Received: 7 March 2023 | Revised: 18 May 2023 | Accepted: 29 June 2023

DOI: 10.1002/tox.23880

RESEARCH ARTICLE

ENVIRONMENTAL TOXICOLOGY WILEY

Ohwia caudata aqueous extract attenuates doxorubicin-induced mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells

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Funding information
Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Grant/Award Number:

Abstract

Mitochondrial dysfunction has been linked to many diseases, including organ degeneration and cancer. Wharton's jelly-derived mesenchymal stem cells provide a valuable source for stem cell-based therapy and represent an emerging therapeutic approach for tissue regeneration. This study focused on screening the senomorphic properties of *Ohwia caudata* aqueous extract as an emerging strategy for preventing or treating mitochondrial dysfunction in stem cells. Wharton's jelly-derived mesenchymal stem cells were incubated with 0.1 μM doxorubicin, for 24 h to induce mitochondrial dysfunction. Next, the cells were treated with a series concentration of *Ohwia caudata* aqueous extract (25, 50, 100, and 200 μg/mL) for another 24 h. In addition, an untreated control group and a doxorubicin-induced mitochondrial dysfunction positive control group were maintained under the same conditions. Our data

Tsung-Jung Ho and Chih-Yang Huang contributed equally to this work.

IMAR-110-01-12; Ministry of Science and Technology, Taiwan, Grant/Award Number: MOST 111-2314-B-303-008-MY3; China Medical University and Asia University, Grant/Award Numbers: CMU102-ASIA-04-08, CMU102-ASIA-04

showed that *Ohwia caudata* aqueous extract markedly suppressed doxorubicin-induced mitochondrial dysfunction by increasing Tid1 and Tom20 expression, decreased reactive oxygen species production, and maintained mitochondrial membrane potential to promote mitochondrial stability. *Ohwia caudata* aqueous extract retained the stemness of Wharton's jelly-derived mesenchymal stem cells and reduced the apoptotic rate. These results indicate that *Ohwia caudata* aqueous extract protects Wharton's jelly-derived mesenchymal stem cells against doxorubicin-induced mitochondrial dysfunction and can potentially prevent mitochondrial dysfunction in other cells. This study provides new directions for the medical application of *Ohwia caudata*.

KEYWORDS

Desmodium caudatum, mitochondrial dysfunction, mitochondrial stability, *Ohwia caudata*, Wharton's jelly-derived mesenchymal stem cells

1 | INTRODUCTION

Mitochondria is the "powerhouse" of the cell and is the main source of energy production in the cells. However, research in the last decade has shown mitochondria not only produce energy but also involve in the regulation of several biological mechanisms, including cellular metabolism, cell signaling pathways, energy production, cell survival, and apoptosis in cells.[1–4] Mitochondrial abnormality can lead to the suppression of mitochondrial respiration, reduction in energy synthesis, increase in the production of free radicals, and damage to mitochondrial dynamics. Eventually, mitochondrial dysfunction changes cellular metabolism and consequently leading to cell death and apoptosis and contributing to pathological phenotypes.[1–4] Mitochondrial dysfunction is a hallmark of aging and chronic diseases, including neurodegenerative diseases, neurobehavioral and psychiatric disorders, cardiovascular disorders, autoimmune disorders, metabolic syndromes, diabetes, gastrointestinal illnesses, fatiguing illnesses, musculoskeletal disorders, chronic infections, and tumorigenesis.[5–7] The mitochondria also regulate the fate of stem cells and senescence. Mitochondrial DNA mutagenesis and reactive oxygen species accumulation disrupt stem cell homeostasis.[8,9] Besides, The balance between mitochondrial fusion and fission and reduced mitophagy affects mitochondrial dynamics in stem cells and modulates stem cell fate.[10,11] Moreover, metabolites produced through mitochondrial metabolism have also been shown to modify the DNA and proteins to control stem cell fate via epigenetic modulation.[12,13] Mitochondria can also modulate the gene expression in the nucleus to alter cell fate decisions and physiological functions of stem cells.[14,15] Therefore, maintaining mitochondrial function in stem cells is essential to improve stem cell homeostasis and keep normal function of stem cells. Tumorous imaginal disc 1 (Tid1), a mitochondrial co-chaperone protein of mitochondrial Hsp70, is located mainly in the mitochondrial compartment. Tid1 stabilizes the integrity of mitochondrial DNA, maintains the steady-state homogeneity of the mitochondrial membrane potential ($\Delta\psi$), and reduction of oxygen consumption.[16] Translocase of outer mitochondrial membrane 20 (Tom20), a central component of the receptor complex, that responsible for import recognition and translocation of cytosolically synthesized mitochondrial preproteins into the mitochondrial matrix.[17] These proteins are important to ensure the mitochondrial homeostasis and we decide to find traditional Chinese medicines to regulate mitochondrial homeostasis in stem cells via these proteins.

Traditional Chinese medicines, predominantly comprising herbal medicines and natural products, has been recognized as complementary and alternative therapy for several diseases for thousands of years. The efficacy of traditional Chinese medicines and their components has also been shown to maintain stem cell function.[18–21] *Ohwia caudata*, also known as *Desmodium caudatum*, belonging to the family *Fabaceae*, is a traditional Chinese medicine that is widely used to treat colds, febrile diseases, gastroenteritis, bacillary dysentery, rheumatic arthritis, and icterohepatitis.[22,23] The bioactive compounds of *Ohwia caudata* include alkaloids, triterpenoids, and flavonoids, among which flavonoids have been shown to exert anti-inflammatory and antioxidant activities.[24] Moreover, *Ohwia caudata* can treat Alzheimer's disease and inhibit Influenza A Virus infection.[23,25] Caudatan A, derived from *Ohwia caudata*, can be used for cancer prevention as it is a potential inhibitor of human kidney-type glutaminase, which is overexpressed in many cancer cells to up-regulate oncogenes and raise the rate of cell proliferation.[26] However, the protective ability of *Ohwia caudata* against other type of stress in stem cells, has not been investigated yet.

Doxorubicin, a cytotoxic chemotherapeutic agent used to treat a variety of cancers, can cause mitochondrial dysfunction.[27] Therefore, we used doxorubicin as a stress factor to induce mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells, and investigated the possible protective effects of *Ohwia caudata* treatment through the maintenance of mitochondrial function.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

All chemicals used in this study were of analytical grade, and were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), unless otherwise indicated. Dry, healthy, and mature *Ohwia caudata* leaves were sourced from a local traditional Chinese medicine pharmacy in Hualien City (Taiwan). Doxorubicin hydrochloride (44583) was obtained from Sigma-Aldrich. The primary antibodies against Tid1 (sc-18 820), Tom20 (sc-17 764), GAPDH (sc-47 724), and Bcl-xL (sc-8392) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies corresponding to each primary antibody were acquired from Sigma-Aldrich.

