

Nicotiana benthamiana-derived epidermal growth factor rescues human oral keratinocytes exposed to 5-fluorouracil cytotoxicity

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Objectives: 5-fluorouracil (5-FU) is commonly used as a chemotherapy drug for head and neck cancers. However, it often produces an adverse cytotoxic effect on the oral epithelia leading to oral mucositis (OM) in cancer patients. Thus, reversing this cytotoxic effect locally with novel bioactive drugs or factors is necessary. This study aimed to investigate the rescue effect of *Nicotiana benthamiana* plant-derived epidermal growth factor (P-EGF) on human normal oral keratinocytes (NOKs) exposed to 5-FU-induced cytotoxicity.

Materials and Methods: Cytotoxicity and ATP-dependent proliferation and metabolism of NOK monolayer cultures were assessed after 48 hours of 5-FU exposure and 48 hours of P-EGF treatment using MTT and luciferase-based ATP assays, respectively. Cell death was determined with Live/Dead staining using calcein-AM and propidium iodide. Cell recolonization was evaluated by *in vitro* scratch assay. Five biological replicates were run. One-way ANOVA with post hoc Dunnett's test was used for statistical analysis with GraphPad Prism 9.5.1 software.

Results: P-EGF at 80ng/ml significantly promoted cell proliferation and metabolism after 5-FU-induced cytotoxicity (p -value=0.0006). Additionally, the recolonization of NOKs was also significantly enhanced by the addition of P-EGF (20, 40, and 80 ng/ml, p -value<0.0001).

Conclusion(s): P-EGF rescued the proliferation of 5-FU-exposed NOKs as well as induced epithelial cell recolonization. Thus, P-EGF could be a potential therapeutic approach for OM.

Keywords: chemotherapy, epidermal growth factor, *Nicotiana benthamiana*, oral keratinocytes, plant molecular farming, 5-fluorouracil

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Introduction

Oral mucositis (OM) is a condition driven by the detrimental effects of anti-cancer interventions including chemotherapy with 5-fluorouracil (5-FU), cisplatin or other pharmaceuticals. A study

estimated that more than 59.4% of individuals with head and neck cancer experience OM [1]. This condition significantly impairs patients' quality of life. Mucositis progresses through five distinct stages [2]: (1) initiation marked by DNA breaks, oxidative stress, and immune responses; (2) primary damage response activating various

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pathways, including the NF- κ B; (3) signal amplification; (4) ulceration; and (5) healing. Currently, there is no effective management strategy for OM [3]. Basic oral care strategies remain the best approach to prevent OM [3], but the options are limited and mainly focused on palliative care, which is ineffective and costly [3, 4].

Keratinocyte growth factor (KGF) or palifermin is the only available intravenous drug proven to ameliorate OM [5, 6] by promoting epithelial cell proliferation, differentiation, and migration [7]. However, the market withdrawal by some regulatory agencies and the high cost and inconvenience of systemic administration of palifermin have prevented its use in OM management. The proven concept of stimulating oral epithelial repair by bioactive cues like KGF/palifermin has led researchers to investigate other epithelial cues such as the epidermal growth factor (EGF). EGF could enhance epithelial proliferation and augment epithelium thickness in a murine model of OM [8]. Preclinical trials also demonstrated positive outcomes employing EGF for OM treatment [9, 10]. The commercially available recombinant human EGF, produced by *Escherichia coli*, exhibits various advantages, including its cost-effectiveness, rapid and large-scale production capabilities, and established sustainable manufacturing practices. However, a bacterial platform has its own drawbacks such as the absence of post-translational modifications (PTM), protein aggregation and misfolding, and the potential for toxin contamination [11]. Hence, plant molecular farming has arisen as a promising alternative. Advantages of plant-based system include ease of harvest, limited potential for pathogen contamination, lower manufacturing costs, high scalability, rapid production process, and the possibility of incorporating PTM [12]. *Nicotiana benthamiana* with its fast growth rate and easy genetic intervention is commonly

exploited for recombinant protein production. EGF derived from *N. benthamiana* was successfully generated by one of our research teams, yielding promising outcomes in promoting the proliferation and migration of skin epithelial keratinocytes and in the biofabrication of salivary gland organoids [13, 14]. However, it is not known whether this plant-derived EGF can revert the oral epithelia cytotoxicity produced by chemotherapeutic drugs like 5-FU.

