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OBM Genetics

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Research Article

Effects of Coenzyme Q10 on H_2O_2-Induced Oxidative Stress of Human Keratinocytes

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Academic Editor: Lunawati L Bennett

OBM Genetics
2025, volume 9, issue 1
doi:10.21926/obm.genet.2501279

Received: July 05, 2024
Accepted: December 22, 2024
Published: January 06, 2025

Abstract

Keratinocytes are particularly vulnerable to oxidative stress due to their function as the primary natural protective barrier exposed to chemicals, rays, and pollution to stimulate the formation of free radicals in the body. Two factors of interest correlate with cellular senescence under oxidative stress. These include regulating antioxidant synthesis, which maintains free-radical homeostasis through the *Nrf2* gene, and the prevention of telomere shortening, which involves the collaboration of human telomerase genes, including the *hTERT* and *hTR* genes. Coenzyme Q10 (CoQ10) is a wildly used antioxidant in dietary supplements and the cosmetic industry. Therefore, this study aims to investigate the effect of CoQ10 in inhibiting the oxidation process induced by hydrogen peroxide on cell viability, intracellular ROS content, mRNA expression of the *Nrf2* gene, *hTERT* gene, and *hTR* gene, and relative telomere lengths (RTL). When cells were treated with hydrogen peroxide at LC_{50} for 24 hours, followed by a 24-hour treatment with 1.00 µM CoQ10, cell viability increased by 16.57 ± 2.25%. Additionally, intracellular ROS content decreased by 37.20 ± 13.22%, *Nrf2* gene expression significantly reduced ($p < 0.001$), and *hTERT* gene expression significantly increased ($p < 0.01$). No significant difference in the *hTR* gene expression and the RTL was detected. In conclusion, this study expands the knowledge of CoQ10 potential as an active ingredient in approaching anti-oxidant and anti-aging treatment.

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Keywords
Keratinocyte; antioxidants; coenzyme Q10; telomere

1. Introduction

Several environmental stressors encountered in daily life originate from exogenous and endogenous sources. These factors can precipitate oxidative stress by inducing the generation of reactive oxygen species (ROS), such as superoxide anion radicals (O_2-•), hydroxyl radicals (•OH), singlet oxygen (1O_2), and hydrogen peroxide (H_2O_2) in the skin [1, 2]. Antioxidants serve as electron acceptors to maintain free radical homeostasis by inhibiting oxidation chain reactions between free radicals and other biomolecules. Typically, cells can generate sufficient antioxidants to neutralize free radicals and prevent oxidative damage. However, imbalanced antioxidant content can be triggered by stress, medications that reduce antioxidant enzymes, and clinical manifestations, which all end up making cells unable to maintain free radical homeostasis [3]. Coenzyme Q10 (CoQ10) or ubiquinone is a lipophilic antioxidant that cells can metabolize throughout the body. Furthermore, CoQ10 is an integral component of the mitochondrial oxidative phosphorylation system, rendering it essential for cells susceptible to oxidative stress or possessing high energy demands. Several research indicated that various factors, including cardiovascular activity, substance consumption, and medical conditions, can diminish CoQ10 levels. Consequently, CoQ10 is frequently utilized in nutritional supplements and treatments [4].

The epidermis layer of skin contains 95% keratinocytes, which function as the first natural barrier against exogenous oxidative stressors. Thus, keratinocytes are highly susceptible to oxidative stress, resulting in photoaging and skin damage, undergoing several cellular processes such as DNA damage, lipid peroxidation, immune system stimulation such as cytokine secretion, etc. [5]. The HaCaT cell line is widely used to standardize *in vitro* investigations on keratinocytes, which are long-lived, spontaneously immortalized with positive telomerase activity, and susceptible to *in vitro* differentiation. Additionally, it serves as a suitable model to identify the release of inflammatory and repair molecules [6-8].

