

# Inhibition of Ultraviolet B-induced Apoptosis in Fibroblasts by Human Umbilical Cord Blood Mesenchymal Stem Cell Conditioned Media via the Phosphatidylinositol-3-Kinase/Akt Pathway

Ferry Sandra<sup>1</sup>, PhD; Dian Andriani Ratna Dewi<sup>2</sup>, PhD; I Nyoman Mantik Astawa<sup>3</sup>, PhD; Wimpie Pangkahila<sup>4</sup>, PhD; Angliana Chouw<sup>5</sup>, PhD; Maria Celinna<sup>6</sup>, BSc; Nurrani Mustika Dewi<sup>6</sup>, MPharm

<sup>1</sup>Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia;

<sup>2</sup>Gatot Soebroto Army Hospital, Jakarta, Indonesia;

<sup>3</sup>Faculty of Veterinary Medicine, Universitas Udayana, Denpasar, Indonesia;

<sup>4</sup>Department of Andrology and Sexology, Faculty of Medicine, Universitas Udayana, Denpasar, Indonesia;

<sup>5</sup>PT Prodia StemCell Indonesia, Jakarta, Indonesia;

<sup>6</sup>The Prodia Education and Research Institute, Jakarta, Indonesia

## Correspondence:

Ferry Sandra, PhD;  
Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Postal code: 11440, Jakarta, Indonesia

**Email:** ferry@trisakti.ac.id

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## What's Known

- Ultraviolet B (UVB) irradiation induces photoaging and apoptosis in cells.
- Conditioned media (CM) obtained from mesenchymal stem cells (MSCs) contain various potential growth factors.

## What's New

- CM obtained from human umbilical cord blood (hUCB)-MSC (CM-hUCB-MSC) inhibits UVB-induced apoptosis in fibroblasts.
- CM-hUCB-MSC induces Akt phosphorylation of UVB-irradiated fibroblasts.

## Abstract

**Background:** Ultraviolet B (UVB) irradiation induces photoaging and apoptosis in various cell types. Inhibition of UVB-induced apoptotic pathways has been explored in different apoptotic cascades. Conditioned media from human umbilical cord blood mesenchymal stem cells (CM-hUCB-MSC) contain important substances for cell regeneration. However, the potential of CM-hUCB-MSC in preventing UVB-induced apoptosis has not been clearly elucidated. Therefore, the current research was conducted to investigate the potential of CM-hUCB-MSC in inhibiting UVB-induced apoptosis and its role in the antiapoptotic signaling pathway.

**Methods:** An experimental *in vitro* study was conducted at PT. Prodia StemCell Indonesia, Jakarta, Indonesia, 2019-2022. Initially, hUCB-MSCs were isolated and cultured to produce CM-hUCB-MSC. NIH3T3 cells were pretreated with/without 50  $\mu$ M LY294002, treated with/without 10% CM-hUCB-MSC, and then irradiated with/without UVB. Subsequently, the cells were analyzed using sub-G1, immunofluorescence, and immunoblotting assays. One-way analysis of variance (ANOVA) was used for data analysis, followed by Tukey's honest significant difference (HSD) test or the Kruskal-Wallis test, followed by the Dunn-Bonferroni test using IBM SPSS Statistics software version 21. Statistical significance was determined at  $P < 0.05$ .

**Results:** CM-hUCB-MSC significantly inhibited UVB-induced apoptosis in NIH3T3 cells ( $P = 0.002$ , Dunn-Bonferroni test). CM-hUCB-MSC significantly induced Akt phosphorylation at Ser 473 in UVB-irradiated NIH3T3 cells ( $P < 0.001$ , Tukey's HSD test). The CM-hUCB-MSC-induced phosphorylation of Akt was significantly inhibited by LY294002 ( $P < 0.001$ , Tukey's HSD test).

**Conclusion:** Taken together, it can be concluded that CM-hUCB-MSC inhibits UVB-induced NIH3T3 cell apoptosis via the activation of phosphatidylinositol-3-kinase (PI3K)/Akt signaling cascades.

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## Introduction

Ultraviolet (UV) irradiation induces photoaging<sup>1,2</sup> and apoptosis in various cell types, including skin cells.<sup>3-6</sup> Some cellular mechanisms are involved in UVB-mediated apoptosis, including deoxyribonucleic acid (DNA) damage and activation of cell death receptors. DNA damage is commonly detected among numerous UVB effects because DNA is the major chromophore for UVB. Meanwhile, UV could directly activate death receptors bound to the cell membrane.<sup>3,7</sup>

