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Wharton's Jelly Mesenchymal Stem Cells-derived Exosomes and Imipenem in Combination Reduce Apoptosis and Inflammatory Responses in E.coli-infected HepG2 Cells

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ABSTRACT

Antibiotics are used to treat bacterial liver infections and the resulting inflammation. However, their use is limited due to their side effects, especially the development of antibiotic resistance.

Mesenchymal stem cells (MSCs) are recognized for their immunomodulatory properties. In this study, we investigated the immunomodulatory effect of Wharton's jelly MSC-derived exosomes in combination with imipenem on HepG2 cells infected with *Escherichia coli*.

MSC-derived exosomes were separated from MSCs, which were isolated by flow cytometry. Scanning electron microscopy and dynamic light scanning were used to confirm the presence of exosomes. Quantitative real-time PCR, ELISA, and nitric oxide assay were used to assess the inflammatory response in the infected cells. Annexin-PI was used to measure the extent of apoptosis.

The results showed that the combination of imipenem and MSC-derived exosomes were more effective than imipenem or exosomes alone in reducing the production and secretion of inflammatory cytokines, nitric oxide, and apoptotic rate in *E Coli*-infected HepG2 cells.

Keywords: Escherichia coli; Exosomes; Immunomodulation; Inflammation; Mesenchymal stem cells

INTRODUCTION

Hepatocytes make up about 90% of liver cells and play a role in the inflammatory response.[1,2] They can identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by expressing various pattern recognition receptors (PRRs).[3,4] They also activate other hepatic and immune cells, including hepatic stellate cells (HSCs), through cytokines.[5] Activated HSCs actively contribute to liver fibrosis. Therefore, controlling the hepatocyte-induced inflammatory response can prevent liver damage.[6]

One way to prevent liver damage is the use of

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antibiotics.[7] This effect can be enhanced by adding complementary therapies.[8] Mesenchymal stem cells (MSCs) are used extensively in cell therapy research due to their differentiation, self-renewal, and immunomodulatory properties.[9] MSCs improve liver function and prevent further damage by affecting other liver cells, such as HSCs[10] and liver sinusoidal endothelial cells (LSECs).[11] Using MSC-derived extracellular vesicles (EVs) can provide a more convenient approach with fewer undesirable effects.[12] The three types of EVs include exosomes (EXO),[13] microvesicles (MVs), and apoptotic bodies (ApoBDs). EXOs are the smallest EVs that range in size from 30 nm to 100 nm.[14,15]

Wharton's jelly-derived MSCs (WJ-MSCs) are better candidates for transplantation than other tissue-derived MSCs. WJ-MSCs are younger and less susceptible to damage, environmental toxins, and diseases. They also have a high proliferative potential, are easier to isolate, make up a larger population of stem cells compared to other tissues, and have a higher transplantation success rate after in vitro expansion.[16] We, hypothesized that the concurrent use of MSC-derived exosomes (MSC-EXOs) and imipenem can reduce cellular stress, apoptosis, and inflammatory response in the HepG2 cell line.

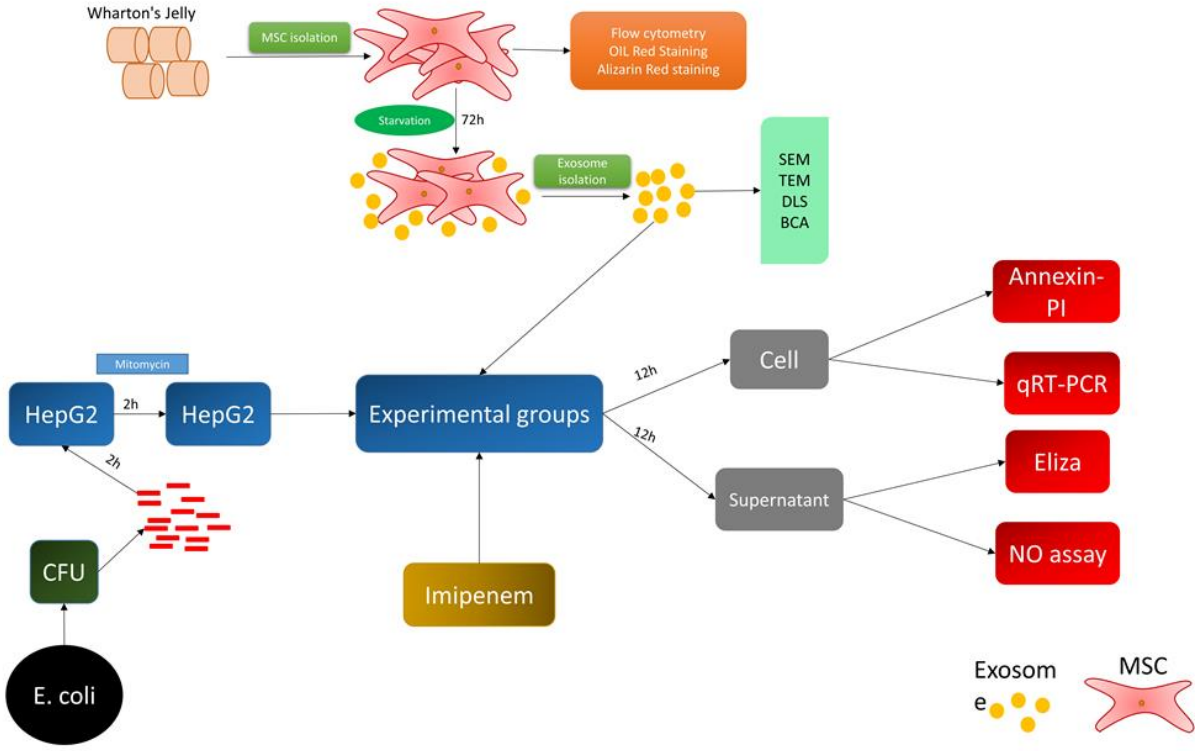

Figure 1. Study design. Mesenchymal stem cells (MSCs); Colony-forming unit (CFU); Nitric oxide (NO); Scanning electron microscope (SEM); Dynamic light scattering (DLS); Bicinchoninic acid (BCA).

Escherichia coli is a gram-negative bacillus of the Enterobacteriaceae family,[17] that have a role in liver infection during sepsis and can induce inflammation via its lipopolysaccharide (LPS). Toll-like receptor 4 (TLR-4) is a receptor for LPS[18] that exists on the surface of hepatocytes. Therefore, we used *E coli* to induce an inflammatory response in HepG2 cells.[19]

This study examines the effects of WJ-MSC-derived exosomes in combination with imipenem in *E Coli*-infected HepG2 cells (Figure 1).

