LAA and CoC_{l2} Pretreated Exosomes Enhance Chondrogenic Differentiation of Mesenchymal Stem Cell

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Research Article

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Abstract

Background

Cells produce extracellular vesicles, such as exosomes and microvesicles, which are used for intracellular communication. Cell-free therapies could be enhanced by using mesenchymal stem cell-derived exosomes. Preconditioning parental cells affects the properties of their exosomes. This study aimed to investigate the role of L-ascorbic acid (LAA) and CoCl2 in the exosomes produced by human Wharton's jelly mesenchymal stem cells (hWJ MSC) and its potential to induce chondrogenic differentiation of stem cells was also studied..

Method

The cells were obtained from umbilical cords and characterized based on mesenchymal stem cell criteria. The cells were cultured in a serum-free medium containing LAA and CoCl2. Exosomes produced by the cells were isolated and their morphology observed with Transmission Electron Microscopy. The presence of CD 63 was confirmed using ELISA. The particle size distribution and exosome concentration were analyzed with Nanoparticle Tracking Analysis (NTA). The ability of exosomes to induce stem cell differentiation into chondrocytes was investigated using the Alcian blue assay and immunocytochemistry.

Results

Stem cells were successfully isolated from the human umbilical cord. The cells can differentiate into adipocytes, chondrocytes, and osteocytes. Flowcytometry analysis showed the specific surface marker of mesenchymal stem cells. Exosomes isolated from pretreatment cells showed round-shaped morphology and confirmed the presence of CD 63. NTA analysis revealed that pretreatment of cells with LAA increases exosome yields. LAA supplementation in cell medium under hypoxic conditions induced by CoCl2 produces exosomes that can induce the chondrogeic differentiation of stem cells, confirmed by the presence of glycosaminoglycan and collagen type 2.

Conclusion

Exosomes produced by preconditioning hWJ-MSC with LAA in hypoxic conditions have the potential to enhance human Wharton Jelly stem cell differentiation into chondrocytes.

Background

Mesenchymal stem cell (MSC) has multipotent ability and can be found in some organs. MSC can be obtained from adipose [1], the umbilical cord, and bone marrow [2]. MSC can be a source for cell-based therapy due to its properties--for instance, anti-inflammatory and immunomodulatory [3]. Stem cells derived from Wharton's jelly (WJ-MSC) have more benefit than other types of MSC because of the less invasive isolation method, a higher proliferation rate, and wider plasticity [4].

Exosomes are a subgroup of extracellular vesicles released by the cell for cell communication. The difference between exosomes and other extracellular vesicles concerns size and the biogenesis. The size of exosomes, about 30 – 100 nm [5], make it the smallest extracellular vesicle [6]. The biogenesis of exosomes is inward budding of endosomes into multivesicular bodies, while other extracellular vesicles originate from direct budding of plasma membrane [7]. Exosomes derived from MSC carry similar therapeutic properties as their parent cell [8]. In addition, the nano-scale dimension of exosomes enables exosomes to deliver message remotely and across biological barriers [9].

Pre-treatment stem cells can influence both the quantity and the quality of released extracellular vesicle including exosomes. L-ascorbic acid (LAA) is a derivate of vitamin C [10] and has been known to enhance stem cell proliferation [10]–[12]. LAA also induces the differentiation of osteoblast to form bone tissue [10]. Pre-treatment of hWJ-MSC with LAA and investigation of the effect on exosome characterization has never been done. Hypoxia condition increase the proliferation of mesenchymal stem cells [2] and hypoxia precondition of cells have an effect on exosomes' secretion. $CoCl_2$, a chemical that inhibits HIF1a degradation, is a hypoxia-inducing agent [3]. The aim of this study was to compare the effect of LAA and hypoxia precondition of mesenchymal stem cells in the propeties of their exosome and the response to chondrogenic induction by its exosome.

Methods

Cell Isolation and Characterization of Mesenchymal Stem Cells

Cells were obtained from human umbilical cord samples with ethical approval. Cells were isolated using the explant method. Characterization of mesenchymal stem cells was analyzed with multipotency assay and flow cytometry.

For multipotency assay, the passage of cells 5 were seeded at 20,000 cells/well in a 24-well plate. After the cells reached 70-80% confluence, the culture medium was changed to chondrogenic medium (StemPro Chondrogenic Differentiation Kit), adipogenic medium (StemPro Adipogenic Differentiation Kit), and osteogenic medium (StemPro Osteogenic Differentiation Kit). The culture was incubated at 37 ^{0}C, 5% CO2 for 21 days, and the medium was changed every 2 days. After 21 days of culturing, the remaining medium was discarded and the culture washed with PBS and fixed with 4% formaldehyde. The cells were stained with Alcian Blue, Oil Red O, and Alizarin Red. The culture was observed with an inverted microscope.