Human keratinocytes contain several genes responsible for antioxidant production, notably the NF-E2-related factor 2 (*Nrf2*) gene, crucial for intracellular stress response and redox signaling. The keratinocyte growth factor (KGF) is essential for preventing cellular damage and facilitating the release of *Nrf2* protein into the cytoplasm under oxidative stress resulting from wounds or other stimuli. Nrf2s translocate to the nucleus, which binds to the antioxidant response element (ARE) and promotes the expression of various antioxidant genes. Consequently, *Nrf2* plays a pivotal role in inhibiting excessive skin inflammation and mitigating the development of skin cancers in response to stressors, particularly ROS [9, 10].

Oxidative damage on cellular components is associated with telomere shortening and genome instability, contributing to premature aging. The progressive shortening of telomeres causes the onset of cellular senescence until they are insufficient length for cell division. The inverse association between age and telomere length suggests that telomere length may be a potential cellular marker for biological aging. Telomeres are untranslated tandem repeat sequences at the ends of eukaryotic chromosomes and contain the hexameric repeat of 5′-$TTAGGG_n$. They serve as protective DNA caps

on chromosomes, ensuring the integrity of information-bearing DNA throughout the cell cycle [11-13]. Telomerase is a ribonucleoprotein complex enzyme that extends cell longevity by adding nucleotide repeats with high guanine content to target DNA strands [14]. Telomerase is typically present in all tissues during early development and in the adult body tissues that undergo continuous or periodic proliferation, such as the epidermis and the hematological system [8, 15]. Telomerase regulated gene mainly consists of the *human telomerase RNA component gene* (*hTR*), which codes for the telomerase RNA (*hTR*) template, and the *human telomerase reverse transcriptase (hTERT)*, which coded for the telomerase reverse transcriptase (*hTERT*) enzyme. The two gene products, *hTR* and *hTERT*, collaborate to generate the telomere replication process [13]. CoQ10 has been reported to play a crucial role in DNA damage and repair. Several studies found that CoQ10 affects mitochondrial DNA damage, mutations, epigenetics, and telomere shortening [16].

In this study, we aim to investigate the effects of using CoQ10 as an antioxidant to inhibit H_2O_2-induced oxidative stress in human keratinocytes. We also aim to evaluate the expression of the *Nrf2* gene, which regulates antioxidant production, as well as the *hTERT* and *hTR* genes, which regulate telomerase synthesis, and to determine the CoQ10 effect on telomere length, thereby enhancing our understanding of how to utilize CoQ10 for cellular senescence prevention.

2. Materials and Methods

2.1 Cell Viability Assay

The human immortalized keratinocyte cell line, HaCaT (Cell Lines Service, Germany), was cultured in DMEM supplemented with 10% FBS and 1% Antibiotic-antimycotic (GIBCO®, USA), and maintained at 37°C with 5% CO_2. For H_2O_2-induced oxidative stress induction, cells were seeded onto 96-well plates (SPL Life Sciences, Republic of Korea) at a density of 1.5×10^3 cells/well and incubated for 24 h. Subsequently, the oxidative stress was induced using H_2O_2 at concentrations ranging from 600 to 1000 µM for 24 h. The LC_{50} of H_2O_2 was analyzed and used for subsequent H_2O_2-induced oxidative stress induction steps.

Four experimental groups were established to investigate the effects of CoQ10 on cell viability. Cells were treated with CoQ10 simultaneously with or 24, 48, or 72 h post-stress induction at concentrations of 0, 0.1, 1.0, 5.0, and 10.0 µM. A sample without hydrogen peroxide induction and CoQ10 treatment was the negative control.

To assess cell viability, the AlamarBlue® assay (Gold Biotechnology, USA) was conducted following the manufacturer's instructions. Light absorbances at 570 nm and 595 nm were measured by SpectraMax M3 microplate reader (SpectraMax M3, Molecular Devices, USA).

2.2 Intracellular ROS Measurement

To assess intracellular ROS content, the 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) assay was conducted following the protocol described by Mao et al. [17]. Briefly, HaCaT cells were seeded onto 96-well plates at a density of 1.5×10^3 cells/well and incubated for 24 h. Cells were treated with CoQ10 ranging from 0 to 10.0 µM for 24 h post-stress-induction and then were incubated with 25 µM DCFH-DA in phosphate-buffered saline (PBS) (GIBCO®, USA) for 30 minutes for ROS detection.