MATERIALS AND METHODS

HepG2 Cell Culture

The HepG2 cell line was purchased from the Iranian biological resource center (IBRC, Tehran, Iran) and was maintained as an adherent cell line in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1× nonessential amino acids at 37°C in a 5% CO2:95% air-humidified atmosphere. Cells were passaged when they rich 85-90 confluence as needed using 0.5% trypsin-EDTA. All the cell culture solutions were purchased from Gibco (Gibco, NY, USA). The Medical Ethics Committee of Shahid Beheshti University of Medical Sciences approved this study (Code: IR.SBMU.MSP.REC.1399.776).

Experimental Groups

After 24 hours of cell passage and transfer to plate 6 cells, 10 to 1 bacteria (2×10^7) were added to HepG2 cells (2×10^6). After 2 hours of incubation, the wells were washed twice with PBS. Then 500 µL of culture medium containing 10% FBS and 20 µL of mitomycin at a concentration of 10 µg/mL were added. After two hours, the culture medium containing mitomycin was replaced with 750 µL of a new DMEM/F12 medium. Cells were examined after 12 hours for further assays. The supernatant of cells in this step was collected to check cytokine and nitric oxide levels and stored at -70°C until measurement. After replacing the mitomycin-containing medium with a new medium, imipenem (20 µg/mL) and WJ-MSC-EXO (100 µg/mL) was added to the experimental groups. In this study, experimental groups were defined as 1) HepG2 cells, 2) HepG2 + *E. coli*, 3) HepG2 + *E. coli* + Imipenem, 4) HepG2 + *E. coli* + WJ-MSC-EXO and 5) HepG2 + *E. coli* + Imipenem + WJ-MSC-EXO. All contaminated wells were washed three times with PBS to eliminate unattached bacteria. Each experimental group had a duplicate set of wells lysed with 0.01% Triton X 100 and grown onto LB agar at different serial dilutions to determine the total number of adhering bacteria. Thus, it confirmed the presence of bacteria and infection in HepG2 cells. This study was performed independently five times in triplicates for all tests on each experimental group. Results were presented as mean±SD values of the obtained data.

WJ-MSCs Isolation

The human umbilical cord was collected from normal-born full-term infants and cut into 0.5 - 1 cm pieces. The pieces were washed with PBS and a culture medium, and three vessels, including arteries and veins, were removed carefully. Each piece was placed in 25 cm^2 flasks. The flasks were supplemented with Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Gibco, Grand Island, NY) containing 20% fetal bovine serum (FBS) (Gibco, Grand Island, NY). The cells' expansion and growth were monitored constantly, and when the confluence was about 85%, they were passaged using 0.5% trypsin-EDTA.

MSCs Characterization

Wharton jelly isolated MSCs cell surface marker expression was assessed from the third passage using a FACSCalibur flow cytometer (BD Biosciences, USA). Anti-human antibodies used for staining the WJ-MSCs were against CD73, CD34, CD29, CD90, and CD45 (all from eBioscience).

To examine the differentiation potential of these cells into osteocyte lineage, 4×10^4 (for each well) WJ-MSCs from the third passage were cultured on 6-well plates. Then, supplemented media containing glycerol phosphate (10 mM), dexamethasone (100 mM), and ascorbic acid-2 phosphate (5 g/mL) were added to the wells. After about 15–21 days, the wells were washed with PBS, and after cell fixation, they were stained with Alizarin Red-S (ARS). Similarly, to evaluate the adipogenic differentiation ability of isolated cells, WJ-MSCs were cultured at the same density. Supplemented media containing dexamethasone (250 mM), indomethacin (100 mM), insulin (5 mM), and 3-isobutylmethylxanthine (0.5 mM) was added to wells. (all from Sigma-Aldrich, Missouri, USA). Media were changed every three days. After about 2–3 weeks, the adipogenic induction media were discarded, and phosphate-buffered saline (PBS) was used for washing the wells. The differentiated cells were fixed by paraformaldehyde. After removing the fixation solution, the PBS was used for washing, and finally, the differentiated cells were confirmed by Oil Red-O (ORO) staining.

Exosome Isolation

After culturing and expanding WJ-MSCs, the supernatants of MSCs at passage two or three were collected and exchanged with FBS free medium with a

lower level of FBS. These steps were performed twice a week. Finally, the cells were adapted with an FBS-free medium. After 72 hours of MSCs culture in an FBS-free medium, their supernatant was collected and filtered using 0.22 μm filters. Then, the pure exosomes were utilized for the exosome-treated groups.

MSC-EXO isolation from the prepared supernatants was done using modified guidelines of the Exocib kit (Cib Biotech Co.). First, 15 mL of the filtered supernatants were mixed with 3 mL of viscose reagent (reagent A). After overnight incubation at 4°C, the tubes were centrifuged at 1000 g for 40 min. Finally, depending on the size of the pellet, 100 to 200 μL PBS was added to the pellet, and the mixture was resuspended. After isolation, the concentration of the exosomes was assessed using a Bicinchoninic Acid (BCA) kit (DNAbiotech Co.).

Exosome Characterization

WJ-MSC-EXO were evaluated using scanning electron microscopy (MIRA3 TESCAN); they were fixed with glutaraldehyde in PBS. After washing and dehydrating with PBS and ethanol, the samples were analyzed by SEM. To measure the exosomes' average size, 10 μl of each sample was diluted in PBS. After mixing, they were transferred into a cuvette and then analyzed by DLS (Malvern Instruments, UK).

Real-time PCR Gene Expression Quantification

HepG2 cells were exposed for 2 hours to 2×10^7 CFU *E. coli*. After washing and eliminating the bacteria, the cells were treated for an additional 120 minutes with mitomycin 10 μg/mL (Sigma, USA) to inhibit the attached bacteria growth. The fresh FBS containing DMEM/F12 medium was eventually replaced with mitomycin-containing media. In this stage, different treatments such as imipenem alone, WJ-MSC-EXO alone, and the combination of imipenem and WJ-MSC-EXO are added to the culture medium. After supernatant collection, cells were washed twice with PBS, and cells were collected; using 0.5% trypsin-EDTA. The GeneAll RibospinTM total RNA purification kit was used to separate total RNA from the experimental groups (GeneAll Biotechnology, Seoul, South Korea). The RevertAid First Strand cDNA Synthesis Kit was used to make cDNA from 1 mg of total RNA (Thermo Fisher, Northumberland, UK). On a Corbett Rotor-Gene 6000 Light Cycler, gene expression was evaluated using a quantitative real-time polymerase chain reaction (qRT-PCR) assay using SYBR Green RealQ Plus 2 Master Mix Green (Ampliqon, Skovlunde, Denmark) (Qiagen, Hilden, Germany). The data were normalized against the b-actin transcript level. The delta-delta CT method was used to calculate the relative gene expression. The characteristics of the primers used in this study are summarized in Supplementary Table.

