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Original article

Protective effect of alpha lipoic acid loaded calcium citrate nanoparticles on oxidative stress-induced cellular damage in human epidermal keratinocytes

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Background: Prolonged oxidative stress leads to apoptosis of keratinocytes and, consequently, results in skin cell damage.
Objective: To investigate the *in vitro* effects of alpha lipoic acid (LA) and lipoic acid loaded calcium citrate nanoparticles (LA loaded CaCitNPs) in terms of cell protection against oxidative stress-induced cellular damage.
Methods: HaCaT cells were preincubated with and without LA and LA loaded CaCitNPs before oxidative stress induction by H_2O_2. The protective effects of both LA and LA loaded CaCitNPs on HaCaT cells were assessed by the percentage of cell viability, percentage of proliferated cells, type of cell death, and ROS generation. In addition, we evaluated the effect of LA and LA loaded CaCitNPs in regulating expression of the Sirtuin 1 *(SIRT1)* gene.
Results: Our result has revealed that both LA and LA loaded CaCitNPs have an effect on increasing cell proliferation. H_2O_2 increases ROS production and decreases *SIRT1* expression levels, whereas LA and LA loaded CaCitNPs pretreatment reverses the situation. The treatment with LA loaded CaCitNPs is more effective than LA.
Conclusion: Our study demonstrates the cytoprotective effects of LA and LA loaded CaCitNPs against oxidative stress induced human epidermal keratinocyte damage.

Keywords: Alpha lipoic acid, calcium citrate nanoparticle, cellular damage, oxidative stress.

Skin, the largest and most important protective organ in the human body, is a dynamic protective shell that prevents water evaporation and the entry of toxins. [1, 2] However, this preventive ability is reduced by the aging process of skin cells (cellular aging), which leads to inflammation of the skin, slow wound repair, and an increased risk of skin cancer. [3, 4] The aging process of skin cells is a complex process induced by a multitude of internal and external factors, such as gene mutations, hormones, ultraviolet irradiation, and pollution. [5] Skin degradation compromises the integrity of skin's structure, leading to the loss of body function. [6] Previous studies reported that excessive stimulation of reactive oxygen species (ROS), i.e. more than the cellular antioxidant protection system can balance, results in oxidative damage to cells. [7, 8] At the same time, excessive ROS accumulation can cause damage to large molecules in cells with possible outcomes of DNA changes and the signaling of apoptosis. [9 - 11] These damages are often accompanied by an increase in skin cell deaths (keratinocyte apoptosis) and result in a decreased rate of epidermal cell proliferation, a factor that can accelerate skin damage and increase the risk of skin disorders. [12]

Many studies have shown that antioxidants can maintain the structure and function of skin, as well as help slow skin's aging process by protecting skin cells from ROS related damages. [13] Therefore, various antioxidants, whether synthetic or naturally derived, have been incorporated in the formulations of cosmetic products in order to prevent wrinkles. [14, 15] However, when comparing synthetic and natural antioxidants, it was found that natural antioxidants are widely available and tend to cause fewer adverse reactions. [7] Thus, interest in the development of natural products are increasing.

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Among the alternatives, alpha lipoic acid (LA) was found a to be potent antioxidant. [16] LA is easily found in natural resources and especially abundant in spinach, cabbage, broccoli and tomatoes. [17] Many studies have shown that LA has various physiological effects, including antioxidant activity, anti-inflammatory and anti-cancer. [18 - 20] LA has the potential to resist the oxidative damage of cells, leading to reduced cell death. [21, 22] In addition, LA has been reported to increase expression of the Sirtuin 1 gene (*SIRT1*), which is a gene associated with aging at the cellular level.[23]Previous reports have shown that the increase in *SIRT1* protects cells from oxidative stress.[24] However, it was shown that the efficiency of LA uptake was lowered by oral administration.[25] Therefore, the use of an effective drug delivery system should be considered in order to increase the effectiveness of LA.