2.2 | Preparation of *Ohwia caudata* aqueous extract

Total *Ohwia caudata* leaves (50 g) were washed to remove dirt and were blended into a fine powder. Thereafter, the powder was mixed with 500 mL of reverse osmosis water, and the mixture was boiled until its volume was reduced to 50 mL. The solid impurities were eliminated from the solution through centrifugation at 10000 rpm for 15 min at 4°C. The *Ohwia caudata* aqueous extract in the supernatant was further filtered to remove all residual debris. The filtered *Ohwia caudata* aqueous extract was stored at −20°C for future use.

2.3 | Cell culture

Wharton's jelly-derived mesenchymal stem cells (RM60596) were procured from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were cultured in low-glucose Dulbecco's modified eagle's medium (D5523, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Gibco™ 26 140 079, Thermo Fisher Scientific Inc., Waltham, MA, USA), 1.5 g/L sodium bicarbonate, 1× non-essential amino acids (M7145, Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (P4333, Sigma-Aldrich), and were further incubated at 37°C with 5% CO_2, in a humidified incubator.

2.4 | Cell viability assay

3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide (MTT) was applied to determine cell viability. Wharton's jelly-derived mesenchymal stem cells were seeded in 96 well plates were treated with various concentrations of Doxorubicin (0.1–1 μM) for 24 h and then the culture medium was replaced by 100 μL of MTT (0.5 mg/mL) and incubated at 37°C for 4 h. The medium was then removed and the purple formazan crystals was solubilized in 100 μL of dimethyl sulfoxide. Absorbance was measured at 590 nm using a spectrophotometer (Figure S1).

2.5 | Assessment of the effect of *Ohwia caudata* aqueous extract on doxorubicin-treated Wharton's jelly-derived mesenchymal stem cells

Wharton's jelly-derived mesenchymal stem cells were seeded at a density of 1×10^6 cells in a 10 cm culture dish and were cultured in a 5% CO_2 incubator. After 24 h, the cells were washed with phosphate-buffered saline (PBS), supplied with fresh medium, and then incubated with 0.1 μM doxorubicin, for 24 h, to induce mitochondrial dysfunction. Next, the cells were treated with a series concentration of *Ohwia caudata* aqueous extract (25, 50, 100, and 200 μg/mL), and incubated at 37°C for another 24 h. Additionally, an untreated control group and a doxorubicin-induced mitochondrial dysfunction positive control group were maintained under the same conditions. The experimental design followed for each experiment was the same.

2.6 | Whole cell lysis

The cells were further washed with PBS and lysed in a cell lysis buffer containing 50 mM Tris-base, 0.5 M NaCl, 1 mM EDTA, 1% NP40, 1% glycerol, and 1 mM 2-mercaptoethanol, phosphatase inhibitor cocktail 2 (Sigma-Aldrich), and protease inhibitor cocktail (Sigma-Aldrich). Whole cell lysates were placed on ice for 1 h and then centrifuged at 14000 rpm for 30 min at 4°C, and the clear supernatants were collected and frozen at −20°C for future use.

2.7 | Western blotting

Bradford protein assay was used to determine the total protein content of the whole-cell extracts.[28] The protein (40 μg) was separated using 8%–15% sodium dodecyl sulfate-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (GE, Amersham, UK). The membrane was blocked with 5% non-fat milk for 1 h and probed overnight with primary antibodies at 4°C. Protein signals were evaluated using horseradish peroxidase-conjugated secondary antibodies (1:10000) (GE Healthcare, Amersham, UK) and further detected using the Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA). As previously described, the immunoblots were analyzed using the iBright Analysis program on the Alpha Imager 4000 digital imaging system (Digital Imaging System, San Diego, CA, USA)[29,30]

2.8 | Mitochondria morphology staining

MitoTracker Red (M7512, Invitrogen™, Waltham, MA, USA) was applied to assess mitochondrial morphology. All experimental protocol

was following the instructions. Photomicrographs were obtained using an OLYMPUS® BX53 microscope coupled with an image analysis system (Olympus® Corporation, Shinjuku-ku, Tokyo, Japan).

2.9 | Immunofluorescence assay

The cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min, and further permeabilized with 0.1% Triton X-100 for 5 min at 4°C. Then the cells were blocked at room temperature for 30 min, using 10% fortified bovine calf serum in PBS, followed by incubation with the Tid1 primary antibody (sc-18 820, Santa Cruz Biotechnology) for 24 h at 4°C. Next, the cells were washed and incubated with bright green fluorescent-conjugated (Alexa Fluor® 488, Thermo Fisher Scientific) goat anti-mouse IgG (A11001, Invitrogen) at room temperature for 1 h and further washed with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. Images were captured using an OLYMPUS® BX53 microscope coupled with an image analysis system (Olympus® Corporation).

2.10 | Detection of mitochondrial reactive oxygen species

Molecular probes (MitoSOX, Invitrogen) were used to measure mitochondrial superoxide production in the cells. After 24 h of doxorubicin exposure and *Ohwia caudata* aqueous extract treatment, the cells were incubated with MitoSOX at 37°C for 30 min, followed by incubation with DAPI for 5 min, to investigate the cell nucleus. Mitochondrial reactive oxygen species production was measured using an OLYMPUS® BX53 microscope with an image analysis system (Olympus® corporation), with excitation and emission wavelengths of 510/580 nm.

2.11 | JC-1 staining

The cells were stained using JC-1 (CS03D, Sigma Aldrich) per the manufacturer's instructions. The cells were stained with 1× JC-1 staining solution at 37°C in a humidified incubator containing 5% CO_2 for 20 min. JC-1 monomers and aggregates were observed under a fluorescence microscope equipped with fluorescein isothiocyanate (FITC) and propidium iodide dual band-pass filters, to detect them after two rounds of rinsing with a complete growth medium. Photomicrographs were obtained using an OLYMPUS® BX53 microscope (Olympus® Corporation).

