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The Properties of Exosomes Derived from Mesenchymal Stem Cells Preconditioned with L-Ascorbic Acid and Cobalt (II) Chloride

Anggraini Barlian[1,5*], Rizka Musdalifah Amsar[1], Salindri Prawitasari[1], Christofora Hanny Wijaya[2], Ika Dewi Ana[3], Triati Dewi Kencana Wungu[4,5]

[1]*School of Life Science and Technology, Institut Teknologi Bandung, Bandung 40132, Indonesia*
[2]*Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, IPB University, Bogor 16680, Indonesia*
[3]*Department of Dental Biomedical Sciences, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[4]*Nuclear Physics and Biophysics Research Group, Department of Physics, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung 40132, Indonesia*
[5]*Research Center for Nanoscience and Nanotechnology, Institut Teknologi Bandung, Bandung 40132, Indonesia*

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ABSTRACT

Extracellular vesicles including exosomes, are produced by cells for intracellular communication. Preconditioning of parental cells influences exosome properties. The purpose of this study was to examine the effects of L-ascorbic acid (LAA) and cobalt (II) chloride ($CoCl_2$) on human Wharton's jelly mesenchymal stem cell (hWJ-MSC)-derived exosomes and their ability to promote stem cell differentiation into chondrocytes. The cells were isolated from the umbilical cord and characterized according to the criteria for mesenchymal stem cell. The cells were cultured in a serum-free medium containing LAA and $CoCl_2$. Cell-produced exosomes were isolated and characterized. hWJ-MSCs can grow in serum-free medium containing LAA and $CoCl_2$. Exosomes derived from hWJ-MSCs had a round morphology, particle size within the exosome range, CD 63 expression, and the capacity to be internalized by cells. The production of exosomes by hWJ-MSCs was enhanced by LAA treatment. LAA and $CoCl_2$ promoted stem cell differentiation into chondrocytes, as indicated by the production of collagen type II and glycosaminoglycans. LAA and $CoCl_2$ affect the properties of MSC-derived exosomes. LAA induces cells to produce exosomes in greater quantities, which have the potential to promote chondrogenic differentiation of stem cells.

1. Introduction

Exosomes are a subset of extracellular vesicles used for cell communication. Exosomes are distinguished from other extracellular vesicles by their size and biogenesis. Exosomes' size approximately 30-100 nm (D'Aniello *et al.* 2017), make it the smallest extracellular vesicle (Guerreiro *et al.* 2018). Other extracellular vesicles, such as microvesicles, are produced by direct budding of the plasma membrane; however, exosomes originate from the inward budding of endosomes into multivesicular bodies (Haraszti *et al.* 2019). MSC-derived exosomes possess therapeutic properties similar to those of progenitor cells (Shelke *et al.* 2014). Moreover, the nanoscale size of exosomes enables them to transmit signals remotely and across various biological barriers (Wu *et al.* 2009).

Pre-conditioning of parental cells can alter the quality and quantity of the released exosomes. L-ascorbic acid (LAA) is a vitamin C derivative (Choi *et al.* 2008), known to promote stem cell proliferation (Choi *et al.* 2008; Zhang *et al.* 2016; Teti *et al.* 2018). LAA also induced osteoblast differentiation to generate bone tissue (Choi *et al.* 2008). The effect of pre-conditioning hWJ-MSCs with LAA on its derived exosomes has not yet been investigated. Hypoxia can increase the proliferation of mesenchymal stem cells (Grayson *et al.* 2007) and hypoxic preconditioning of cells influences exosomes secretion. $CoCl_2$, a hypoxia-inducing agent, inhibits HIF-1α degradation (Hirsilä *et al.* 2005).

The purpose of this study was to investigate the effects of hypoxia and LAA on the properties of

* Corresponding Author
E-mail Address: aang@sith.itb.ac.id

exosomes derived from mesenchymal stem cell and their responses to chondrogenic induction.

2. Materials and Methods

2.1. Ethics Statement

This study was approved by the Padjajaran University Research Ethics Commission (Number: 983/UN6.KEP/EC/2020) and Rumah Sakit Khusus Ibu dan Anak (RSKIA) Kota Bandung (070/0079–RSKIA).

2.2. Cell Culture and Characterization

Human umbilical cord samples were used to obtain cells with ethical written informed consent and ethical approval. Cells were isolated using an enzymatic method (Leng *et al.* 2019). Multipotency assay and flow cytometry were used to characterize the cells (Dominici *et al.* 2006). Using passages 3–8 of human Wharton's jelly stem cells (hWJ-MSCs), an extracellular vesicles study was undertaken (Khanh *et al.* 2021). In this experiment, only cells from passages 4–6 were used to ensure cell viability.

In a 24-well plate, 200,000 cells (P 5) were cultured for multipotency analysis. After 70-80% cells confluence, the culture medium was changed to differentiation medium (StemPro™ Chondrogenic Differentiation Kit, StemPro™ Adipogenic Differentiation Kit, StemPro™ Osteogenic Differentiation Kit). Cells were grown at 37°C and 5% CO_2 for 21 days. Every two days the medium was changed. The remaining culture medium was discarded after 21 days. Cultures were washed with PBS and fixed with 4% formaldehyde. Alizarin Red, Oil Red O, and Alcian Blue were used to stain the cells (Abouelnaga *et al.* 2022). The culture was examined under an inverted microscope.

Flow cytometry was used to observe the specific surface markers of MSC using the Human MSC Analysis Kit (BD Stemflow™), in accordance with the manufacturer's instructions. The passage number of hWJ-MSCs used in the flow cytometry assay was four. Flow cytometry was performed using BD FACSAria III and BD FACSDiva Version 6.1.3 software.