The specific surface marker of mesenchymal stem cell was analyzed with flow cytometry assay using Human MSC Analysis Kit (BD Bioscience) according to manufacturerprotocols. The hWJ-MSC used for flow cytometry assay is passage 5. Flow cytometry was performed using BD FacsLyric.

Proliferation of Cell in Serum Free Medium

The hWJ-MSC passage 5 were seeded at 2,000 cells per well in 96-well plate and cultured in DMEM-FBS or DMEM or RPMI 1640. The culture cells were incubated at 37 ^{0}C, 5 % CO_2. Assessment of cell proliferation was measured in terms of optical absorbance (OD) by using MTT assay in 6 days of incubation. The absorbance was measured every day with Biorad Microplate Reader at the wave length of 595 nm.

Cell Viability in $CoCl_2$ Medium

The hWJ-MSC passage 5 were cultured in growth medium containing $CoCl_2$ with the concentration 25 µM, 50 µM, 100 µM, 200 µM and 400 µM. The culture cells were incubated at 37 ^{0}C, 5 % CO_2. The MTT assay was used to assess cell viability in terms of optical absorbance (OD) after 24 and 48 hours of incubation. At a wavelength of 595 nm, the absorbance was measured using a Biorad Microplate Reader.

Exosomes Isolation

The hWJ-MSC passage 5 were cultured in a medium consisting of DMEM LG, antibiotic antimycotic, and FBS 10%. At near 70% of confluence, the cells were washed with PBS. The medium was changed to a culture medium without FBS and the cells were incubated in a humified incubator (37 ^{0}C, 5% CO_2) for 48 hours. Supernatant was collected and exosomes were isolated. Exosomes were isolated using Invitrogen Total Exosomes Isolation according to manufacturer instructions. Briefly, the conditioned medium was collected and centrifuged at 2000 x g for 30 minutes. The supernatant was collected, added with 0.5 volume Invitrogen reagent, and vortexed to mix. Samples were incubated at 4^{0}C overnight. The samples were centrifuged 10,000 x g for one hour at 4^{0}C. The supernatant was discarded and the pellet was resuspended in 1x PBS.

Characterization of Mesenchymal Stem Cells-derived exosomes

The presence of exosomes and its morphology visualized with Transmission Electron Microscope. Briefly, ten microliters of exosomes suspension in PBS were placed onto a 300 mesh copper grid with carbon-coated film. The grids were blotted with filter paper and washed with double-distilled water. The samples were stained with uranyless and observed with a Hitachi HT7700 Transmission Electron Microscope.

ELISA was performed to analyze exosomes marker (CD 63). Human CD63 ELISA kit (Thermo Scientific) was used according to manufacturer instructions. Briefly, 100µL of each standard and sample were put into the appropriate well and incubated for 2.5 hours at room temperature. The solution was discarded and washed with wash buffer. The biotinylated antibody was added to each well and incubated for 1 hour at room temperature. The solution was discarded and washed again with wash buffer. Streptavidin-HRP was added into each well, incubated for 45 minutes at room temperature, and the solution then discarded and washed with wash buffer. TMB substrate was added into each well and incubated for 30 minutes at room temperature. Stop solution was added and the absorbance was read with Biorad Microplate Reader at 450 nm.

Exosomes in PBS were visualized by Horiba Scientific ViewSizer 3000 / 0053. Collected data were analyzed with Nanoparticle Tracking Analysis (NTA) software and provide particle size disribution profiles and concentration measurements.

Alcian Blue Assay

Alcian Blue assay was performed to evaluate the presence of glycosaminoglycan (GAG) in the cell culture. The medium of cell culture was aspirated and washed with phosphate buffer saline twice. The cells were fixated with acetone-methanol in 4 C for three minutes. 1% Alcian Blue staining (in 3% acetate acid) was added and incubated in room temperature for 30 minutes. The samples were washed with 3% acetate acid and the washed again with deion. The samples were added with 1% SDS and incubated for 30 minutes with shaker 200 rpm. The absorbance of samples were measured with UV-Vis spectrophotometry at a wavelength of 605 nm.

Immunocytochemistry of Collagen type 2

Medium of cell cultures were discarded and the cells were washed with phosphate buffer saline (PBS). All samples were fixed with paraformaldehyde and permeabilized with PBST (0,05% tween 20 in PBS). BSA was used as blocking agent and the samples were added with primary antibody collagen type 2 and incubated overnight. After incubation, the samples were washed with PBS and added secondary antibody Alexa Flour 488 and counterstaining with DAPI for nucleus staining. All samples were observed with Confocal Laser Scanning Microscope Olympus Fv1200.

