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Hepatoprotective effect of hUC-MSC secretome in LPS-induced HepG2 cells

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ABSTRACT

Objective(s): This study aimed to evaluate the cytotoxicity and hepatoprotective effects of human umbilical cord mesenchymal stem cell–derived secretome (hUCMSC-Sec) in lipopolysaccharide (LPS)-induced HepG2 cells as an in vitro model of liver inflammation.

Materials and Methods: hUCMSC-Sec was obtained from conditioned media of hUCMSCs at passage four. Inflammation was induced in HepG2 cells using LPS. Cytotoxicity was assessed using the WST-8 assay. Hepatoprotective effects of hUCMSC-Sec at concentrations of 12.5%, 4.17%, and 1.39% were evaluated by measuring alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), and tumor necrosis factor-alpha (TNF-α). Gene expression levels of α-smooth muscle actin (α-SMA), SMAD-7, collagen type I alpha 1 (COL1A1), and matrix metalloproteinase-1 (MMP-1) were analyzed using quantitative real-time PCR.

Results: hUCMSC-Sec concentrations ranging from 1.6% to 25% were non-toxic, maintaining cell viability above 90%. Treatment with hUCMSC-Sec significantly reduced ALT, AST, GGT, and TNF-α levels in LPS-induced HepG2 cells. In addition, hUCMSC-Sec down-regulated α-SMA, COL1A1, and MMP-1 expression, while up-regulating SMAD-7 expression. The concentration of 4.17% showed the most pronounced hepatoprotective effect.

Conclusion: hUCMSC-derived secretome demonstrated hepatoprotective effects by attenuating inflammatory and fibrotic responses in LPS-induced HepG2 cells. However, as this study was limited to an in vitro model, further in vivo and clinical studies are required to confirm its therapeutic potential and translational applicability.

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Introduction

Numerous factors, including non-alcoholic fatty liver disease (NAFLD), viral infections, and excessive amount of alcohol consumption, can induce damage to the hepatocytes (1, 2). These circumstances trigger an inflammatory reaction in which immune cells—in particular, macrophages—are essential for both reacting to tissue damage and controlling the healing process (3). Hepatocyte damage is made worse by the production of inflammatory mediators during the inflammatory phase, which causes an accumulation of inflammatory cells in liver tissue and a rise in oxidative stress (4, 5).

Chronic inflammation can participate in forming liver fibrosis (LF), a chronic liver disease marked by hepatic stellate cells (HSCs) initiation. LF involves functional liver replacement parenchymal cells with fibrous connective tissue (6). The condition is marked by excessive extracellular matrix (ECM) protein accumulation, abnormal activation of HSCs, and other pathological changes in liver tissue (7). Around 40% of cases may progress to terminal liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) (8). Current treatments for LF primarily focus on managing

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the underlying cause or directly targeting fibrosis. These strategies aim to reduce inflammation, inhibit oxidative stress, and promote collagen degradation (9, 10). While both conventional and synthetic drugs are employed, many fail to provide sufficient liver protection and are associated with significant long-term side effects. Therefore, exploring alternative therapies is essential. One promising approach is hepatoprotection, which involves protecting liver cells and preventing the onset or exacerbation of liver disease (11).

Key liver enzymes such as aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), and alanine aminotransferase (ALT) play crucial roles as indicators of hepatic function. While GGT is often associated with liver injury, both ALT and AST are widely recognized as biomarkers for liver damage (12). The progression of LF involves numerous complex signaling pathways and molecular interactions. Among the key proinflammatory cytokines involved is tumor necrosis factor-alpha (TNF-α), which significantly contributes to liver inflammation and fibrosis. TNF-α also induces the expression of α-smooth muscle actin (α-SMA), a hallmark of activated myofibroblasts. This is particularly important due to its role in linking LF with the epithelial-mesenchymal transition (EMT) process. One major signaling cascade involved is the SMAD pathway, commonly activated via TNF-α signaling. This pathway regulates the transcription of several fibrotic markers, including collagen type I alpha 1 (COL1A1), which encodes type I collagen—one of the main structural components of the extracellular matrix (ECM). In contrast, SMAD-7 acts as a suppressor by inhibiting Transforming Growth Factor-beta (TGF-β)/SMAD signaling, thereby reducing fibrotic responses. Inflammatory processes triggered by TNF-α can also increase the activity of collagen-degrading enzymes such as matrix metalloproteinase-1 (MMP-1), which contributes to ECM remodeling (13).