2.3. Cell Viability in Medium Containing $CoCl_2$

hWJ-MSCs at passage five were cultured in growth medium containing $CoCl_2$ at concentrations of 25, 50, 100, 200, and 400 µM. Cultured cells were incubated at 37°C and 5% CO_2. After 24 and 48 h of incubation, cell viability was measured using the MTT assay. The absorbance was measured at a wavelength of 595 nm using a microplate reader (Bio-Rad). LAA concentration used in this study is 250 µM (Choi *et al.* 2008).

2.4. Exosome Isolation

hWJ-MSCs (P 5) were grown in a growth medium containing DMEM LG (Gibco), antibiotic antimycotic (Gibco), and 10% Fetal Bovine Serum/FBS (Gibco). After the cells reached approximately 60% confluence, the cells were washed with phosphate-buffered saline (PBS). The medium was replaced with serum-free culture medium (control), medium containing 25 µM $CoCl_2$, medium containing 250 µM LAA, or medium containing both 25 µM $CoCl_2$ and 250 µM LAA. Cells were cultured in a humidified incubator (37°C, 5% CO_2) for 48 h. Exosomes were extracted from the supernatant using the Invitrogen™ Total Exosome Isolation Kit according to the manufacturer's instructions. Briefly, the conditioned medium was collected and centrifuged at 2,000 x g for 30 min. The supernatant was collected, 0.5 volume of Invitrogen™ reagent was added, and the mixture was vortexed. The samples were then incubated overnight at 4°C. The samples were centrifuged at 4°C for one hour at 10,000 × g. The pellet was resuspended in 1x PBS.

2.5. Characteristic of Exosomes

The presence and shape of exosomes were examined using Transmission Electron Microscopy (TEM). Briefly, exosomes in PBS (10 µL) were placed on a 300-mesh copper grid with a carbon-coated film. The grids were blotted with a filter paper and washed with ddH_2O. Uranyless was used to stain the samples (Shu *et al.* 2020) and the samples were examined using a Transmission Electron Microscope (Hitachi HT7700).

Exosomes markers (CD 63) were analyzed using ELISA. The Invitrogen Human CD 63 ELISA Kit (Thermo Scientific) was used according to the manufacturer's instructions. 100 µL of each standard and sample were added to each well and incubated for 2.5 hours at room temperature. The solution was discarded, and samples were washed with wash buffer. Biotinylated antibodies were added to each well and incubated at room temperature for one hour. The solution was discarded, and samples were washed with wash buffer. Streptavidin-HRP was added to each well, incubated for 45 min at room temperature, and washed with the wash buffer. Each well was added with TMB substrate and incubated

at room temperature for 30 min. Stop solution was added, and the absorbance was measured at 450 nm using a BioRad microplate reader.

2.6. Nanoparticle Tracking Analysis

Exosomes were observed using a Horiba Scientific ViewSizer 3000/0053. Using Nanoparticle Tracking Analysis (NTA) software, the collected data were analyzed to produce particle size distribution profiles and concentration measurements.

2.7. Cellular Uptake of Exosomes

PKH 67 Flourescent Cell Linker Kits (Sigma) was used to study the uptake of exosomes in hWJ-MSCs. To 1 ml of Diluent C, 100 µL of exosomes were added. The samples were incubated for 4 minutes. 2 ml Bovine Serum Albumin/BSA 1% (Sigma) was added and centrifugated at 20,000 x g, 4°C for 1 hour. Two centrifugations of 15 minutes at 4°C at 20,000 x g were performed with the addition of Phosphate Buffer Saline/PBS for each wash sample. The concentrates were collected and diluted in DMEM (Gibco) with the addition of Antibiotic-antimycotic (Gibco) and used as culture medium. After 1h, 3h, 6h, and 24h of incubation, the samples were fixed with paraformaldehyde and the nuclei were stained with DAPI. The Olympus FV1200 Confocal Laser Scanning Microscope was used to observe the cellular uptake.

2.8. Alcian Blue Assay

hWJ-MSCs cultured in 24-well plate. After 60% confluence, the culture medium is removed and replaced with medium containing 2% FBS (Gibco), 1% antibiotic-antimycotic, and exosomes. As a control, chondrogenic differentiation medium (Gibco's StemPro™ Chondrogenesis Differentiation Kit) was used. The cells were incubated at 37°C, 5% CO_2. After 21 days of incubation, glycosaminoglycan (GAG) levels were determined using the Alcian Blue assay. The cell culture medium was aspirated and washed twice with PBS. The cells were fixed in acetone-methanol for three minutes at 4°C. The samples were stained with Alcian Blue (in 3% acetic acid) and incubated for 30 min at room temperature. The samples were washed with 3% acetic acid solution, followed by deionized water. The samples were treated with 1% SDS and incubated on a shaker at 200 rpm for 30 min. The absorbance of the samples was determined using UV-Vis spectrophotometry at 605 nm.

2.9. The Presence of Collagen Type II

hWJ-MSC cultures were grown on coverslips. After the cell density reaches 60%, the culture media is replaced with DMEM LG (Gibco) supplemented with 2% FBS (Gibco), 1% antibiotic-antimycotic, and exosomes. The cells cultured in chondrogenic differentiation medium (StemPro™ Chondrogenesis Differentiation Kit, Gibco) used as a control. The cells were then incubated in 5% CO_2 at 37°C. The medium was discarded after 21 days of incubation and the cells were washed with PBS. All samples were fixed at -20°C for 5 min with a methanol-DMEM series and permeabilized with PBST (0.05% Tween-20 in PBS). BSA was used as a blocking agent, and primary antibody collagen type II (Rabbit polyclonal to Collagen II, Abcam ab34712, 1:1000) was added to the samples and incubated overnight at 4°C. The samples were washed with PBS, secondary antibody Alexa Flour 488 (Anti-rabbit IgG (H+L), CFTM488A antibody produced in F(ab')2 fragment of goat, Sigma SAB4600234, 1:1,000) was added, and nuclei were stained with DAPI. All samples were examined with an Olympus FV1200 Confocal Laser Scanning Microscope at ITB-Olympus Bioimaging Center.