Results

Characterization of Mesenchymal Stem Cells

In order to know that the isolated cells were mesenchymal stem cell (MSC), the cells were characterized based on MSC characterization. The morphology of the cell was spindle-shaped and adhered to the substrate (Fig. 1A). To investigate the differentiation potential of the cell, the cells were cultured in chondrogenic, adipogenic, and osteogenic differentiation media. Chondrocyte matrix was found in cells cultured in the chondrogenic medium as confirmed by Alcian Blue staining (Fig. 1B). The cells culture in adipogenic medium formed lipid droplet stained with Oil Red O staining (Fig. 1C). Calcium mineralization was found in cells cultured in the osteogenic medium (Fig. 1D). The cells exhibited the surface marker for mesenchymal stem cells, including CD 90^{+}, CD 105^{+}, CD 73^{+} and the negative surface marker CD 45 at less than 2%. (Fig. 1E). Based on characterization results, it can be said that the isolated cells were mesenchymal stem cells and could be called human Wharton's Jelly Mesenchymal Stem Cells (hWJ-MSC).

Cell Growth in Serum Free Medium

The cell growth in serum-free medium was analyzed by MTT assay. The hWJ-MSC that cultured in DMEM and RPMI without serum addition was lower than the control (medium contain FBS). As shown in Figure 3, the optical density (OD) value rose gradually from day 1 to day 2 but slightly decreased on day 3. The hWJ-MSC cultured in DMEM serum-free showed better proliferation than hWJ-MSC cultured in RPMI serum-free. DMEM without serum was used for further hWJ-MSC culture.

Cell Viability in $CoCl_2$ Medium

MTT assay was performed to evaluate the cytotoxicity of $CoCl_2$ on hWJ-MSC. MTT assays were performed with various $CoCl_2$ concentrations from 25 M to 400 M for 24 hours and 48 hours. The percentage of cell viability under hypoxic condition induced by $CoCl_2$ were high in all concentration, however 25 μM of $CoCl_2$ showed the highest cell viability compare others (Fig. 3A). Those concentration were used in the following experiment for $CoCl_2$ treatment.

The hWJ-MSC was then grown in DMEM serum-free with the addition of LAA, $CoCl_2$, LAA and $CoCl_2$ and DMEM serum-free as control. There was no difference in cell morphology among mediums (Fig. 3B).

Characterization of Mesenchymal Stem Cells-derived exosomes

The morphology of the exosomes was investigated with a negative staining Transmission Electron Microscope (TEM). TEM analysis revealed that the three group treatments had a round structure of various sizes (Fig. 4A). The medium without cells was observed with TEM to make sure the medium did not contain any external exosomes.

Using ELISA analysis, a marker for exosomes (CD 63) was detected in all of the treatments. The concentration of CD 63 detected in the medium containing LAA and $CoCl_2$ was higher than medium containing LAA or $CoCl_2$ alone. (Fig. 4B).

Nanoparticle Tracking Analysis results showed that the average size of exosomes isolated from hWJ-MSCs without treatment (control) is 138,98 nm with modal size 125,33 nm; pre-treated using $CoCl_2$ is 116,56 nm with modal size 24,98 nm; pre-treated using LAA is 116,97 nm with modal size 96,38 nm; and pre-treated with both LAA+ $CoCl_2$ is 134,07 nm with modal size 149,3 nm (Fig. 4C). The concentration of exosomes in culture cell supplemented with LAA showed the highest concentration compare to other treatment (Fig. 4D).

GAG Analysis

GAG accumulation in hWJ-MSC treated with exosomes was analyzed using Alcian Blue staining, then absorbance was measured using uv-vis spectrophotometry at a wavelength of 605 nm. The presence of GAG in hWJ-MSC treated with exosomes can be observed in the day 7 and 14, and 21. The absorbance measurement of GAG showed that exosome treatment can induce chondrogenic differentiation (Fig. 5A).

Collagen Type II Analysis

Collagen Type II analyzed by immunocytochemistry and visualized using a confocal laser scanning microscope. The results show the intensity of collagen type II (green) increased after 14 and 21 days exosome treatment (Fig. 5B)

Discussion

Mesenchymal stem cell (MSC) has been proved to be a good resource for cell-based therapy due to its regenerative potential. A previous study showed that MSC can be used to treat bone defects [1], [4] and regeneration periodontal. The therapeutic efficacy of MSC comes from paracrine secreted by MSC and mediated by extracellular vesicles, including exosomes. LAA has been demonstrated to induce osteogenic differentiation of stem cells [5]. This study investigated exosomes released from MSC treated with medium containing LAA and $CoCl_2$.