Measurement of Cytokine Concentration

HepG2 cells were exposed for 2 hours to 2×10^7 CFU *E. coli*. After washing and eliminating the bacteria, the cells were treated for an additional 120 minutes with mitomycin 10 g/mL (Sigma, USA) to inhibit the attached bacteria growth. The fresh FBS containing DMEM/F12 medium was eventually replaced with mitomycin-containing media. In this stage, different treatments such as imipenem alone, WJ-MSC-EXO alone, and the combination of imipenem and WJ-MSC-EXO are added to the culture medium. The supernatant of the treated cells was collected in a 15 ml falcon and centrifuged for 5 minutes at 1800 rpm to remove dead cells and cell debris and stored at -70°C until measurement. Finally, the levels of IL-10, IL-6, IL-1β, and TNF-α in the different treatment groups' supernatant were measured using ELISA commercial kits (R&D Systems, USA) according to the manufacturer's instructions. The optical density of each well was evaluated at 450 nm after each sample was distributed in triplicate.

Nitric Oxide Assay

HepG2 cells were exposed for 2 hours to 2×10^7 CFU *E. coli*. After washing and eliminating the bacteria, the cells were treated for an additional 120 minutes with mitomycin 10 g/mL (Sigma, USA) to inhibit the attached bacteria growth. The fresh FBS containing DMEM/F12 medium was eventually replaced with mitomycin-containing media. In this stage, different treatments such as imipenem alone, WJ-MSC-EXO alone, and the combination of imipenem and WJ-MSC-EXO are added to the culture medium. In this stage, other therapies such as imipenem alone, WJ-MSC-EXO alone, and the combination of imipenem and WJ-MSC-EXO are added to the culture medium. The supernatant of the treated cells was collected in a 15 mL falcon and centrifuged for 5 minutes at 1800 rpm to remove dead cells and cell debris and stored at -70°C until measurement. The NO production was also measured using the Griess test. The

supernatant was mixed 1:1 with Griess reagent (Cibbiotech, Iran), and their optical absorbance was determined at 540 nm using this approach. The NO production was calculated using a sodium nitrite standard curve.

Apoptosis Assay

HepG2 cells were exposed for 2 hours to 2×10^7 CFU *E. coli*. After washing and eliminating the bacteria, the cells were treated for an additional 120 minutes with mitomycin 10 g/mL (Sigma, USA) to inhibit the attached bacteria growth. The fresh FBS containing DMEM/F12 medium was eventually replaced with mitomycin-containing media. In this stage, different treatments such as imipenem alone, WJ-MSC-EXO alone, and the combination of imipenem and WJ-MSC-EXO are added to the culture medium. After collecting the supernatant, the cells were washed twice with PBS before being collected with 0.5% trypsin-EDTA. The impact of direct contact with WJ-MSC-EXO on the apoptosis of HepG2 cells in exposure to *E. coli* was investigated using the AnnexinV-PI staining kit (BioLegend, USA). The FACS CanII device was used to assess the fluorescence intensity of AnnexinV and PI according to the manufacturer's recommendations.

Statistical Analysis

Statistical data analysis was performed using a T-test and one-way ANOVA in Graph Pad Prism 8.3.2 software. The significance level was considered less than 0.05 ($p<0.05$), and the data were expressed as Mean±SD.

RESULTS

WJ-MSC Characterization

The proliferation and shape of the cultured MSCs were monitored using an inverted microscope, and it was observed that they were plastic adherent and were large and thin fibroblast-like cells. Cell surface marker analysis was performed by flow cytometry to characterize the WJ-MSCs. The majority of WJ-MSCs were positive for CD90 (85%), CD73 (65%), and CD29 (97%) markers and relatively negative for CD34 (4.5%) and CD45 (6.6%) markers (Figure 2A). In addition, after about three weeks, the osteogenic or adipogenic lineages differentiation capacity of WJ-MSCs was evaluated. Calcium phosphate accumulation and Lipid droplets were visualized after Alizarin Red-S and Oil Red-O staining (Figures 2B and C).

WJ-MSC-EXO Characterization

The shape and approximate size of the isolated exosomes were visualized by SEM analysis (Figure 3A). DLS assay was performed to determine the precise size distribution of the exosomes. According to the DLS results, it was observed that the mean size of the isolated exosomes was 44.21 nm (Figure 3B).

Effect of Different Treatments on the Cytokines mRNA Expression

After incubation of HepG2 cells with *E. coli* (Figure 4), the relative expression of *IL-1β* is shown in Figure 5A. The level of FC related to *IL-1β* expression in HepG2 cells increased to 4.31±0.303 ($p<0.0001$) 12 hours after exposure to *Escherichia coli*. The FC related to *IL-1β* mRNA expression in the treatment group with imipenem, exosome and combination were recorded 2.3±0.32 ($p=0.0009$), 3.8±0.21 ($p=0.0282$) and 1.915±0.229 ($p= 0.0002$), respectively.

Twelve hours after incubation, *IL-6* mRNA expression in all *E. coli* infected groups increased significantly ($p<0.01$) in comparison to the HepG2 groups (Figure 5B). The highest level of *IL-6* mRNA expression was in *E. coli-infected* HepG2 cells (2.21±0.051, $p=0.0002$). The *IL-6* mRNA expression in the combination-treated group was the lowest among *E. coli-infected* groups ($p<0.0001$).

As shown in Figure 5C, the *TNF-α* expression in the HepG2 cell line in exposure to *E. coli* increased by 4.05±0.258 fold (<0.0001). The *TNF-α* expression in the imipenem, exosome, and combination-treated groups was decreased by 2.12±0.32 fold ($p=0.0012$), 3.324±0.249 ($p=0.0243$), 1.84±0.354 ($p=0.0009$), respectively.