Samples without hydrogen peroxide induction and CoQ10 treatment served as the negative control. A microplate reader measured Fluorescence intensity at 485 nm (excitation) and 530 nm (emission).

2.3 Quantitative Real-Time PCR

HaCaT cells were cultured in T75 flasks to 80% confluent and then incubated with LC_{50} H_2O_2 for 24 h. Subsequently, cells were treated with 1.0 µM CoQ10 for 24 h. Samples without hydrogen peroxide induction and CoQ10 treatment served as the negative control.

mRNA extraction was carried out using the RNeasy® Mini Kit (Qiagen, Germany), and the extracted mRNA was stored at -80°C. cDNA synthesis was performed using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Italy) and stored at -20°C.

Gene expression levels were assessed using a quantitative real-time polymerase chain reaction (qPCR) method with CFX Opus 96 system (Bio-Rad, USA) using CAPITAL™ qPCR Green Mix (Biotechrabbit, Germany). qPCR cycling conditions consisted of 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds. *GAPDH* served as the internal control. Primers for the *Nrf2*, *hTERT*, *hTR*, and *GAPDH* amplification were according to previous studies [18, 19]. Gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method [20].

2.4 Relative Telomere Length (RTL) Measurement

For H_2O_2-induced oxidative stress induction, HaCaT cells were cultured in T75 flasks to 80% confluent and then incubated with LC_{50} H_2O_2 for 24 h. Three experimental groups were established to investigate the effects of CoQ10 on RTL, including normal conditions, oxidative stress induction, and CoQ10 treatment after oxidative stress induction. Cells were treated simultaneously with or 24, 48, and 72 h post-stress induction with 1.0 µM CoQ10. Cells cultured in standard media were used as the negative control. Following cell harvesting, the genomic DNA was isolated using the G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology, Republic of Korea). DNA concentrations were determined by spectrophotometry and adjusted to 25 µg/ml. The ratio of the telomeric repeat sequence to the single-copy gene was determined using real-time PCR, following the methodology described by Cawthon (2002) [21]. The *36B4* gene was employed as the reference single-copy gene for this analysis.

2.5 Statistical Analysis

All CoQ10-treated HaCaT keratinocyte groups, along with the control group, were subjected to 3 independent replicates for the assessment of cell viability, intracellular ROS, and gene expression, and their differences between the percentage of in each treatment group and the control group were statistically analyzed using the independent sample t-test. Multiple group comparisons in the intracellular ROS reduction and relative telomere length were performed using one-way and two-way ANOVA (post hoc test: Tukey's multiple comparisons test), respectively. All statistical analyses were performed using IBM SPSS Statistics V22.0 (SPSS, USA), with a significance level set at $P < 0.05$. Graphs were generated using GraphPad Prism version 8 (GraphPad, USA).

3. Results

3.1 CoQ10 Enhanced HaCaT Cell Viability Under Stress with Hydrogen Peroxide

HaCaT cells were treated with H_2O_2 at concentrations ranging from 600 to 1,000 µM for 24 h to test for LC_{50}. The outcomes revealed the LC_{50} to be 691.83 µM, which was subsequently adopted as the H_2O_2-induced oxidative stress condition for subsequent experiments (Figure 1A).