Mucous connective tissue located in the umbilical cord (UC), positioned between the amniotic epithelium and the umbilical blood vessels, serves as the origin of human umbilical cord mesenchymal stem cells (hUCMSCs) (14). These MSCs possess several beneficial traits, including immunosuppressive properties, immune evasion mechanisms, diminished expression of human leukocyte antigen (HLA) class II, (HLA-DR). Due to these distinctive traits, human Wharton's Jelly mesenchymal stem cells (hWJMSCs) present a diminished likelihood of immunological rejection, rendering them an ideal candidate for cellular therapies (15).

MSCs release a range of bioactive substances called secretome, which are found in the conditioned medium. Growth factors (GF), cytokines, macromolecules, and extracellular vesicles are among the secretome that promote a range of biological reactions, particularly those that control tissue formation (14). Because hUCMSC-derived secretome (hUCMSC-Sec) modulate the local immunological milieu, reduce tissue damage, and promote epithelial healing, they show great therapeutic potential for liver injury and disease (16).

Despite increasing evidence supporting the therapeutic potential of MSC-derived secretome in liver injury and fibrosis, data using LPS-induced HepG2 inflammatory models remain limited. In particular, the hepatoprotective effects of hUCMSC-derived secretome in LPS-induced HepG2 cells, as well as its modulation of key fibrotic markers such as α-SMA, COL1A1, MMP-1, and SMAD-7, have not been sufficiently investigated. This present study provides direct experimental evidence by evaluating the cytotoxicity and hepatoprotective effects of hUCMSC-Sec in an LPS-induced HepG2 inflammatory model through integrated analysis of protein expression levels (ALT, AST, GGT, and TNF-α) and gene expression profiles (α-SMA, SMAD-7, COL1A1, and MMP-1). Accordingly, this study primarily focuses on the immunomodulatory effects of hUCMSC-derived secretome in an LPS-induced HepG2 inflammatory model and its downstream impact on fibrosis-related gene expression. By emphasizing inflammation-driven fibrotic regulation, this work aims to provide a focused and mechanistic understanding of secretome-mediated hepatoprotection. The hUCMSCs used in this study had been previously characterized and differentiated, with surface marker analysis confirming strong expression of CD44, CD90, CD105, and CD73, and absence of HLA-DR, CD11b, CD34, CD45, and CD19 (17).

Materials and Methods

Isolation and culture of hUCMSCs

The hUMSCs were collected and cultured from fresh human UC tissue sourced from a 25-year-old woman who experienced a normal vaginal delivery (UC; n=1) at full term. The research received endorsement from the Indonesia Ethics Committee in Maranatha (Approval No. 002/I/S.Kep./FK-UKM/2023), and the donor provided informed consent (Form No. 033/KNEPK/2008). Following a thorough rinse of the UC with 10x Phosphate Buffered Saline (PBS, Biowest, X0520-500), it was meticulously sectioned into diminutive explants measuring 1-2 mm in size. As noted earlier (18), the explants were grown in 6-well plates utilizing MEM-α (Biowest, L0475-500) enriched with 20% FBS (S1810-500). Incubation was carried out at 37 °C under humid conditions with 5% CO_2 for 24 hr to maintain optimal growth conditions. for a duration of 14 days. Each week, a novel culture medium was employed. The cells were obtained and replanted at $8x10^3$ cells/cm^2 density upon achieving 70–80% confluence. hUCMSCs were nurtured in MEM-α enriched with 15% FBS to promote their continued development. Every two to three days, the medium was replaced.