The type of medium for cell culture can have a contribution to cell proliferation and morphology. Cells that do not grow properly can have an effect on exosomes cargo released by the cells. The stress environment may influence both the quantities and function of the extracellular vesicle produced by cells [6]. Both DMEM and RPMI 1640 are common media for cell culture. DMEM is widely used to grow adherence cells while RPMI is generally used for suspension cell culture. While hWJ-MSC is an adherence cell, it can be cultured in both medium. Haraszti et al. [7] cultured MSC in RPMI medium and observed the production of the exosomes. Serum, for instance Fetal Bovine Serum (FBS), provides rich nutrients to maintain cell growth. However, exosomes can be found in serum [8]. The use of a serum free medium in cell culture for exosomes isolation will not lead to bias in the experiment results. In this study, the cell proliferation of hWJ-MSC cultured in DMEM serum-free was higher than with RPMI serum-free even though it was lower than the control (medium culture supplemented with FBS). The different medium in this research did not influence the cellular morphology of hWJ-MSC after being cultured in serum free medium for 48 hours. DMEM and RPMI have a different composition. DMEM LG (low glucose) has a lower sugar and phosphate concentration than RPMI 1640, but DMEM LG has a concentration of calcium than RPMI 1640 [9]. L-ascorbic acid (LAA) can induce mesenchymal stem cell proliferation [10], [11]. However, serum-free medium supplemented with LAA does not influence the morphology of hWJ-MSC. The cell viability was quite high after being treated for 24 hours and 48 hours. There was a decrease in cell viability after 48 hours with a concentration of 400 µM $CoCl_2$, this is in accordance with research conducted by Teti and collegues [12]. Concentration of 25 µM $CoCl_2$ showed the highest cell viability at 24 h and decreased slightly after 48 h, so this concentration was used for the next experiment.

The existence of exosomes in conditioned medium and the morphological shape of exosomes were visualized with negative staining-TEM. The vesicles shape was spherical although the size distribution varied. The size distribution of vesicles observed in TEM for LAA treatment varied among 30 – 70 nm. Due to membrane integrity, the size measurement from TEM imaging was confirmed using NTA analysis. The NTA data from all treatments showed one peak with wide distribution of vesicles size. Extracellular

vesicles show heterogeneity in size, as confirmed from a broad population analyzed with NTA. According to Sun et.al [13], the size of exosomes is between 30 – 100 nm. A range of exosomes size was detected in NTA data, and it can be said that exosomes were successfully isolated among other extracellular vesicles.

The presence of exosomes also can be indicated by the presence of CD 63. CD 63 is a tetraspanin found abundantly in exosomal membranes [14]. The concentration of CD 63 in LAA + $CoCl_2$ treatment was highest. The addition of LAA in hWJ-MSC medium with hypoxic environment enhanced exosomes secretion by hWJ-MSC. However, the mechanism of LAA with the combination of $CoCl_2$ in increasing extracellular vesicles secretion still needs further research.

The presence of GAG accumulation in hWJ-MSC showed that on the 7th day of exosome treatment, there was potential for chondrogenic differentiation, which was strengthened by visualization with an inverted microscope. A study conducted by Yoo and collegues [15] stated that the administration of $CoCl_2$ can trigger chondrogenic differentiation, so that exosomes isolated from cells treated with $CoCl_2$ are expected to trigger chondrogenic differentiation. In addition, LAA supplementation of 100 µg/mL can trigger chondrogenic differentiation in ADSC which is characterized by an increase in GAG accumulation [16]. On the 14th day, based on the absorbance results, it was shown that cells treated with Exo-Control and Exo-LAA+CoCl2 experienced a significant increase in GAG accumulation.

Type II collagen is an important component secreted during chondrogenic differentiation. Collagen Type II was observed on the 7th day through a confocal microscope. Based on the observations, administration of exosomes-LAA+CoCl2 to hWJ-MSCs could trigger chondrogenic differentiation, which was indicated by the formation of Collagen Type II. These results are in accordance with a previous study [16] that after 7 days of LAA administration, type 2 collagen localization began to occur around the cytoplasm. After 14 days and 21 days exosomes treatment, the intensity of Collagen Type II (green) increased. Based on research conducted by Bae and collegues [17], hypoxic conditions increase the expression of GAG and Collagen Type II compared to normoxia conditions.

Conclusion

In conclusion, the current study demonstrates that adding LAA to hWJ-MSC medium under hypoxic conditions affects the cells' exosome yield. Exosomes produced by preconditioning stem cells with LAA in a hypoxic environment can induce stem cell chondrogenic differentiation.

Declarations

Ethics approval

This research conducted with the approval of the Padjajaran University Research Ethics Commission (Number: 983/UN6.KEP/EC/2020).

Consent for publication

Not applicable

Avaibility of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

A Barlian design the research and helping in analyzed and interpreted data. RM Amsar performed the characterization of extracellular vesicle and contribute in writing the manuscripts. S Prawitasari performed the functional study and contribute in writing the manuscript. CH Wijaya , ID Ana, AC Hidayah, HB Notobroto and TDK Wungu reviewed and edited the manuscripts. All authors read and approved the final manuscripts.