UVB could induce both extrinsic and intrinsic apoptotic pathways.<sup>8</sup> The extrinsic apoptotic pathway involves tumor necrosis factor receptor type 1-associated death domain (TRADD) and Fas-associated death domain (FADD), which are activated by the death receptors. Meanwhile, the intrinsic apoptotic pathway depends on mitochondrial damage and cytochrome c release and is regulated by a collection of antiapoptotic and proapoptotic proteins, which are the members of the B-cell lymphoma 2 (Bcl-2) family.<sup>9</sup> Akt, which is also known as protein kinase B (PKB), plays a key role in controlling the function of Bcl-2 family members. For example, the proapoptotic protein Bcl-2 associated agonist of cell death (Bad) binds to and inhibits the antiapoptotic effects of Bcl-2. In the presence of survival factors, Bad is phosphorylated by Akt, thereby inactivating it, and inducing the release of active Bcl-2.<sup>10, 11</sup>

Skin damage caused by UVB-induced apoptosis can be prevented by using growth factors, such as plasma rich in growth factors (PRGF), which possess regenerative effects to reduce UVB-induced photooxidative stress and prevent fibroblast damage *in vitro*.<sup>12, 13</sup> Recently, mesenchymal stem cell (MSC) therapy are also used to promote skin regeneration and rejuvenation. Furthermore, MSC administration not only helps to repair and regenerate UVB-damaged skin but also potentially protects the skin from UVB-induced photoaging. MSCs could prevent skin photoaging by inhibiting matrix metalloproteinases (MMPs) activity, increasing collagen production, attenuating oxidative stress, promoting dermal fibroblast proliferation, and reducing apoptosis.<sup>14, 15</sup> The mechanism underlying these protective roles of MSCs on skin photoaging is thought to be the paracrine secretion of extracellular vesicles and a wide range of soluble factors, including various growth factors, cytokines, and chemokines, which are collectively defined as secretome.<sup>15, 16</sup> MSC culture media containing secretome are referred to as conditioned media (CM). Because CM is potent in proliferation induction and apoptosis

inhibition,<sup>16</sup> CM could be potentially used as an agent to prevent skin photoaging.

The potential and cellular mechanisms of CM obtained from human umbilical cord blood (hUCB)-MSC in preventing UVB-induced apoptosis have not been elucidated. Therefore, the current research was conducted to investigate the potential of CM-hUCB-MSC in inhibiting UVB-induced apoptosis and its role in the antiapoptotic signaling pathway.

## Materials and Methods

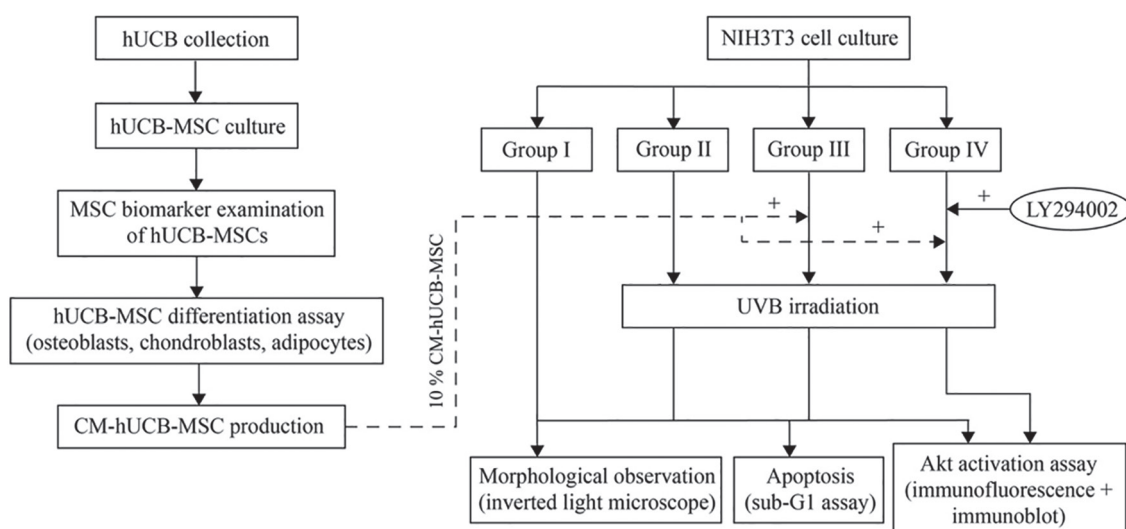
An experimental *in vitro* study was conducted at PT. Prodia StemCell Indonesia, Jakarta, Indonesia, between 2019-2022. hUCB sample was collected from healthy subjects who underwent normal delivery at Moh. Ridwan Meuraksa Hospital, Jakarta, Indonesia. Each subject signed an informed consent before study enrollment. Ethical approval for this study was obtained from the Ethical Committee of the Faculty of Medicine Universitas Udayana/Sanglah Central General Hospital (No. 1979/UN14.2.2.VII.14/LP/2018). Figure 1 shows a flowchart summarizing the study.