This study aimed to investigate the rescue effect of *Nicotiana benthamiana* plant-derived epidermal growth factor on human normal oral keratinocytes (NOKs) exposed to 5-FU-induced cytotoxicity.

Materials and Methods

Cell culture and epithelial characterization

Human normal oral keratinocytes (NOKs) harvested from gingiva were provided by Prof. Chidchanok Leethanakul (Prince of Songkhla University, Thailand). This study was approved by the Research Ethics Committee and the Institutional Biosafety Committee of the Faculty of Dentistry, Chulalongkorn University with reference numbers HREC-DCU2023-03 and DENT CU-IBC 012/2023. NOKs were cultured in defined keratinocyte growth media (DKSFM, Gibco, Thermo Fisher Scientific, MA, USA), and the media was changed every two days. NOKs were dissociated with TriPLE (Gibco). Cell numbers in each passage were determined by trypan blue exclusion method, and population doubling time was calculated based on a formula previously described [15]. Primary antibodies against Ki-67 (Invitrogen, catalog number PA51942), p63 (Cell Signalling Technology, catalog number 4892S), E-cadherin or E-cad (Cell Signalling Technology, catalog number 3195S), cytokeratin-14 or K14 (Abcam, catalog number ab181595) were used to phenotypically

characterize NOKs by immunofluorescence. To induce the expression of E-cad, NOKs were exposed to 1.5mM CaCl_2 (VWR Chemicals BDH, USA) for 48 hours [16]. NOKs were fixed with 4% paraformaldehyde for 10 min. After fixation, Triton-X 0.1% was added for permeabilization, and cells were subsequently washed thrice with 1x PBS before a 2-hour incubation with a blocking buffer. Primary antibodies were incubated overnight at 4°C, followed by a PBS wash step and the addition of the anti-rabbit secondary antibodies labeled with AlexaFluor 488 or AlexaFluor 594 (Abcam, catalog numbers ab150077 and ab150080). Finally, Hoechst 33342 (Thermo Fisher Scientific, MA, USA) was added and incubated for 20 minutes. EVOS FL Auto II fluorescence microscope (Thermo Fisher Scientific) was used to visualize the above markers.

5-Fluorouracil exposure and P-EGF treatment

NOKs were seeded at 2,500 cells/well overnight for attachment in 96-well regular plates. On the next day, 5-Fluorouracil (5-FU) (Sigma-Aldrich) with concentration of 0, 2.5, 5, 10, 20, 40 µg/ml was added. NOK viability was determined after 48 hours of culture. To evaluate the effect of *Nicotiana benthamiana* plant-produced EGF (P-EGF) in rescuing NOK cell viability after 5-FU cytotoxic exposure, media with 5-FU was replaced with media containing varying concentrations of P-EGF (20, 40, 80ng/ml). 5-FU treated cells without P-EGF were the control. Cell viability and proliferation were determined after 48 hours.

Tetrazolium reduction assay and Live/Dead staining

NOK general cytotoxicity and metabolism after 5-FU exposure was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Thermo Fisher Scientific). Cell culture media was replaced with MTT 1.2mM and incubated for 4 hours. After

incubation, the MTT solution was replaced by 100 µl of DMSO (VWR Chemicals BDH, USA), and the plate was shaken on an orbital shaker at 450rpm for 10 minutes to dissolve the crystal formazan. Absorbance was read using a Glomax Discover Microplate Reader at a wavelength of 560 nm. Calcein-AM (Thermo Fisher Scientific) and propidium iodide (Thermo Fisher Scientific) (PI) were used to determine the viable and necrotic cells. NOKs were cultured at a density of 10,000 cells/well in a 96 well-plate. On the next day, 20 µg/ml 5-FU was added and incubated for 48 hours, followed by treatment of different concentrations of P-EGF (0, 20, 40, 80ng/ml) for another 48 hours. After that, cells were incubated with calcein-AM and PI for 30 mins at room temperature before being observed by EVOS FL Auto II fluorescence microscope (Thermo Fisher Scientific).