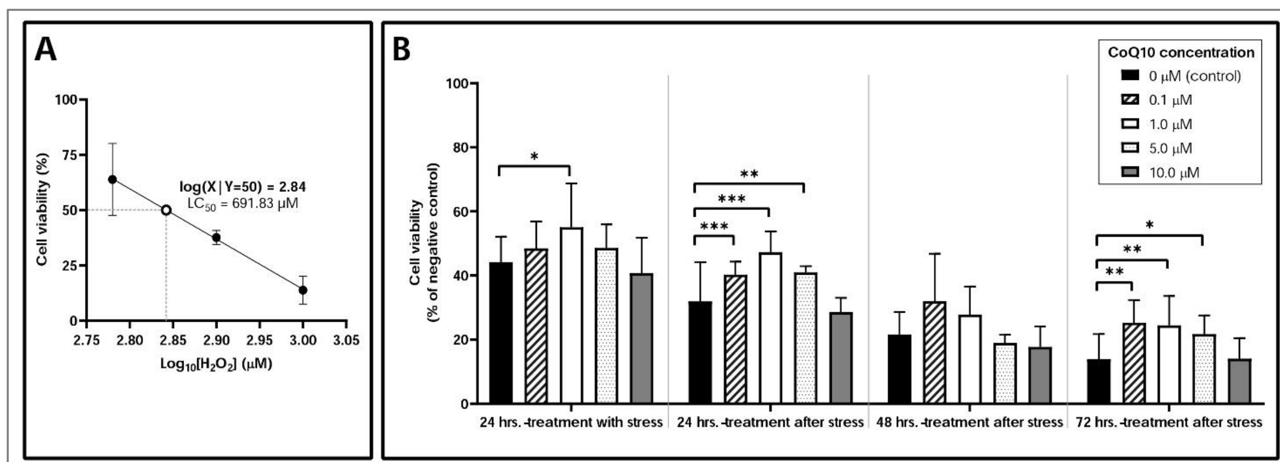

Figure 1 Effects of CoQ10 on HaCaT cell viability under H_2O_2-induced oxidative stress. (A) The LC_{50} of hydrogen peroxide on HaCaT keratinocytes over a 24 h period. (B) The viability of keratinocytes was investigated on cells incubated with various concentrations of CoQ10 (0, 0.1, 1.0, 5.0, and 10.0 µM). All data are represented as mean ± SD. Mean different test between cell viability of each CoQ10-treated condition and CoQ10-untreated control using independent sample t-test. Statistical significance levels are represented by *, **, and ***, which correspond to p-values < 0.05, <0.01, and <0.001, respectively.

To analyze the effects of CoQ10 on cell viability, cells were treated with CoQ10 immediately or 24, 48, or 72 h post-stress induction. The results demonstrated that the 1.0 µM CoQ10 significantly increased cell viability when treated simultaneously with ($p < 0.05$) or 24 h ($p < 0.001$) and 72 h ($p < 0.01$) post-stress induction compared to the stressed control. CoQ10 at 0.1 and 5.0 µM also significantly increased cell viability when treated for 24 and 72 h post-stress induction. The condition of 1.0 µM CoQ10 treatment for 24 h post-stress induction was selected for subsequent experiments due to its substantial enhancement of cell viability, increasing by 16.57 ± 2.25% ($p < 0.001$) compared to the control (Figure 1B).

Dunnett's multiple comparisons test was used to support the selected concentration to compare three treatment groups (0.1, 1.0, 5.0, and 10.0 µM) against a single control group. Results indicated that only 1.0 µM of CoQ10 significantly increased cell viability ($p < 0.05$) across all four timepoints.

3.2 CoQ10 Decreased the Intracellular ROS Content and the Nrf2 Gene Expression

To analyze the effects CoQ10 on intracellular ROS reduction, the intracellular ROS contents were determined in cells treated with CoQ10 for 24 h post-stress induction. The intracellular ROS content in keratinocytes under each CoQ10-treated condition was demonstrated compared to the negative

control. All the tested CoQ10 concentrations significantly decreased the intracellular ROS content compared to the CoQ10 non-treated control, with cells receiving 1.0 µM CoQ10 exhibiting the most significant decrease by 37.20 ± 13.22% (Figure 2A). No significant difference was observed between cells treated with different concentrations of CoQ10.