Unlike other cytokines expression patterns, the mRNA expression of *IL-10* in HepG2 cells increased in the treatment groups compared to *E. coli* infected group (Figure 5D). This study showed that the related expression of *IL-10* in the combination therapy group with exosomes and imipenem increases significantly compared to the *E. coli* infected group (1.125±0.045, $p<0.0001$).

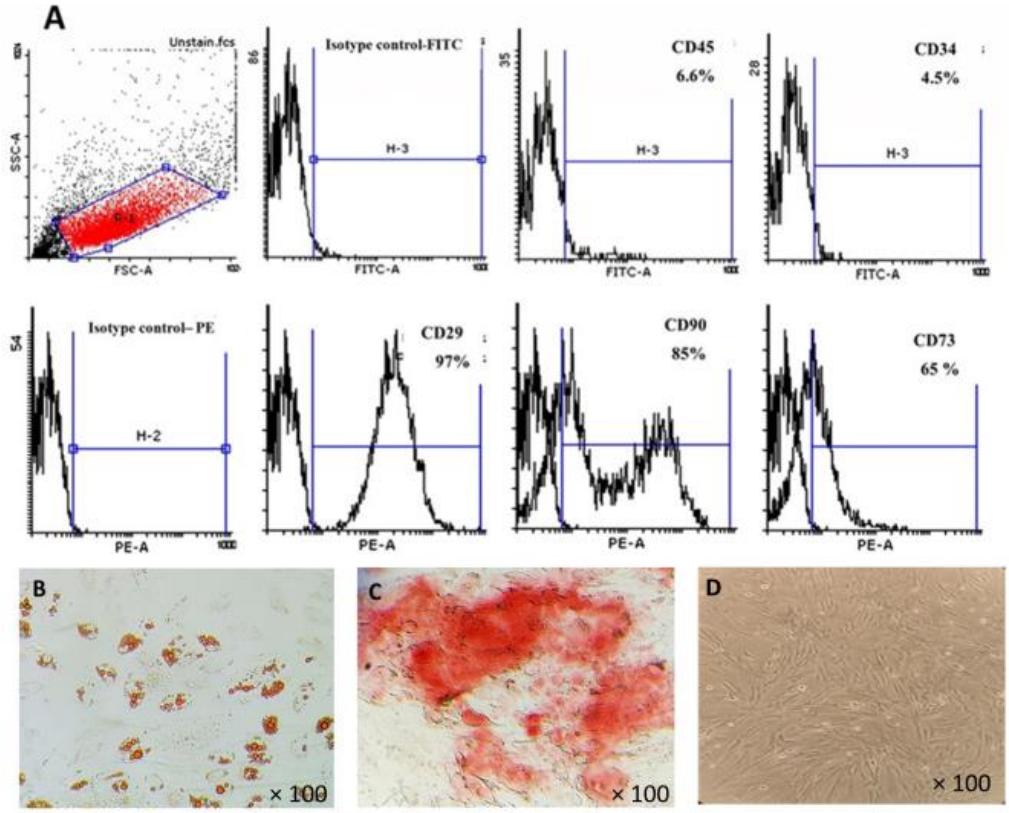

Figure 2. Characterization of WJ-MSCs. (A) Specific surface markers expression in WJ-MSCs, including CD73, CD14, CD105, CD90, and CD45, analysis by flow cytometry. The differentiation potential of WJ-MSCs into (B) adipogenic and (C) osteogenic lineages is confirmed by Oil red O staining of lipid droplets and Alizarin red S staining of calcium phosphate accumulation, respectively. (D) WJ-MSCs spindle fibroblast-like shape monitored by light microscopy. Wharton's jelly-Mesenchymal stem cells (WJ-MSCs).

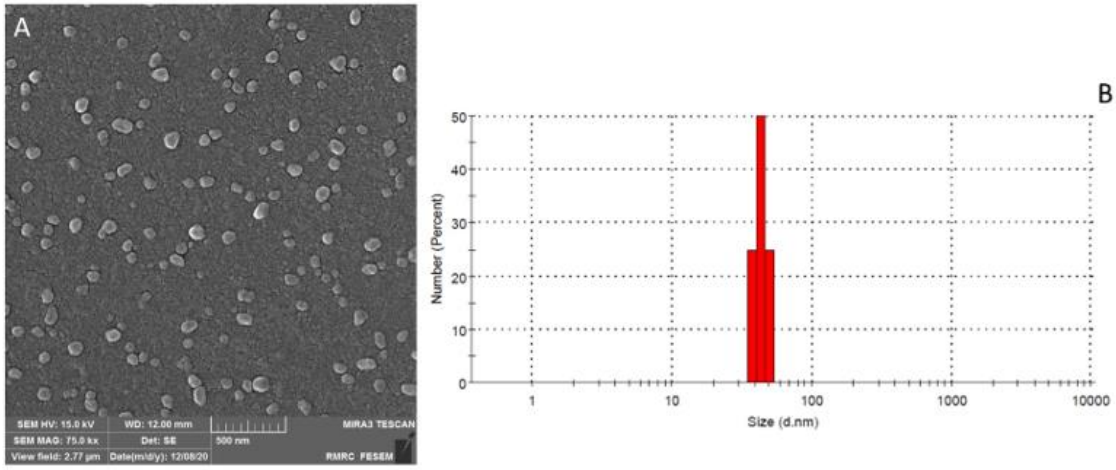

Figure 3. Characterization of WJ-MSC-EXO. (A) The shape of isolated exosomes was confirmed using SEM. (B) Using DLS), the average size of WJ-MSC-EXO was 44.21 nm, as determined. Wharton's jelly-Mesenchymal stem cells (WJ-MSCs); Scanning electron microscope (SEM); Dynamic light scattering (DLS).