2.10. Statistical Analysis

The obtained data were expressed in terms of mean ± standard deviation (SD). Data were analyzed using analysis of variance (ANOVA). Post-hoc analysis done through Tukey's multiple comparison test. Statistical test performed using the GraphPad Prism 9 software.

3. Results

3.1. Characteristics of Mesenchymal Stem Cells

The cells were characterized based on MSC characteristics to confirm that the isolated cells were MSCs. The cells were spindle-shaped and adhered to the substrate (Figure 1A). Cells were cultured in chondrogenic, adipogenic, and osteogenic differentiation media to investigate their multipotency. The extracellular matrix produced by chondrocytes was present in cells cultured in chondrogenic medium, as confirmed by Alcian Blue staining (Figure 1B). Cells cultured in the adipogenic medium formed lipid droplets, as confirmed by Oil Red O staining (Figure 1C). Calcium mineralization was observed in cells grown in the osteogenic

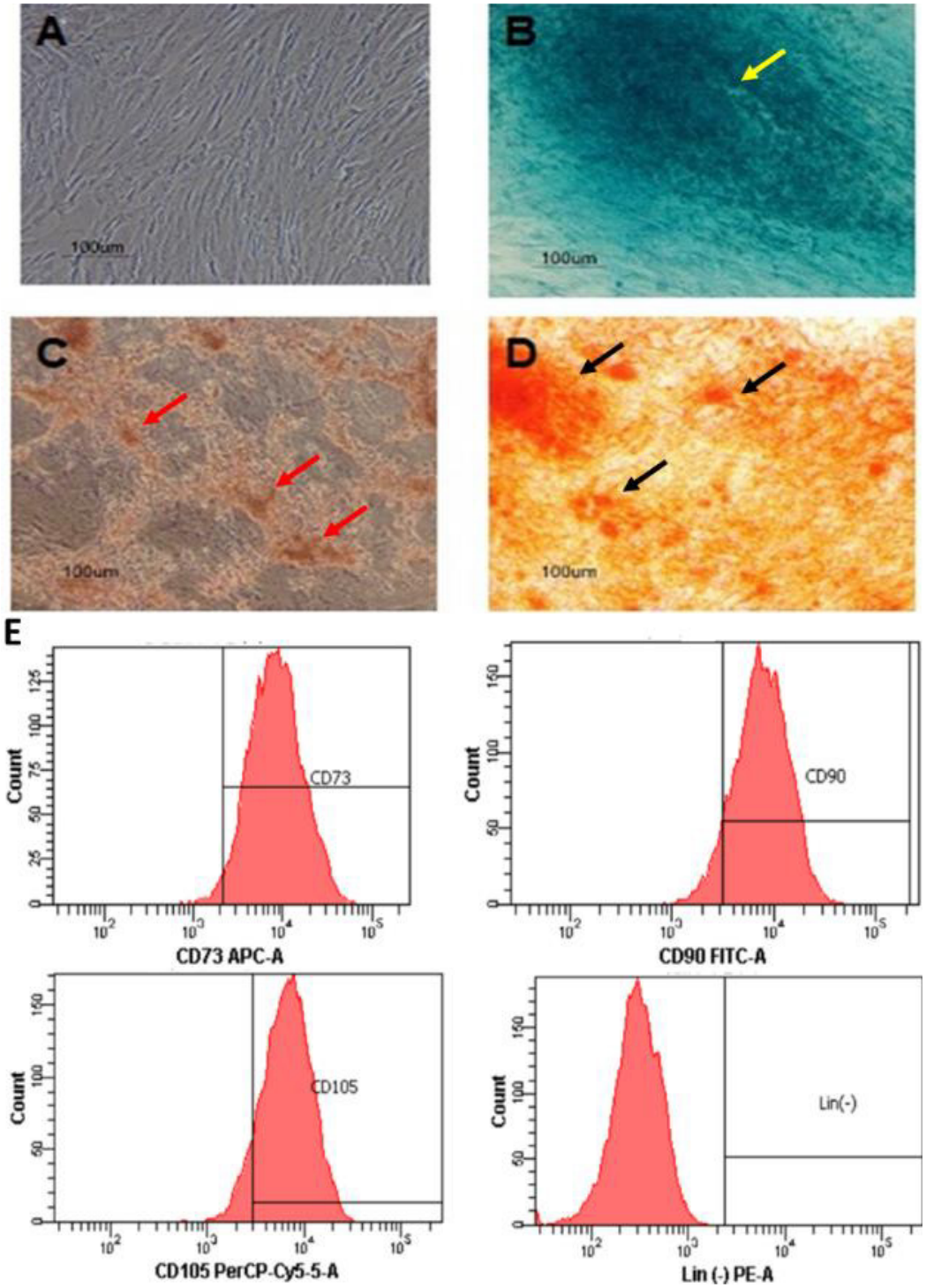

Figure 1. Stem cell characterization. (A) Cell morphology was fibroblast-like. (B) Cells can differentiate into chondrocytes (shown by the chondrocyte matrix; yellow arrow), (C) adipocytes (represented by the formation of lipid droplets; red arrow), and (D) osteocytes (indicated by the calcium mineralization; black arrow). (E) Cell surface markers reveal mesenchymal stem cells-specific surface markers

medium (Figure 1D). The cells present MSC surface markers, including CD 90+, CD 105+, CD 73+, and the negative surface marker CD 45 at less than 2% (Figure 1E). Based on these results, we conclude that the isolated cells were MSCs and could be identified as hWJ-MSCs.