In recent years, there has been a widespread interest in the utilization of nanotechnology in drug delivery systems in order to increase treatment efficiency. Therefore, in this study, the proposition is to implement a drug delivery system to increase the efficacy of LA. There are many types of drug carriers under investigation for drug delivery systems. Among various nanoparticles, calcium nanoparticles have emerged as promising drug carriers because of their novel properties, such as availability, low toxicity, and low biodegradability.[26] Such properties make calcium citrate nanoparticles (CaCitNPs) suitable for the encapsulation and transportation of substances to active areas. Prior to this, no research has been done on the efficacy of LA loaded CaCitNPs in the reduction of ROS. Thus, in this study we aimed to investigate the effects of antioxidant activities of LA and LA loaded CaCitNPs against ROS production and cellular damage of human epidermal keratinocytes.

Materials and methods

Synthesis and characterization of LA loaded CaCitNPs

Following the standard citrate reduction procedure, 650 µl of 5 M calcium chloride ($CaCl_2$) and 375 µl of 5% lipoic acid were gently mixed for 10 min; then 2.5 ml of 1 M trisodium citrate ($Na_3C_6H_5O_7$) was added and stirred vigorously for 10 min. After the addition of 5 ml of distilled water and discarding the large particles of $Na_3C_6H_5O_7$ nanoparticles which precipitated without centrifugation, the suspension was centrifuged (2000 rpm, 5 min) and the precipitate collected. The particle sizes of LA loaded CaCitNPs was characterized by transmission electron microscope (TEM) (Hitachi High-Technologies Corporation, Tokyo, Japan) and a ZetasizerNano machine (Malvern, UK).

Cell culture

An immortalized human keratinocyte cell line (HaCaT) was purchased from American Type Culture Collection (ATCC, USA). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). The cells were maintained at 37°C in an incubator (Esco, Singapore) with 5% carbon dioxide.

Cell viability assay

To determine the cytotoxic effect of LA loaded CaCitNPs on HaCaT cells, PrestoBlue® cell viability reagent (Invitrogen, USA), which is a cell permeable resazurin-based solution, was employed. A cell viability assay was conducted in 96-well plates at a density of 1×10^4 cells/ well. After seeding and incubating overnight, the cells were treated with 1 mM H_2O_2 for a positive control and various concentrations of LA and LA loaded CaCitNPs (1, 5, 10, 25, 50, 100, 250, 500, 1000 µg/ml) for other conditions; subsequently, the cells were incubated for 48 hours. After this process, 10 µl of PrestoBlue® cell viability reagent (Invitrogen, USA) was added to each well; then, the samples were incubated for 30 minutes. Cell viability was determined by measurement of the fluorescence intensity using a Varioskan Flash microplate reader (Thermo Scientific, USA) at 530 nm excitation and 590 nm emission.

Cell death pattern analysis

For cell death pattern analysis, cells were seeded at 1×10^6 cells/well into 6-well plates. The cells were treated with LA and LA loaded CaCitNPs for 48 hours. Then, the treated cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) (BD Biosciences, USA) for 15 minutes at room temperature in the dark. Stained cells were analyzed by flow cytometry (Beckman Coulter CytoFlex, USA).

Cell proliferation assay

A cell proliferation study was performed using CellTrace™ Cell Proliferation Kits (Invitrogen, Waltham, Massachusetts, USA), according to the manufacturer's protocols. In brief, cells were seeded

at 1×10^6 cells/well into 6-well plates and incubated overnight. Before treating with LA and LA loaded CaCitNPs, the cells were stained with carboxyfluorescein succinimidyl ester (CFSE) reagent for 20 minutes at 37°C in the dark. Following CFSE staining, the cells were incubated with 10 µg/ml LA and LA loaded CaCitNPs for 48 hours at 37°C. After this process, the cells were harvested by trypsinization and washed twice with phosphate buffer saline (PBS) before flow cytometry analysis (Beckman Coulter CytoFlex, USA).

Intracellular ROS generation

To evaluate intracellular ROS levels, 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) reagent (Invitrogen, USA) was employed according to the manufacturer's instructions. Briefly, the cells were seeded into a 96- black well plate at a density of 1×10^4 cells/ well. The cells were preincubated with 10 µg/ml LA and LA loaded CaCitNPs for 48 hours. Then, the cells were incubated with DCFH-DA reagent for 30 minutes at 37°C in the dark prior to administration of H_2O_2. Fluorescence intensity was detected at 10 minutes intervals after the addition of H_2O_2 for a total duration of 60 minutes; measurements was made at 485 nm emission wavelength and 528 nm excitation wavelength using a Varioskan Flash microplate reader (Thermo Scientific, USA). Cells treated with H_2O_2 represented a positive control.