ATP-dependent cell proliferation assay

To evaluate ATP-dependent cell proliferation, CellTiter-Glo™ 2.0 Assay (Promega Corporation, WI, USA) and ATPLite™ 1 step (PerkinElmer, MA, USA) were used following manufacturer instructions. Briefly, each reagent was added directly to media in each well of a 96-well plate with a ratio of 1:1, followed by 2 minutes of 700 rpm shaking on an orbital shaker. The plate was subsequently left at room temperature for 10 minutes for equilibration. Afterwards, 50 µl of the media solution was transferred to an opaque plate for bioluminescence quantification with Glomax Discover Microplate Reader.

In vitro wound healing assay to evaluate epithelial recolonization

Cells were seeded at 40,000 cells/well in 96-well plates for overnight attachment. On the next day, a pipette tip (diameter: 1/32 inch) was used to create a scratch on each well. Cell recolonization was observed under EVOS FL Auto II fluorescence microscope (Thermo Fisher Scientific)

at time points 0 and 48 hours. The gap was analyzed using ImageJ software with a wound healing tool plugin as previously described [17].

Statistical analysis

Data were collected and plotted as mean $\pm$ standard deviation (SD). Shapiro-Wilk normality test was performed to determine whether data has a normal distribution. Differences between groups were evaluated by one-way ANOVA analysis followed by Dunnett post hoc tests. Statistical analysis was performed with GraphPad Prism 9.5.3.

Results

Phenotypic characterization of NOKs

The large majority of NOKs in monolayer (two-dimensional) cultures exhibited the pro-mitotic marker Ki-67, the marker for epithelial lineage p63, and the marker for proliferative basal keratinocytes K14 (Figure 1). Upon the addition of CaCl_2 , the presence of E-cad, a pivotal

intercellular adhesion molecule in differentiated epithelial cells, was also detected, particularly in confluent areas (Figure 1). Population doubling time (PDT) of NOKs was stable (51.7 ± 56.23 hours). These results underscored the active mitotic status and proliferative nature of NOKs used in this study.

Cytotoxicity, ATP-dependent proliferation, and metabolism of NOKs after 5-FU exposure

Next, 5-FU was supplemented to the media for 2 days in NOK monolayer cultures. Treatments with 5, 10, 20, and 40 $\mu\text{g/ml}$ of 5-FU resulted in 50% general cytotoxicity for NOKs (Figure 2a), while the ATP-dependent proliferation was decreased to 50% at the concentration of 20 and 40 $\mu\text{g/ml}$ (Figure 2b). At 2.5, 5, and 10 $\mu\text{g/ml}$, NOKs exhibited shrunken pyknotic nuclei and cytoplasmic debris (Figure 2c), while at 20 and 40 $\mu\text{g/ml}$, nuclei were absent, and cell membranes were compromised. These findings confirmed 5-FU's toxicity to NOK cells, and 20 $\mu\text{g/ml}$ 5-FU was chosen for subsequent experiments to investigate the rescue effect of P-EGF.