3.2. Cell Viability in $CoCl_2$ Medium

$CoCl_2$ cytotoxicity on hWJ-MSCs was assessed using an MTT assay. 24 hour and 48 hour MTT assays were performed with $CoCl_2$ concentration ranging from 25 µM to 400 µM. All concentrations of $CoCl_2$ induced high cell viability under hypoxic conditions; however, 25 µM $CoCl_2$ exhibited the highest cell viability, as depicted in Figure 2A. In this study, 25 µM $CoCl_2$ was used in subsequent experiments.

Our previous study showed that hWJ-MSCs can be cultured in serum-free media (See Figure 1 in Supplementary Materials). In this study, the cells were then grown in serum-free DMEM containing LAA or LAA and $CoCl_2$. Serum-free DMEM was used as the control (Figure 2). There was no difference in cell morphology between the media.

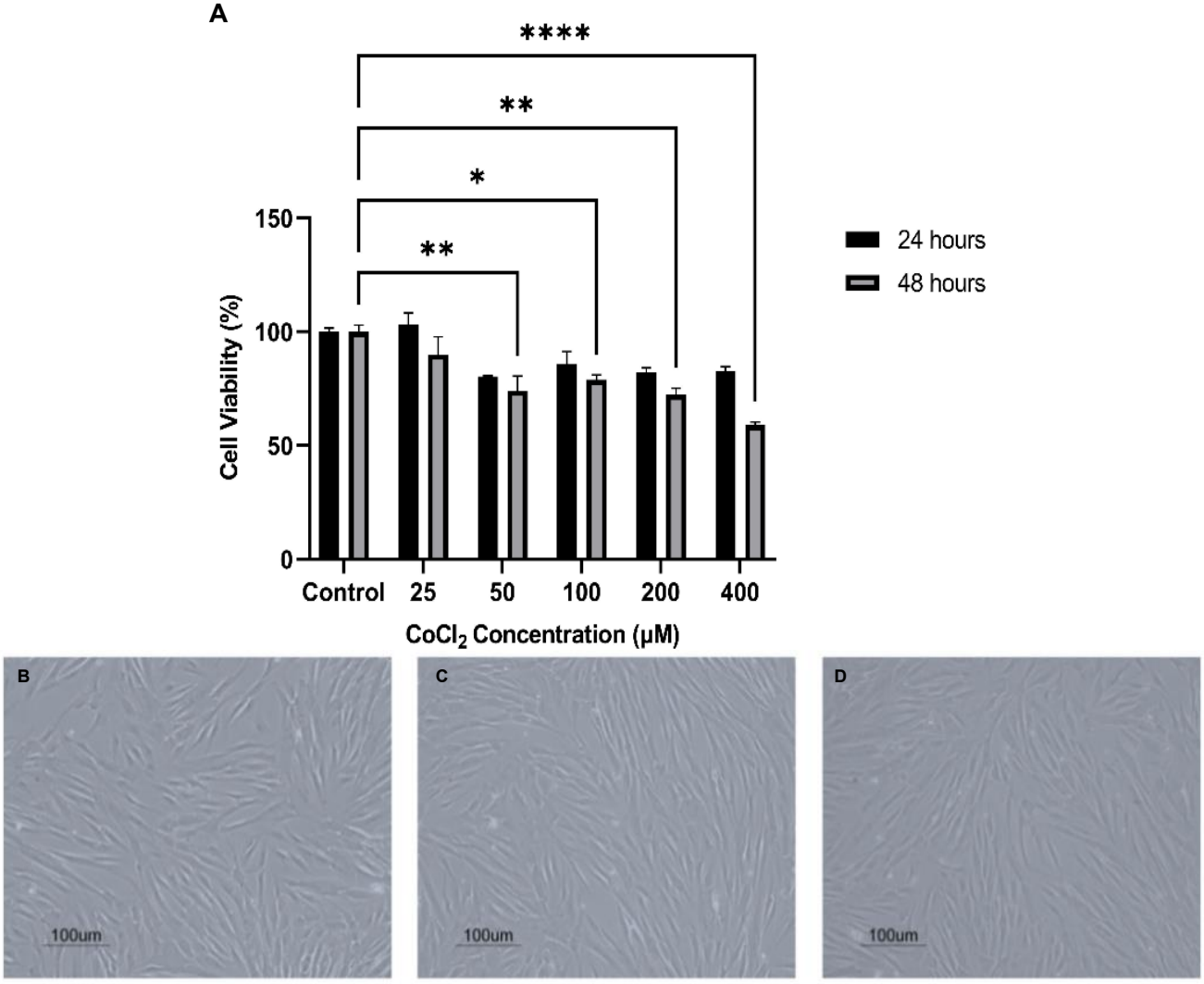

Figure 2. Effect of $CoCl_2$ on hWJ-MSCs. (A) Viability of cells as determined by the MTT assay. (B) Cells were cultured in serum-free DMEM media for 48 h. (C) Cells cultured in serum-free DMEM with LAA. (D) Cells cultured in serum-free DMEM containing LAA and $CoCl_2$. Data are presented as the mean ± SD with significance indicators * ($p<0.05$), ** ($p = 0.0016$ and 0.0025), and **** ($p<0.0001$)

3.3. Characterization of Exosomes

The morphology of exosomes as examined using negative staining TEM. Exosomes of varying diameters were found to have spherical structures in all three experimental groups, as shown in Figure 3. The medium without cells was examined using TEM to ensure that it did not contain exosomes.