Isolation of secretome

At the fourth passage, when the cells had achieved 80–90% confluency, the secretome was extracted from hUCMSCs. Osteogenic differentiation and multipotency were confirmed prior to secretome collection. The hUCMSCs were then incubated at 37 °C under humid conditions with 5% CO_2 for 24 hr following the replacement of the culture medium with a serum-free medium to allow the release of secreted bioactive factors into the conditioned medium. After that, the conditioned medium was gathered and centrifuged using a chilled centrifuge (MWP 260R) at 3000 × g, 37 °C for 4 min to remove cellular debris. The resulting supernatant, containing the secreted bioactive factors, was then filtered using a Durapore membrane filter unit (Millipore Corporation, SLGV 03397 RS) to obtain the purified secretome and ensure sterility before experimental use (19–21).

HepG2 cell culture and inflammatory induction

Aretha Medika Utama provided the HepG2 (ATCC HB-8065TM) cell line from human hepatocyte cancer.

Complete Dulbecco's Modified Eagle Medium (DMEM; Biowest, MSN0011007) was used to cultivate the cells, and 10% FBS was added for enrichment (22). The media was refreshed every 2–3 days while the cells were kept at 37 °C under humid conditions containing 5% CO_2. The cells were incubated in the presence of 4 µg/ml of *Escherichia coli* O55:B5 lipopolysaccharides (LPS, Sigma-Aldrich, L2880) for 18 hr based on previous studies demonstrating effective induction of inflammatory responses without excessive cytotoxicity in order to create an *in vitro* inflammatory model (23). Untreated cells served as negative controls, while LPS-treated cells without secretome supplementation were used as inflammatory controls. Following that, the cells received treatment in accordance with the assigned treatment groups.

Cytotoxicity assay

Following LPS induction, cells were dispensed into 96-well microplates at a concentration of 1×10^4 cells per well. Once attachment was achieved, 180 µl of DMEM containing 2% FBS was added, followed by 20 µl of hUCMSC-Sec at different concentrations: 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.39%. Following the manufacturer's instructions, cytotoxicity was evaluated using the WST-8 assay (Elabscience, E-CK-A362) (18). The treatment groups comprised Vehicle Control (VC)-LPS-induced cells treated with serum-free media, the Positive Control (PC)-LPS-induced HepG2 cells, Negative Control (NC)-untreated cells, and Treatment Groups-LPS-induced cells treated with hUCMSC-Sec at varying doses of 12.5%, 4.17%, and 1.39%.

Measurement of ALT, AST, GGT, and TNF-α levels

LPS was utilized to activate HepG2 cells in 6-well plates subsequent to their plating ($5x10^5$ cells/well). Subsequent to induction, 1800 µl of DMEM supplemented with 200 µl of hUCMSC-Sec at several concentrations (12.5%, 4.17%, and 1.39%) and 2% FBS was included into the media. Levels of ALT, AST, and GGT were quantitatively assessed using assay kits (Elabscience: GGT (E-BC-K126-M), AST (E-BC-K236-M), ALT (E-BC-K235-M), and TNF-α protein) via calorimetric methods, following the manufacturer's guidelines (12, 24).

Measurement of gene expression of SMAD-7, COL1A1, α-SMA, and MMP-1

The genes SMAD-7, COL1A1, α-SMA, and MMP-1 were examined in the HepG2 cell pellets that had undergone treatment. Total RNA was isolated using the Direct-zol RNA Miniprep Plus Kit (Zymo, R2073), following the instructions provided by the manufacturer. Details on RNA purity and concentration can be found in Table 1. The synthesis of complementary DNA (cDNA) was conducted with the Sensi-FAST DNA Synthesis Kit, adhering to the manufacturer's protocol. The qRT-PCR process was carried out on the AriaMx 3000 Real-Time PCR System (Agilent, G8830A), using the SensiFast Syber NO-ROX Kit (Meridian, BIO-98005), following the recommended procedures (23). The primers specific to each gene target are listed in Table 2. To ensure accurate quantification, GAPDH served as the housekeeping reference gene to normalize expression levels.

Statistical analysis

Data presented as mean ± standard deviation (SD). Statistical evaluation was performed using one-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons, employing the Statistical Package for the Social Sciences (IBM®, version 20.0). A *P*-value less than 0.05 was regarded as statistically significant (25).

