Original article

Anti-inflammatory effect of alpha lipoic acid loaded calcium citrate nanoparticle on human keratinocyte HaCaT cells

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Background: Alpha lipoic acid (LA) is potent anti-oxidant and anti-inflammation natural compound. To enhance the efficacy and stability of LA, LA-loaded calcium citrate nanoparticles were developed.
Objective: To investigate the anti-inflammatory effects of LA-loaded calcium citrate nanoparticles (LA-NPs) in HaCaT keratinocytes.
Methods: HaCaT cells were exposed under two inflammation stimuli conditions, lipopolysaccharide (LPS) and $Pb(NO_3)_2$, and then were treated with or without LA and LA-NPs for 24 h. Cell viability and pattern of cell death were evaluated. In addition, the expression levels of inflammatory cytokines including IL-1β, IL-6, and TNF-α as well as inflammatory mediator COX-2 were determined by real-time polymerase chain reaction.
Results: LA and LA-NPs decreased the percentage of cell death in both LPS and $Pb(NO_3)_2$-induced conditions. In LPS-induced cells, LA and LA-NPs attenuated the fold of gene expression levels in IL-1β, IL-6, TNF-α, and COX-2. Decreased expression in proinflammatory cytokines, including IL-1β and IL-6, was also observed in $Pb(NO_3)_2$-induced cells.
Conclusion: Our study demonstrated the anti-inflammatory effects of LA and LA-NPs on LPS and $Pb(NO_3)_2$-induced human keratinocytes.

Keywords: Alpha lipoic acid, calcium citrate nanoparticle, anti-inflammation.

The skin immune response is an importance mechanism to prevent irritation and injury from toxic or foreign substances such as microorganism infection, UV irradiation, pollutants exposure.[1] Although acute inflammation in skin cells is simply a protective reaction towards foreign objects and various stresses, the alteration of skin structure or function from excessive and long-term stress exposure results in chronic inflammation that may lead to development of skin diseases including cancer and premature aging.[2, 3] Owing to the structure of skin, epidermal keratinocytes plays a crucial role in receiving environmental signals and acts as initiator of inflammation through the expression of cytokines and chemokines in activating signaling cascades.[4]

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Protective and therapeutic skin drugs, including cosmeceuticals in topical application, are one of the solutions to treating inflammatory skin diseases. One example of a natural compound with potential therapeutic properties is α-Lipoic acid (LA), as it shows potent antioxidant effects and also acts as anti-inflammatory agent under various experimental conditions.[5] In *in vitro* studies, LA has been shown to attenuate the level of inflammatory cytokines, including TNF-α, IL-1β and IL-6 and level of NF-κB, in H_2O_2-treated fibroblast cells and has also demonstrated the ability to reduce inflammatory cytokine secretion in human keratinocytes.[6, 7] In *in vivo* studies, LA has shown an anti-inflammation effect on acute and chronic inflammation in rats because of its strong anti-oxidative potency.[8] Moreover, LA showed anti-inflammatory and antiapoptotic effects by reducing TNF-α, iNOS, COX-2 and caspase-3 levels in liver tissue rat hepatic injury.[9]

Due to LA's unique characteristics, it has potential to be therapeutically applied in a wide range of clinical conditions, especially to treat oxidative stress-associated diseases.[10] However, LA still possesses various limitations that inhibits its medical applicability: this includes its low stability, low cellular uptake, fast biodegradability, and low penetration capacity in topical administration at the skin.[11] Therefore, calcium citrate nanoparticles (CaCitNPs) were developed in order to enhance LA's therapeutic benefits and its control release properties. Previously, we have reported that LA-NPs demonstrate the cytoprotective property against H_2O_2– induced oxidative stress in human keratinocytes.[12] However, the anti-inflammatory properties of modified LA-NPs on cells have yet to be investigated. Thus, this study aims to investigate the anti-inflammatory response of human keratinocyte (HaCaT) cells that have been induced by two different types of inflammation trigger agents, lipopolysaccharide (LPS) and lead (II) nitrate ($Pb(NO_3)_2$), to synthesized LA-NPs.