CD 63, an exosomes' marker, was present in all treatments, as confirmed by ELISA (Figure 4A). The concentration of CD 63 in the medium of hWJ-MSCs containing LAA and $CoCl_2$ was higher than that in the medium of hWJ-MSCs containing LAA or $CoCl_2$ alone.

The exosome size of the control (untreated hWJ-MSCs) was 114 nm, with a modal size of 125 nm. The exosome size of hWJ-MSCs pre-treated with $CoCl_2$ was 100 nm with a modal size 24.98 nm. The size of exosomes derived from hWJ-MSCs pre-treated with LAA was 101 nm, with a modal size 96.38 nm. Pre-treatment of hWJ-MSCs with both LAA+ $CoCl_2$ produced exosomes with a size of 110 nm and a modal size of 149 nm (Figure 4B). A size range of 30-150 nm confirmed that the isolated exosomes were indeed exosomes.

3.4. Exosomes Concentration

The concentration of exosomes produced by cells was investigated using NTA. Our results, as shown in Figure 5, revealed that preconditioning hWJ-MSCs with LAA produced the highest concentration of exosomes compared to other treatments.

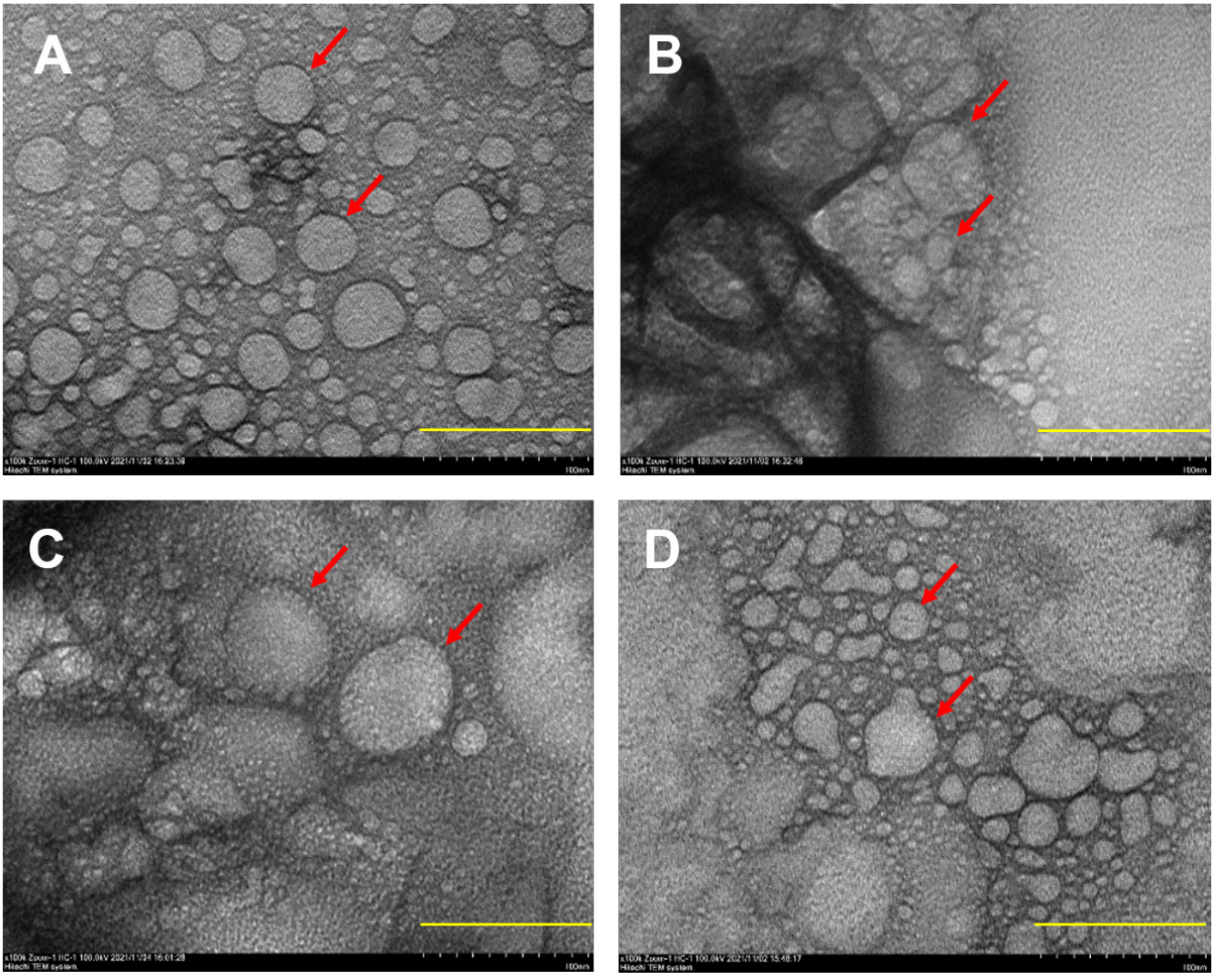

Figure 3. TEM visualization of exosomes isolated from hWJ-MSCs grown in (A) serum-free DMEM, (B) $CoCl_2$, (C) LAA, and (D) LAA+$CoCl_2$. The red arrow indicated exosomes. Scale bar = 100 nm