Results

Cytotoxicity test of various hUCMSC-Sec concentrations in LPS-induced HepG2 cells

Figure 1 shows the cytotoxic effects of various concentrations of hUCMSC-Sec on LPS-induced HepG2 cells. A dose-dependent relationship was observed, where increasing concentrations of hUCMSC-Sec corresponded with increase in cytotoxicity. The highest hUCMSC-Sec concentration (50%) resulted in the lowest cell viability

Table 1. RNA concentration and purity of samples

Sample		Concentration (ng/ µl)	Purity (λ260/ λ280 nm)
I	NC (untreated cell)	92.8800	2.3092
II	PC (LPS-induced HepG2 cell)	120.8800	2.2999
III	VC (PC + serum-free medium)	77.8400	2.2758
IV	hUCMSC-Sec 1.39 (PC + hUCMSC-Sec 1.39%)	72.3200	2.2605
V	hUCMSC-Sec 4.17 (PC+ hUCMSC-Sec 4.17%)	102.1600	2.3290
VI	hUCMSC-Sec 12.50 (PC+ hUCMSC-Sec 12.50%)	29.5200	2.3768

hUCMSC: Human umbilical cord mesenchymal stem cell; LPS: Lipopolysaccharide; NC: Negative control; PC: Positive control; VC: Vehicle control

Table 2. Primer sequences for Real-Time PCR

Gene	Primer Sequences (5'- 3')	Annealing (°C)	Cycle	Reference
COL1A1 (*Homo sapiens*)	F: GAATTCGGCTTCGACGTTGG R: AGGGGGTTCAGTTTGGGTTG	58	40	NM_000088.4
MMP-1 (*Homo sapiens*)	F: GCCATCTGCTCCTGGATCTC R: CCACATCAGGCACTCCACAT	58	40	NM_002421.4
SMAD-7 (*Homo sapiens*)	F: GCCATCTGCTCCTGGATCTC R: GGCACTGGGTGAGCAATACT	64	40	NM_005904.4
α-SMA (*Homo sapiens*)	F: TGCCTTGGTGTGTGACAATG R: TTTGCTCTGTGCTTCGTCAC	58	40	NM_001141945.3
GAPDH (*Homo sapiens*)	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	58	40	NM_001289745.3

