JARB
Journal of Animal Reproduction and Biotechnology

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Original Article

CDDO-Me alleviates oxidative stress in human mesenchymal stem cells

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ABSTRACT   Mesenchymal stem cells (MSCs) have been recognized as a therapeutic tool for various diseases due to its unique ability for tissue regeneration and immune regulation. However, poor survival during *in vitro* expansion and after being administrated *in vivo* limits its clinical uses. Accordingly, protocols for enhancing cell survivability is critical for establishing an efficient cell therapy is needed. CDDO-Me is a synthetic C-28 methyl ester of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, which is known to stimulate nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway. Herein, report that CDDO-Me promoted the proliferation of MSCs and increased colony forming units (CFU) numbers. No alteration in differentiation into tri-lineage mesodermal cells was found after CDDO-Me treatment. We observed that CDDO-Me treatment reduced the cell death induced by oxidative stress, demonstrated by the augment in the expression of Nrf2-downstream genes. Lastly, CDDO-Me led to the nuclear translocation of NRF2. Our data indicate that CDDO-Me can enhance the functionality of MSCs by stimulating cell survival and increasing viability under oxidative stress.

Keywords: CDDO-Me, mesenchymal stem cell, oxidative stress

INTRODUCTION

Mesenchymal stem cells (MSCs) have been used as a novel tool for treating various diseases due to their immune-regulatory and tissue-regenerative potential (Leyendecker et al., 2018; Xia et al., 2018). Additionally, they are relatively immunologically tolerable, which makes them immunologically inactive upon being administrated *in vivo* (Fitzsimmons et al., 2018). Despite their unique usefulness, the therapeutic potential has not been consistent, and sometimes no improvement can be seen (Holan et al., 2016; Lohan et al., 2017).

Given these difficulties, studies have shown that various chemical, physical, and genetic modification have improved the trophic effects of MSCs, e.g., adhesion, migration, survival, while limiting the senescence during an extended culture period (Jo et al., 2019; Jeon and Rho, 2020; Ocansey et al., 2020). Specifically, unfavorable microenvironment upon being cultured *in vitro* and being administrated *in vivo* precludes the clinical uses of cell-based therapeutic products (Li et al., 2019).

2-Cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid methyl ester (CDDO-Me), an analogue of oleanolic acid, the first orally available NRF2 activator, has been known to inhibit inflammation, tumorigenesis, neural cell damage, and oxidative damage (Kim et al., 2019). Importantly, it was demonstrated that CDDO-Me stimulated the Nrf2 pathway, as shown by an up-regulation in NQO1 in while

blood cells (Hong et al., 2012) Another study demonstrated that CDDO-Me has potential to reduce inflammation as well as oxidative stress, thus reducing serum creatinine level in chronic renal failure (Pergola et al., 2011; Ruiz et al., 2013).

Herein, we investigated whether CDDO-Me can reduce the oxidative stress in MSCs under oxidative stress.

MATERIALS AND METHODS

Experimental design

We first investigated whether the growth of MSCs can be promoted by CDDO-Me. Next, we ascertained whether the basic characteristics of human MSCs remain unaltered upon CDDO-Me treatment. Subsequently, the survival of MSCs under oxidative stress in the presence or absence of CDDO-Me was compared. Finally, the nuclear translocation of NRF2, an ant-oxidative transcription factor, was examined in the MSCs after being treated with or without CDDO-Me.

Culturing human umbilical cord-derived MSCs

Human umbilical cord tissue-derived mesenchymal stem cells (MSCs) was purchased from ATCC (Manassas, VA). MSCs were cultured in MEM-α (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS, atlas Biologicals, Fort Collins, USA), antibiotics-antimycotics (Genedirex, Taoyuan, Taiwan). CDDO-Me was purchased from Sigma-Aldrich (St Louis, MO). 50 nM of CDDO-Me was used.