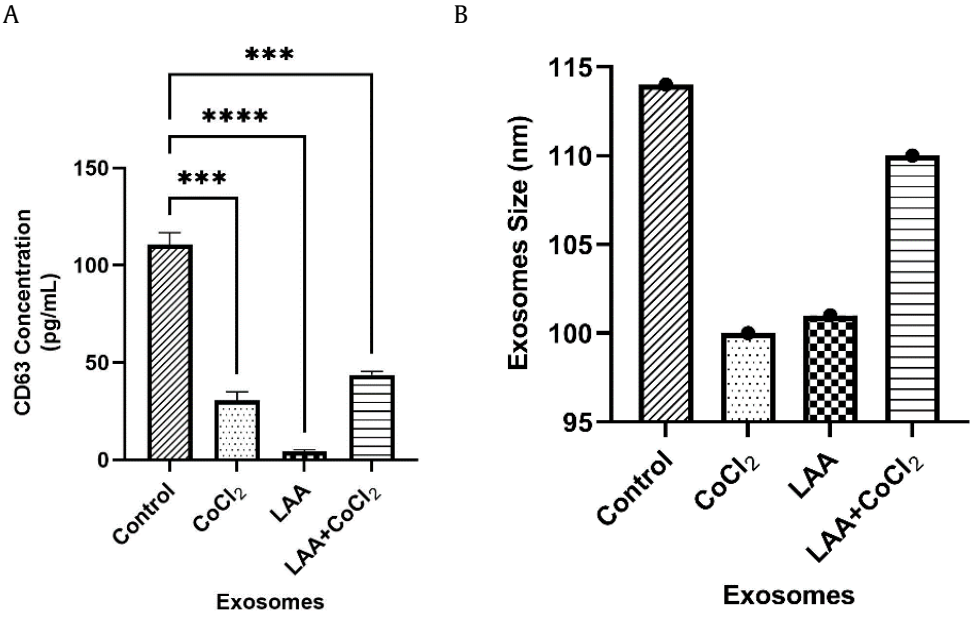

Figure 4. Characterization of exosomes. (A) Presence of CD 63 in hWJ-MSCs-derived exosomes. Exosomes from hWJ-MSCs, without LAA or $CoCl_2$, were used as controls. (B) Exosomes size distribution

3.5. Exosomes Internalization

To examine whether exosomes could be taken up by cells, PKH-67 labelled exosomes were incubated with hWJ-MSCs at different time points and observed with a confocal laser scanning microscope. Exosomes were taken by cells and detected in the cytoplasm after one hour, as depicted in Figure 6. Exosomes uptake by mesenchymal stem cells rises as the incubation period lengthens. Following 24 hours of incubation, exosomes completely fill the cytoplasm.

3.6. Chondrogenic Differentiation Potential of Exosomes

Alcian Blue staining detected GAG production in hWJ-MSCs treated with exosomes. The absorbance of the blue color was measured using UV-Vis spectrophotometry at 605 nm. On days 7, 14, and 21, GAG were detectable in hWJ-MSCs treated with exosomes. GAG absorbance measurement revealed that exosomes could induce chondrogenic differentiation (Figure 7A).