Materials and methods

LA loaded CaCitNPs (LA-NPs) preparation

Synthesis of LA loaded CaCitNPs has been clearly explained in our previous study. Briefly, calcium chloride and lipoic acid were mixed together and then trisodium citrate was added and stirred for 10 min. Distilled water was added to discard large particles. The suspension was centrifuged in order to collect the precipitate and store it as stock in powder form. Characterization of LA loaded CaCitNPs had been demonstrated in our previous report. [12] Before usage in the experiment, fresh LA-loaded CaCitNPs were dissolved in distilled water and sonicated.

Cell cultures

Human keratinocyte cell line, HaCaT, was purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10.0% fetal bovine serum (FBS) and 1.0% antibiotics at 37 °C in 5.0% CO_2 humidified atmosphere.

Viability measurement

Viability of cells were determined by PrestoBlue™ cell viability reagent (Invitrogen, USA), and cell permeable reasazurin-based solution. After incubation, 10 µl of PrestoBlue® were added to each well and then the samples were incubated at 37°C for 30 min. Fluorescence intensity of viable cells were detected by Varioskan Flash microplate reader (Thermo Scientific, USA) at 560 nm emission and 590 nm excitation.

Cell death pattern

To determine the pattern of cell death after exposure to inflammatory conditions and treatment with LA and LA-NPs, Annexin V and propidium iodide (PI) staining were used. After 24 h of treatment, the cells were collected and resuspended in 1x phosphate buffer saline (PBS). The pellet of cells after centrifugation were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI (BD Biosiciences, USA) for 15 min at room temperature in dark place. The cells were analyzed by flow cytometry instrument (Backman Coulter CytoFlex, USA).

Inflammatory gene expression

Quantitative real-time polymerase chain reaction (PCR) was performed on ABI step-one plus using SensiFAST™ SYBR® One-Step Kit (Bioline, UK). Total RNA was converted into cDNA and target gene expression was amplified in one-step Real-Time PCR using the following conditions: reverse transcription at 45°C for 10 min, polymerase activation at 95°C for 2 min and 40 cycles of denaturation and annealing were performed at 95°C for 5 s and 60°C for 30 s, respectively. Sequence of gene-specific primers were as follows: IL-1β forward, 5'-AGA-TGA-TAA-GCC-CAC-TCT-ACA-G-3' and reverse, 5'ACA-TTC-AGC-ACA-GGA-CTC-TC-3'; IL-6 forward, 5'-GTGTGAAAGCAGCAAAGAG-3' and reverse, 5'-CTCCAAAAGACCAGTGATG-3'; TNF-α forward, 5'-TCCTTCAGACACCCTCAACC-3' and reverse, 5'-AGGCCCCAGTTTGAATTCTT-3; COX-2 forward, 5'TTCTCCTTGAAAGGACT TATGGGTAA-3' and reverse, 5'-AGAACTTGCA TTGATGGTGACTGTTT-3; GAPDH forward, CAT-CAC-CAT-CTT-CCA-GGA-GCG and reverse, GAG-GGG-CCA-TCC-ACA-GTC-TTC. Melt curve analysis was performed to confirm the specificity of the amplified product. Fold changes of gene expression were calculated using comparative Ct method ($2^{-\triangle\triangle Ct}$) and relative expression of inflammatory genes were calculated using GAPDH as a housekeeping control.

Statistical analysis

All data were analyzed using GraphPad Prism and the results were expressed as mean ± standard deviation (SD). One-way analysis of variance

(ANOVA) was used to detect significant differences between treatment groups followed by Tukey's post hoc multiple comparison test. Results were considered to be significantly different when $P < 0.05$.