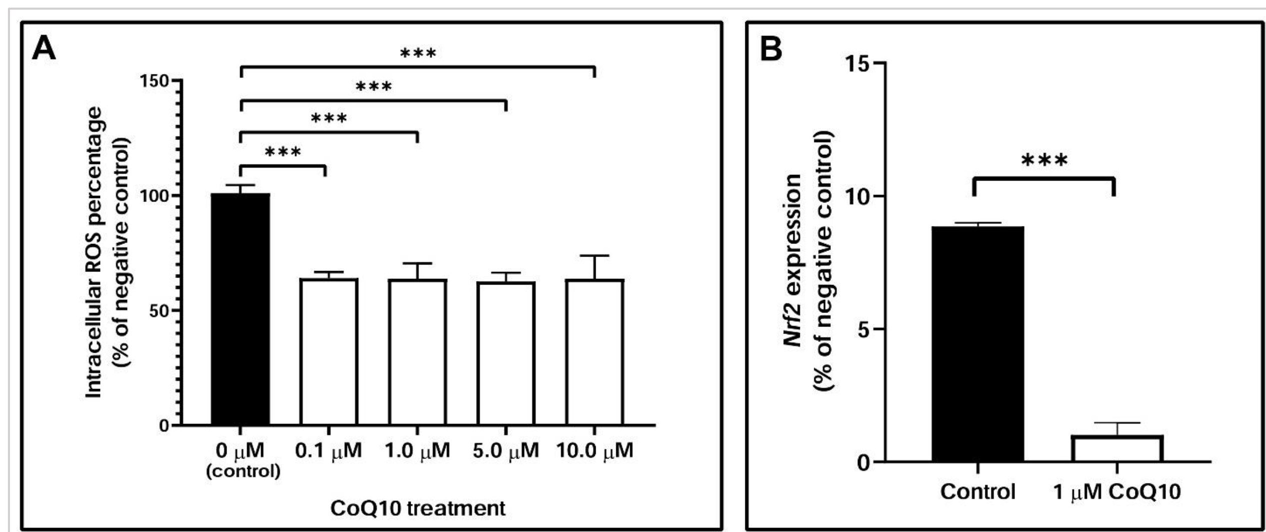

Figure 2 Effect of CoQ10 on the intracellular ROS contents and the *Nrf2* gene expression in HaCaT cells under H_2O_2-induced oxidative stress. (A) Average intracellular ROS contents. (B) Relative *Nrf2* expression in HaCaT. Cells in both (A) and (B) were treated with CoQ10 for 24 h post-stress induction. All data are represented as mean ± SD. Mean different test between each CoQ10-treated condition and CoQ10-untreated control using independent sample t-test statistical analysis. *** represents p-value < 0.001.

To gain insights into the underlying mechanisms of ROS reduction, *Nrf2* mRNA levels were analyzed. The *Nrf2* mRNA levels were compared between the cells treated with 1 µM CoQ10 and the non-treated control group. The results revealed that *Nrf2* expression significantly decreased from 8.85 ± 0.14% in the control group to 1.02 ± 0.46% in the CoQ10-treated group (Figure 2B).

3.3 CoQ10 Showed No Effects on RTL, but Increased the hTERT Gene Expression

As CoQ10 was suggested to have telomere shortening prevention properties, relative telomere lengths (RTLs) were compared between cells treated with 1.0 µM CoQ10 simultaneously with stress or at 24, 48, and 72 h post-stress induction, CoQ10 untreated cells, cells under normal condition. RTLs were significantly decreased in cells under stress conditions for 24 h (Figure 3A). Treatment with CoQ10 did not rescue the RTLs at that time point. However, when cells were under stress conditions for 48 and 72 h, the RTLs returned to normal, treated and untreated with CoQ10, compared to cells without stress.