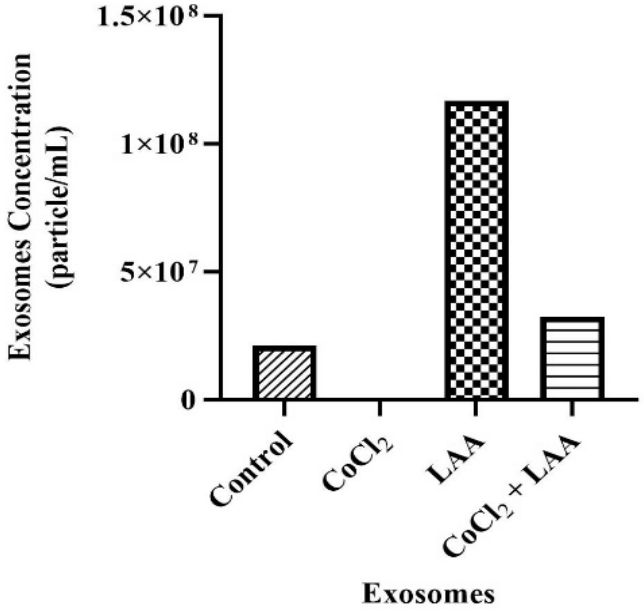

Figure 5. Concentration of exosomes produced by hWJ-MSCs

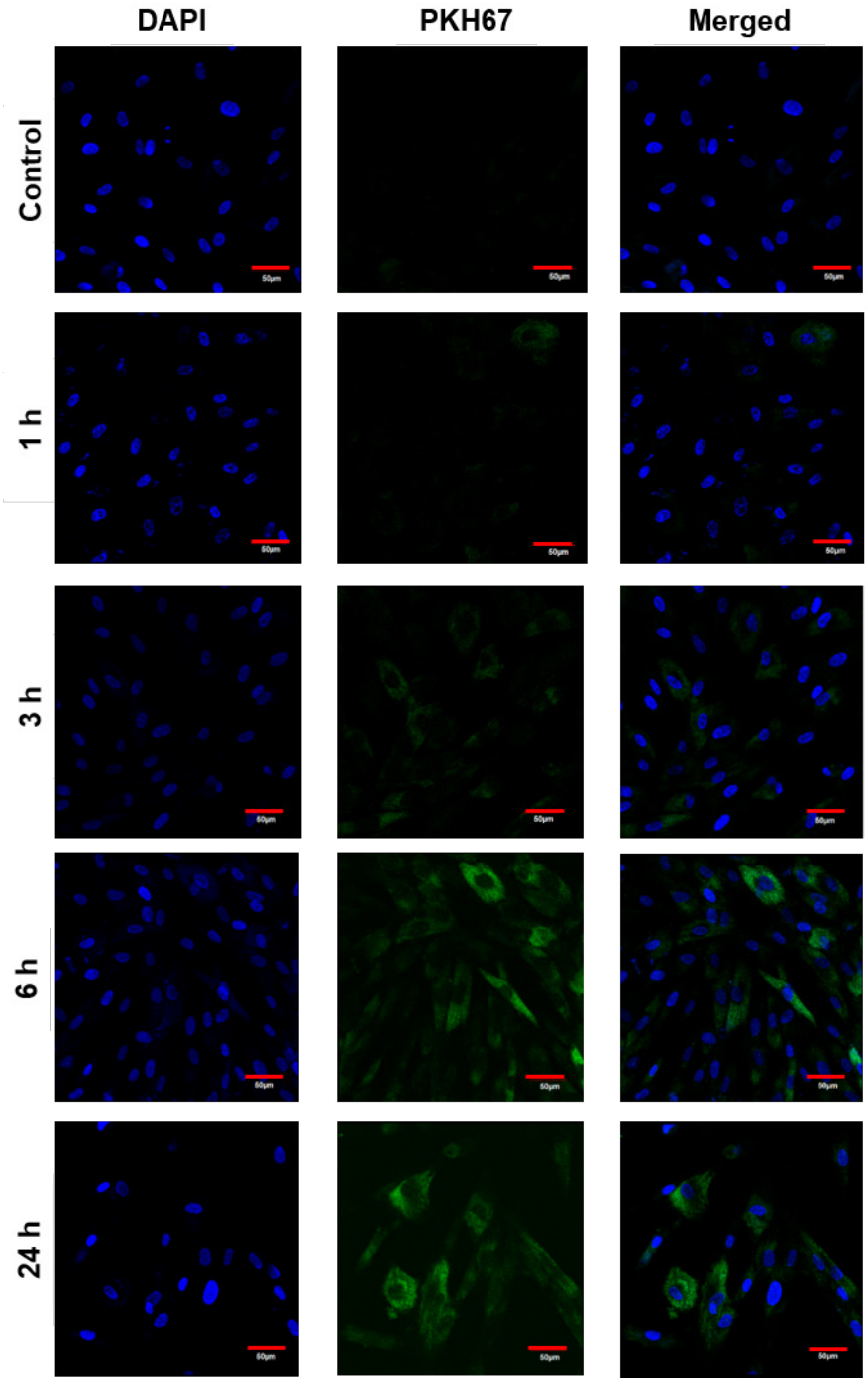

Figure 6. Uptake of exosomes by hWJ-MSCs. Exosomes were labelled with PKH-67 (green) and nucleus were stained with DAPI (blue). Scale bar = 50 µm

Immunocytochemistry and confocal laser scanning microscopy were used to analyze and visualize collagen type II. Figure 7B shows that collagen type II (indicated by the green color) increased after exosomes treatment for 14 and 21 days.

4. Discussion

Pretreatment (preconditioning/priming) MSC aims to improve the survival, function, and therapeutic efficacy of MSC (Zhou *et al.* 2021). Exosomes from MSCs secreted *in vivo* under certain conditions have