Results

Cell viability

To determine the cytotoxicity of the inflammation-inducing agents and the effects of LA and LA-NPs on keratinocytes under inflammation-triggered condition, cells were exposed with 10 μg/ml LPS and 100 μg/ml $Pb(NO_3)_2$ and were treated with or without LA and LA-NPs at concentration 5 and 10 μg/ml (doses were selected based on the previous study) for 24 h (Figure 1).[12] LPS and $Pb(NO_3)_2$ slightly decreased the percentage of cell viability. However, the cells exposed with LPS and LA at 10 μg/ml showed a significant increase in percentage of viability than LPS treatment alone. Also, the cells exposed to $Pb(NO_3)_2$ with LA at 10 μg/ml and LA-NPs at 5 and 10 μg/ml showed significantly increased percentage of viability compared to $Pb(NO_3)_2$ alone.

Cell death pattern

To evaluate the effect of LA and LA-NPs on the cell death pattern of cells treated with LPS or $Pb(NO_3)_2$, annexin V-FITC/PI staining was performed using flow cytometry analysis. Cells were induced with 10 μg/ml of LPS (Figure 2A-B) or 100 μg/ml of $Pb(NO_3)_2$ (Figure 2C-D) and were treated with LA or LA-NPs 10 μg/ml for 24 h. The results showed both inflammatory-induced agents can induce necrosis in HaCaT cells as 25.2% and 29.3% of total cells in LPS and Pb treatment, respectively. On the other hands, in treatment with LA and LA-NPs 10 μg/ml with LPS can decrease percentage of necrotic cells into 11.23 and 12.24, respectively. The same results were found in $Pb(NO_3)_2$–induced group as 10.3% and 8.4% of necrotic cells in LA and LA-NPs treatment, respectively.

Gene expression level of inflammatory cytokines

Real-time PCR was performed to detect the mRNA expression level of inflammatory cytokines, including IL-1β, IL-6, TNF-α, and COX-2, in cells exposed to LPS or $Pb(NO_3)_2$ alone compared to the groups that combined treatment with LA or LA-NPs. As shown in Figure 3., LPS-induced cells demonstrated significantly increased genes fold changes of IL-6, TNF-α and COX-2 from the control. IL-1β in LPS-induced cells also demonstrated a slight increase in expression level. Combined treatment of LPS and LA attenuated the levels of IL-6, TNF- α and COX-2. LA-NPs with LPS-treated cells showed suppression of IL-1β, IL-6 and TNF-α levels. However, the expression of all inflammatory cytokines showed no statistically significant difference between LA and LA-NPs treatment groups. The same results showed in $Pb(NO_3)_2$-treated groups for expression of IL-1β and IL-6 (Figure 4 A and B).