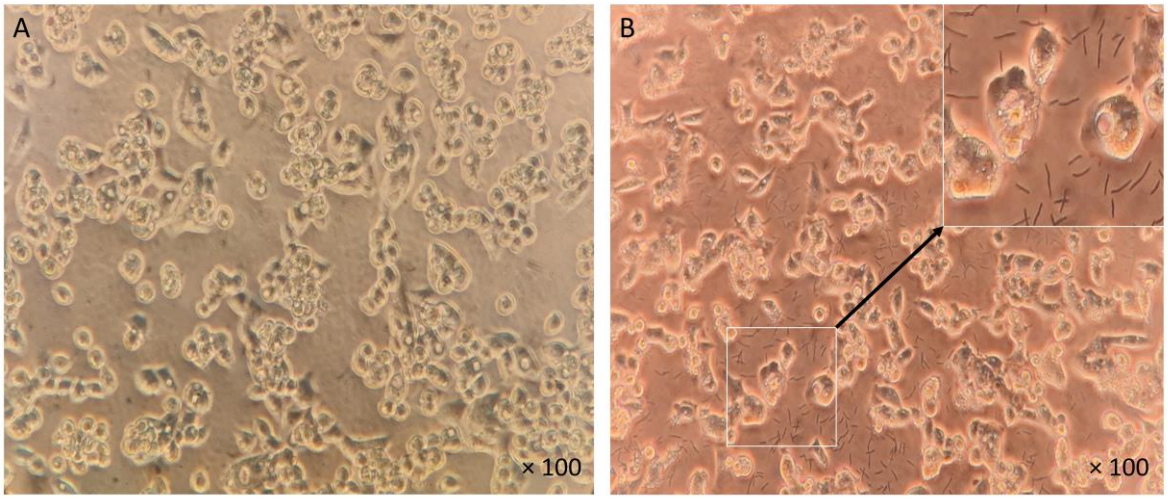

Figure 4. Comparison of HepG2 cell morphology in Escherichia coli-infected and uninfected cells. (A) shows normal cells and when they are in logarithmic growth. (B) shows infected cells with bacteria that have become granular.

Evaluation of Cytokines Level in the Supernatant

The concentration of IL-1β in the supernatant of HepG2 cells was 342.101±41.82 pg/mL, which increased to 1603.78±120.356 pg/ml in the presence of bacteria (p<0.0001). The production of IL-1β in imipenem-treated HepG2 cells was 640.47±61.871 pg/ml, significantly reduced compared to the *E. coli* infected group (p=0.0002). Also, the production of this cytokine in the WJ-MSC-EXO treated group and the combination of exosome and imipenem were reported to be 1001.326±40.43 pg/mL (p=0.012) and 290.609±41.259 pg/mL (p<0.0001), respectively (Figure 6A).

According to Figure 6B, the mean concentration of IL-6 in the supernatant of HepG2 cells was reported to be 120.1±25.001 pg/mL, which increased to 962.01±61.20 pg/ml in the presence of bacteria 12 hours after exposure (p<0.0001). This increase was statistically significant. IL-6 production in imipenem-treated cells was 419.80±31.12 pg/mL, significantly reduced compared to the bacterial-infected group (p=0.0002). Also, the production of this cytokine in the group treated with exosomes and the combination of exosome and imipenem antibiotics were reported to be 650±49.989 pg/mL (p=0.0023) and 212.31±25.15 pg/mL (p<0.0001), respectively (Table 1).

It was also seen that the mean concentration of TNF-α in the supernatant of the HepG2 cell was 250.06±60.23 pg/mL, which increased to 1398.20±110.312 pg/mL in the presence of bacteria (p<0.0001). TNF-α production in imipenem-treated cells was 719.80±81.60 pg/mL (p=0.0010). Also, the production of this cytokine in the exosome and the combination-treated group were reported at 1098.021±81.65 pg/mL (p= 0.0188) and 624.21±32.87 pg/mL (p=0.0003), respectively 12-hour after treatment (Figure 6C).

According to the results, the mean concentration of IL-10 as an anti-inflammatory cytokine in the supernatant of HepG2 cells was 941.25±71.60 pg/mL, which decreased to 342.87±32.65 pg/mL in the presence of bacteria (p=0.0002). IL-10 production in imipenem-treated cells was 680.48±45.63 pg/mL (p=0.0004). Also, the production of this cytokine in the group treated with exosome and imipenem antibiotics was reported as 604.786±35.33 pg/mL (p=0.0006) and 752.301±61.37 pg/mL (p<0.0001), respectively (Figure 6D).

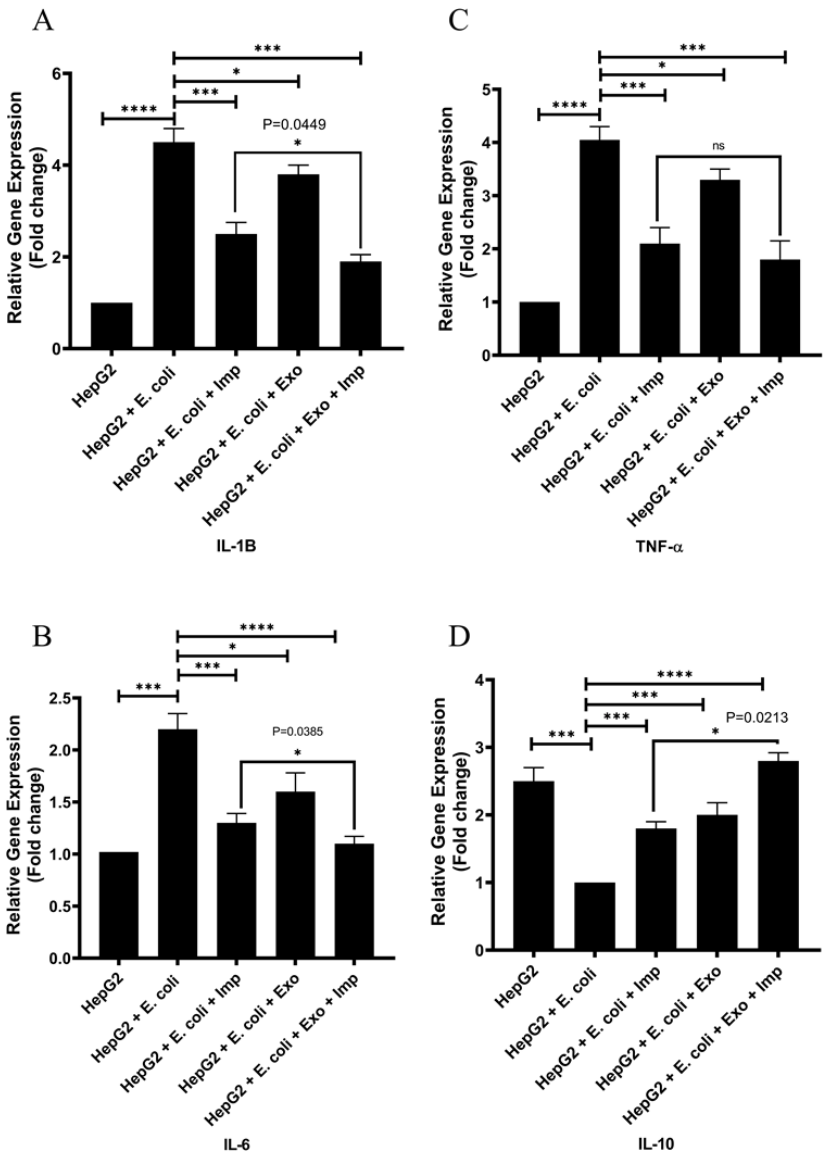

Figure 5. mRNA expression of inflammatory/anti-inflammatory cytokines. The relative expression of these cytokines in the different experimental groups were measured; using RT-PCR 12h after treatment. * p< 0.05, ** p<0.01, *** p<0.001, and **** p<0.0001. Imipenem (Imp); Tumor necrosis factor-α (TNF-α); Interleukin (IL).