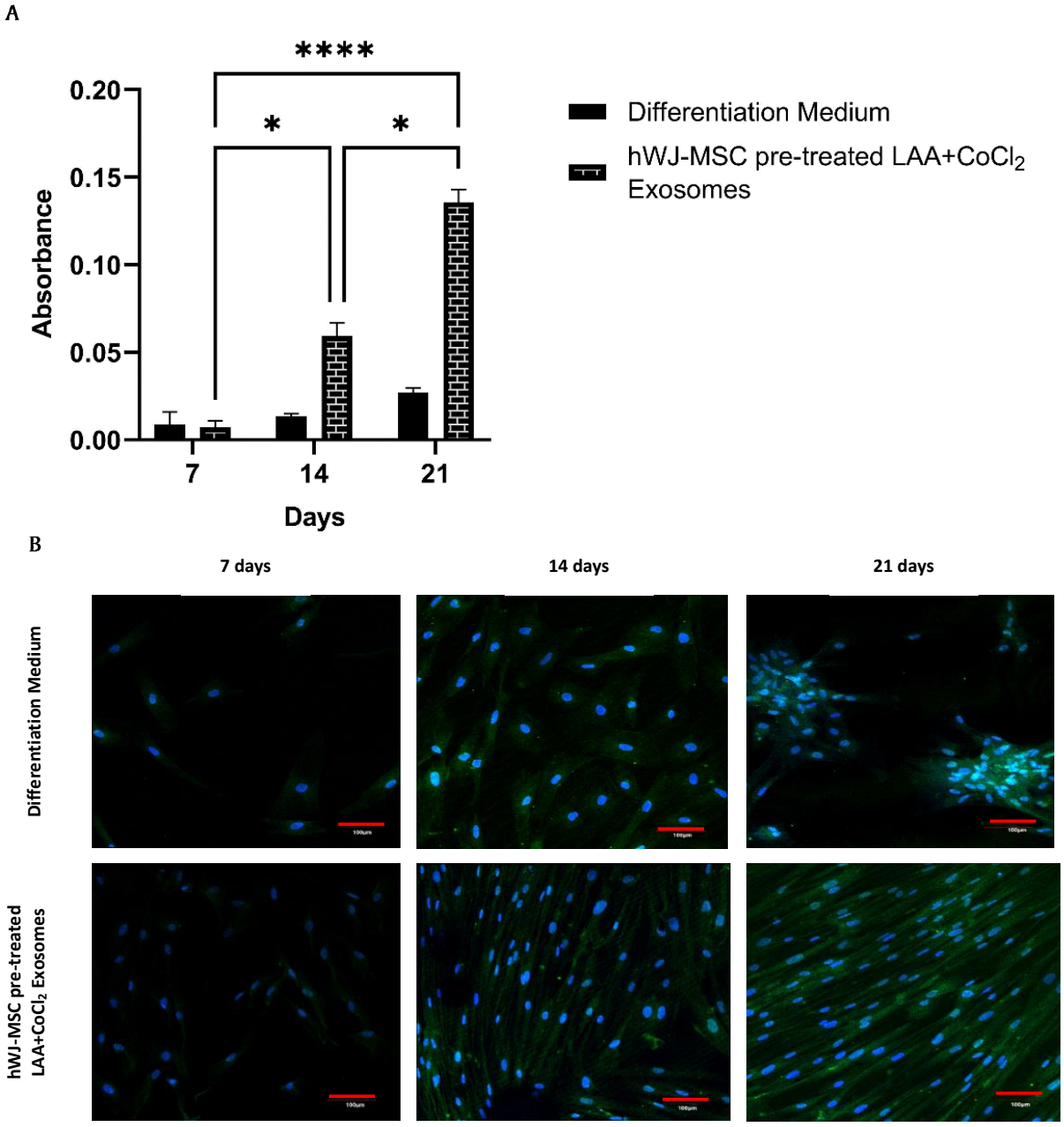

Figure 7. Exosomes enhance chondrogenic differentiation of hWJ-MSCs. (A) Production of glycosaminoglycan (GAG) in hWJ-MSC cultures. (B) Visualization of collagen type II produced by monolayer cultured cells. Blue indicates the nucleus and green indicates collagen type II. Data are presented as mean ± SD, with significance indicators *($p<0.05$), ****($p<0.0001$). Scale bar = 100 µm

different characteristics from exosomes produced *in vitro*, so that priming of MSCs cultured *in vitro* is expected to mimic the *in vivo* conditions of MSCs and produce more effective exosomes for therapy of a disease (Ren 2019). Research conducted by Zhang *et al.* showed that exosomes from MSCs pretreated with TGF-β/IFN-γ were more efficient in transforming mononuclear cells into T-regs (Zhang *et al.* 2018). In this study, we investigated the effects of LAA and chemical hypoxia induced by $CoCl_2$ on exosomes produced by MSCs.

LAA exerts proliferative and differentiation effects on mesenchymal stem cells (Choi *et al.* 2008). Research conducted by Mekala *et al.* showed that administration of LAA with a concentration of 250 µM significantly increased MSC proliferation

(Mekala *et al.* 2013). In addition, another study showed that administration of LAA at a concentration of 50 µg/ml increased the proliferation rate of MSCs and correlated with an increase in the chondrogenic differentiation capacity of MSCs (Barlian *et al.* 2020). $CoCl_2$ is a hypoxia mimicking agent that can trigger the differentiation of MSCs into chondrocytes. Giving $CoCl_2$ treatment to MSCs triggers chondrogenic differentiation by increasing the synthesis of chondrogenesis markers including aggrecan, Sox9, and type II collagen (Yoo *et al.* 2016). Exosomes secreted by MSCs will have properties and functions similar to those of the parent cell, so that the administration of LAA and $CoCl_2$ to MSCs is expected to produce exosomes containing cytokines, growth factors, and miRNAs that play a role in the chondrogenesis process (Toh *et al.* 2017).

Our results showed that pretreatment of LAA and $CoCl_2$ on hWJ-MSCs did not affect the morphology of hWJ-MSCs. The exosomes produced by hWJ-MSCs pretreated with LAA, $CoCl_2$, and LAA+$CoCl_2$ have a circular shape, but vary in size. The isolated exosomes were confirmed as exosomes because they have a size of 100-114 nm and express the CD 63 marker (Théry *et al.* 2018). The addition of LAA increased the concentration of exosomes, while the concentration of exosomes in hWJ-MSCs pretreated with $CoCl_2$ was not detected. The increase in the concentration of exosomes after LAA pretreatment is thought to occur through an increased expression of genes that play a role in cell proliferation and survival, which are then carried by exosomes as cargo (Fujisawa *et al.* 2018). hWJ-MSCs pretreated with LAA and $CoCl_2$ produced lower concentrations of exosomes than hWJ-MSCs treated with LAA. LAA and $CoCl_2$ are thought to have competing mechanisms of action. Cobalt in $CoCl_2$ acts as a chelating agent to replace iron (Fe) in the prolyl hydroxylase (PHD) enzyme and prevent it from binding HIF-1α so that the HIF-1α transcriptional complex is stabilized and accumulated (Yoo *et al.* 2016). Research conducted by Fujisawa *et al.* showed that the addition of LAA increased mitochondrial activity through the degradation of HIF-1α (Fujisawa *et al.* 2018).

Exosomes' functional properties in inducing chondrogenic differentiation were also investigated in this study. Exosomes from pretreated hWJ-MSCs can trigger chondrogenesis, as evidenced by the increased accumulation of GAG and the presence of collagen type II. However, based on the results of the ICC, it appears that hWJ-MSCs treated with exosomes have not yet formed cell condensation, which indicates that differentiation into chondrocytes has begun (Yamashita *et al.* 2018). Therefore, further studies are needed to determine the mechanism of LAA and $CoCl_2$ in inducing chondrogenesis. With this research, it is hoped that it will be a starting point to study the effect of treatment, especially LAA and chemically induced hypoxia, on exosomes.

LAA and $CoCl_2$ affect the properties of exosomes produced by mesenchymal stem cells. LAA induced mesenchymal stem cells to produce exosomes in higher yield and the exosomes have the potential to enhance chondrogenic differentiation of stem cells.

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