Analysis of mRNA expression

Total RNA was extracted using TRIzol reagents (invitrogen); 1 µg was converted to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, Lithuania). Quantitative Real-time PCR was performed on an ABI step-one plus using EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen, USA). Forward and reverse primer of *SIRT1* were F, 5' GCC-TCA-CAT-GCA-AGC-TCT-AGT-GAC 3'; R, 5' TTC-GAG-GAT-CTG-TGC-CAA-TCA-TAA 3'. The GAPDH gene with forward primer 5' CAT-CAC-CAT-CTT-CCA-GGA-GCG 3'and reverse primer 5' GAG-GGG-CCA-TCC-ACA-GTC-TTC 3' was used as a reference gene. Threshold cycle (Ct) values of *SIRT1* were normalized for the GAPDH and compared with a calibrator using the $2-\Delta\Delta Ct$ method.

Statistical analysis was performed using the statistical package for the Social Sciences (SPSS) version 20.0 (SPSS, Inc, Chicago, IL, USA) Data are expressed as the mean ± standard deviation (SD) Statistical differences among groups were determined using one-way analysis of variance (ANOVA). A $P < 0.05$ was considered statistically significant.

Results

Characterization of LA loaded CaCitNPs

LA loaded CaCitNPs particle size measurement was done by TEM and a Zetasizer machine. The average LA loaded CaCitNPs particle size approximately 20 nm according to TEM analysis (Figure 1A). The hydrodynamic diameter of the particles measured by a Zetasizer machine also approximately 20 nm (Figure 1B).

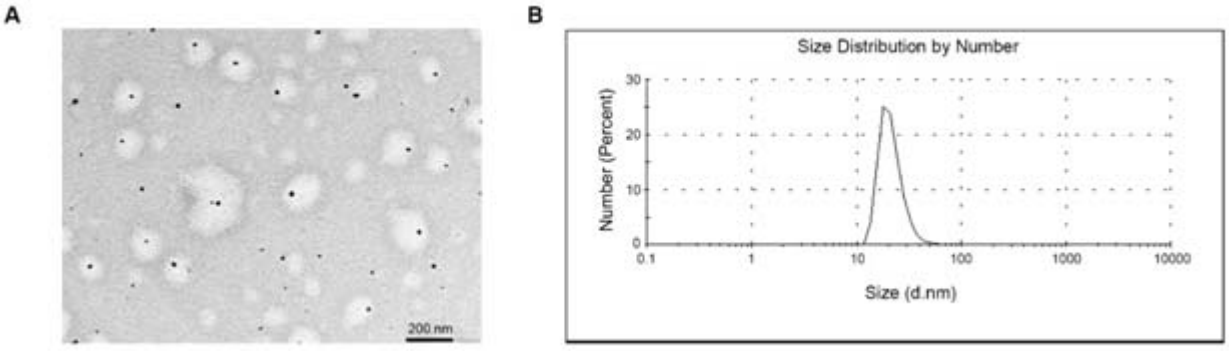

Figure 1. Nanoparticles size measurement: (A) TEM micrograph reveals the average size of LA loaded CaCitNPs (scale bar: 200 nm). (B) Hydrodynamic diameter of LA loaded CaCitNPs from a Zetasizer machine.

Cell viability

After LA loaded CaCitNPs characterization, the cytotoxic effects of LA and LA loaded CaCitNPs on HaCaT cells was determined. Cell viability was evaluated by the activity of mitochondrial dehydrogenase using a resazurin-based technique. Our results revealed that the cells exposed to both LA and LA loaded CaCitNPs at the concentrations of 1, 5, and 10 μg/ml did not sustain significant cytotoxic effects (Figure 2). In our subsequent experiments, the concentration of 10 μg/ml LA and LA loaded CaCitNPs were selected to assess protective effects against oxidative stress-induced cellular damage.