Nitric Oxide Production

As shown in Figure 7, the production of NO in *E. coli* infected HepG2 cells in 12-hour supernatant was increased compared to the control group (4.2±0.5 μM), and the amount of nitric oxide produced was (8.30±0.3 μM, p=0.002). This showed that exposure of HepG2 cells to *E. coli* significantly increased nitric oxide production and cellular stress. In the imipenem treated group, the amount of nitric oxide production decreased compared to the positive control group, and its value in the present study was reported to be 5.8±0.58 μM (p=0.0013). In the WJ-MSC-EXO treated group, the reduction of nitric oxide production was not statistically significant, and its value was reported to be 7.03±0.35 μM (p=0.0198). In the primary treatment group in which the simultaneous effects of the WJ-MSC-EXO treated group and imipenem were applied, the amount of nitric oxide production and cellular stress decreased more than in the imipenem treated group, and its amount in the treatment supernatant was 4.48±0.48 μM (p= 0.0005).

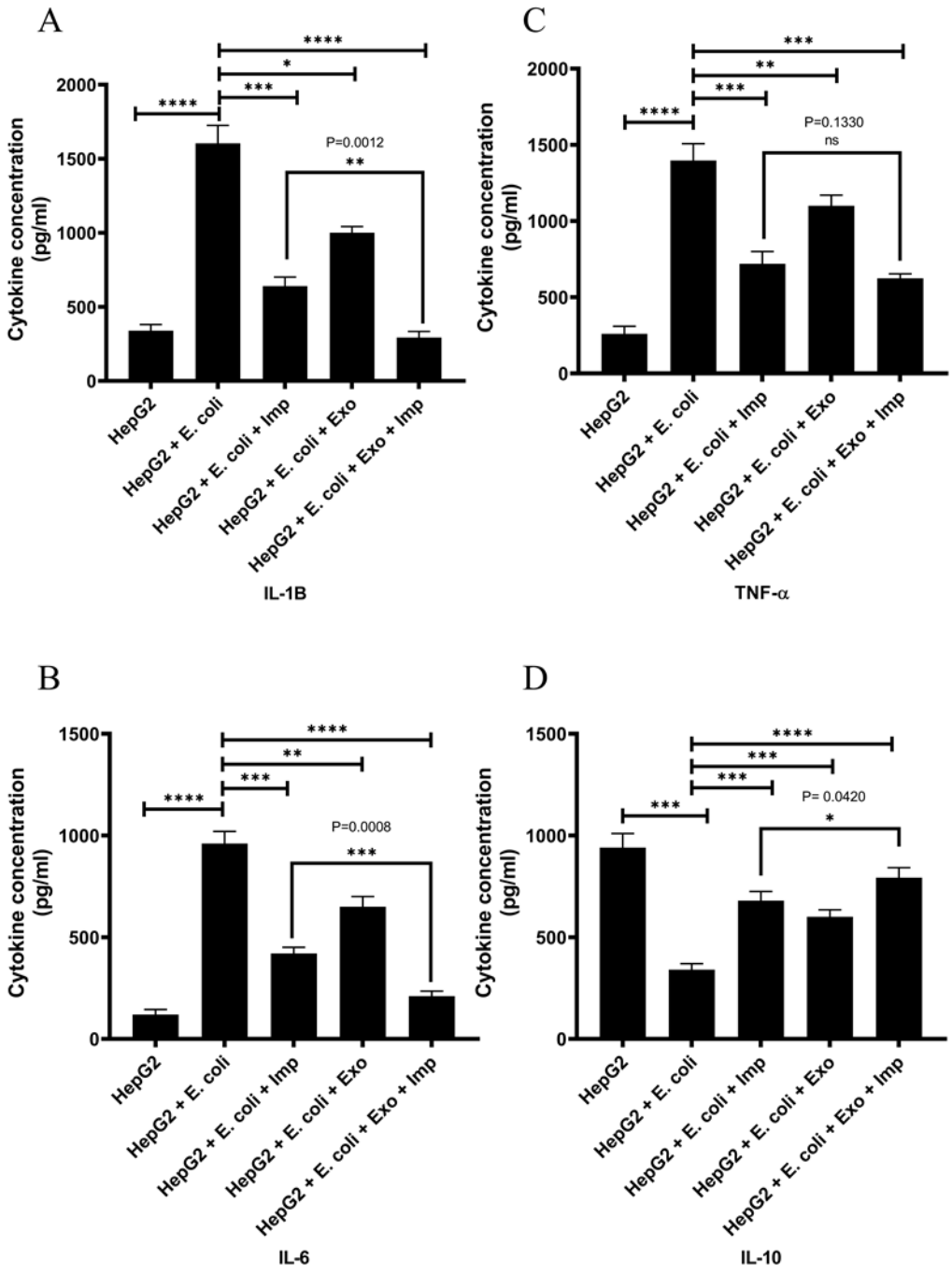

Figure 6. The concentration of inflammatory cytokines (IL-β, IL-6, and TNF-α) and anti-inflammatory cytokines (IL-10). The concentration of these cytokines in the supernatants of different experimental groups was measured using ELISA 12 h after treatment. * p< 0.05, ** p<0.01, *** p<0.001, and **** p<0.0001. Imipenem (Imp); Tumor necrosis factor-α (TNF-α); Interleukin (IL).

Evaluation of Apoptosis

Flow cytometric analysis was performed to evaluate HepG2 apoptosis after 12 hours of treatment. Based on this method, the percentages of viable and apoptotic cells were reported in Figures 8A and Figure 8B. The mean apoptosis rate in HepG2 cells was 12.68±2.16%, reaching 47.59±2.36% in treatment with *E. coli* (p<0.0001). The mean percentage of apoptosis in the imipenem treated group, WJ-MSC-EXO treated group, and combination therapy was 23.84±1.69% (p<0.0001), 35.982±2.312% (p=0.0037), and 13.84±1.265%, respectively. As shown in Figure 8B, the apoptosis rate in the combination therapy group was associated with a 70.918% decrease compared to the bacterial treatment group, which is statistically significant (p<0.0001).