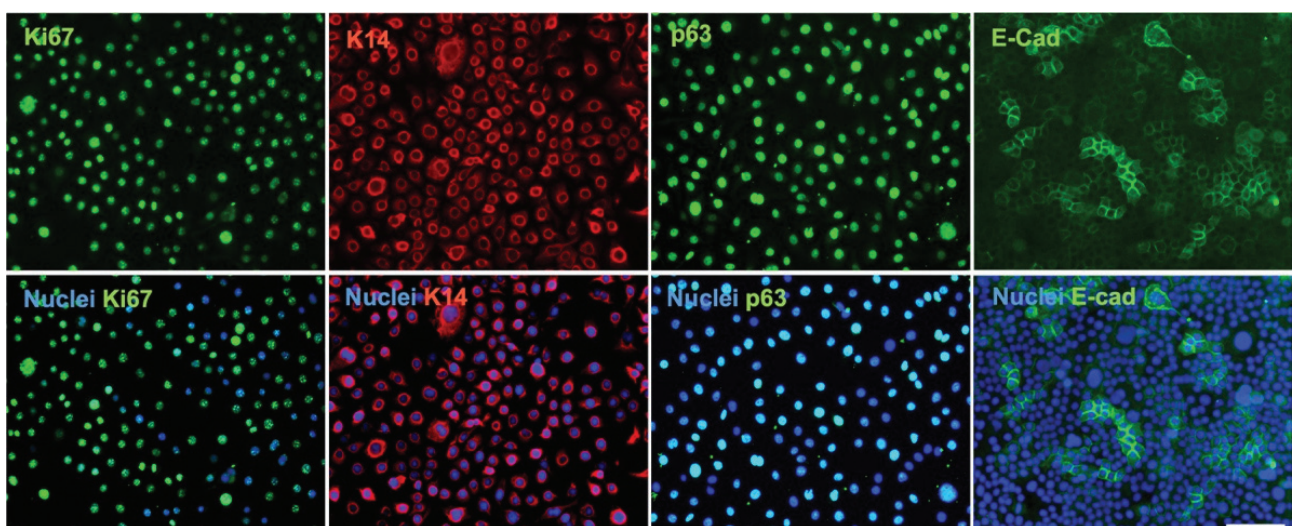


Figure 1 Epithelial characterization of NOKs via immunofluorescence. Magnification: 10X, scale bar: 200 μm