Cell proliferation assay

Cell proliferation was assessed by Cell Counting Kit-8 (CCK-8; Dojindo). MSCs were seeded into the 96-well plates prior to analysis. 10 μL of the CCK-8 solution was added to each well. After 3 hours of incubation, the absorbance was measured at 450 nm using a microplate reader.

Characterization of MSCs

MSCs were incubated at 4℃ for 1 h with the following specific primary antibodies; CD34 Mouse anti-Human (Invitrogen), PE Mouse Anti-Human CD73 (BD Pharmingen), CD29 antibody (BioLegend), HLD-DR (Santa Cruz Biotechnology). After binding, the cells were washed three times in 1mL of PBS containing 4% FBS. For secondary

Abs, goat anti-mouse IgG H&L Dylight 488 was used. Data was acquired by the BD FACS Canto II Cytometer and FACS DIVA software (Ver6.1.3, BD Bioscience, Franklin Lakes, NJ, USA). Protocols for the differentiation of MSCs was based previous studies (Moon et al., 2018; Lee et al., 2019). In brief, cells were seeded in 4-well plates (SPL, Korea) and cultured for 2 weeks using StemPro chondrogenesis or osteogenesis differentiation medium (Thermo Fisher Scientific, Waltham, MA, USA). The differentiated cells were then stained with Alcian Blue or Alizarin Red staining kit (Lifeline Cell Technology, Frederick, MD, USA). For quantification, cells were incubated overnight in 0.1N HCl containing 0.1% Alcian blue, and rinsed three times in distilled water. After extraction using 200 μL of 0.1 N HCl, and absorbance was measured at 620. For quantifying Alizarin red, 10% acetic acid was added, and the supernatant was collected. After washing with 10% acetic acid, they were collected again. After adding 250 μL of mineral oil to the tube, the samples were incubated at 85℃ for 10 min, followed by being incubated in ice. After spinning-down, 250 μL of supernatant was mixed with 100 μL of NH_4Cl, and the absorbance was measured at 405. For colony-forming unit (CFU) assay, MSCs and FD-MSC (2,400/well) were plated in 6-well plate. After fourteen days, colonies were stained with crystal violet. For quantification, dye was eluted using acetic acid and the absorbance was measured at 590nm using microplate reader (TECAN, Mannedorf, Switzerland).

Examining the function of CDDO-Me in MSCs under oxidative stress

MSCs were seed on 6-well plates and cultured overnight. Next day, 200 or 300 μM of hydrogen peroxide (H_2O_2) (Daejung, Korea) was used to treat the cells for 12 or 24 h with or without CDDO-Me (50 nM). Cell viability was analyzed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

qRT-PCR

Total RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA, USA). A total 2 μg of RNA was used for input, and cDNA was synthesized using cDNA synthesis Kit (Philekorea, Daejeon-si, Korea). qRT-PCR was conducted using the Accupower 2X GreenStar qPCR Master Mix (Bioneer, Korea) in CFX96 Touch Real-Time PCR Detection

System (Bio RAD, Hercules, California). After the expression of each gene was normalized against Gapdh, and the relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Immunocytochemistry

In a Poly-D-Lysine-coated 8-well chamber slide (SPL Lifesciences, Pochun, Korea), MSCs (five thousand cells) were seeded and incubated overnight. Cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 10 min. After being incubated for 10 min with PBS containing 0.1% Triton X-100, cells were washed 3 times with cold PBS. The cells were then blocked using 1% BSA in PBST (PBS + 0.1% Tween20) for 30 min. After incubated with primary antibodies overnight, cells were washed 3 times. After being bound with secondary antibody for 1 hour, cells were washed three times with PBS. Before analysis, cells were

stained with 0.1 μg/mL DAPI for 30 seconds. All images were analyzed and obtained using a confocal microscope (Leica TCS SP8 STED, Wetazlar, Germany).

Statistical analysis

Statistical analysis was performed using analysis of variation (ANOVA). Where statistical significance was found, an unpaired Student's t-test was conducted between two groups. All analysis was performed by using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). Significance was defined as $p < 0.05$.