Evaluation of the cell death pattern

To confirm whether the pattern of cell death is involved, double staining of FITC-conjugated annexin V and PI was employed to investigate the patterns of cell death apoptosis and necrosis. As presented in Figure 3, flow cytometry analysis showed that 98.15% of untreated HaCaT cells were viable, whereas the H_2O_2 treated cells were found to be only 49.34% viable. At the concentration of 10 μg/ml LA and LA loaded CaCitNPs, the percentages of viable cells were 86.25% and 97.98%, respectively. LA-exposed cells had a higher percentage of both apoptotic and necrotic cells (6.01% and 7.74%, respectively) when compared to LA loaded CaCitNPs; the difference was, however, not significant.

Cell proliferation

In an attempt to evaluate cell proliferation levels after exposure to LA and LA loaded CaCitNPs, the exposed cells were stained with CFSE reagent and analysed by flow cytometry. As shown in Figure 4, H_2O_2 had a negative effect on cell proliferation, while LA and LA loaded CaCitNPs-exposed cells had significantly increased percentages of proliferated cells.

Amelioration of intracellular ROS

To explore the role of LA and LA loaded CaCitNPs in the reduction of ROS generation, we evaluated the levels of ROS production using the cell-permeant 2',7'-dichlorodihydrofluorescein dicetate (H2DCFDA) reagent. After treatment with H_2O_2, HaCaT cells exhibited a significant increase in ROS generation compared to the untreated control. However, intracellular ROS, which were induced by H_2O_2, were significantly diminished by LA and LA loaded CaCitNPs pre-treatments (Figure 5).

SIRT1 mRNA expression

To evaluate the ability of LA and LA loaded CaCitNPs in preventing ROS induced cellular aging, *SIRT1* expression, which is a promising target in slowing down the aging process, was evaluated by real-time reverse transcription-PCR. As shown in Figure 6, the levels of *SIRT1* expression in HaCaT cells exposed to H_2O_2 were decreased. Our results revealed that treatment with LA increases *SIRT1* expression levels. Moreover, LA loaded CaCitNPs treatment is shown to increase *SIRT1* expression levels to a greater extent (Figure 6).

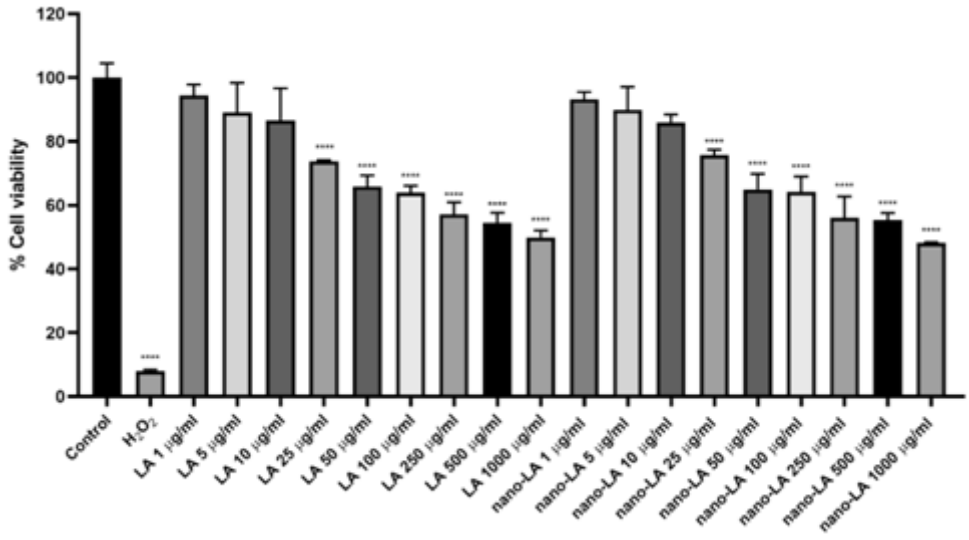

Figure 2. Effects of various concentration of LA and LA loaded CaCitNPs (1, 5, 10, 25, 50, 100, 250, 500, 1000 μg/ml) on cell viability after incubation for 48 hours.