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Not applicable

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Figures

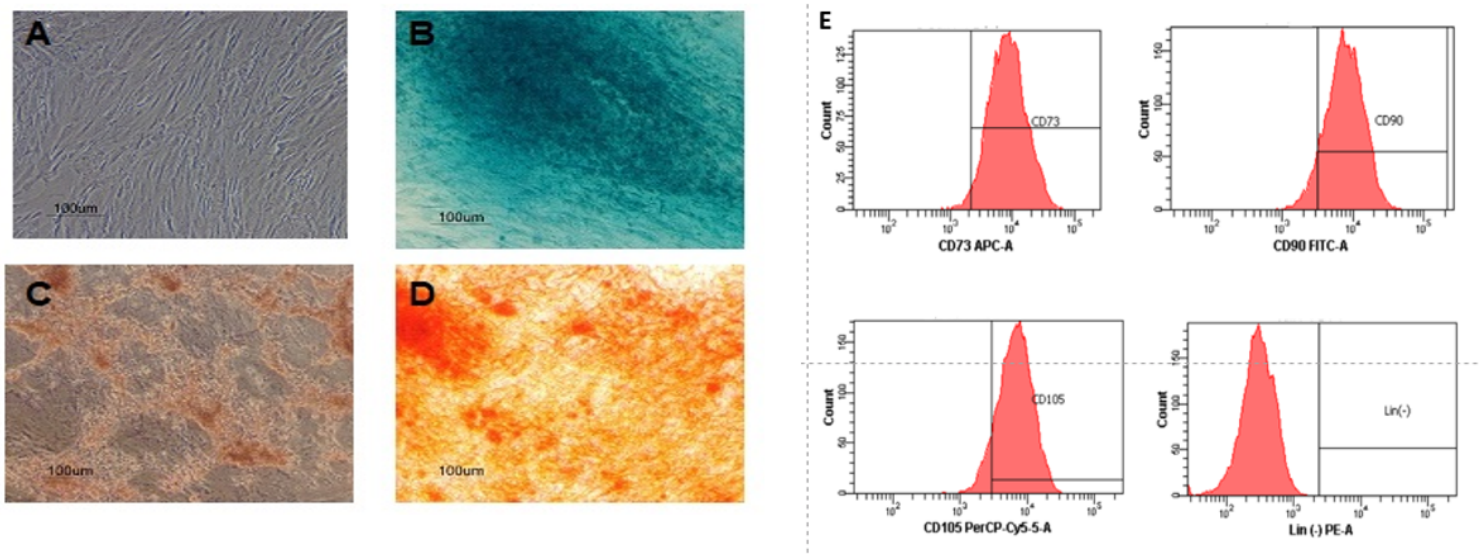

Figure 1

The characterization of stem cells. **A** The morphology of cell is fibroblastic-like. **B** Cells can differentiate into chondrocytes, **C** adipocytes and **D** osteocytes. **E** The cell surface marker showed specific surface marker for mesenchymal stem cells.

