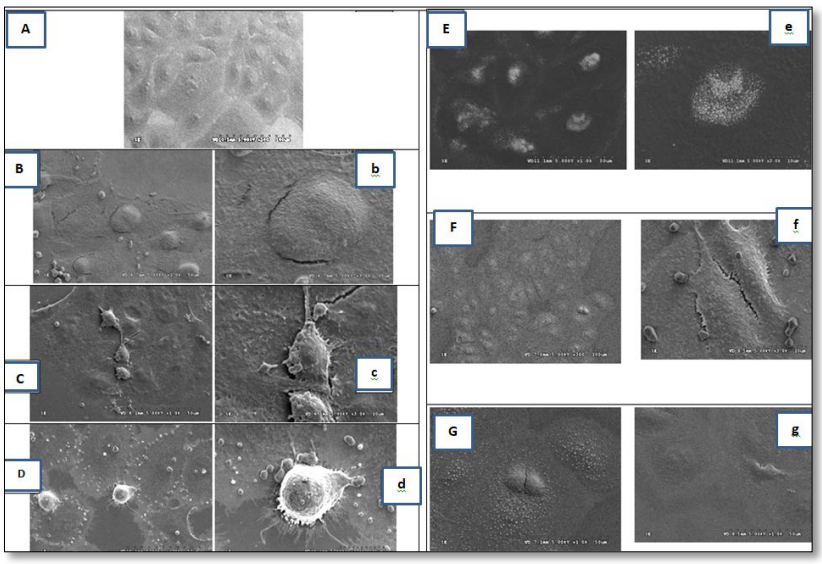


Fig. 11
SEM of HOK cells; DMEM 24 hrs (A), Ery conc. 440 µM observed after 24 hrs PDT (B,b), Ery conc. 440+ TiO_2 µM observed after 24 hrs PDT (C,c), Ery conc. 440+ TiO_2 µM observed after 6 hrs PDT (D,d), Ery conc.440 µM observed after 6 hrs PDT (E,e), Ery conc. 440 µM observed after 1 hr PDT (E,f), Ery conc. 440+ TiO_2 1 hr PDT (G,g) by SEM 1000x and 3000x (S3000N, Hitachi, Japan).

Discussion

Erythrosine generates singlet oxygen in photochemical reactions.[31] Erythrosine is used in dentistry as a dental plaque disclosure on the teeth. In some previous study, Erythrosine B final conc. of 20 µM Xenon light source (MAX-301, Asahi Spectra, Tokyo, Japan, 5-20 J/cm^{-2}), tested on neutrophils cell viability was about 50-70% after PDT[32]. Some studies showed Erythrosine B (330μM = 275.84 μg.mL^{-1}) induced chromosome aberrations in Syrian Hamster Embryo (SHE) cells.[33] Higher dose (0.1–70.0 μg.mL^{-1}) of Erythrosine B could damage DNA structure of HepG2 cells[34]. The present study used relatively higher amount of Erythrosine and the result showed conc. 440 µM activated by blue light 16-19 J/CM^{-2} could damage both HGF-1 and HOK cells. However 220 µM could slightly damage HGF-1 cell lines after 24 hrs. observation. The LED blue curing material light is commonly used in dentistry mainly because the cost is low and it has narrow range of wave length which can effectively penetrate the target cells. [30] Nano-TiO_2 has been widely studied as various electronic applications, utilizing the photo-catalytic nature and transparent conductivity, which strongly depend on the crystalline structure, morphology, and crystallite size[35] and even earns an antibacterial agent due to Nano-TiO_2 photo-semiconductor properties for organism

decomposition[36]. In the previous study, 1% w/w Nano-TiO_2 as a catalyst can increase efficiency of methylene blue and Anthocyanin (+LED 625 - 635nm, dose of 1 J/cm^{-2}) on killing *Streptococcus spp.*[37] The PDT with Blue LED light + Erythrosine 440 μM mixed with Nano-TiO_2 is also effective in killing *C. Albicans*.[30] . In this study, 1% w/w TiO_2 of the maximum concentration of Ery conc. 440 µM, (or having Nano-TiO_2 only 0.35 μg/mL)[30] had negative effect on cell viability. The small amount of TiO_2 1% w/w (maximum 4μg/mL) used as the photo-catalytic agent to PDT showed positive result has relatively in line with the previous study that Nano-TiO_2 (200μg/mL) that it had negative effects on cell viability, proliferation, and the cell dose-dependent and size-dependent manner[39]. In this study, TiO_2 1% w/w, used as an accelerating agent for PDT did not affect percentage of cell viability, in line with some previous studies showed positive effects of TiO_2, that TiO_2–rutile permits acceptable survival of mammalian CNS neurons for at least 10 days in culture.[40] which is because TiO_2 actively inhibits the inflammatory response by breaking down reactive oxygen species (ROS),[41,42] TiO_2 micro- and nanoparticles induced significant decreases in cell survival if used at concentrations higher than 1 μg/mL[38]

The primary HOK cells and HGF-1 cell-lines were studied. Both cells are the major cells in oral tissue where most oral lesion occurs. In this in vitro study, the primary cells and cell lines require different additional nutrients since the primary cells are not genetically modified for the in intro environment, and also needed special media. In contrast to cell lines, primary cells extracted from tissues have a finite lifespan and a limited expansion capacity. However, the primary cells have normal cell morphology and maintain many of the important markers and functions seen in vivo[43] that is why both type of cells were tested in this study. The results showed that in 440 μM cell viability of HGF reduced to 25% and HOK reduced 40% which can be assumed that the primary cells used could be more sensitive that HGF-1 cell lines as stated from previous studies.

6. Conclusion

Erythrosine with Nano-titanium dioxide, a novel PDT photosensitizer, in the range of the present study exerted no toxicity to HGF-1 cell and HOK cells.

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