RESULTS

The effect of CDDO-Me on the proliferation of MSCs

As shown in Fig. 1A, CDDO-Me-treated MSCs had an increased number of CFUs compared with non-treated
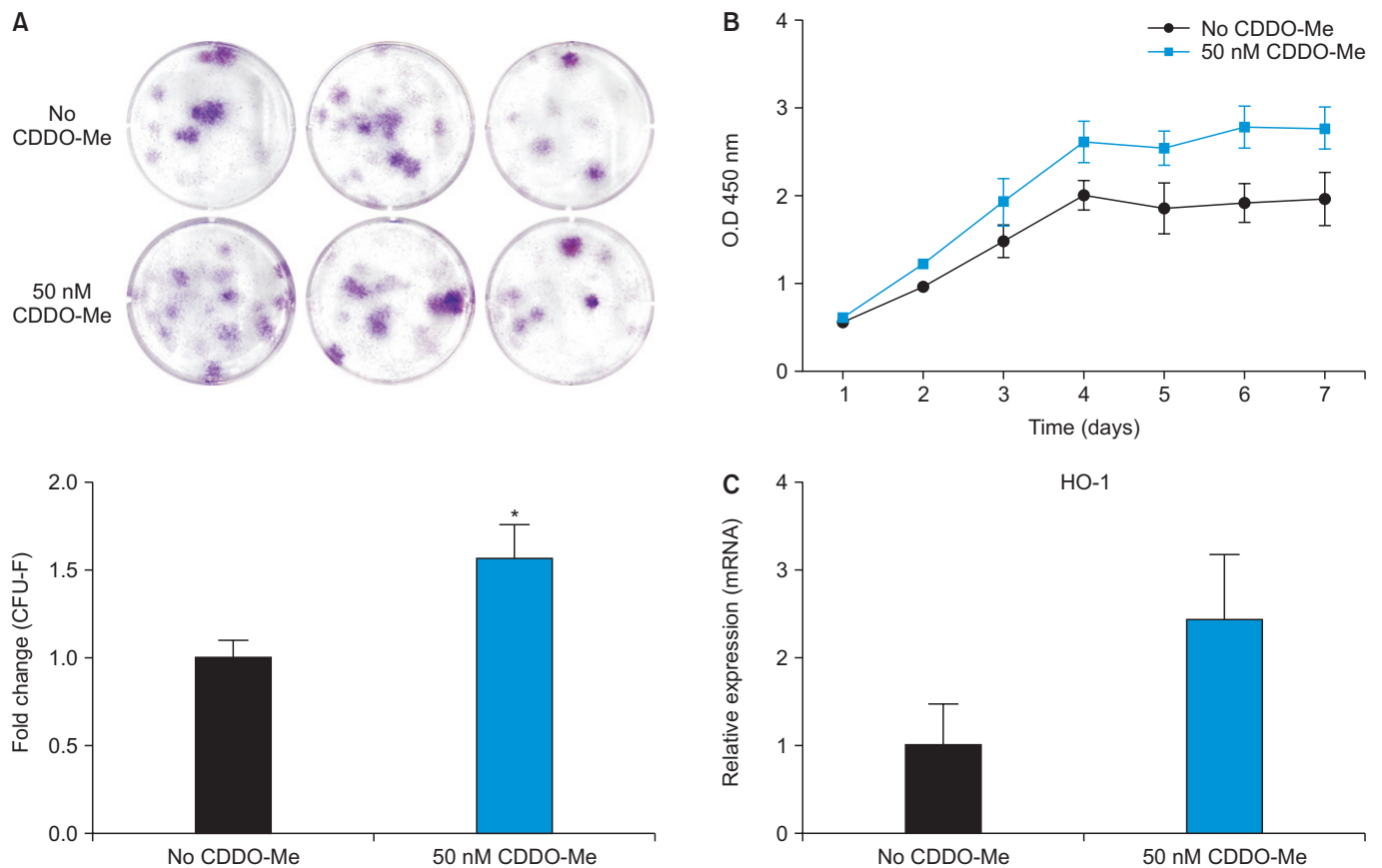
Fig. 1. The growth kinetics of CDDO-Me-treated MSCs. (A) The colony-forming unit (CFU) ability was analyzed by measuring the optical density (OD_{595}). (B) Growth kinetics of MSCs treated with 50 nM of CDDO-Me for a week. The optical density (OD_{450}) was measured for calculating the growth of MSCs. (C) Expression analysis of HO-1 in MSCs treated with or without CDDO-Me. Expression was normalized as those of GAPDH. All data are presented as the mean ± SEM. *$p < 0.05$ compared to untreated MSCs.