### Isolation and Culture of hUCB-Mononuclear Cell (MNC)

To isolate hUCB-MNCs, 50 mL hUCB was procured *ex utero* from each subject immediately after baby delivery. MNCs were isolated from the hUCB sample using a Lymphoprep medium (StemCell Technologies, Canada). Subsequently, the isolated hUCB-MNCs were then cultured in MesenCult media with a stimulatory supplement (StemCell Technologies, Canada) containing antibiotic-antimycotic (Sigma-Aldrich, USA) in a humidified incubator (5% CO<sub>2</sub>, 37 °C).

### Examination of the MSC Biomarkers

To examine whether MSC biomarkers were expressed on passage 5 hUCB-MSCs, a BD Stemflow hMSC Analysis Kit (BD Biosciences, USA) was used following the manufacturer's instructions. Briefly, passage 5 hUCB-MSCs were harvested by trypsinization, and then incubated separately with antibodies for MSC-positive markers including fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD90, peridinin chlorophyll protein (PerCP)-cyanine (Cy) 5.5-conjugated mouse anti-human CD105, allophycocyanin (APC)-conjugated mouse anti-human CD73, and an MSC-negative marker cocktail comprising phycoerythrin (PE)-conjugated CD45/CD34/CD11b/CD19/human leukocyte antigen (HLA)-DR antibodies. After incubation, the labeled hUCB-MSCs were



**Figure 1:** Flowchart of the study. Left panel: Flow of the conditioned media obtained from human umbilical cord blood-mesenchymal stem cell (MSC) (CM-hUCB-MSC) production, starting from hUCB collection until the production. Right panel: Flow of CM-hUCB-MSC, ultraviolet B (UVB) and LY294002 treatments, and related assays for all groups.

loaded into a FACSCanto II flow cytometer (BD Biosciences, USA), and FACSDiva software (BD Biosciences, USA) was used to analyze the acquired flow cytometry data.

#### Multilineage Differentiation of hUCB-MSCs

A multilineage differentiation assay was performed to assess the capacity of hUCB-MSC to differentiate into osteoblasts, chondroblasts, and adipocytes *in vitro*. Passage 5 hUCB-MSCs were differentiated into osteoblasts, chondroblasts, and adipocytes using the MesenCult Osteogenic Differentiation Kit (Human) (StemCell Technologies, Canada), MesenCult-ACF Chondrogenic Differentiation Kit (StemCell Technologies, Canada), and MesenCultAdipogenic Differentiation Kit (Human) (StemCell Technologies, Canada), following the manufacturer's protocols. After culture in the differentiation media for 14 days, hUCB-MSCs were fixed with 4% paraformaldehyde (Wako, Japan), and stained with alizarin red (Sigma-Aldrich, USA), alcian blue (Sigma-Aldrich, USA), and oil red O (Sigma-Aldrich, USA) stains to assess osteogenic, chondrogenic, and adipogenic differentiation, respectively. Differentiated hUCB-MSCs were then observed and documented under an Axiovert inverted light microscope (Zeiss, Germany).

#### Production of CM-hUCB-MSC

To prepare CM-hUCB-MSC, passage 5 hUCB-MSCs were first allowed to reach 80% confluency. The culture media were then removed and replaced with MesenCult MSC basal medium (StemCell Technologies, Canada). After 24-hour incubation, the media were processed

by centrifugation. The supernatant obtained from the centrifugation process was collected as CM-hUCB-MSC and stored at -20 °C.

#### NIH3T3 Cell Culture and Treatment

To culture NIH3T3 cells, Dulbecco's Modified Eagle Medium (Sigma-Aldrich, USA), 10% fetal bovine serum (Sigma-Aldrich, USA), and antibiotic-antimycotic agents were used. NIH3T3 cells were then maintained in a humidified incubator (5% CO<sub>2</sub>, 37 °C) and subcultured in flasks and chamber slides for further assays. After overnight culture, NIH3T3 cells were pretreated with/without 50 μM LY294002 (Cell Signaling, USA), treated with/without 10% CM-hUCB-MSC, then irradiated with/without UVB. UVB irradiation was performed for 30 min in an incubator using a UTX 20M UVIvue transilluminator (UVItec, United Kingdom) at a wavelength of 312 nm.

#### Sub-G1 Assay

The sub-G1 assay was performed to explore the potential of CM-hUCB-MSC to inhibit UVB-induced NIH3T3 cell apoptosis. Twenty-four hours after the treatment, NIH3T3 cells were harvested, suspended in the solution of 0.1% sodium citrate (Wako, Japan), 0.1% Triton X-100 (Sigma-Aldrich, USA), and 50 μg/mL propidium iodide (Sigma-Aldrich, USA), then kept in the dark at 4 °C for 2 hours. Subsequently, the NIH3T3 cell suspension was then subjected to flow cytometric analysis using a FACSCanto II flow cytometer (BD Biosciences, USA). The fluorescence intensity of the cell nuclei was measured at fluorescence length (FL)-2 for 10,000 events.