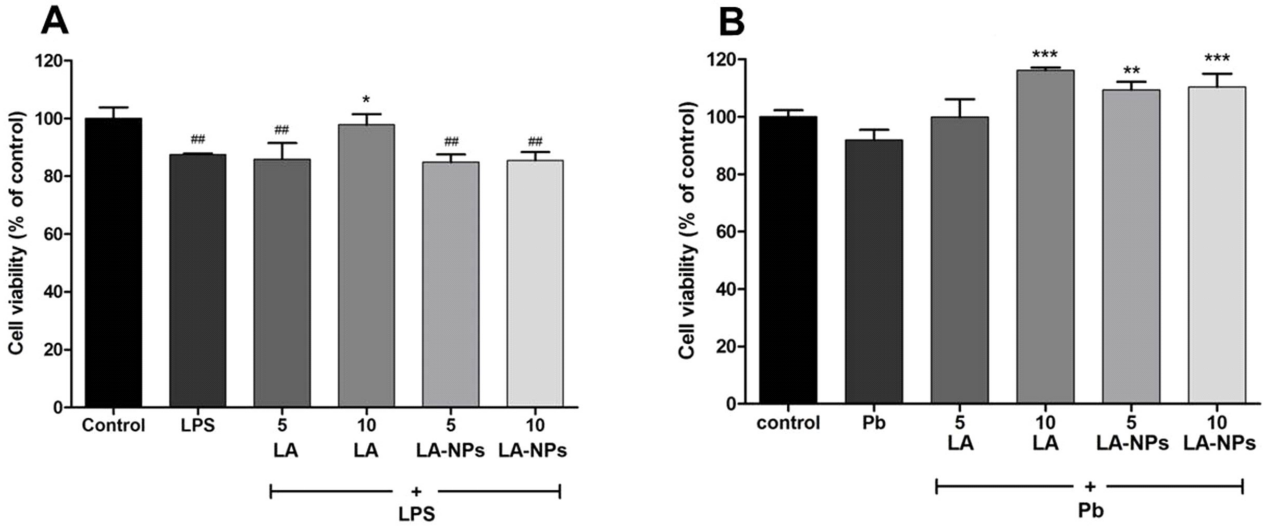

Figure 1. Effects of LA and LA-NPs on viability in HaCaT cells treated with LPS (A) and $Pb(NO_3)_2$ (B). Cells were treated with 10 and 100 μg/ml LPS and $Pb(NO_3)_2$, respectively and then treated with 5 and 10 μg/ml LA or LA-NPs for 24 h. Fluorescence of viable cells were detected. Data represent the mean value of triplicate ± SD. $^{\#\#}P < 0.01$ versus control and $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ versus LPS or Pb.