control cells (Fig. 1B). Also, CDDO-Me led to an increased growth of MSCs (Fig. 1C). Also, qRT-PCR analysis revealed that the expression of HO-1 was up-regulated by CDDO-Me in MSCs, compared with non-treated control cells.

Characterization of MSCs treated with CDDO-Me

Flow cytometric analysis indicated that treatment of CDDO-Me did not alter the expression of MSC cell surface markers, as shown by positive activity against CD73, 29 in both cell types. Similarly, CDDO-Me treatment did not induce the expression of negative markers CD34 and HLA-DR (Fig. 2A). No difference was found in osteogenic differentiation between cells treated with or without CDDO-Me (Fig. 2B).

Nuclear translocation of NRF2 by CDDO-Me

Confocal analysis showed that NRF2 expression was detected exclusively in the cytoplasm of MSC in the absence of CDDO-Me (Fig. 3). In contrast, CDDO-Me treatment (50 nM) for 24 hours led to the translocation of NRF2 protein into the nucleus of MSCs.

The effect of CDDO-Me on the survival of MSCs under oxidative stress

As shown in Fig. 4, pre-treatment of MSCs with CDDO-Me potently increased the survival of MSCs undergoing oxidative cell damage. No change was observed when MSCs were treated with CDDO under oxidative stress.

DISCUSSION

This study aims to investigate whether CDDO-Me, a triterpene analogue of oleanolic acid, can promote anti-oxidative role in MSCs. CDDO-Me has been used for treatment of inflammation, cancer and chronic kidney diseases (Wang et al., 2014). Specifically, this drug has been known as efficient inhibitor of the production of inflammatory enzymes, e.g., cyclooxygenase-2 and inducible nitric oxide synthase (Honda, 2000; Liby et al., 2007).

Also, CDDO-Me contributes to activation of Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1/nuclear factor erythroid 2-related factor 2/antioxidant response element (Keap1/Nrf2/ARE) axis, contributing to cell protection under abundant electro-
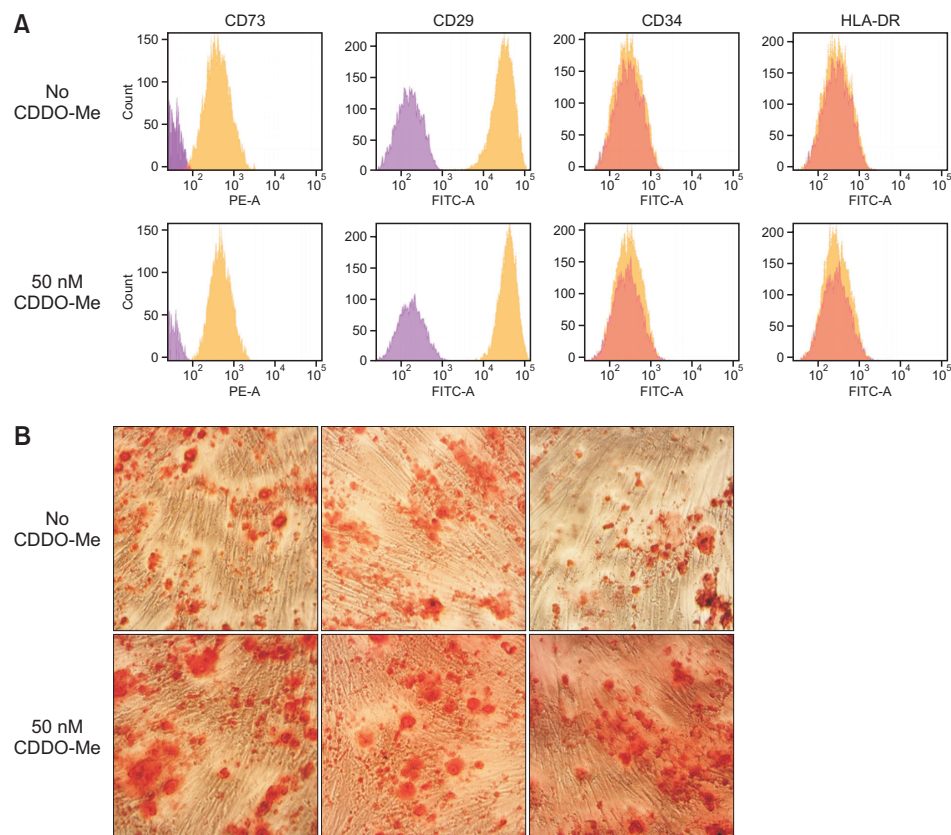
Fig. 2. Characterization of MSCs treated with CDDO-Me. (A) Flow cytometry analyses of MSCs. (B) Differentiation potential of MSCs treated with CDDO-Me. Cells were differentiated into osteogenic lineages for 10 days.