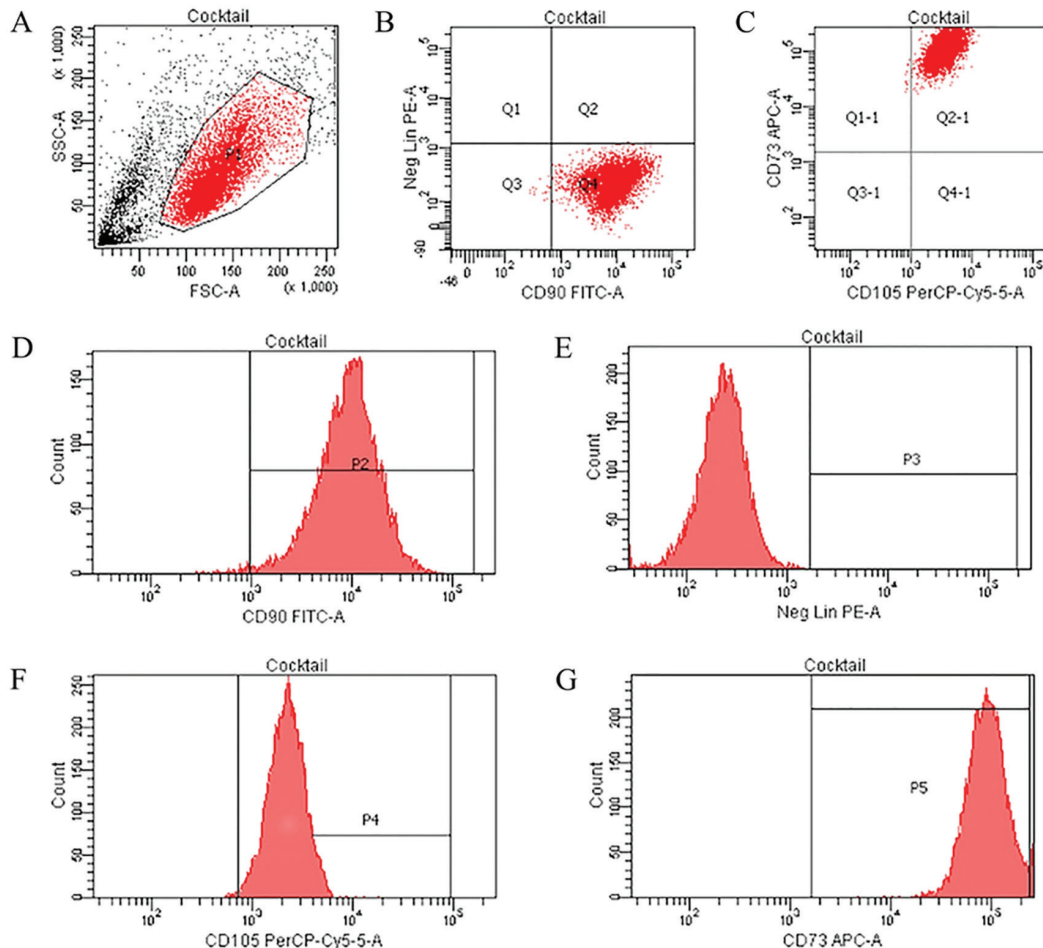
### Immunofluorescence Analysis

To observe whether CM-hUCB-MSCTreatment induced Akt phosphorylation in NIH3T3 cells, immunofluorescence analysis was employed. Thirty min after the treatment, NIH3T3 cells were fixed with ice-cold 10% trichloroacetic acid (Sigma-Aldrich, USA) and increasing concentrations of ethanol. For membrane permeabilization, the cells were treated with 0.2% Triton X-100 and 100 mM glycine (Wako, Japan). To block non-specific binding, the cells were incubated in 0.1% bovine serum albumin (Wako, Japan). For the primary antibody, 1:400 diluted rabbit anti-phospho-Akt (Ser473) (D9E) monoclonal antibody (Cell Signaling, USA) was used. After that, a 1:160 diluted FITC-linked goat anti-rabbit immunoglobulin G (IgG) (whole molecule) antibody (Sigma-Aldrich, USA) was applied as the secondary antibody. After washing, the cells were incubated in 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Merck, Germany), coverslipped with ProLong Gold

Antifade Mountant (ThermoFisher, USA), and documented with an Axio Observer inverted fluorescence microscope (Zeiss, Germany).

### Immunoblot

The immunoblotting assay was performed to measure the expression level of phosphorylated Akt in CM-hUCB-MSCTreated NIH3T3 cells. Thirty min after the treatment, NIH3T3 cells were lysed using ReadyPrep Protein Extraction Kit (Bio-Rad, USA) and Protease and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, USA). The lysate was subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated lysate proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA), which was then blocked with 5% skim milk and labeled with 1:1000 diluted rabbit polyclonal anti-Akt antibody (Cell Signaling, USA) or 1:2000 diluted rabbit monoclonal anti-phospho-Akt (Ser473) (D9E) antibody.