2.12 | Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed to analyze cell apoptosis using the In Situ Cell Death Detection Kit (11 684 809 910, Roche, Basel, Switzerland), per the manufacturer's instructions. The Wharton's jelly-derived mesenchymal stem cells were rinsed with PBS, fixed with 4% PFA at room temperature for 1 h, and then permeabilized with 0.1% Triton X-100 in sodium citrate for 2 min. The cells were stained with the TUNEL solution for 60 min at 37°C in the dark. The slides were rinsed with PBS and incubated with DAPI for 10–15 min, as described previously. Fluorescence microscopy was used to examine the cells (Olympus CKX53, Olympus® corporation).[31]

2.13 | Flow cytometry

The cells were treated with doxorubicin for 24 h, and further treated with *Ohwia caudata* aqueous extract, and washed after two times PBS. The cells were then resuspended in 1× binding buffer and treated with the CD90 (11-0909-42, Invitrogen) and CD34 (11-0341-82, Invitrogen) monoclonal antibodies for 15–30 min, to investigate the cell surface markers and FITC, annexin V fluorescein, and propidium iodide dye using the FITC Annexin V detection kit, as per manufacturer's instructions (BD Biosciences). For statistical analysis, 10 000 cells were used for each occurrence. The test was performed by the FACSCalibur flow cytometer.[32]

2.14 | Statistical analysis

Data are presented as the mean ± standard deviation from three independent experiments. One-way analysis of variance (ANOVA) with Tukey's test was used to determine the statistical significance for multiple experiments. Statistical significance was defined as: $^{*}p < .05$; $^{**}p < .01$; and $^{***}p < .001$ versus the control; and $^{\#}p < .05$; $^{\#\#}p < .01$; and $^{\#\#\#}p < .001$ versus 0.1 μM doxorubicin.

3 | RESULTS

3.1 | *Ohwia caudata* promoted Tid1 to maintain mitochondrial stability in Wharton's jelly-derived mesenchymal stem cells

Since Tid1 plays a critical role in maintaining a homogeneous distribution of mitochondrial membrane potential and the integrity of mitochondrial DNA,[16] western blot and fluorescent image assays to investigate whether the effects of *Ohwia caudata* induced Tid1 expression in doxorubicin-stimulated Wharton's jelly-derived mesenchymal stem cells. The blot image showed that doxorubicin significantly reduced Tid1 expression; however, a high dose (200 μg/mL) of *Ohwia caudata* aqueous extract restored Tid1 levels in Wharton's jelly-derived mesenchymal stem cells (Figure 1A). Unexpectedly, other dose of *Ohwia caudata* aqueous extract presented less effect on Tid1 expression in blot image. Furthermore, fluorescent imaging showed that Tid1 (green color) and mitochondria (red color) were considerably decreased in the doxorubicin-treated group. On the contrary,

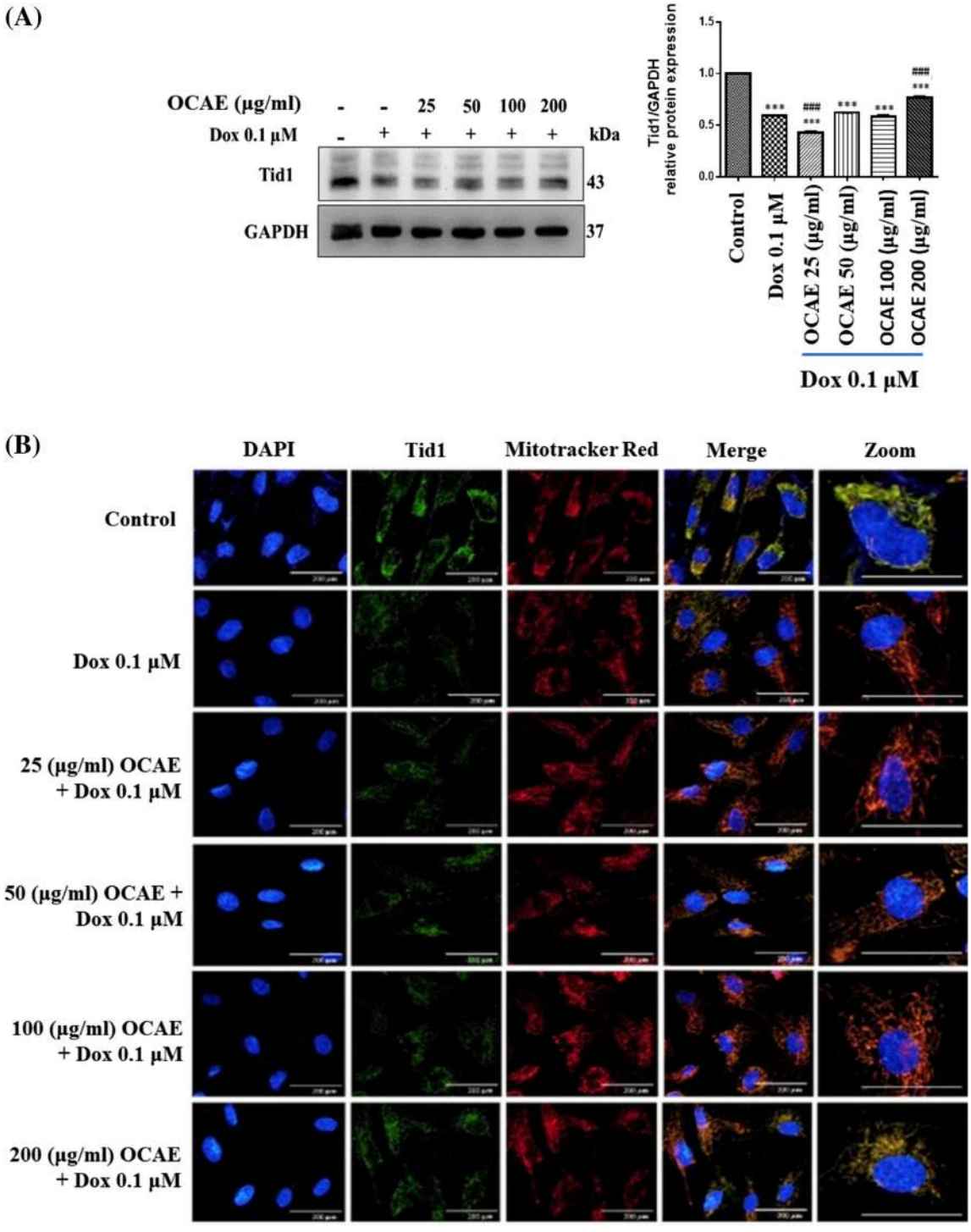

FIGURE 1 *Ohwia caudata* aqueous extract promoted Tid1 to maintain mitochondrial stability in doxorubicin-stimulated Wharton's jelly-derived mesenchymal stem cells. (A) Tid1 expression was analyzed using western blotting. Wharton's jelly-derived mesenchymal stem cells were treated with different doses of *Ohwia caudata* aqueous extract 24 h after they were treated with 0.1 μM doxorubicin. The Tid1 expression in Wharton's jelly-derived mesenchymal stem cells was analyzed using western blotting. A representative blot from three independent experiments is shown. Data are shown as mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA). $^{***}p < .001$ versus control. $^{###}p < .001$ versus 0.1 μM doxorubicin. (B) Tid1 expression detected using immunofluorescence assay, and the mitochondria were stained with MitoTracker Red. Fluorescent imaging showed that Tid1 (green color) and mitochondria (red color) decreased considerably in the doxorubicin-stimulated group. However, following the treatment with *Ohwia caudata* aqueous extract, Tid1 levels and the number of mitochondria in the doxorubicin-treated Wharton's jelly-derived mesenchymal stem cells improved, especially with the high dose of *Ohwia caudata* aqueous extract. The scale bar is 200 μm. Dox, doxorubicin; OCAE, *Ohwia caudata* aqueous extract.

treatment with *Ohwia caudata* aqueous extract improved Tid1 levels and the number of mitochondria in the doxorubicin-treated Wharton's jelly-derived mesenchymal stem cells, especially with the high dose of *Ohwia caudata* aqueous extract (Figure 1B; Figure S2). These results demonstrated that *Ohwia caudata* might have a protective effect and maintains mitochondrial stability under doxorubicin-induced damage by activating Tid1 production.