| DAPI | Nrf2 | Merge |
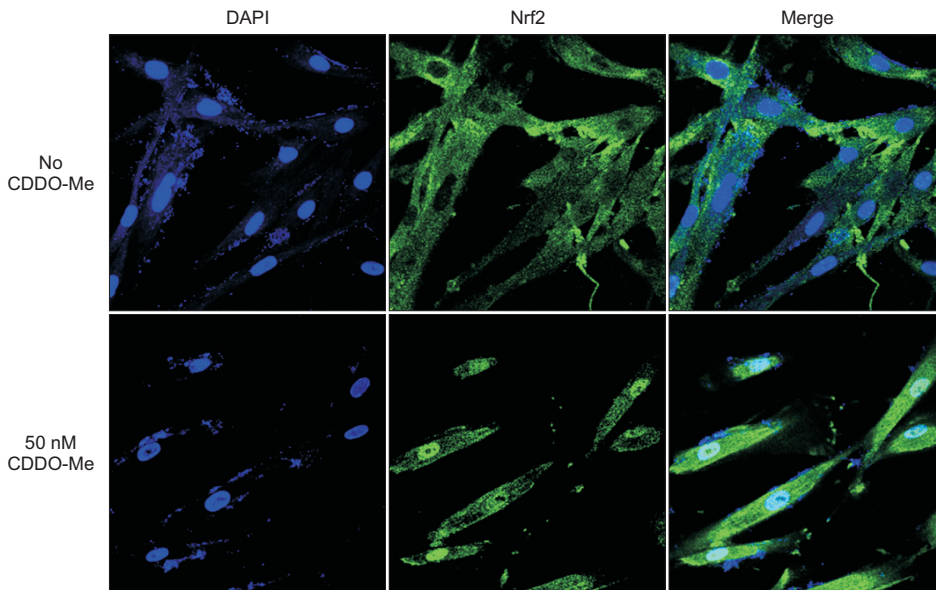
Fig. 3. Confocal analysis of nuclear translocation of NRF2 in MSCs. MSCs were treated with or without CDDO-Me for 24 hours, fixed, and stained with anti-NRF2 antibody.
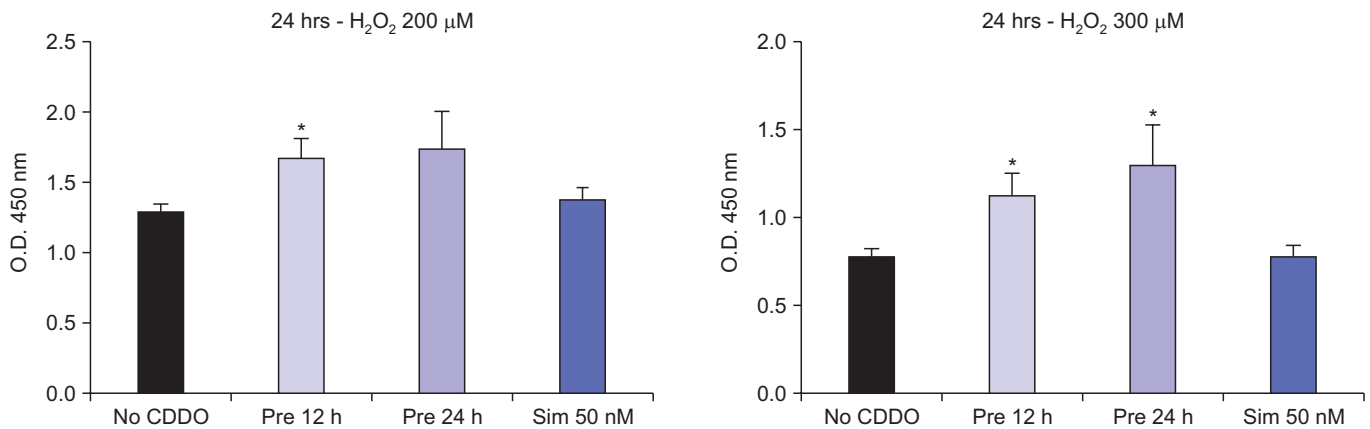
Fig. 4. The effect of CDDO-Me on the survival of MSCs under oxidative cell stress. MSCs were cultured with or without CDDO-Me for 12 or 24 hours prior to being exposed to hydrogen peroxide, or cultured simultaneously with or without CDDO under 200 or 300 μM of hydrogen peroxide for 12 or 24 hours (Sim 50 nM). The optical density (OD$_{450}$) was measured for calculating the survival of MSCs. All data are presented as the mean ± SEM. *$p < 0.05$ compared to untreated MSCs.