**Figure 2:** Flow cytometric result of human umbilical cord blood (hUCB)-mesenchymal stem cells (MSCs). Passage 5 hUCB-MSCs were harvested and labeled with antibodies for MSC-positive and negative markers as described in the Materials and Methods section. A: Dot plot showing forward scatter (FSC) for size and side scatter (SSC) for granularity of hUCB-MSCs; B: Dot plot of CD90 vs. negative lineage (Neg Lin: CD45, CD34, CD11b, CD19, and HLA-DR), showing that hUCB-MSCs were positive for CD90 and lacked Neg Lin; C: Dot plot of CD105 vs. CD73, showing that hUCB-MSCs were positive for both CD105 and CD73; D: Histogram of CD90 positive expression; E: Histogram of Neg Lin negative expression; F: Histogram of CD105 positive expression; G: Histogram of CD73 positive expression.

Subsequently, 1:2000 diluted horseradish peroxidase (HRP)-linked goat anti-rabbit IgG antibody (Cell Signaling, USA) was added as the secondary antibody. For chemiluminescence detection, LumiGlo reagent (Cell Signaling, USA) was used. Alliance 4.7 (UVItech, United Kingdom) and UVIband software (UVItech, United Kingdom) were used to document and quantify the immunoblotting result.

### Statistical Analysis

For statistical analysis, IBM SPSS Statistics for Macintosh version 21 (IBM, USA) was used. Data distribution was assessed with the Shapiro-Wilk test, whereas one-way analysis of variance (ANOVA) was used to test significance, followed by Tukey's honest significant difference (HSD) test or the Kruskal-Wallis test, followed by Dunn-Bonferroni test. Statistical significance was determined at  $P < 0.05$ .

## Results

### hUCB-MSCs Expressed MSC Biomarkers and Had Multilineage Differentiation Capacity

The antibody cocktail-labeled hUCB-MSCs showed positive expression for CD90, CD105, and CD73 (>95%) (figure 2). The hUCB-MSCs showed negative results (<2%) for CD45, CD34, CD11b, CD19, and HLA-DR. Furthermore, hUCB-MSCs were positive for alizarin red,

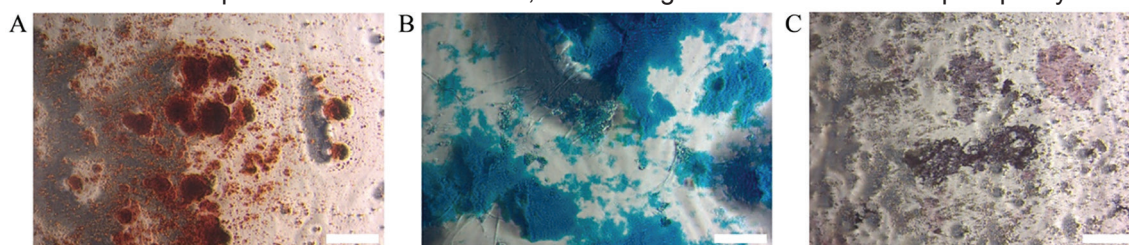
alcian blue, and oil red O staining after culture with osteogenic, chondrogenic, and adipogenic induction media, respectively (figure 3). These findings implied that hUCB-MSCs possessed MSC properties.

### CM-hUCB-MSC Inhibited UVB-induced NIH3T3 Cell Apoptosis

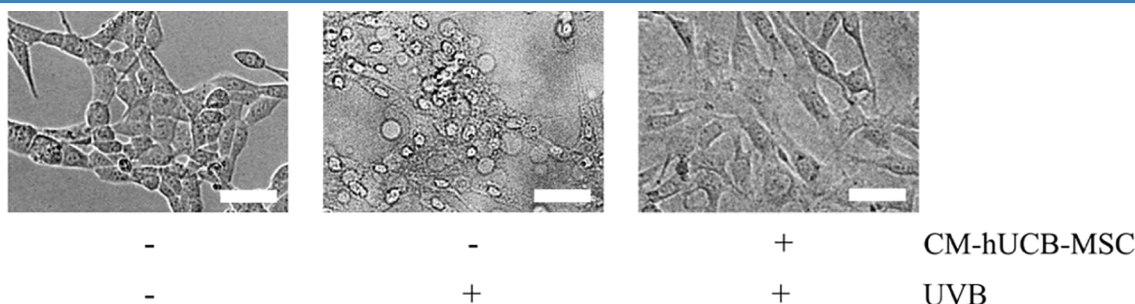
UVB irradiation for 30 min caused apoptosis in NIH3T3 cells, marked by a round shape with nuclear fragmentations (figure 4). Shapiro-Wilk test showed that the percentage of apoptotic cells was not normally distributed in the untreated ( $P=0.003$ ), UVB-irradiated ( $P < 0.001$ ), and UVB+CM-hUCB-MSC-treated ( $P=0.006$ ) groups. Thus, the Kruskal-Wallis test was performed, which showed a statistically significant difference among the three groups ( $P < 0.001$ ). *Post hoc* Dunn-Bonferroni test showed that the percentage of apoptotic cells in the UVB-irradiated group was significantly higher than the one in the untreated group ( $P < 0.001$ ) (figure 5). With the treatment of CM-hUCB-MSC, a lower percentage of apoptotic cells resulted (figure 5), which was significantly lower than the one in the UVB-irradiated group ( $P=0.002$ ).