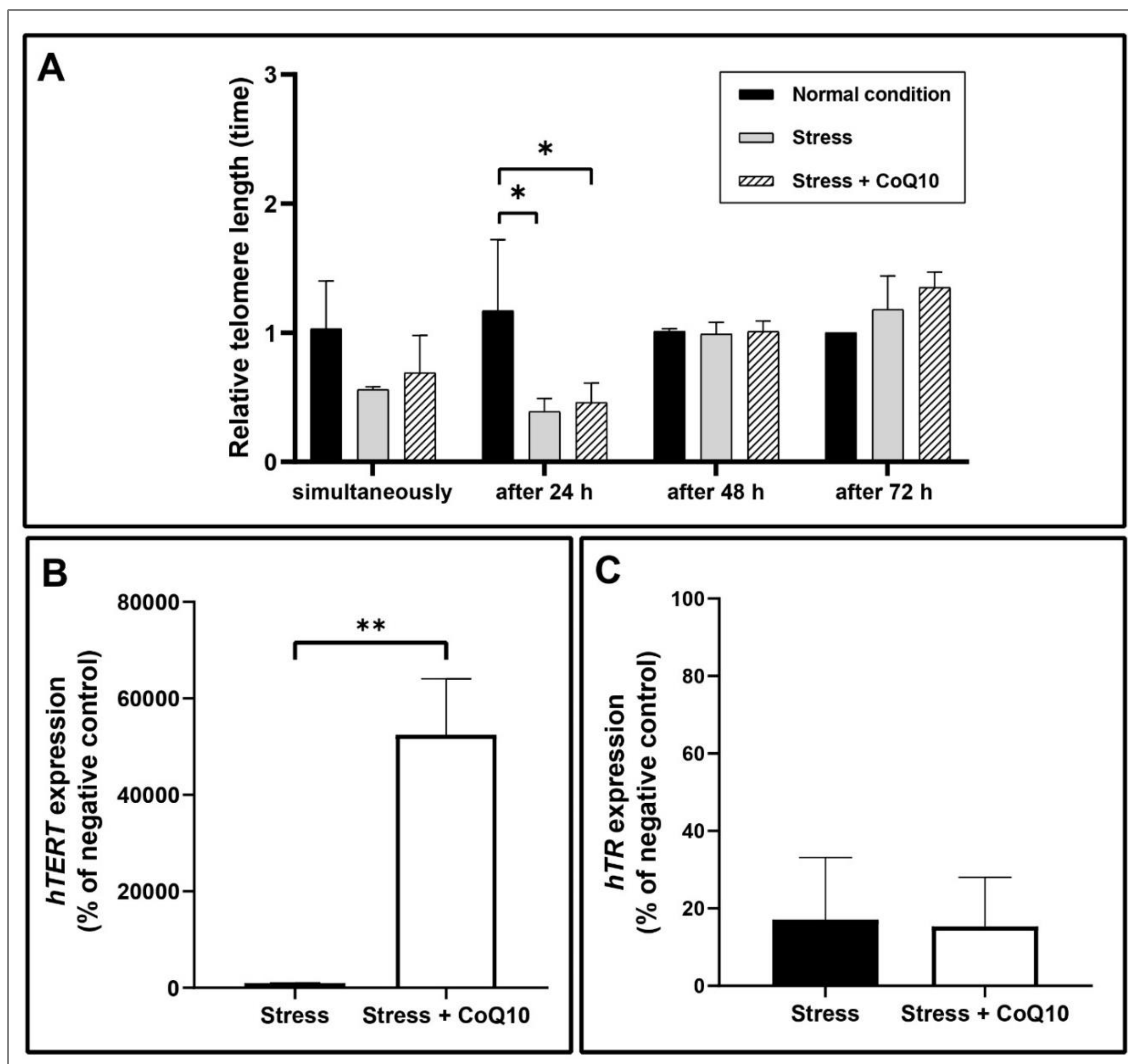

Figure 3 Effect of CoQ10 on RTL and the *hTERT* and *hTR* telomerase gene expression in HaCaT cells under H_2O_2-induced oxidative stress. (A) RTL in HaCaT cells treated with 1.0 µM CoQ10 simultaneously with and 24, 48, 72 h post-stress induction. Two-way ANOVA was used for statistical analysis to demonstrate RTLs' response in each treated condition. (B) Relative *hTERT* expression and (C) Relative *hTR* expression in HaCaT cells treated with 1.0 µM CoQ10 for 24 h post-stress induction. Data are represented as mean ± SD. Mean different test between each CoQ10-treated condition and CoQ10-untreated control using independent sample t-test statistical analysis. *, ** represents p-value < 0.05, 0.01, respectively.

To evaluate the effects of CoQ10 on preventing telomere shortening through telomerase function, the *hTERT* and *hTR* were used as telomerase marker genes. The expression of the *hTERT* and *hTR* showed different results. The *hTERT* expression level significantly increased by 51,527.19 ± 5775.72% ($p < 0.01$) compared to the control (Figure 3B), while the *hTR* expressions showed no significant difference ($p = 0.889$) (Figure 3C).