philes or oxidative stress (Dinkova-Kostova et al., 2005; Yates et al., 2007).

We found that 50 nM of CDDO-Me did not alter the expression of MSC cell surface markers, while stimulated the growth and CFU-forming ability. No difference was found in osteogenic differentiation of MSCs treated with CDDO-Me. CDDO-Me up-regulated the expression of HO-1. Also, nuclear translocation of NRF2 was detected under confocal imaging analysis. Based on these results, we suggest that CDDO-Me functions as a positive regulator of anti-oxidative role in MSCs, increasing cell survivability under oxidative stress.

Studies have demonstrated that the function or survival of MSCs can be improved by various strategies. Most commonly, culture protocols have been optimized for MSCs. Other methods include drug treatment, gene engineering, for promoting their survival and migration toward lesion (Noronha et al., 2019). Based on our results, utilizing CD-DO-Me may become a useful, simple, and potent protocol for culturing MSCs. In addition, CDDO-Me are now being used clinically, making this drug readily applicable for clinical use. Also, its signaling mechanism is well-defined (Ahmad et al., 2006; Borella et al., 2019; Kim et al., 2019).

The role of CDDO-Me in mammalian cell is manifested by a cytoprotective strategy to reduce cell damage induced by abrupt increase of ROS (Wu et al., 2011). Spe-

cifically, the ARE downstream of NRF2 activation may have contributed this role, since ARE functions as an efficient antioxidant, leading to cellular balance (Alam et al., 1999; Loboda et al., 2016). As an ARE-regulated phase II detoxifying enzyme, HO-1 is a detox enzyme under control of ARE, making NRF2/HO-1 axis an efficient antioxidative mechanism (Alam et al., 1999). Consistently, our study showed that NRF2 was exclusively found in nucleus after CDDO-Me treatment. It would be needed to analyze whether the expression of other antioxidant target genes are increased.

CONCLUSION

CDDO-Me improves the functionality of MSCs by increasing viability and protecting against oxidative damages.

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