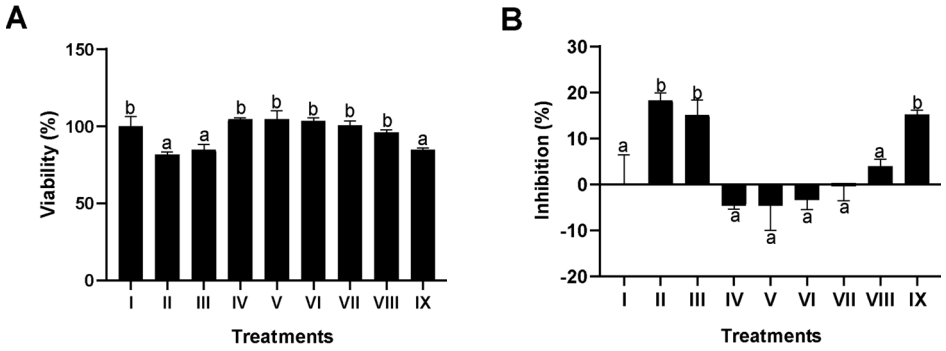


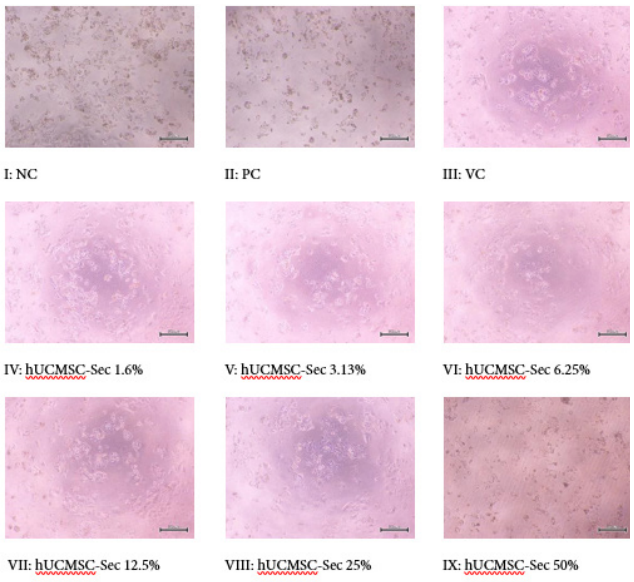
Figure 1. Cytotoxicity test of various concentrations of human umbilical cord mesenchymal stem cell-derived secretome (hUCMSC-Sec) on lipopolysaccharide (LPS)-induced HepG2 cells toward cell viability (A) and cell inhibition (B).
I: Negative control (NC; HepG2 cells without LPS induction and treatment); II: Positive control (PC; LPS-induced HepG2 cells treated with Dulbecco's Modified Eagle Medium (DMEM) containing 2% fetal bovine serum (FBS); III: Vehicle control (VC; LPS-induced HepG2 cells treated with serum-free DMEM); IV–IX: LPS-induced HepG2 cells treated with hUCMSC-Sec at concentrations of 1.6%, 3.13%, 6.25%, 12.5%, 25%, and 50%, respectively. Each value represents the mean ± standard deviation (SD). Different letters indicate significant differences among groups based on Tukey's Honestly Significant Difference (HSD) *post hoc* test (P<0.05).

(84.82%) and the highest level of cell inhibition (15.18%). Conversely, the lowest hUCMSC-Sec concentration (1.6%) exhibited the highest cell viability (104.73%) and the lowest cell inhibition (-4.73%). The hUCMSC-Sec concentrations ranging from 1.6% to 25% maintained cell viability above 90%, classifying them as non-toxic. The cell morphology of HepG2, as noted throughout the cytotoxicity testing, is summarized in Table 3.

Effect of hUCMSC-Sec on ALT, AST, GGT, and TNF-α levels in LPS-induced HepG2 cells

Figure 2 depicts the effect of hUCMSC-Sec treatment toward ALT, AST, and GGT levels (Figure 2 B-D) and TNF-α level (Figure 2A) in LPS-induced HepG2 cells. LPS induction markedly elevated the levels of AST, GGT, ALT, and TNF-α compared to the negative control (P<0.05). Treatment with hUCMSC-Sec at concentrations of 12.5%, 4.17%, and 1.39% reduced significantly the levels of AST, GGT, ALT, and TNF-α in LPS-induced HepG2 cells (P<0.05). Among the concentrations tested, hUCMSC-Sec at 4.17% demonstrated the most potent effect, normalized TNF-α level and it was comparable with NC.

Table 3. Morphological changes of LPS-induced HepG2 cells following treatment with hUCMSC-Sec



Magnification 10×. I: Negative control (NC; HepG2 cells without lipopolysaccharide (LPS) induction and treatment);
II: Positive control (PC; LPS-induced HepG2 cells treated with Dulbecco's Modified Eagle Medium (DMEM) containing 2% fetal bovine serum (FBS)); III: Vehicle control (VC; LPS-induced HepG2 cells treated with serum-free DMEM); IV–IX: LPS-induced HepG2 cells treated with human umbilical cord mesenchymal stem cell-derived secretome (hUCMSC-Sec) at concentrations of 1.6%, 3.13%, 6.25%, 12.5%, 25%, and 50%, respectively.

Effect of hUCMSC-Sec toward gene expression of α-SMA, SMAD-7, COL1A1, and MMP-1 in LPS-induced HepG2 cell

The qRT-PCR analysis demonstrated that hUCMSC-Sec treatment induced differential expression of key regulatory genes α-SMA, SMAD-7, MMP-1, and COL1A1 (Figure 3A-D). The data revealed that stimulation with LPS resulted in a significant elevation in the expression of α-SMA, COL1A1,