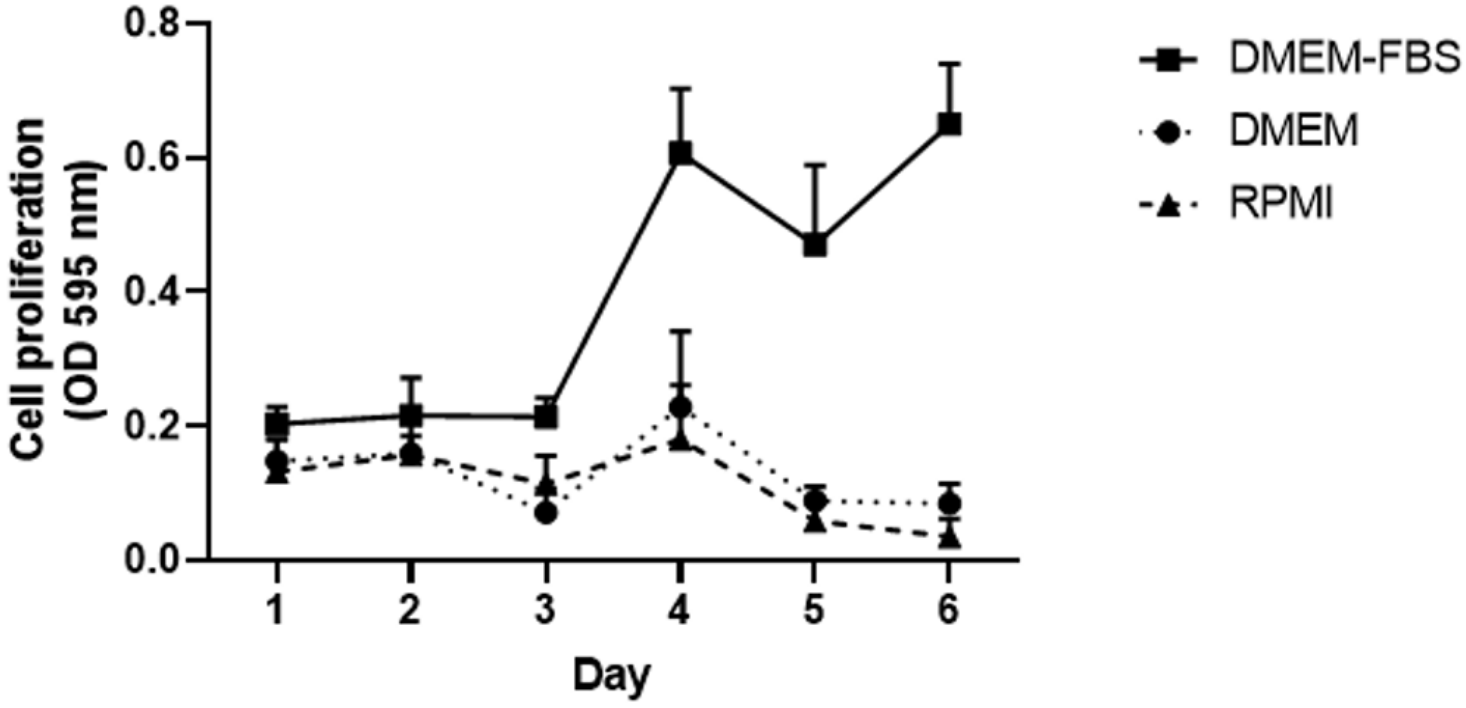

Figure 2

hWJ-MSC proliferation in serum free medium

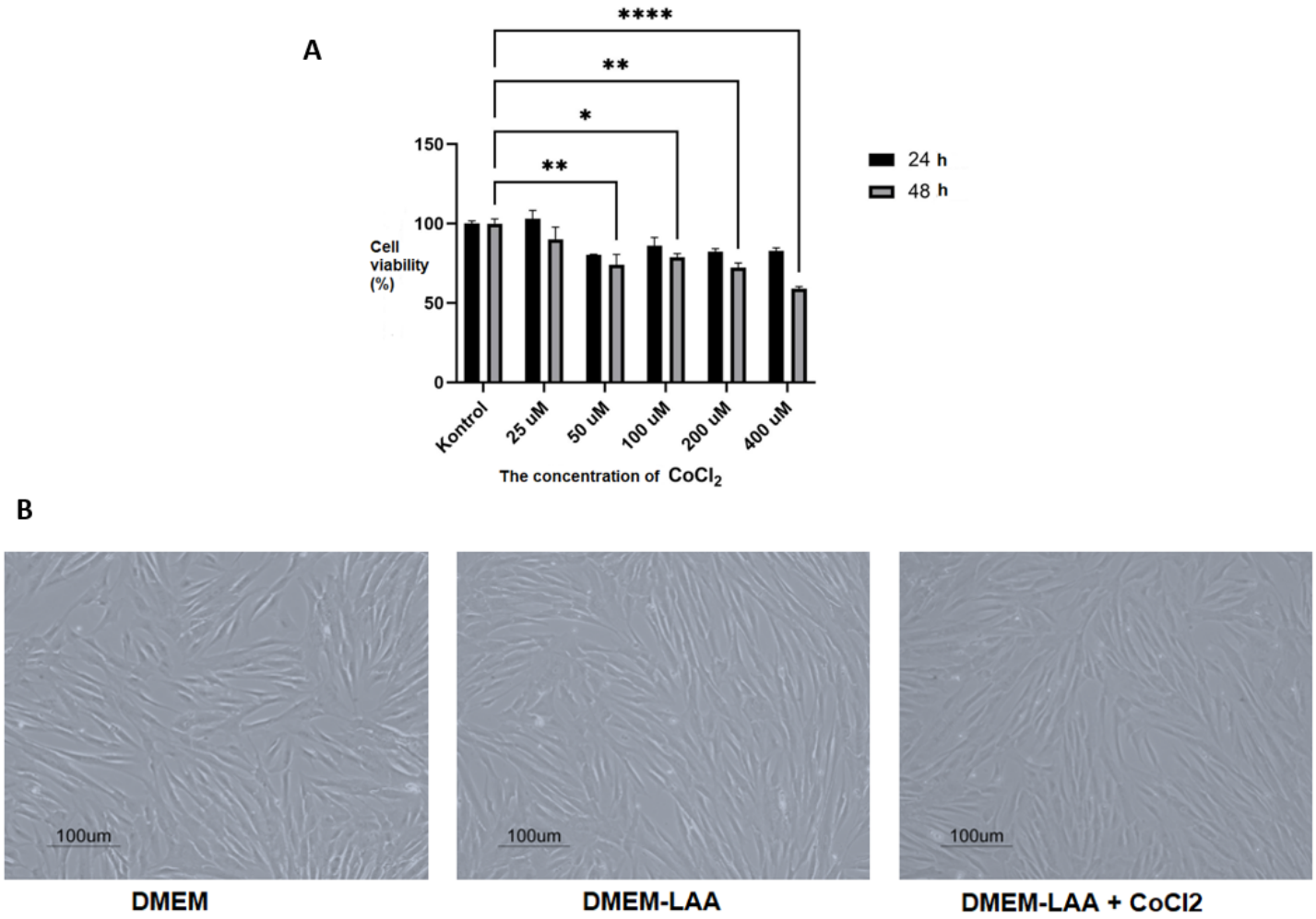

Figure 3

The effect of $CoCl_2$ on hWJ-MSC. **A** Cell viability determined by MTT assay. **B** The morphology of the hWJ-MSC maintained in DMEM serum free for 48 hours of incubation

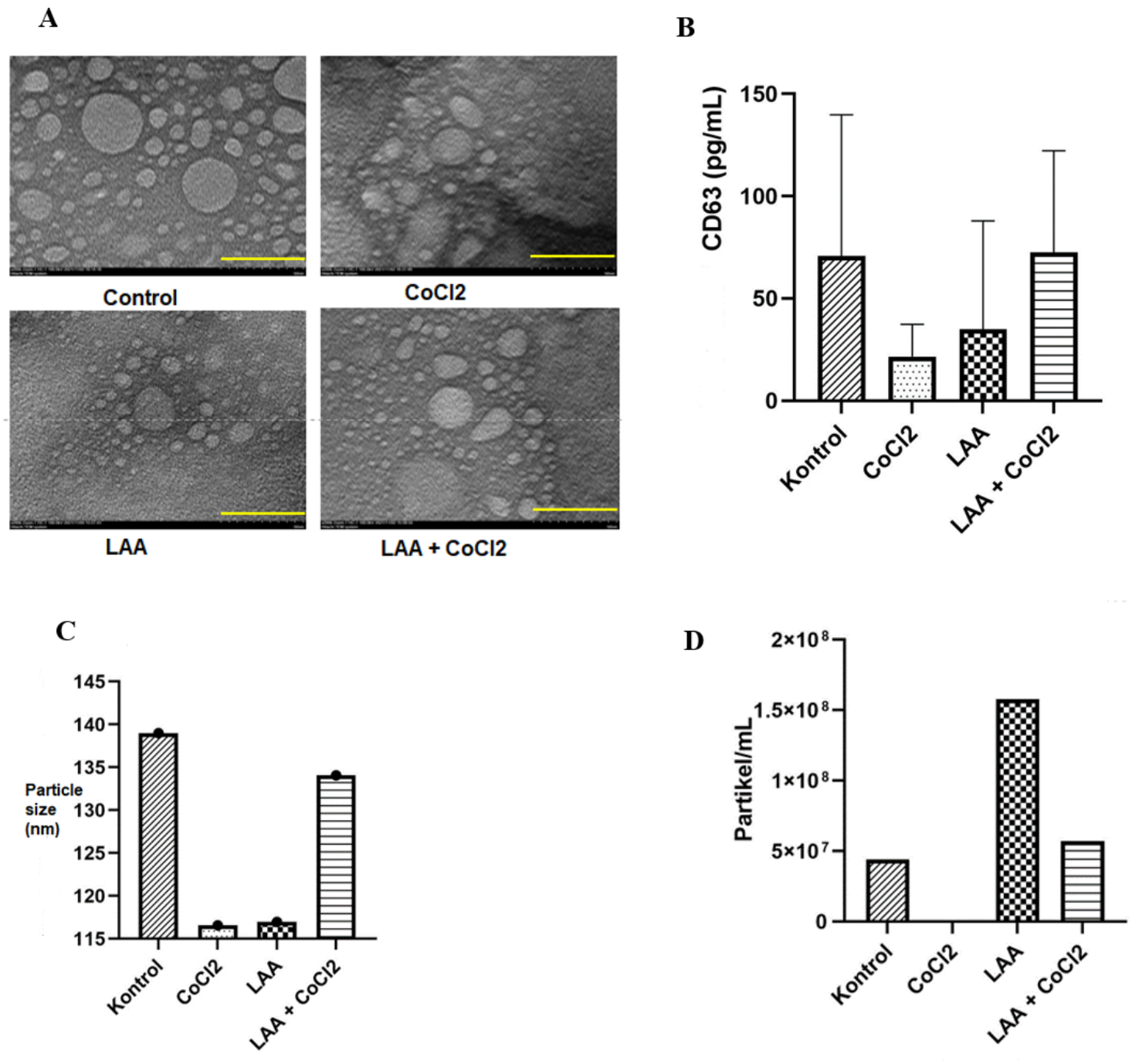

Figure 4

Characterization of exosomes from hWJ-MSC. **A** TEM images of exosomes showing circular morphology. **B** The presence of CD 63 in in conditioned medium of hWJ-MSC that treated with LAA, CoCl2 and LAA+ CoCl2. Conditioned medium of hWJ-MSC without LAA and CoCl2 was used as control. **C** Mean particle sizes analysis from NTA data. **D** The concentration of exosomes measured with NTA.