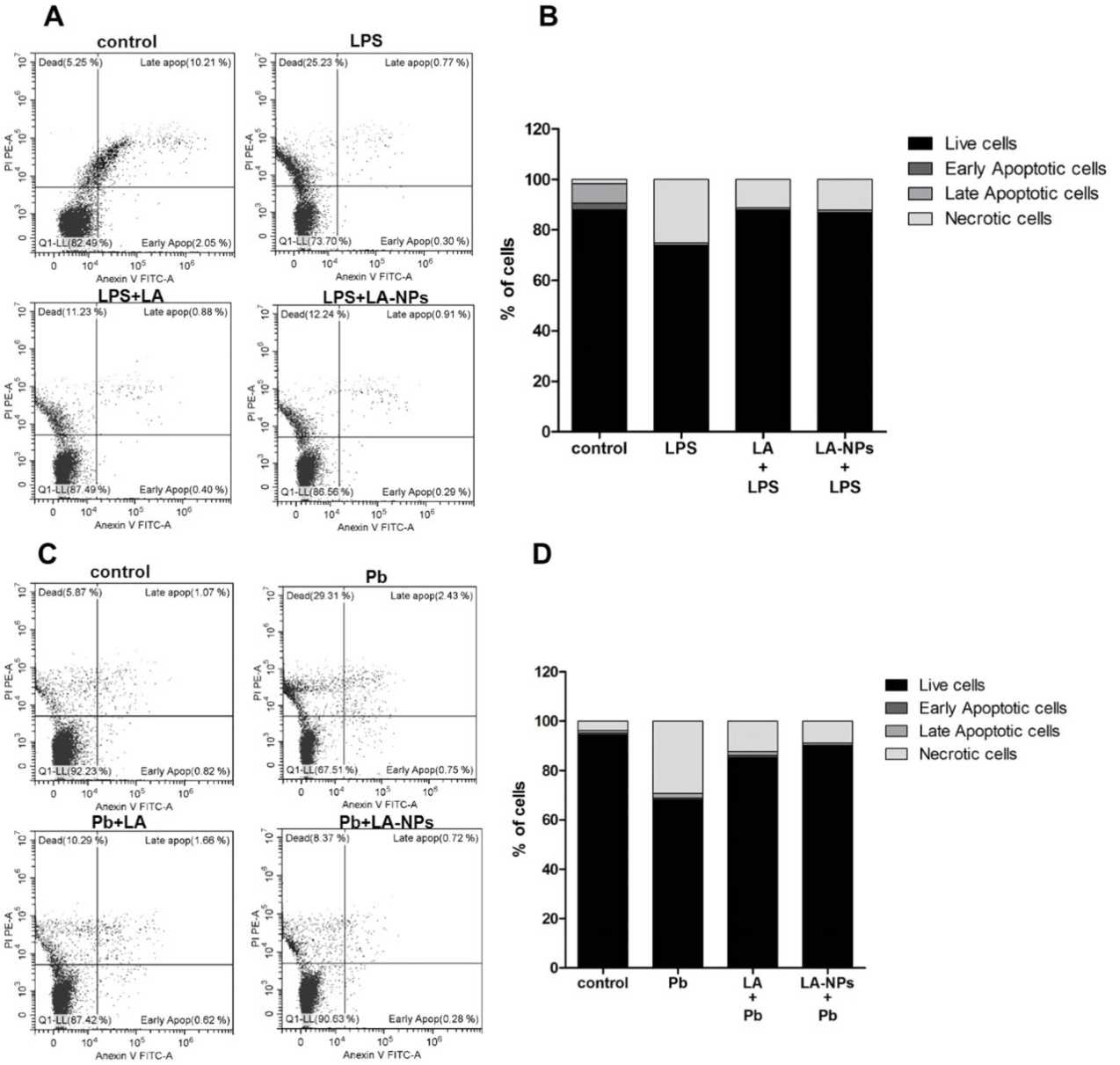

Figure 2. Effects of LA and LA-NPs on death pattern of HaCaT cells treated with LPS (A and B) and $Pb(NO_3)_2$ (C and D). Cells were treated with 10 and 100 μg/ml LPS or $Pb(NO_3)_2$, respectively and then treated with 10 μg/ml LA or LA-NPs for 24 h. Cell dead pattern were detected by annexin V-FITC/PI staining. A) and C) showed scatter plots of annexin V-FITC/PI staining. B) and D) represented quantitative analysis.