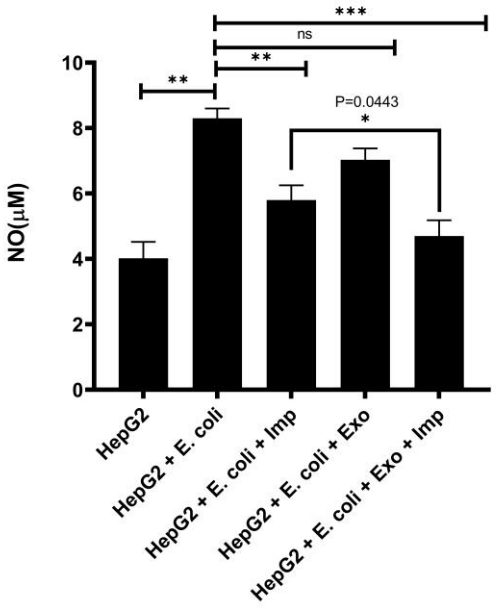

Figure 7. Measurement of Nitric Oxide concentration. The concentration of this metabolite in the supernatants of different experimental groups was measured using the Griess test 12h after treatment. * p<0.05, ** p<0.01, and *** p<0.001, . Nitric oxide (NO); Imipenem (Imp).

Table 1. The concentration of inflammatory and anti-inflammatory cytokines in the supernatant of experimental groups

Groups	IL-1β concentration (pg/mL)		TNF-α concentration (pg/mL)		IL-6 concentration (pg/mL)		IL-10 concentration (pg/mL)	
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
HepG2	342.101	42.82	250.06	60.23	120.10	25.001	941.25	71.60
HepG2 + E. coli	1603.78	120.356	1398.20	110.312	962.01	61.20	342.87	32.65
HepG2 + E. coli + Imp	640.470	60.871	719.80	81.6	419.80	31.12	680.48	45.63
HepG2+ E. coli + Exo	1001.326	40.430	1098.021	81.65	650	49.989	604.786	35.33
HepG2+E. coli+ Exo+Imp	290.609	41.259	624.21	32.87	212.31	25.15	752.301	61.37

Tumor necrosis factor-α (TNF-α); Interleukin (IL); Standard (Std); Imipenem (Imp).

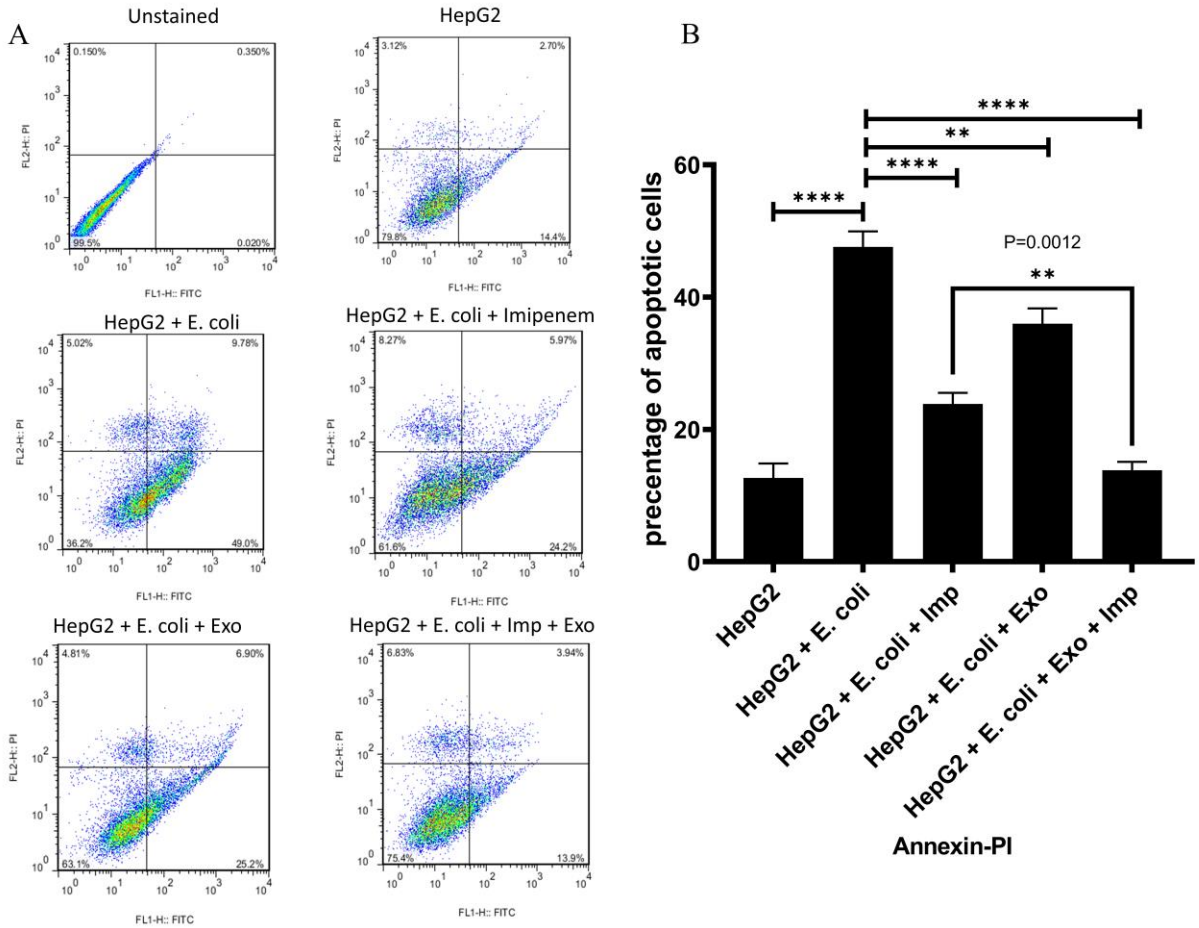

Figure 8. Evaluation of the effect of treatments on the rate of apoptosis in E.coli infected HepG2 cells by Annexin V-PI staining. (A) Dot plot diagrams show the percent of necrotic cells (Q1), late apoptotic cells (Q2), early apoptotic cells (Q3), and viable cells (Q4). (B) The bar chart shows the mean percentage of apoptosis. Results were presented as means±standard deviation (SD). * *p*< 0.05, ** *p*<0.01, * *p*<0.001, and **** *p*<0.0001.**

DISCUSSION

This study aimed to evaluate the immunomodulatory effects of WJ-MSC-EXO on the *E. coli*-mediated inflammatory response of hepatocytes in the In-vitro model. There is no specialized medicine for treating liver disease, and most of the existing therapies are only used in the most difficult situations due to their adverse side effects.[20] As a result, finding effective treatment approaches to prevent liver disease development and restore liver tissue is critical.