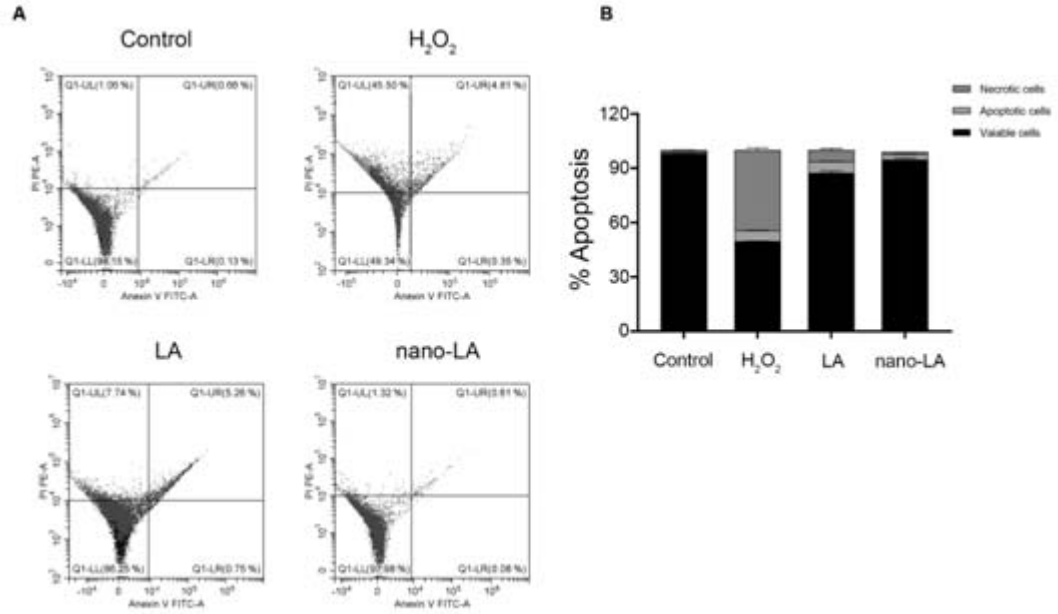

Figure 3. Death patterns of HaCaT cells treated with H_2O_2, LA, and LA loaded CaCitNPs: (A) Representative scatter plots of annexin V-FITC/PI staining and (B) quantitative analysis. Data are the average of three replicate samples.

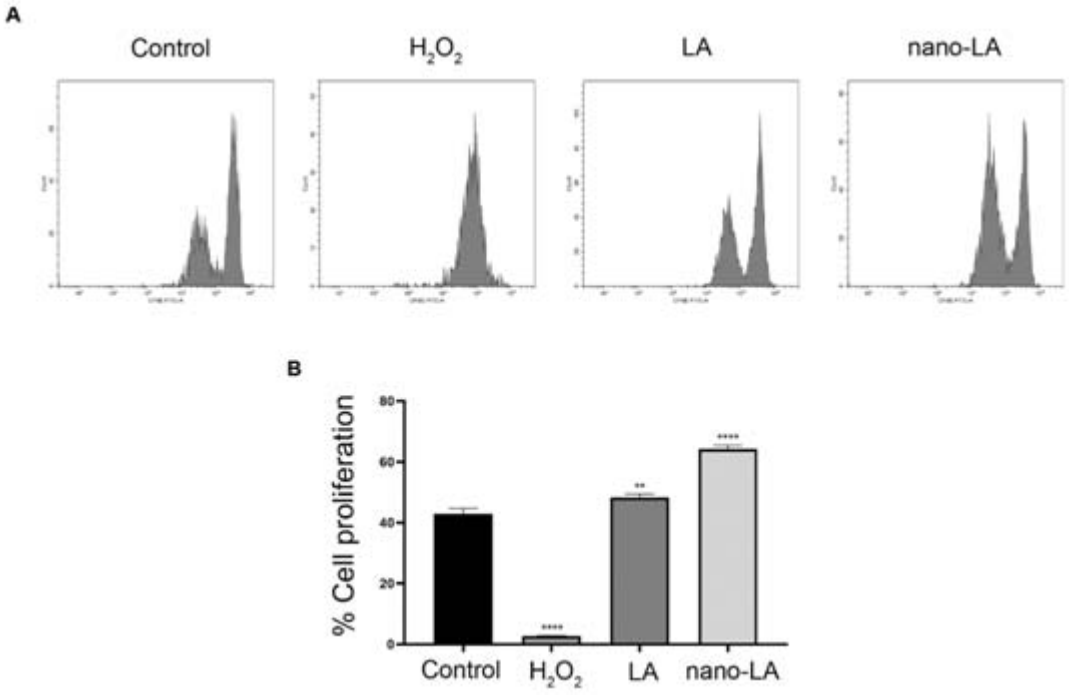

Figure 4. Cell proliferation analysis. (A) Flow cytometric analysis of HaCaT cells stained with CFSE and incubated with LA and LA loaded CaCitNPs for 48 hours after staining. (B) Quantitative analysis of three replicate samples.
(**$P < 0.001$ and **** $P < 0.0001$ compared with the control group).