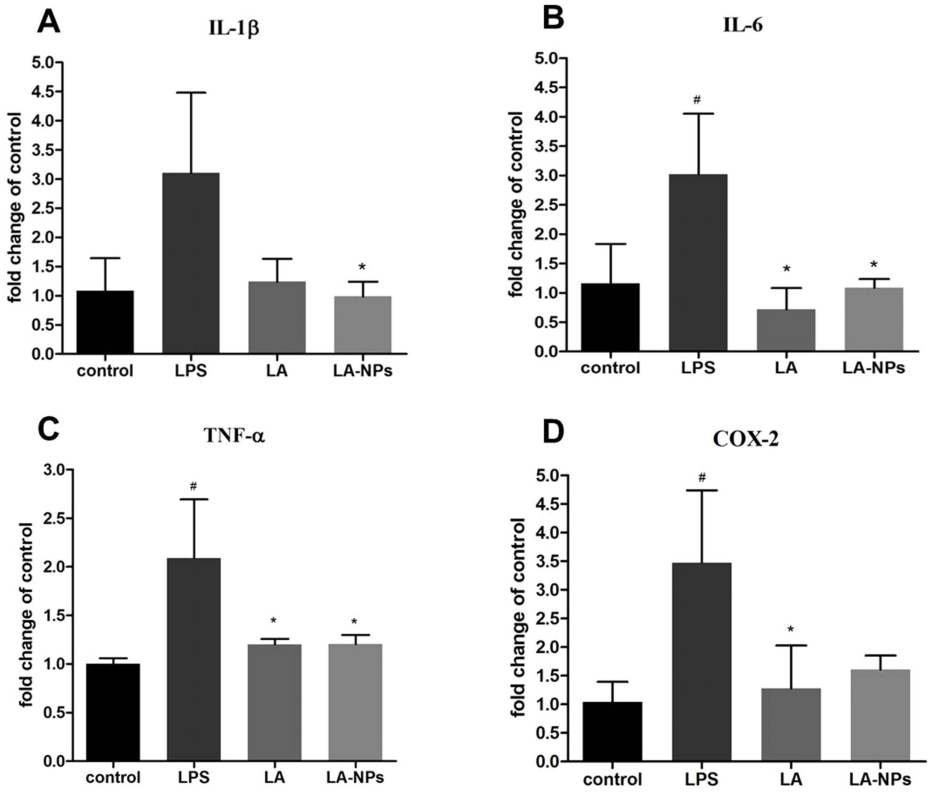

Figure 3. Effects of LA and LA-NPs on mRNA expression level of pro-inflammatory cytokine in LPS-induced HaCaT cells. Cells were treated with 10 μg/ml LPS and then treated with 10 μg/ml LA or LA-NPs for 24 h. The mRNA levels of IL-1β (A), IL-6 (B), TNF-α (C), and COX-2 (D) were detected by Real-time PCR. Data represent the mean value of triplicate ± SD. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ versus LPS.

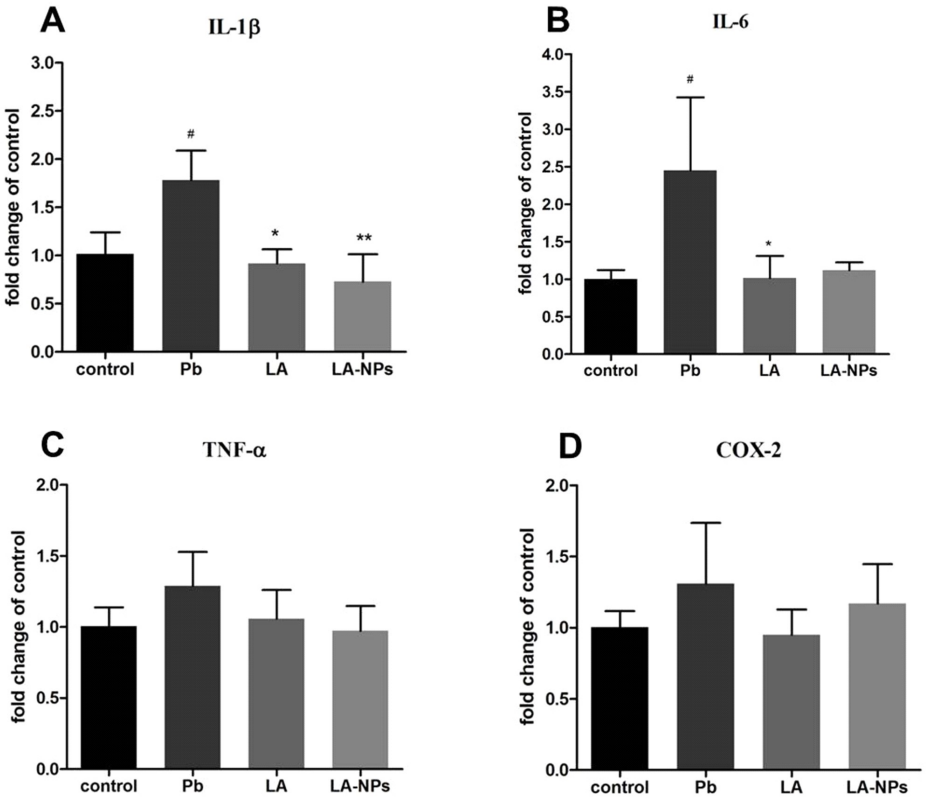

Figure 4. Effect of LA and LA-NPs on the mRNA level of pro-inflammatory cytokine in $Pb(NO_3)_2$-induced HaCaT cells. Cells were treated with 100 μg/ml $Pb(NO_3)_2$ and then treated with 10 μg/ml LA or LA-NPs for 24 h. The mRNA levels of IL-1β (A), IL-6 (B), TNF-α (C), and COX-2 (D) were detected by Real-time PCR. Data represent the mean value of triplicate ± SD. $^{\#}P < 0.05$ versus control.
$^{*}P < 0.05$ and $^{**}P < 0.01$versus Pb.