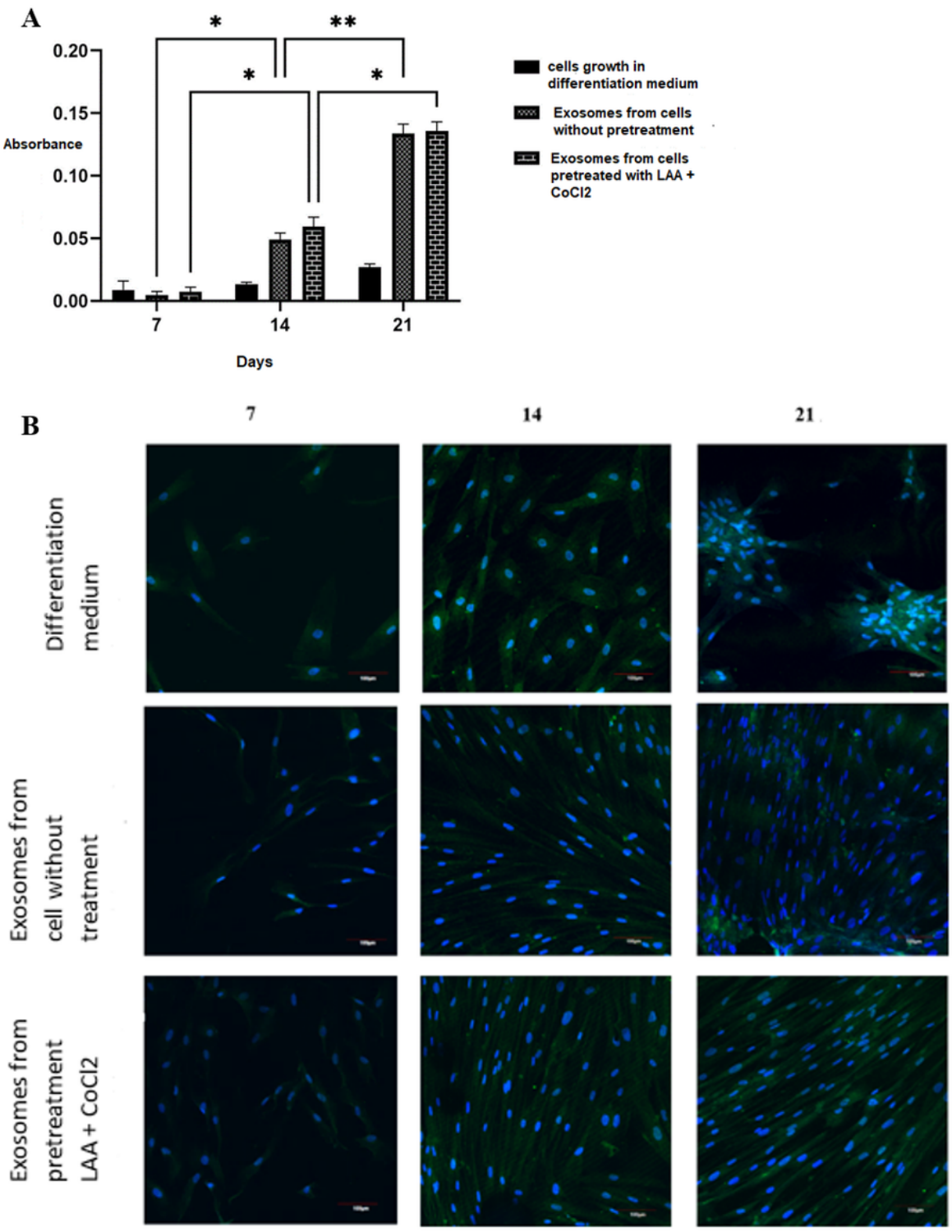

Figure 5

The effect of exosomes treatment on chondrocyte differentiation of hWJ-MSC. **A** The presence of glycosaminoglycan (GAG) in hWJ-MSC culture. **B** Laser scanning confocal microscope visualization of collagen type 2 produced by cells in monolayer culture. DAPI nuclear staining is blue and green for collagen type 2.