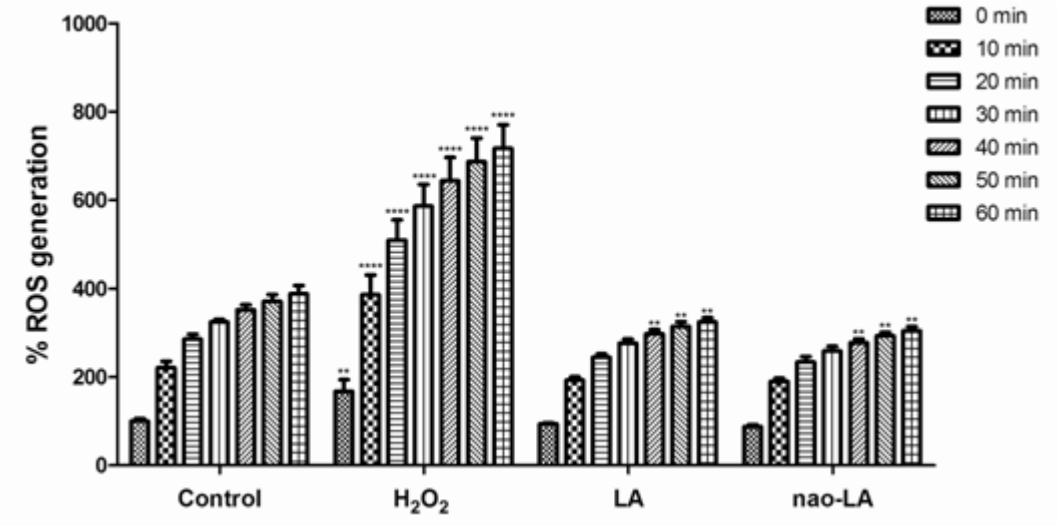

Figure 5. Percentage of ROS generation in HaCaT cells preincubated with LA and LA loaded CaCitNPs for 48 hours before administration of H_2O_2. Data are the average of three replicate samples. (**$P < 0.001$ and **** $P < 0.0001$ compared with the control group).

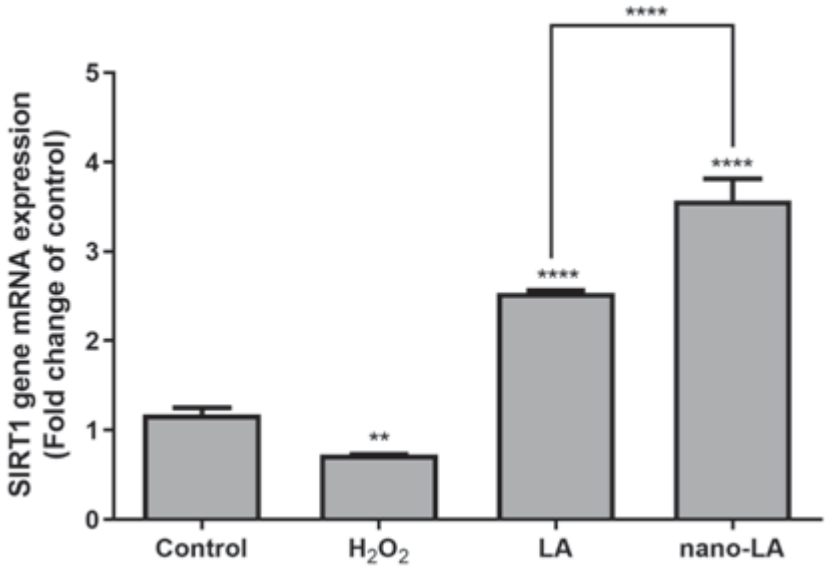

Figure 6. LA and LA loaded CaCitNPs induced the changes in *SIRT1* mRNA expression. Cells were treated with untreated control, H_2O_2, LA, and LA loaded CaCitNPs for 48 hours and processed with real time PCR.