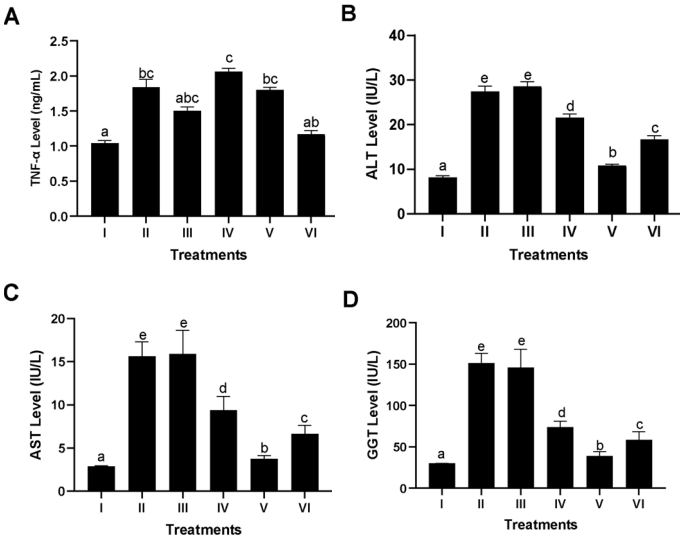


Figure 2. Effect of human umbilical cord mesenchymal stem cell-derived secretome (hUCMSC-Sec) on tumor necrosis factor-alpha (TNF-α)
(A), alanine aminotransferase (ALT) (B), aspartate aminotransferase (AST) (C), and gamma-glutamyl transferase (GGT) (D) levels in lipopolysaccharide (LPS)-induced HepG2 cells.
I: Negative control (NC; HepG2 cells without LPS induction and treatment); II: Positive control (PC; LPS-induced HepG2 cells treated with Dulbecco's Modified Eagle Medium (DMEM) containing 2% fetal bovine serum (FBS)); III: Vehicle control (VC; LPS-induced HepG2 cells treated with serum-free DMEM); IV–VI: LPS-induced HepG2 cells treated with hUCMSC-Sec at concentrations of 1.39%, 4.17%, and 12.50%, respectively. Each value represents the mean ± standard deviation (SD). Different letters indicate significant differences among groups based on Tukey's Honestly Significant Difference (HSD) *post hoc* test (P<0.05).