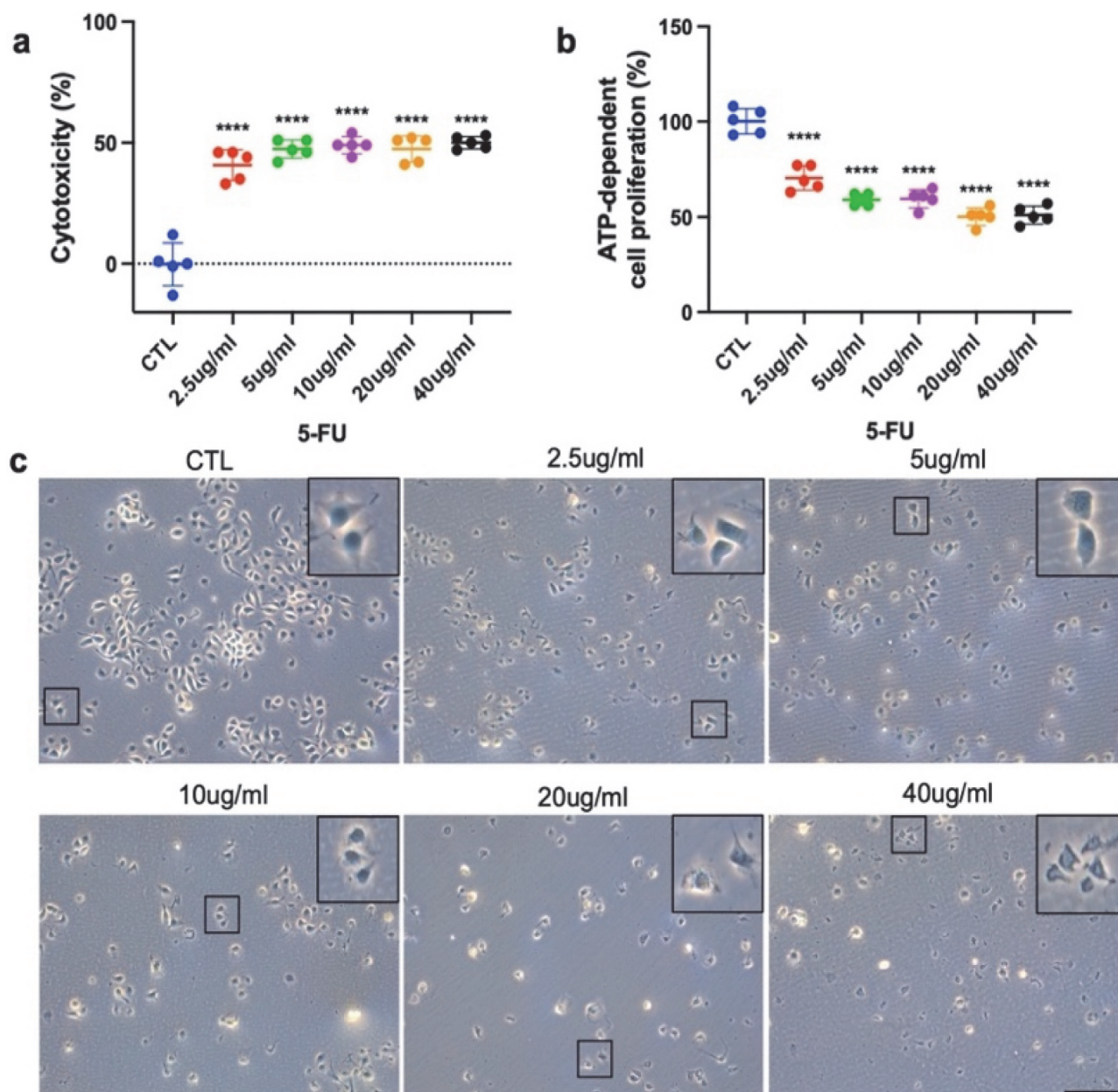


Figure 2 Cytotoxicity, ATP-dependent proliferation and metabolism of NOKs after 5-FU exposure using MTT assay (a) and luciferase-based ATP assay (b). Data are presented as means±SD and one-way analysis of variance (ANOVA) with post-hoc Dunnett's test was performed: **** $p < 0.0001$ when compared with NOKs not exposed to 5-FU, $n = 5$. NOK morphology was observed under light microscopy with phase contrast (c) at 10X magnification, scale bar: 200µm. Inset micrographs are displayed at the top right corner and are highly focused areas from smaller black frame squares.

P-EGF rescued the viability and proliferation of 5-FU-exposed NOKs

The introduction of plant-derived EGF (P-EGF) after 5-FU treatment exhibited a trend in impacting cell viability in a dose-dependent manner. P-EGF at high dose (80ng/ml) significantly stimulated NOK proliferation compared to the untreated control (Figure 3a). This pattern of response was also evident when using Live/Dead staining, where necrotic cells were observed less in 80 ng/ml P-EGF-treated group (Figure 3c). These findings indicated that P-EGF rescued the survival and proliferation of NOKs after 5-FU exposure.

After P-EGF treatment, recolonization of 5-FU-exposed NOKs significantly increased compared to untreated cells. At concentrations of 40 and 80ng/ml P-EGF, NOKs substantially recolonized, reaching approximately 70% of the gap within 48 hours (Figure 3b). This outcome indicated that the treatment of plant-derived EGF (P-EGF) following 5-FU cytotoxic exposure promoted the recolonization of NOKs.

Discussion

The findings from this study revealed new insights into the *in vitro* effects of *N. benthamiana*-derived EGF (P-EGF) treatment on the behavior of NOKs following 5-FU cytotoxic exposure. Upon 5-FU exposure, P-EGF tamed its cytotoxicity by enhancing the proliferation and ATP production and inducing recolonization of oral keratinocytes *in vitro*.