Discussion

Numerous reports have shown that natural product-derived agents such as LA provide cytoprotective effect on oxidative stress induced skin damage due to their antioxidant capacity. [27, 28] Moreover, LA has been added to various skin products for anti-photoaging of facial skin with a low rate of side effects. [29, 30] Under normal conditions, ROS caused by oxidative phosphorylation is usually be balanced by radical scavenging systems. Production of ROS observed in keratinocytes, which were induced by H_2O_2, promotes processes related to skin aging by decreasing skin regeneration and increasing wrinkle formation. [31, 32] In this regard, we evaluated the cytoprotective effect of our LA-nanoparticle based-delivery system.

In this study, we investigated the effects of LA loaded CaCitNPs on cellular features such as cellular cytotoxicity, cell death pattern, cell proliferation, intracellular ROS generation, and gene expression in human epidermal keratinocytes. Our results revealed that H_2O_2 exposed cells had cytotoxic effects,

increased ROS generation, and decreased *SIRT1* expression levels. The administration of LA and LA loaded CaCitNPs was able to prevent oxidative stress-induced cellular aging as demonstrated by the attenuation of ROS generation and the increase of *SIRT1* expression levels. Moreover, our results have shown that the use of LA loaded CaCitNPs is more effective than the use of LA.

In addition to providing protection against cellular aging, based on our results, preincubation with LA loaded CaCitNPs before H_2O_2 treatment decreases induced ROS generation; ROS is one of the major causes of oxidative stress-induced cellular aging. [33] In this study, *SIRT1*, which is a crucial regulator in cellular defence against oxidative stress, was upregulated by LA and LA loaded CaCitNPs. This finding is in accordance with a previous study that reported LA served as a cytoprotective agent in acrylamide-induced neurotoxicity via upregulation of *SIRT1*. [34] From these findings, we infer that *SIRT1* is one of the target genes of LA loaded CaCitNPs in providing the protective effect against oxidative stress-induced cellular damage.

Efforts in the development of topical applications for LA have been considered difficult due to its chemical instability.[35] The strength of our study is that the efficacy of LA loaded CaCitNPs in preventing H_2O_2 induced cellular oxidative stress and in stimulation of *SIRT1* expression is more effective than in the LA-treated group. Although it is unclear how LA loaded CaCitNPs are more effective than LA, a previous study has shown that calcium nanoparticles have good sustained-release performance and high stability. [36] In addition, due to the slow biodegradability, accessibility, safety, and high stability of calcium nanoparticles, they have been utilized in controlled drug delivery, biosensing, and encapsulation for different types of drug. [26, 37] A previous study reported that calcium nanoparticles exhibited significantly improved gene delivery efficiency due to the enhanced cellular uptake. [38] Therefore, it is possible that the protective effects of LA loaded CaCitNPs are more potent than LA due to efficacious characteristics of calcium nanoparticles for drug delivery.

This study demonstrates the ability of LA loaded CaCitNPs in the prevention of oxidative stress induced keratinocyte damage. These findings increase our understanding of calcium nanoparticles as a carrier of lipoic acid for cellular aging prevention. However, the mechanisms involved in LA loaded CaCitNPs' reduction of ROS and the upregulation of the *SIRT1* gene are still unknown. Further studies are necessary in order to conclude the precise mechanism of LA loaded CaCitNPs in cellular aging prevention.

Conclusion

The developed LA nanoparticle-based delivery system has better therapeutic potential for the effective treatment of skin aging. Our LA loaded CaCitNPs achieved better results in the prevention of oxidative stress induced cellular damage in human epidermal keratinocytes; with efficacy improvements of LA at lower concentrations in the form of LA nanoparticle-based delivery system. An implication of this finding is that the use of LA loaded CaCitNPs may be considered a potential candidate for developing a drug for the treatment of the degeneration of skin cells which can lead to the problem of wrinkles.

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Conflict of interest

The authors, hereby, declare no conflicts of interest.

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