### CM-hUCB-MSC Induced Phosphorylation of Akt

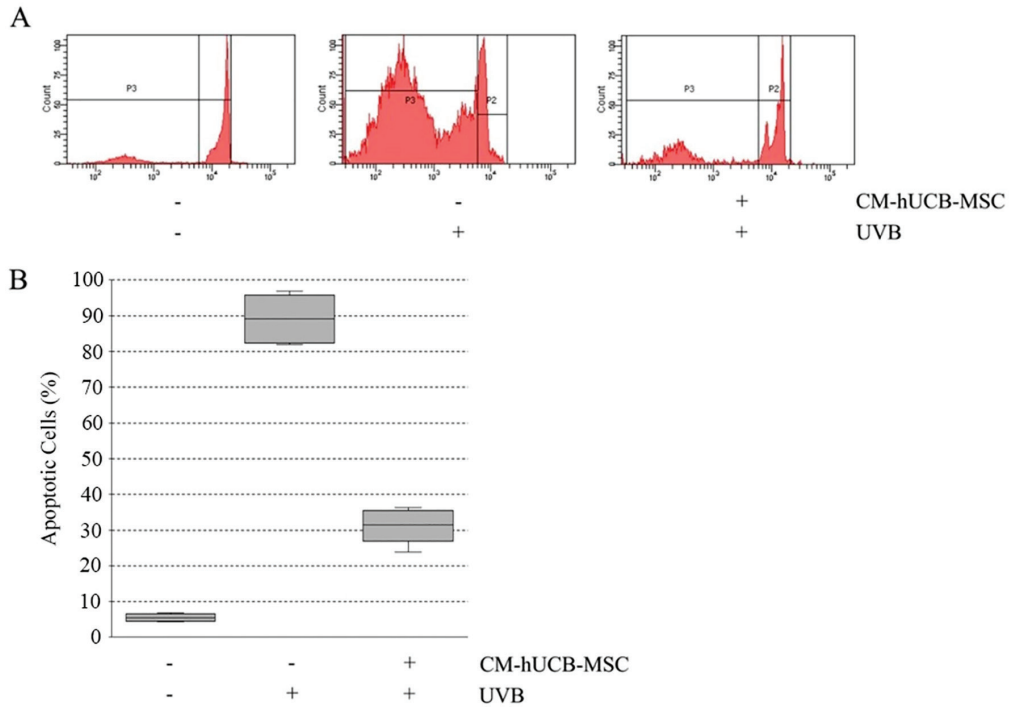
Under an inverted fluorescence microscope, the green fluorescence of phosphorylated Akt



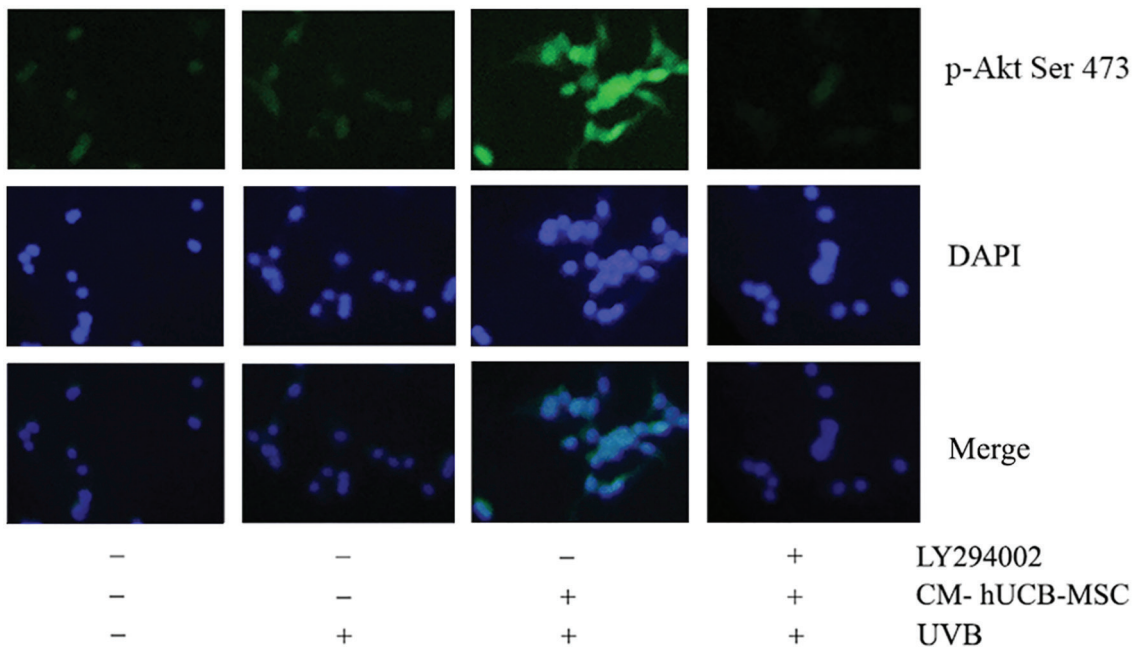
**Figure 3:** Multilineage differentiation staining of human umbilical cord blood (hUCB)-mesenchymal stem cells (MSCs) for osteogenic, chondrogenic, and adipogenic differentiation, which were performed in *in vitro* culture. Passage 5 hUCB-MSCs were cultured in osteogenic, chondrogenic, and adipogenic differentiation media. Osteogenic, chondrogenic, and adipogenic differentiation were evaluated using alizarin red, alcian blue, and oil red O staining, respectively as described in the Materials and Methods section. A: Osteogenic staining result; B: Chondrogenic staining result; C: Adipogenic staining result. White bar=50  $\mu$ m.