EGF is a soluble protein acting as an extracellular signal in the human body. The binding of EGF to its receptors (ErbB) activates tyrosine kinase activity and triggers various cascades of

signaling pathways related to cell growth and migration such as PI3K/AKT and ERK/MAPK [18]. In a previous report, bacteria-produced EGF showed comparable effects in terms of oral epithelial proliferation and wound healing *in vivo* [8]. However, recombinant proteins produced by bacteria systems lack PTM, promote protein aggregation, and can lead to potential toxin contamination [19]. On the other hand, those produced from mammalian cells can be post-translationally modified but this process is highly complex, time-dependent, and costly for production at a large scale [20]. Hence, plant-derived EGF combines the advantages of both mammalian cells and bacteria production systems and may constitute an appealing alternative. Herein, at a high concentration, P-EGF effectively partially restored NOK viability and proliferation following 5-FU treatment. This observation is consistent with previous studies emphasizing P-EGF's impact on cell proliferation of skin keratinocytes and primary porcine salivary glandular epithelial cells [13, 14]. It is noteworthy that the concentration required for this effect in our study surpasses that of previous research. This discrepancy might arise from the cytotoxicity of 5-FU that P-EGF ultimately needs to overcome, as well as the different cell seeding densities and cell lines. Hainitinan *et al.* reported that high concentrations of P-EGF, 50 and 100 ng/ml, promoted the proliferation in immortalized skin keratinocytes [13]. In primary glandular epithelial cells, Phan *et al.* observed that P-EGF at low concentrations of 5-20ng/ml was effective for cell proliferation [14]. Therefore, we chose 20, 40, and 80ng/ml of P-EGF for testing in our study. Interestingly, the scratch assay results provided robust evidence of P-EGF's ability to enhance the