3.2 | Doxorubicin-induced mitochondrial superoxide in Wharton's jelly-derived mesenchymal stem cells was ameliorated with *Ohwia caudata*

Excessive production of reactive oxygen species is an important indicator of oxidative stress-induced mitochondrial dysfunction.[33] Hence, to detect the capability of *Ohwia caudata* to scavenge free radicals, the MitoSOX Red reagent was used to detect the generation of intracellular reactive oxygen species induced by doxorubicin. The fluorescence images showed that the signal for reactive oxygen species (red color) intensity increased markedly when Wharton's jelly-derived mesenchymal stem cells were incubated with 0.1 μM doxorubicin for 24 h. However, treatment with *Ohwia caudata* aqueous extract significantly reduced the reactive oxygen species intensity (red color) (Figure 2A). Similarly, the exposure of Wharton's jelly-derived mesenchymal stem cells to 0.1 μM doxorubicin increased intracellular reactive oxygen species levels. However, treatment with *Ohwia caudata* aqueous extract dramatically inhibited doxorubicin-induced reactive oxygen species in Wharton's jelly-derived mesenchymal stem cells in a dose-dependent manner (Figure 2B). These results suggested that *Ohwia caudata* reduced doxorubicin-induced mitochondrial dysfunction by eliminating intracellular reactive oxygen species. Therefore, we further assessed the effect of *Ohwia caudata* on mitochondrial membrane potential (ΔΨm).

3.3 | *Ohwia caudata* improved mitochondrial membrane potential (ΔΨm) in Wharton's jelly-derived mesenchymal stem cells under doxorubicin stress

Depolarization of the transmembrane potential (Δψm) in mitochondria leads to apoptosis.[3435] To assess whether *Ohwia caudata* affected mitochondrial function, the changes in mitochondrial membrane potential were analyzed using JC-1 dye, which stains the mitochondria in a ΔΨm dependent manner.[36] As shown in Figure 3A, Wharton's jelly-derived mesenchymal stem cells exposed to 0.1 μM doxorubicin for 24 h exhibited a significant decrease in the JC-1 aggregate (Red color), and an increase in green JC-1 monomers (Green color). However, *Ohwia caudata* aqueous extract treatment prevented the loss of JC-1 aggregate and increased the red JC-1 monomers. Exposure to 0.1 μM doxorubicin for 24 h significantly decreased the ratio of the aggregate to monomers, whereas *Ohwia caudata* aqueous extract (25, 50, 100, 200 μg/mL) treatment prevented the decrease of the aggregate to monomers ratio in a dose-dependent manner (Figure 3B). These results implied that *Ohwia caudata* attenuated doxorubicin-induced dissipation of the mitochondrial membrane potential.

3.4 | Stemness of Wharton's jelly-derived mesenchymal stem cells was maintained with *Ohwia caudata* treatment

We further analyzed the cell surface markers on the Wharton's jelly-derived mesenchymal stem cells using flow cytometry analysis to check their stemness. Since the Wharton's jelly-derived mesenchymal stem cells were CD90-positive ($CD90^+$) and CD34-negative ($CD34^-$),[37] we assessed the percentage of cells that were $CD90^+$ (Figure 4A) and $CD34^-$ (Figure 4B). The expression of CD90 reduced in doxorubicin-treated cells, within 24 h. However, CD90 expression was increased in more than 88% of the Wharton's jelly-derived mesenchymal stem cells after *Ohwia caudata* aqueous extract treatment (Figure 4A). In contrast, doxorubicin treatment significantly raised the percentage of $CD34^+$ cells, wherein *Ohwia caudata* aqueous extract treatment reduced the percentage of $CD34^+$ cells (Figure 4B). These results indicated that *Ohwia caudata* might maintain the stemness of Wharton's jelly-derived mesenchymal stem cells under doxorubicin treatment.

3.5 | Effects of *Ohwia caudata* on the expression of Tom20 reduced doxorubicin-induced apoptosis in Wharton's jelly-derived mesenchymal stem cells

Tom20 is the essential import of proteins into the matrix and outer membrane.[38] For example, survivin is imported into mitochondria to inhibit apoptosis and Tom20 has been implicated in its import.[39] Hence, we analyzed the expression of Tom20 in the different groups. Our results showed that with doxorubicin treatment, Tom20 levels reduced within 24 h. However, *Ohwia caudata* aqueous extract treatment restored Tom20 levels (Figure 5A). Next, we confirmed the protective effect of *Ohwia caudata* aqueous extract on doxorubicin-induced cell apoptosis through TUNEL assay and flow cytometry. The results showed that TUNEL positive cells increased after treatment with 0.1 μM doxorubicin. However, treatment with *Ohwia caudata* aqueous extract significantly decreased the number of apoptotic cells (Figure 5B; Figure S3). In addition, the data from the flow cytometric analysis also showed that *Ohwia caudata* aqueous extract treatment reduced the percentage of doxorubicin-induced apoptotic cells, but not in a dose-dependent manner (Figure S4). These results suggested that *Ohwia caudata* might reduce apoptosis in doxorubicin-stimulated Wharton's jelly-derived mesenchymal stem cells and the mechanism might involve the import of proteins into the mitochondria via Tom20.