**Figure 4:** Conditioned media obtained from human umbilical cord blood-mesenchymal stem cell (CM-hUCB-MSC) attenuated morphological changes of ultraviolet B (UVB)-induced NIH3T3 cells. NIH3T3 cells were treated with/without 10% CM-hUCB-MSC then irradiated with/without UVB for 30 min. Twenty-four hours after the UVB irradiation, the cells were documented under an inverted light microscope. White bar=10  $\mu$ m.



**Figure 5:** Conditioned media obtained from human umbilical cord blood-mesenchymal stem cell (CM-hUCB-MSC) inhibited ultraviolet B (UVB)-induced NIH3T3 cell apoptosis. NIH3T3 cells were treated with/without 10% CM-hUCB-MSC then irradiated with/without UVB for 30 min. Twenty-four hours after the treatment, the apoptosis percentage of NIH3T3 cells was analyzed with sub-G1 assay as described in the Materials and Methods section. A: Flow cytometric results of NIH3T3 cells; B: Apoptosis percentage of NIH3T3 cells; P2: Gating area of viable NIH3T3 cells; P3: Gating area of apoptotic NIH3T3 cells.

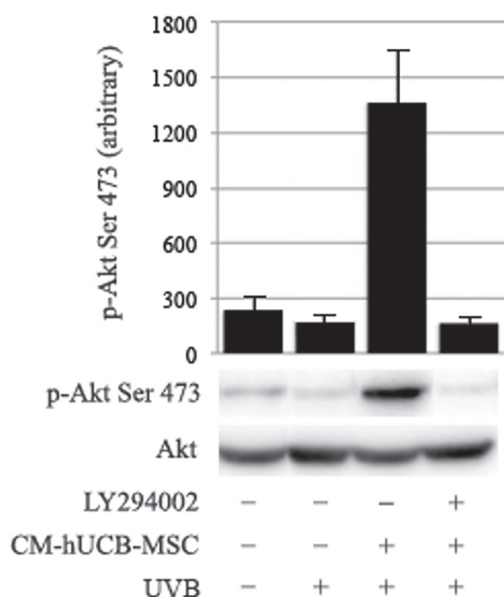


**Figure 6:** Conditioned media obtained from human umbilical cord blood-mesenchymal stem cell (CM-hUCB-MSC) induced phosphorylation of Akt in ultraviolet B (UVB)-irradiated NIH3T3 cells. NIH3T3 cells were pretreated with/without 50  $\mu$ M LY294002, treated with/without 10% CM-hUCB-MSC, then irradiated with/without UVB for 30 min. Thirty min after the treatment, the cells were subjected to immunofluorescence.

was clearly observed on the cells treated with CM-hUCB-MSC. Low fluorescence intensity of phosphorylated Akt was detected in the untreated cells and the UVB-irradiated cells. Low fluorescence intensity was also observed in the cells pretreated with LY294002 and treated

with CM-hUCB-MSC (figure 6).

For immunoblot results, a low phosphorylation level of Akt at Ser 473 was observed in the untreated cells (figure 7). Shapiro-Wilk test showed that the phosphorylation level of Akt was normally distributed in the untreated ( $P=0.763$ ),



**Figure 7:** Conditioned media obtained from human umbilical cord blood-mesenchymal stem cell (CM-hUCB-MSC) increased the amount of phosphorylated Akt in ultraviolet B (UVB)-irradiated NIH3T3 cells. NIH3T3 cells were pretreated with/without 50  $\mu$ M LY294002, treated with/without 10% CM-hUCB-MSC, then irradiated with/without UVB for 30 min. Thirty min after the treatment, the cells were subjected to immunoblot analysis.