4. Discussion

This study evaluated the potential of CoQ10 in antioxidant and antiaging activities. Treatment with 1 µM CoQ10 for 24 h post-stress induction was the most effective in cell viability. This finding correlates with results from Hseu et al., which reported that CoQ10 had a cytoprotective effect on UVA-irradiated HaCaT cells when pre-treated with 2 µM for 24 hours [22]. However, HaCaT cells treated with CoQ10 for 48 h post-stress induction exhibited a trend but showed no significant change in cell viability at any concentrations. This finding might correlate to the inflammatory stage of the wound healing process, mainly because the highest level of inflammation occurs on the third day [23, 24]. A study by Braun et al. suggested the concept of activities within the keratinocyte triggered by oxidative stress that may correspond to phases of wound healing [9]. Three overlapping stages of cellular and biochemical events happen during wound healing: inflammation within 24-48 h, cell proliferation, and ECM synthesis within 2-3 days, and remodeling within 2-3 weeks or longer [23, 24].

The effect of CoQ10 on cell viability correlated with their ROS reduction results. All conditions of CoQ10 treatment (0.1-10.0 µM) in this study resulted in a significant reduction in the intracellular ROS contents, consistent with the findings of Fasano et al. [25]. Their study demonstrated that oxidative stress was suppressed, and ROS content decreased when HaCaT keratinocytes were treated with CoQ10 solutions ranging from 0.5 to 10.0 µM. Furthermore, the results are aligned with the study by Hseu et al., which reported that CoQ10 concentrations ranging from 1.0 to 4.0 µM, administered for 0.5 to 4.0 h, reduce intracellular ROS content and activate *Nrf2* protein activity in HaCaT cells [22].

To analyze the molecular activity in ROS reduction, this study showed that a 1 µM CoQ10 treatment for 24 hours post-stress induction decreases *Nrf2* expression. The results from this study conflicted with those from Samimi et al. in animal model, in which diabetic rats were treated with 10 mg/kg/day CoQ10 for 6 weeks, resulting in a significant increase in *Nrf2* gene expression in liver tissue. However, these different results can be attributed to factors such as organism, cell type, oxidative stress induction method, CoQ10 concentration, and incubation time [26]. Thus, this finding suggests that 1 µM CoQ10 may not stimulate the *Nrf2*/ARE pathway by increasing the quantity of *Nrf2*. Similarly, the Hseu et al. study revealed that CoQ10 can promote the *Nrf2*/ARE pathway by facilitating the nuclear translocation process of the transcription factor *Nrf2* and supporting the fraction of *Nrf2* and its inhibitory protein Keap-1 in both cytosol and nuclear compartments within 4 h [22]. Nevertheless, many factors are associated with the cellular response to oxidative stress on human keratinocytes and other human cells, except for the *Nrf2*/ARE pathway. For instance, CoQ10 reduced oxidative stress by promoting PI3K/Akt signaling pathway in HaCaT keratinocytes [27] and NO-related pathways in human endothelial cells [28], or by inhibiting caspase-based mitochondria-mediated cell death pathway in human fibroblast [29]. For future research, the long-term effects of CoQ10 on ROS reduction, mediated by *Nrf2* levels, should be examined to determine whether *Nrf2* downregulation indicates sufficient intracellular antioxidant activity or to explore alternative pathways involved in ROS reduction as previously described.

To evaluate the effects of CoQ10 in an anti-aging approach, the results showed no effect on RTL in HaCaT cells treated with CoQ10 for 24, 48, and 72 h post-stress-induction. However, this finding revealed a significant decrease of RLT in stressed cells, no significant difference at 24 h-post-stress induction, and no difference in RTL at 48 and 72 h, suggesting the natural mechanism to rescue the

telomere length. Furthermore, the effects on telomere length in this study are consistent with the clinical research by Barden et al., which found that CoQ10 supplementation with n-3 FA in chronic kidney disease patients did not affect telomere length in neutrophils and peripheral blood mononuclear cells over 8 weeks [30]. Conversely, a long-term randomized clinical study conducted by Opstad et al. on the Swedish population treated with a combination of 200 mg/day CoQ10 and selenium throughout 42-month showed a significantly less shortening in leukocyte telomere length compared to the placebo group [31]. Consequently, an extensive period may be required for CoQ10 to initiate defense against telomere shortening. For further validation of telomere length analysis, including the experimental group treated by an established telomerase activator such as HeLa cell extract or TA-65 will provide more comprehensive comparisons for the degree of RTL change from the effects of CoQ10 [32].