4 | DISCUSSION

In this study, Wharton's jelly-derived mesenchymal stem cells was applied to the investigate the beneficial effect of *Ohwia caudata*

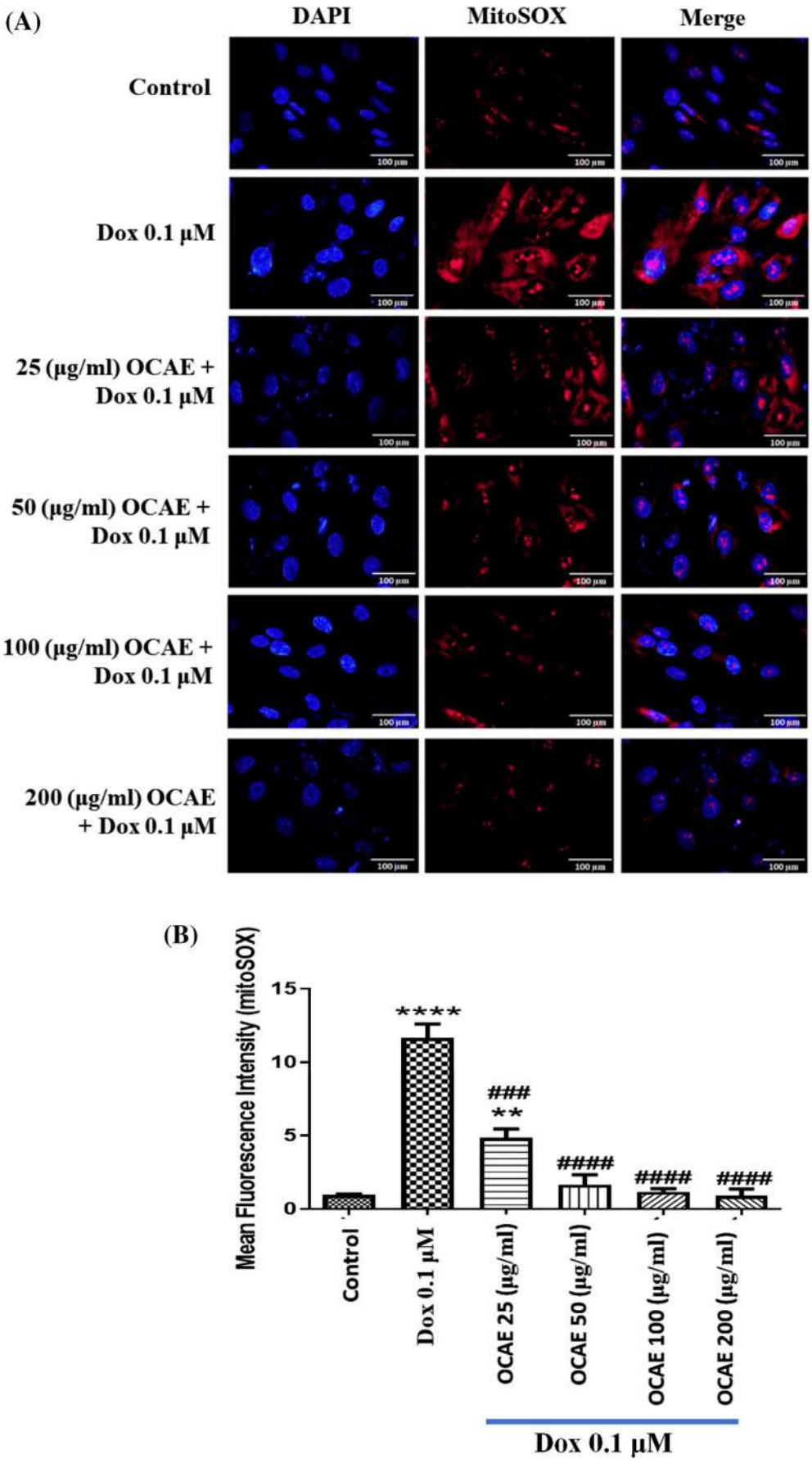

FIGURE 2 *Ohwia caudata* aqueous extract reduced doxorubicin-induced oxidative stress in Wharton's jelly-derived mesenchymal stem cells. (A) The intracellular reactive oxygen species were assessed using the MitoSOX Red reagent. The generation of intracellular reactive oxygen species induced by doxorubicin was assessed by treating Wharton's jelly-derived mesenchymal stem cells with 0.1 μM doxorubicin for 24 h followed by incubation with different doses of *Ohwia caudata* aqueous extract. Fluorescence images were acquired to detect the intensity of reactive oxygen species signals (red color). The scale bar is 100 μm. (B) The exposure of Wharton's jelly-derived mesenchymal stem cells to 0.1 μM doxorubicin increased intracellular reactive oxygen species levels. However, treatment with *Ohwia caudata* limited the production of reactive oxygen species. Interestingly, *Ohwia caudata* treatment inhibited doxorubicin-induced reactive oxygen species in Wharton's jelly-derived mesenchymal stem cells in a dose-dependent manner. The data were analyzed using one-way ANOVA. $^{**}p < .01$ and $^{****}p < .0001$ versus control; $^{\#\#\#}p < .001$ and $^{\#\#\#\#}p < .0001$ versus 0.1 μM doxorubicin. Dox, doxorubicin; OCAE, *Ohwia caudata* aqueous extract.