UVB-irradiated ( $P=0.780$ ), UVB+CM-hUCB-MSC-treated ( $P=0.969$ ), and UVB+CM-hUCB-MSC+LY294002-treated ( $P=0.527$ ) groups. Therefore, one-way ANOVA was performed, which indicated a statistically significant difference among the four groups ( $P<0.001$ ). Tukey's HSD *post hoc* test revealed that the phosphorylation level of Akt in the UVB-irradiated group was not significantly different from the one in the untreated group ( $P=0.953$ ). Thus, UVB irradiation did not affect the phosphorylation level of Akt. Meanwhile, the phosphorylation level of Akt in the UVB+CM-hUCB-MSC-treated group was significantly higher than the one in the UVB-irradiated group ( $P<0.001$ ). This suggested that the phosphorylation level of Akt was significantly increased by CM-hUCB-MSC in UVB-irradiated NIH3T3 cells. The CM-hUCB-MSC-induced phosphorylation of Akt was inhibited by LY294002, as shown by the phosphorylation level of Akt in the UVB+CM-hUCB-MSC+LY294002-treated group that was significantly lower than the one in UVB+CM-hUCB-MSC-treated group ( $P<0.001$ ) (figure 7).

## Discussion

Current results showed that CM-hUCB-MSC inhibited UVB-induced apoptosis. Moreover, CM-hUCB-MSC induced phosphorylation of Akt at Ser 473 in UVB-irradiated NIH3T3 cells.

These results suggested that activation of Akt played an important role in the inhibition of apoptosis provided by CM-hUCB-MSC. These results were in accordance with those of a previous study in adipose-derived stem cell-conditioned media (ADSC-CM).<sup>17</sup> Therefore, the antiapoptotic activity of stem cell CM could be mainly mediated by the Akt/PKB signaling pathway,<sup>17, 18</sup> which enhances cell survival.<sup>11</sup>

Substances in CM-hUCB-MSC might enhance cell survival through the induction of phosphatidylinositol-3-kinase (PI3K). Phosphatidylinositol 3,4,5-triphosphate (PIP3) production from phosphatidylinositol 4,5-diphosphate (PIP2), which is catalyzed by PI3K, could stimulate Akt translocation to the inner layer of the cell membrane and phosphorylation.<sup>10, 19</sup> Our current results confirmed the translocation and activation marked by the green fluorescence expression in CM-hUCB-MSC-treated NIH3T3 cells. The role of PI3K in this signaling pathway was confirmed as well by the inhibition of LY294002, a PI3K inhibitor. It could be observed that LY294002 inhibited the CM-hUCB-MSC-induced phosphorylation of Akt at Ser 473.

Substances in CM have been suggested as a therapy to prevent the skin-damaging effects caused by UVB irradiation.<sup>20, 21</sup> Despite inhibiting apoptosis,<sup>22-24</sup> the substances may have antioxidant activities through decreasing reactive oxygen species (ROS) production,<sup>25-27</sup> thus protecting skin from photooxidative stress. In addition, these substances were reported to play roles in various signaling pathways including MMPs,<sup>25, 27, 28</sup> mitogen-activated protein kinase (MAPK),<sup>21, 27-29</sup> and nuclear factor (NF)- $\kappa$ B.<sup>21, 30</sup> Therefore, CM-hUCB-MSC contains numerous important substances that could have potential for cell regeneration and tissue growth.

Our findings indicate that CM-hUCB-MSC can potentially enhance the survival of UVB-exposed skin cells, thereby preventing UVB-induced skin damage. CM-hUCB-MSC could be an alternative agent for avoiding skin photoaging.

There are several limitations in the current study. Since CM-hUCB-MSC might contain various growth factors, cytokines, and other macromolecules that could potentially promote cell regeneration and tissue growth, additional research is required to identify which component of the CM-hUCB-MSC is directly involved in stimulating antiapoptotic responses. In addition, it is well acknowledged that skin regeneration is a very complex process involving multiple signaling pathways. Although the potential of CM-hUCB-MSC in inhibiting UVB-induced apoptosis through the PI3K/Akt signaling pathway has been shown in the current

research, the involvement of other signaling pathways should be investigated for a better understanding of the antiapoptotic mechanism of CM-hUCB-MSC.

### Conclusion

CM-hUCB-MSC could activate the PI3K/Akt signaling pathway, which inhibits UVB-induced NIH3T3 cell apoptosis. Taken together, CM-hUCB-MSC could be a potential inhibitor of UVB-induced apoptosis.

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### Authors' Contribution

F.S: Study concept and design, Investigation, data analysis, drafting; DAR.D: Investigation, data analysis, drafting; INM.A: Data analysis, drafting; W.P: Data analysis, drafting; A.C: Investigation, drafting; M.C: Data analysis, drafting; NM.D: Data analysis, drafting; All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

**Conflict of Interest:** None declared.

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