Sepsis is a pathological condition involving various organs, including the liver, and liver cells become infected with different bacteria. Some bacteria are intracellular and attack cells, while others are extracellular and perform their pathogenic functions by attaching to cells.[21] *Escherichia coli* is an extracellular gram-negative bacterium that infects human hepatocytes during sepsis and similar infections and can induce death and inflammatory responses in these cells.[22] Also, *Escherichia coli* and other bacterias such as *Klebsiella pneumonia*, *Streptococcus faecalis*, *Streptococcus miller*, and *Proteus Vulgaris* are the most common causes of liver abscesses.[23]

Antibiotics are the leading choices for treating life-threatening bacterial infectious diseases. It is important to note that their unrestricted and uncontrolled prescription has resulted in the appearance of antibiotic-resistant bacteria in patients.[24] One of the other side effects associated with antibiotics overuse is

their impact on the function of immune system cells.[25] These effects can occur directly on the immune cells or indirectly by killing bacteria and over releasation of PAMPs like LPS, etc.[26] The release of these molecules can increase inflammation and lead to unwanted inflammatory damage to the involved tissues. Therefore, antibiotics should be strictly controlled, and as low doses as possible should be used in conjunction with complementary therapies. Cell therapy is one of the disciplines in regenerative medicine that has attracted much attention and optimism. Cells such as MSCs, hepatocytes, hematopoietic cells, immune system cells, and endothelial progenitor cells have been used in various studies to treat liver disease.[27] MSCs are isolated from different sources, including umbilical cord tissue, dental pulp, menstrual blood, adipose tissue, amniotic fluid, bone marrow, and umbilical cord blood.[28] The application of WJ-MSCs in clinical applications is more than other sources due to their favorable characteristics such as providing a large number of MSCs in each isolation procedure, non-invasive isolation method, lower immunogenicity, higher self-renewability, and differentiation capacity of WJ-MSCs in comparison with BM-MSCs.[29]

The MSC suppresses the immune system by producing mediators including indolamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), TGF-b, and IL-10 through unknown mechanisms.[30] The immunosuppressive ability of MSCs can be induced by the presence and combination of proinflammatory cytokines IFN-g, IL-1a or IL-1b, and TNF-a.[31] Another candidate in the MSC-mediated immune suppression mechanism is the production of NO.[32] Cytokines cause a significant increase in induced NO synthase (iNOS), and several leukocytes-derived chemokines that may recruit immune cells, including T cells, B cells, and APCs closer to the MSC, where high levels of NO can suppress immune cells functions.[31] Exosomes derived from these cells can also mimic the immunomodulatory properties of MSCs and help reduce inflammatory responses.[33] The results of various studies show that using immunomodulatory agents in MSC culture can increase their exosome therapeutic efficacy. A survey by Haggai Kaspi et al. showed that exosomes derived from pre-treated MSCs to produce neutrophilic and immunomodulatory factors compared to exosomes isolated from intact MSCs had higher therapeutic efficacy in ARDSs.[34]

Also, studies on an animal clinical model related to swine flu virus showed that MSC-EVs have anti-influenza and anti-inflammatory properties, and EVs may be used as a cell-free treatment for influenza in humans.[35] Due to the antibiotic dose limitations, in this study, two strategies used to control inflammation in *Escherichia coli*-infected HepG2 cells include imipenem as the first line of antibiotic therapy and WJ-MSC-EXO as a complementary treatment. The results of this study show that the expression and production of critical inflammatory cytokines, including IL-1β, IL-6, and TNF-α, in the treated groups decreased significantly, and this decrease was associated with increased expression and production of IL-10. However, it is noteworthy that reducing inflammatory cytokine expression and production in exosomes single-use is less than imipenem single-use. Due to the importance of inflammatory cytokines in the progression of liver disease,[1, 36] reducing these cytokines can help improve the function of hepatocytes and lead to the cessation of liver damage.[37] On the other hand, the study of nitric oxide levels in the supernatant of experimental groups as a marker of cellular stress[38] shows a decrease in the production of this metabolite in the treated groups compared to the *Escherichia coli* infected group. Although normal levels of NO affect the regulation of blood flow, motility, and intestine epithelial secretions, increased levels can lead to apoptosis of various cells and decreased tissue function.[39] During infection, this response occurs to prevent the progression of the infection, but by affecting the host-involved cells, it increases the apoptosis rate in them.[40] Due to the role of inflammation and NO in inducing apoptosis, reducing these mediators' levels can reduce apoptosis in infected cells. Annexin-PI analyses in this study show that with decreasing levels of NO and inflammatory cytokines, the apoptosis rate decreases depending on their concentration. Therefore, in general, it can be said that the treatment strategies in this study increase survival and decrease apoptosis in *Escherichia coli*-infected HepG2 cells by reducing cellular stress, lowering pro-inflammatory cytokines expression and production while enhancing anti-inflammatory cytokine production.

As mentioned earlier, this study aimed to improve the function of the imipenem to control inflammation and reduce its related damage. As shown in Figures 4 and 5, combination therapy with WJ-MSC-EXO and imipenem enhances the production of inflammatory cytokines from *Escherichia coli*-infected cells by

improving antibiotic function and immunomodulatory action. This further decrease in the combination therapy group can be attributed to the characteristics of WJ-MSC-EXO because this vesicle transports immunomodulatory mediators such as cytokines, growth factors, and various microRNAs,[41] improving the function of HepG2 cells. As a result, due to the reduction of inflammation induced by inflammatory cytokines, the cellular stress and apoptosis rate was also significantly lower in the combination therapy group compared to the imipenem treatment group and acted as a better therapeutic strategy. In summary, this study's results show that the combined use of imipenem and WJ-MSC-EXO reduces the production of inflammatory cytokines, nitric oxide, and apoptosis in *E. coli*-infected HepG2 cells. These results also showed that combination therapy is better than each therapy alone.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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