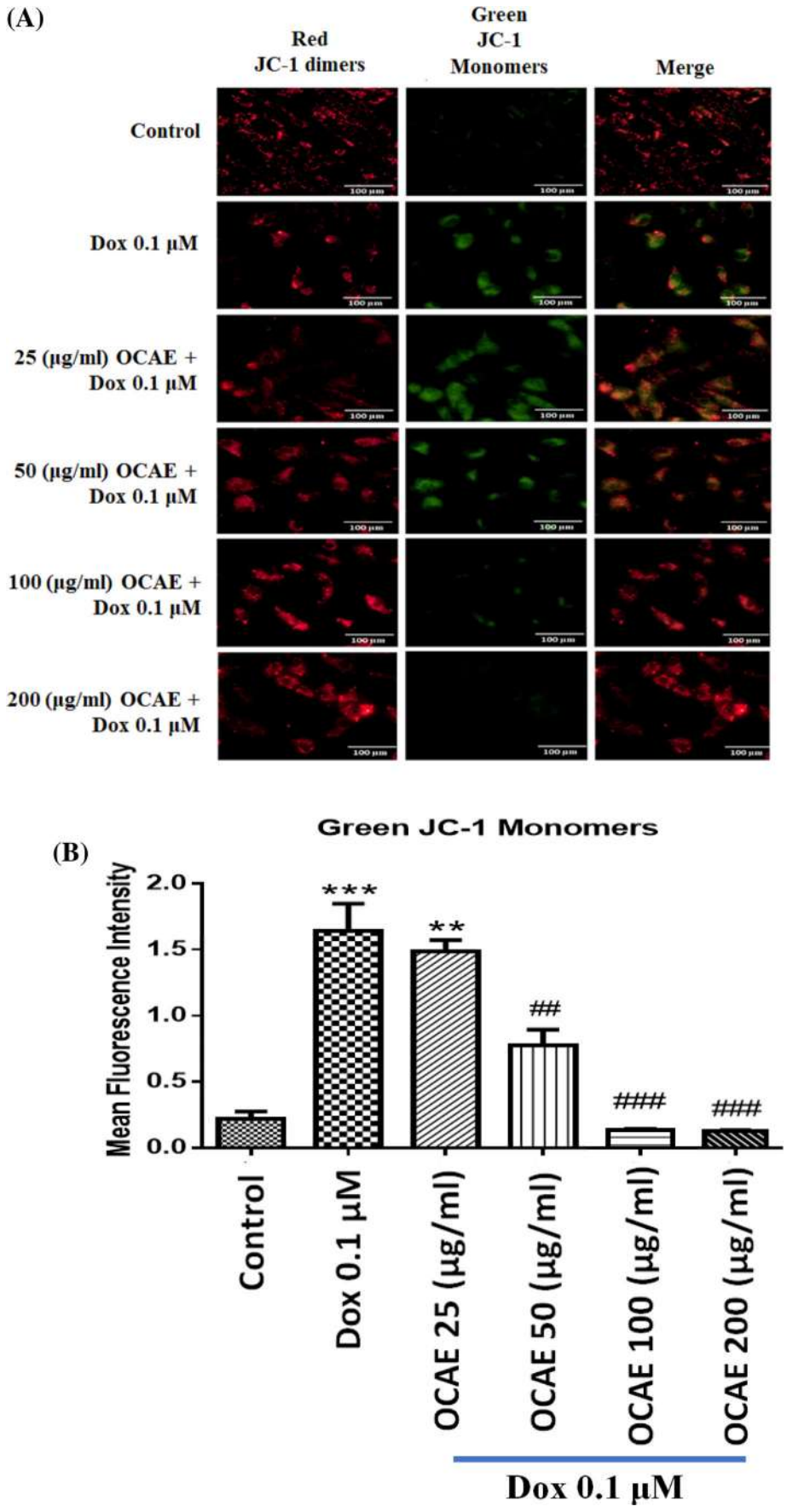

FIGURE 3 *Ohwia caudata* aqueous extract improved Mitochondrial Membrane Potential (ΔΨm) in doxorubicin-stimulated Wharton's jelly-derived mesenchymal stem cells. (A) Mitochondrial membrane potential assessment using JC-1 staining after doxorubicin exposure and treatment with *Ohwia caudata* aqueous extract in Wharton's jelly-derived mesenchymal stem cells. Wharton's jelly-derived mesenchymal stem cells were exposed to 0.1 μM doxorubicin for 24 h, followed by treatment with different doses of *Ohwia caudata* aqueous extract. The intensity of significant decrease in theJC-1 aggregate (Red color), and an increase in green JC-1 and monomers (Green color) after doxorubicin stimulation. However, *Ohwia caudata* aqueous extract. prevented the loss of JC-1 aggregate and increased the red JC-1 monomers. The scale bar is 100 μm. (B) The exposure of Wharton's jelly-derived mesenchymal stem cells to 0.1 μM doxorubicin led to an increase in JC-1 monomers level. However, treatment with *Ohwia caudata* aqueous extract reduced the production of the JC-1 monomers. Exposure to 0.1 μM doxorubicin for 24 h significantly decreased the ratio of the aggregate to monomers, but *Ohwia caudata* aqueous extract (25, 50, 100, 200 μg/mL) treatment prevented the decrease of the aggregate to monomers ratio in a dose-dependent manner. The data were analyzed using one-way ANOVA. $^{**}p < .01$ and $^{***}p < .001$ versus control; $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ versus 0.1 μM doxorubicin. Dox, doxorubicin; OCAE, *Ohwia caudata* aqueous extract.