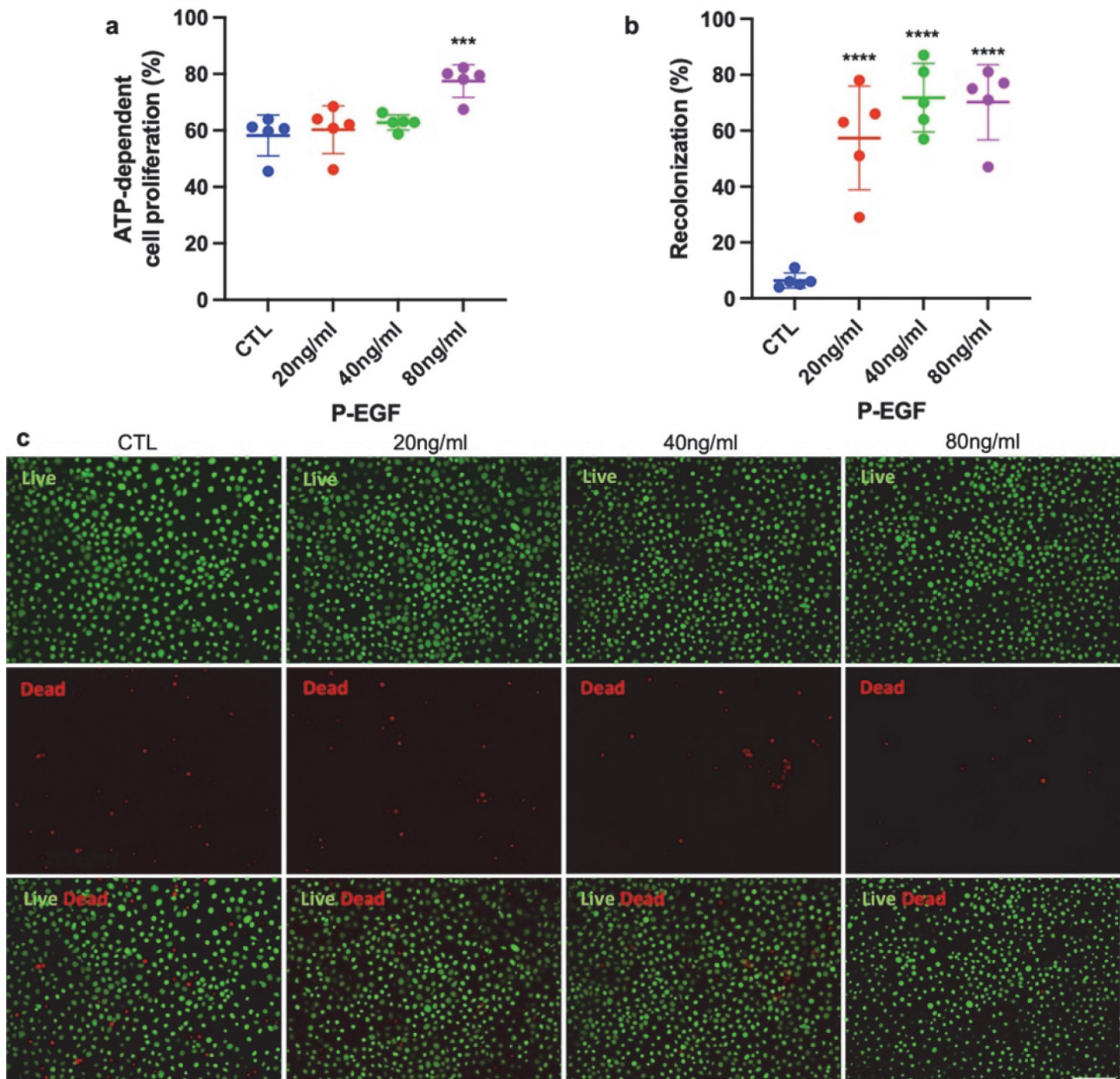


Figure 3 ATP-dependent proliferation of 5-FU-exposed NOKs (20 μ g/ml) after P-EGF treatment using luciferase-based ATP assay (a). Quantitative outcomes of cell recolonization by ImageJ (b). Data are presented as means $\pm$ SD and one-way analysis of variance (ANOVA) is performed: *** p < 0.001, **** p < 0.0001 when compared to 5-FU-exposed NOKs not treated with P-EGF, n = 5. After P-EGF treatment, Live/Dead staining was performed with Calcein-AM (green fluorescence) and propidium iodide (red fluorescence) (c). Magnification: 10X, scale bar: 200 μ m.

recolonization potential of NOKs, particularly at concentrations of 40 and 80ng/ml. This finding is consistent with the prior study where P-EGF promoted migration of skin epithelial cells [13]. The significant recolonization of the wound area within a relatively short timeframe underscores the potential clinical relevance of P-EGF treatment in accelerating the oral epithelial repair process, and this is critical especially when oral ulcers/lesions are present as commonly observed in 5-FU chemotherapy-induced oral mucositis patients.

This study has its own limitations. First, monolayer culture systems were employed, which lack the epithelium complexity present in the *in vivo* oral mucosa. Consequently, cell behaviors may not accurately reflect the *in vivo* situation [21]. Secondly, P-EGF was not compared to mammalian cell-produced EGF due to the limited commercial availability of this EGF. However, the previous comparison with *E. coli*-derived EGF demonstrated comparable effects of both platforms in supporting the proliferation of various cell types, including salivary glandular epithelial cells, kidney epithelial cells, and skin keratinocytes [13, 14, 22]. Finally, pro-inflammatory cytokine expression was not evaluated, which is crucial to understand oral epithelial tissue damage and repair. Future research will investigate the oral epithelial repair in 3D culture systems which better resemble the *in vivo* tissues, and pro-inflammatory cytokines will be assessed to better understand the cellular response of NOKs to 5-FU and P-EGF. Additionally, further research unveiling the underlying molecular mechanisms that govern the herein-reported effects would provide valuable insights into the intricate crosstalk between growth factor signaling, cellular proliferation, and migration.

Conclusion

N. benthamiana-derived EGF rescued cell viability and proliferation as well as promoted recolonization of 5-FU-exposed human oral keratinocytes. Thus, this plant-derived EGF could constitute a potential therapeutic option for oral mucositis treatment.

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