Discussion

LA is a natural compound that exhibits various potent antioxidant and anti-inflammation properties that candidates it to be used as therapeutic drug for several diseases.[13] Our research group has modified the LA drug delivery system through calcium citrate nanoparticles and have demonstrated in the previous report that LA-conjugated calcium citrate nanoparticles can better protect the keratinocyte cells from oxidative stress induced agents than LA alone.[12] In this study, we showed the anti-inflammatory effect of LA-NPs under LPS- and $Pb(NO_3)_2$ induced conditions.

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, has been demonstrated to be a key molecule in triggering an immune response through the activation proinflammatory cytokines and chemokines.[14] Prolonged exposure to LPS can lead to cell injury and necrosis.[15] A previous study has revealed that LPS can activate proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, in keratinocyte cells.[16] COX-2, a prostaglandin-endoperoxide synthase (PTGS), is readily induced in response to inflammatory stimuli, including LPS. Since the critical role of COX-2 in mediating inflammatory processes and involvement in pathogenesis has been verified in skin model, it is determined as anti-inflammatory-targeted molecule.[17] Therefore, LPS were used in this study as the stimuli to construct *in vitro* cell skin inflammation model. In this study, we found that LA-NPs and LA can inhibit expression of IL-1β, IL-6, TNF-α, and COX-2 in LPS-induced HaCaT cells along with improve percentage of cell death. Protective effects of LA in LPS-induced models have been reported. [14, 18, 19] In addition, LA has been shown ability in decrease IL-1β, IL-6, TNF-α level in H_2O_2-induced in rat embryonic fibroblast cells and suppress the expression of IL-1β, IL-6, TNF-α, and COX-2 in LPS-induced kidney cells. [6, 20] A previous study of LPS-induced monocytes demonstrated that LA inhibit the effect of LPS by activating the PI3K/Akt pathway.[21] To investigate effect of LA-NPs on other toxic substances, lead (II) nitrate ($Pb(NO_3)_2$) was selected to stimulate an inflammatory response of an environment pollutant, as the toxic effects of Pb^{2+} are associated with inflammatory diseases.[22] The results of several studies have clearly confirmed that Pb plays a crucial role in development of inflammation by acting on the level of gene expression and the production of proinflammatory proteins.[23] In our study, 100 μg/ml of $Pb(NO_3)_2$ showed an increased percentage of cell death and increased expression levels of IL-1β and IL-6 proinflammatory genes. As expected, LA-NPs and LA can suppress both IL-1β and IL-6 expression and improve the level of viability cells. As a result of this study, LA-NPs have showed potential in attenuating the expression of proinflammatory cytokines in both LPS and $Pb(NO_3)_2$-induced keratinocyte cells.

LA-NPs and LA demonstrated the same strength of anti-inflammatory effect in LPS and $Pb(NO_3)_2$-induced HaCaT cells. However, as the drug loading capacity of LA-NPs limits the amount of LA encapsulated into calcium citrate, the exact amount of LA in LA-NPs must be less than weighted. The previous study has been revealed that calcium nanoparticles can improve the efficiency of drug delivery by enhancing cellular uptake.[26] Moreover, drugs or bioactive proteins in calcium nanoparticles system have reported good sustained-release performance and high stability.[25] Therefore, the equal anti-inflammatory effect of LA and LA-NPs, which is composed of a smaller amount of LA, may be due to calcium nanoparticles' beneficial properties.

In the present study, we demonstrated the anti-inflammatory effect of LA-NPs on keratinocyte cells via attenuated expression of associated inflammatory cytokines in both LPS and $Pb(NO_3)_2$-induced models. These findings provide support for the effect of modified LA on inflammatory responses of skin cells. However, the mechanisms of LA in nanoparticles-based delivery form and the specific properties of calcium nanoparticles that encourage LA-NP's effect on keratinocyte cells are still unknown and needs to be clarified in further research.

Conclusion

Modified LA-loaded calcium citrate nanoparticles demonstrated an anti-inflammatory effect that was same as LA in different toxic substance-induced inflammation conditions in skin cells models. However, the efficacy of controlled release system of nanoparticles-based should be investigated and stability of LA with carrier must be verified in future studies.

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Conflict of interest

The authors report no conflicts of interest.

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