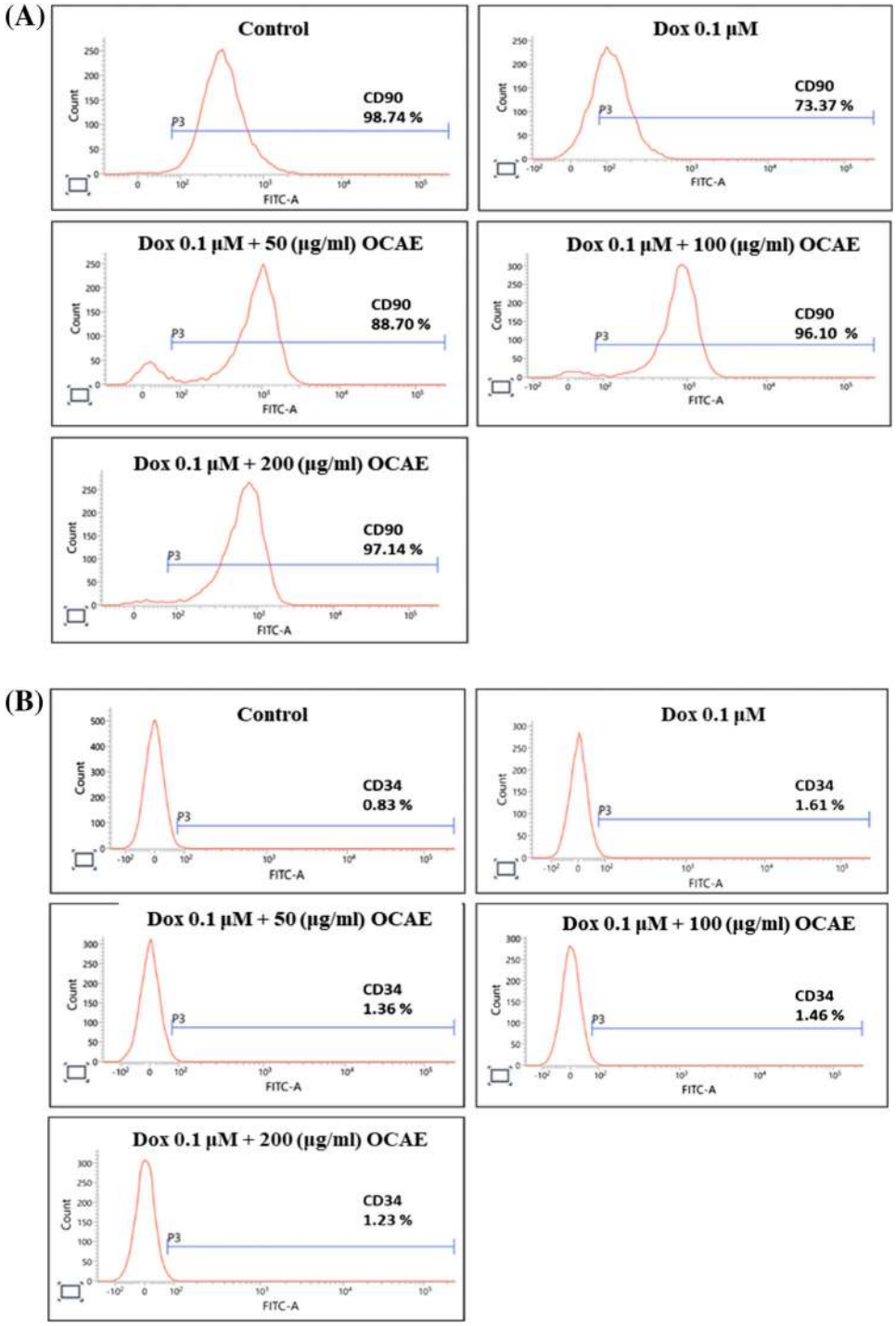

FIGURE 4 Analysis of mesenchymal stem cell marker expression using flow cytometry. (A,B) Comparison of cell surface proteins ($CD90^+$ and $CD34^-$) in Wharton's jelly-derived mesenchymal stem cells using flow cytometry. The $CD90^+$ and $CD34^-$ percentage in Wharton's jelly-derived mesenchymal stem cells was assessed after doxorubicin treatment for 24 h, followed by different doses of *Ohwia caudata* aqueous extract. The expression of CD90 reduced in doxorubicin-treated cells, within 24 h. However, CD90 expression was increased in more than 88% of the Wharton's jelly-derived mesenchymal stem cells after *Ohwia caudata* aqueous extract treatment. In contrast, doxorubicin treatment significantly raised the percentage of $CD34^+$ cells, wherein *Ohwia caudata* aqueous extract treatment reduced the percentage of $CD34^+$ cells. Dox, doxorubicin; OCAE: *Ohwia caudata* aqueous extract.

against doxorubicin-induced damage in stem cells. Wharton's jelly-derived mesenchymal stem cells are isolated from the Wharton's jelly portion of the umbilical cord, providing easy access for collection and fewer ethical and legal problems. Wharton's jelly-derived mesenchymal stem cells need less doubling time and have a high differentiation potential. They also have a greater ability for ex vivo expansion and better immunomodulatory properties, influencing innate and adaptive immune responses. Several studies have demonstrated the applicability of Wharton's jelly-derived mesenchymal stem cells in developing therapeutic strategies against several ailments, including neurological diseases, organ damage (like lung, liver, kidney, and bone damage), diabetes, and cancer.[40–42] Aging, a progressive chronological process, can be affected by environmental and genetic factors as well and limits the stemness of stem cells.[43] In cells, defense mechanisms against reactive oxygen species deteriorate due to aging, especially in the mitochondria.[44] Therefore, mitochondrial dysfunction plays an important role in aggravating cellular aging. Doxorubicin, a first-line cancer treatment, has been shown to induce mitochondrial dysfunction and oxidative stress, consequently leading to cellular senescence.[45,46] As demonstrated in our previous studies, we treated

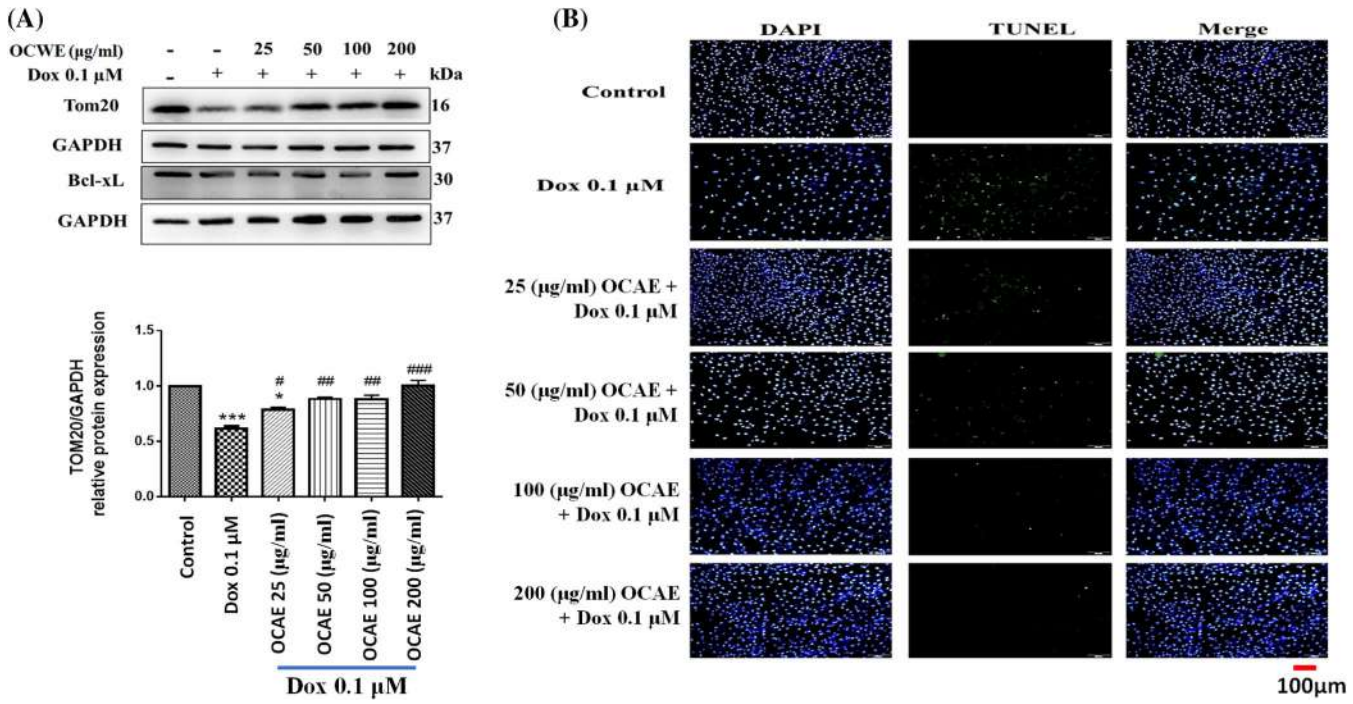

FIGURE 5 Effects of *Ohwia caudata* aqueous extract on Tom20 expression in doxorubicin-induced apoptosis in Wharton's jelly-derived mesenchymal stem cells. (A) Tom20 expression was analyzed using immunoblotting. After doxorubicin stimulation, Tom20 levels reduced within 24 h. However, *Ohwia caudata* aqueous extract treatment restored Tom20 levels. Data were analyzed using one-way ANOVA. $^{*}p < .05$ and $^{***}p < .001$ versus control; $^{\#}p < .05$; $^{\#\#}p < 0.01$; and $^{\#\#\#}p < .001$ versus 0.1 μM doxorubicin. (B) The protective effect of *Ohwia caudata* on doxorubicin-induced cell apoptosis assessed using the TUNEL assay. The results showed that TUNEL-positive cells increased after treatment with 0.1 μM doxorubicin. However, treatment with *Ohwia caudata* aqueous extract significantly decreased the number of apoptotic cells. DAPI (4′,6-diamidino-2-phenylindole) was applied for nuclear staining. The scale bar is 100 μm. Dox, doxorubicin; OCAE: *Ohwia caudata* aqueous extract.