To gain insight into the telomerase mechanism, the effects of CoQ10 on *hTERT* and *hTR* expressions were investigated. This study is the first to demonstrate that CoQ10 affects *hTERT* expression in HaCaT keratinocytes. However, the 1 µM CoQ10 treatment for 24 h significantly upregulated the *hTERT* expression without affecting *hTR* expression. Typically, *hTR* is abundant in most regular cells, resulting in *hTERT* being the limiting factor and strongly corresponding with telomerase activity [33]. However, a subgroup of *hTERT* protein is not incorporated into telomerase complexes. Various *hTERT* molecules (100-700 molecules) can interact with *hTR* at any time in cells with active telomerase [13]. The studies conducted by Cerezo et al. involved generating constitutive overexpression of *hTERT* in HaCaT cells through a retrovirus called HaCaT-TERT. Their findings revealed that *hTERT* plays various roles in HaCaT cells, including promoting telomerase activity and cell proliferation and regulating the pathway of epidermal differentiation in the terminal stage [15, 34]. Cereser consistently reported that the telomere length in HaCaT-TERT cells increased from an average of 4 kb to 6.5 kb after 5 weeks of culture [35]. *hTERT* exhibits various expression patterns depending on the cell type and cell behaviors. The *hTERT* catalytic subunit and the *hTR* template are co-substrates for telomerase synthesis with several associated proteins [15, 36]. The results suggest that only *hTERT* upregulation might not affect telomere length through the telomerase role in HaCaT cells in a short-term period. In future directions, the effect of CoQ10 on telomere-maintaining studies was recommended to vary CoQ10 doses and treatment periods and cooperate with telomerase activity measurements to determine the telomere length and expression pattern of the telomerase gene. However, this two-dimensional model is insufficient for long-term studies of cell confluence. Therefore, alternative *in vitro* models, such as three-dimensional (3D) models, are required for further investigation. For instance, a 21-day culture study of senescence-related changes in adipose-derived mesenchymal stem cells demonstrated that cell morphology, proliferation, telomere length, and telomerase activity were improved in a 3D hydrogel-based model with long-term propagation [37].

This study provides insights into CoQ10's effects while identifying areas for future exploration. Although no significant impact on RTL was observed in short-term treatments, this highlights the potential for further research to uncover long-term benefits, especially for anti-aging applications. While the short-term design has limitations, it provides a solid foundation for studying long-term cellular responses and telomere dynamics. Additionally, the observed downregulation of *Nrf2* suggests that alternative pathways may contribute to CoQ10's antioxidant effects, offering exciting opportunities to elucidate its mechanisms further and expand its therapeutic potential.

5. Conclusions

In summary, H_2O_2 was applied to raise the ROS content in HaCaT keratinocytes. One µM of CoQ10 effectively rescued cell viability and reduced H_2O_2-induced oxidative stress by decreasing intracellular ROS content. However, telomere protection for anti-aging activity remained uncertain, requiring more long-term experiments for accurate validation. Therefore, this finding enhanced the information on the potential of CoQ10 as an active ingredient in approaching anti-oxidant and anti-aging treatment, leading to additional exploration for supplementation and skin care applications.

Acknowledgments

The authors would like to acknowledge the assistance and facilitation of the Department of Botany, Faculty of Science, Chulalongkorn University.

Author Contributions

Nuchanart Suntornnont designed the methodology, performed all experiments, reviewed, discussed, and wrote the original draft. Ammaraluk Kovavisarach performed the relative telomere length experiments, reviewed and discussed. Rachaneekorn Tammachote developed the methods, reviewed, edited, supervised, and approved the final manuscript.

Funding

This research was supported by the His Royal Highness Crown Prince Maha Vajiralongkorn Fund from the Graduate School, Chulalongkorn University.

Competing Interests

The authors have declared that no competing interests exist.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: Primer sequence for gene expression by qRT-PCR.
2. Table S2: Primer sequence for relative telomere length measurement by qPCR.

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