Wharton's jelly-derived mesenchymal stem cells with doxorubicin to induce mitochondrial dysfunction and oxidative stress in the present study.[18] Doxorubicin enhanced mitochondrial damage and reactive oxygen species and reduced mitochondrial membrane potential and mitochondrial related proteins expression to suppress mitochondrial stability. In addition, the stemness of Wharton's jelly-derived mesenchymal stem cells was repressed, and cell apoptosis increased after doxorubicin treatment. These results indicated the successful establishment of doxorubicin-induced mitochondrial dysfunction.

Ohwia caudata is a traditional Chinese medicine and usually used to treat different diseases.[23,25] and several different alkaloids, triterpenoids, and flavonoids have been identified as its constituents.[22,23] Nonetheless, most research focuses on the organic extract instead of the therapeutic properties of the crude aqueous extract of *Ohwia caudata*, providing a new field for basic researchers to explore the health benefits of *Ohwia caudata* aqueous extract. In this study, we showed that the *Ohwia caudata* aqueous extracts reversed doxorubicin-induced mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells. Tid1 plays a critical role in maintaining a homogeneous distribution of mitochondrial membrane potential and the integrity of mitochondrial DNA.[16] Our findings revealed that treatment with *Ohwia caudata* aqueous extracts enhanced mitochondrial stability by promoting Tid1 expression.

The doxorubicin-induced mitochondrial reactive oxygen species generation in Wharton's jelly-derived mesenchymal stem cells was suppressed by *Ohwia caudata*. Previous study presents several phenolic components in *Ohwia caudata* have capability to reduce inflammation and reactive oxygen species in vitro model.[24] Although we lacked analyze the chemical composition of *Ohwia caudata* aqueous extracts and identify the causal bioactive compounds, we speculate that the same compounds as in the previous study are responsible for their doxorubicin-induced mitochondrial dysfunction alleviating effects. Nevertheless, further in-depth pharmacological studies are required to validate our speculations. Furthermore, the mitochondrial membrane potential was also maintained by *Ohwia caudata*. Eventually, the stemness was improved and the number of survival cells was increased by *Ohwia caudata* treatment after doxorubicin damage.

Interestingly, Tom20 is the essential import of proteins into the matrix and outer membrane.[38] For example, Tom20 has been implicated in the import of survivin into the mitochondria to inhibit apoptosis.[39] Our results show that Tom20 levels decreased after doxorubicin treatment; however, it increased with *Ohwia caudata* treatment. Together, we speculated that the protective effects of *Ohwia caudata* could be mediated via protein import by Tom20. According to our data, we conclude that the crude aqueous extract of *Ohwia caudata* might contain components that protect Wharton's

jelly-derived mesenchymal stem cells against doxorubicin-induced stress, so we plan to analyze the constituents in the crude aqueous extract of *Ohwia caudata* in the future. Finally, it can be concluded that the *Ohwia caudata* aqueous extract can possibly eliminate the side effect of doxorubicin, but further research is necessary. Especially high doses of *Ohwia caudata* aqueous extract are particularly effective.

This study had certain limitations. First, we used only Wharton's jelly-derived mesenchymal stem cells isolated from the umbilical cord of a newborn. The beneficial effects of *Ohwia caudata* should be evaluated using other types of stem cells to confirm the findings of the present study. Second, we did not explore the effects of *Ohwia caudata* aqueous extract on other cell survival mechanisms to maintain stem cell stability apart from alleviating mitochondrial dysfunction. Therefore, further investigations are required to understand the broader effects of *Ohwia caudata* aqueous extract. Third, the effect of *Ohwia caudata* aqueous extract on the down-regulation of Tid1 and dose-dependent rescue of doxorubicin-induced apoptosis were not concordant. This experimental instability could have resulted from using crude aqueous extract—the content and composition may have differed in different batches. Therefore, identifying the composition and active components underlying the beneficial effects of *Ohwia caudata* aqueous extracts is necessary. Finally, this study was based on a cell model, not an animal one. To explore new applications of *Ohwia caudata*, it should be further studied using an animal model. Although the present study has some limitations, it still provides new directions for the medicinal applications of *Ohwia caudata*.

5 | CONCLUSION

The present study demonstrated that doxorubicin induced mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells by impairing the mitochondrial membrane potential and mitochondrial stability and increasing reactive oxygen species generation. Moreover, doxorubicin treatment repressed the stemness of Wharton's jelly-derived mesenchymal stem cells and increased cell apoptosis. These effects of doxorubicin were alleviated by treatment with the crude aqueous extract of *Ohwia caudata*, which promoted Tid1 and Tom20 expression to maintain mitochondrial stability and restore the mitochondrial membrane potential. Collectively, the present study suggests that *Ohwia caudata* aqueous extract can potentially prevent mitochondrial dysfunction and provides new directions for the medical application of *Ohwia caudata*. Nevertheless, further studies are required to identify the active components of the aqueous extract of *Ohwia caudata* and understand the underlying mechanism.

AUTHOR CONTRIBUTIONS

Pei-Ying Lee, Tsung-Jung Ho, and Chih-Yang Huang conceptualized and designed the study. Bruce Chi-Kang Tsai and Maria Angelina Sitorus collected and assembled the data. Chia-Hua Kuo provided materials for the study. Bruce Chi-Kang Tsai, Maria Angelina Sitorus, and Shinn-Zong Lin analyzed and interpreted the data. Bruce Chi-Kang Tsai, and Maria Angelina Sitorus wrote the draft of the manuscript. Shinn-Zong Lin, Cheng-Yen Shih, Shang-Yeh Lu, and Yueh-Min Lin reviewed and gave the final approval of the manuscript. Yueh-Min Lin, Tsung-Jung Ho, and Chih-Yang Huang provided administrative support. Pi-Yu Lin, Cheng-Yen Shih, Chih-Yang Huang, and Pei-Ying Lee provided financial support. All authors reviewed the manuscript.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Science and Technology, Taiwan (MOST 111-2314-B-303-008-MY3), Hualien Tzu Chi Hospital (IMAR-110-01-12) China Medical University, and Asia University (CMU102-ASIA-04-08 and CMU102-ASIA-04).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Lee P-Y, Tsai BC-K, Sitorus MA, et al. *Ohwia caudata* aqueous extract attenuates doxorubicin-induced mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells. *Environmental Toxicology*. 2023;1-12. doi:10.1002/tox.23880