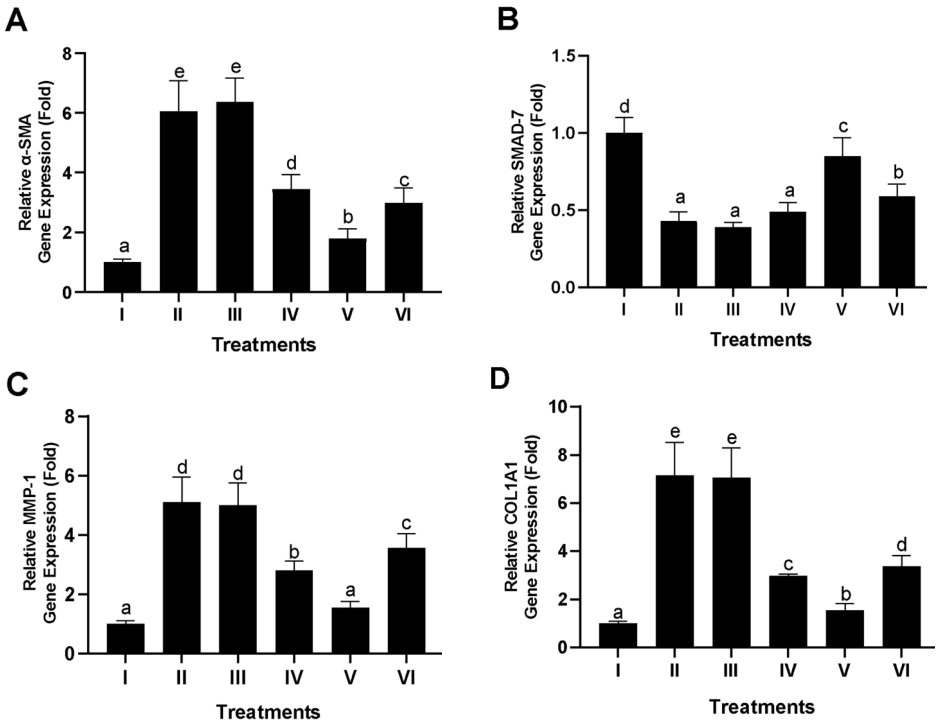


Figure 3. Effect of human umbilical cord mesenchymal stem cell-derived secretome (hUCMSC-Sec) on alpha-smooth muscle actin (α-SMA) (A), SMAD family member 7 (SMAD-7) (B), matrix metalloproteinase-1 (MMP-1) (C), and collagen type I alpha 1 chain (COL1A1) (D) gene expression in lipopolysaccharide (LPS)-induced HepG2 cells.
I: Negative control (NC; HepG2 cells without LPS induction and treatment); II: Positive control (PC; LPS-induced HepG2 cells treated with Dulbecco's Modified Eagle Medium (DMEM) containing 2% fetal bovine serum (FBS)); III: Vehicle control (VC; LPS-induced HepG2 cells treated with serum-free DMEM); IV–VI: LPS-induced HepG2 cells treated with hUCMSC-Sec at concentrations of 1.39%, 4.17%, and 12.50%, respectively. Each value represents the mean ± standard deviation (SD). Different letters indicate significant differences among groups based on Tukey's Honestly Significant Difference (HSD) *post hoc* test (P<0.05).

and MMP-1 (P<0.07), while causing a marked reduction in SMAD-7 expression (P<0.05). In contrast, administration of hUCMSC-Sec at concentrations of 1.39%, 4.17%, and 12.50% resulted in a significant suppression of α-SMA, COL1A1, and MMP-1 expression (P<0.05), along with a significant elevation in SMAD-7 levels (P<0.05). Among all treatment consentrations, the 4.17% hUCMSC-Sec exhibited the most pronounced regulatory effect, restoring gene expression (Figure 3 A-D).

Discussion

Chronic liver inflammation arises from various causes and may advance to chronic liver conditions, including cirrhosis, fibrosis, and HCC. hUCMSC-Sec has proven the existence of several cytokines and GF that promote liver regeneration, also alleviate inflammation (26, 27). Prior to clinical application, it is imperative to assess the cytotoxicity of hUCMSC-Sec to guarantee its safety and mitigate the potential of adverse effects. A therapy is deemed non-toxic if its viability value surpasses 90% (28, 29). In this study, the hepatoprotective effects of hUCMSC-derived secretome are primarily attributed to its immunomodulatory activity, as reflected by reduced TNF-α levels and subsequent suppression of fibrosis-related gene expression. The findings of this study show that hUCMSC-Sec at concentrations ranging from 1.6% to 25% has a non-toxic effect on cells. Higher concentrations of hUCMSC-Sec showed decreased cell viability and increased cell inhibition (Figure 1, Table 3). Table 3 shows cell morphology. Sulaiman *et al.* also documented a notable decline in cell viability percentages as the concentration of SW increased (30).

The hepatoprotective capability of hUCMSC-Sec is demonstrated by its effectiveness in lowering ALT, AST, and GGT levels, as shown in Figure 2B–D. The enzymes ALT, AST, and GGT are widely utilized for the diagnosis of liver damage and the assessment of hepatic function (31). In addition, the combined assessment of these enzymes can aid in the diagnosis of specific types of liver disease. ALT and AST are types of transaminase or aminotransferase enzymes that facilitate the transfer of amino groups. ALT, a hepatic enzyme, facilitates the transformation of L-alanine into α-ketoglutarate, producing pyruvate and L-glutamate. Increased ALT levels signify liver illness. Similarly, AST catalyzes the transfer of L-aspartate to α-ketoglutarate, leading to the production of oxaloacetate and L-glutamate. Like ALT, elevated AST activity signifies tissue damage in the organ where the enzyme is produced, particularly the liver, where heightened AST levels correlate with higher injury or tissue damage (32). This work demonstrated that hUCMSC-Sec administration significantly decreased ALT, AST, and GGT levels in LPS-induced HepG2 cells, highlighting its hepatoprotective potential. In correlation with this study, Zhang *et al.*, reported that SW has strong potent immunomodulatory properties and can reduce inflammation in liver tissue, by lowering the levels of ALT, AST, and GGT (33). Likewise, Zhou *et al.* discovered that the secretome from adipose stem cells transfected with miR-122 enhanced liver function and reduced liver enzyme levels in animal models (34). These findings suggest that components in hUCMSC-Sec can reduce elevated liver enzyme levels and repair liver damage. In regenerative medicine, tissue repair is strongly influenced by the extracellular microenvironment that supports cell survival, proliferation, and functional recovery. Decellularized

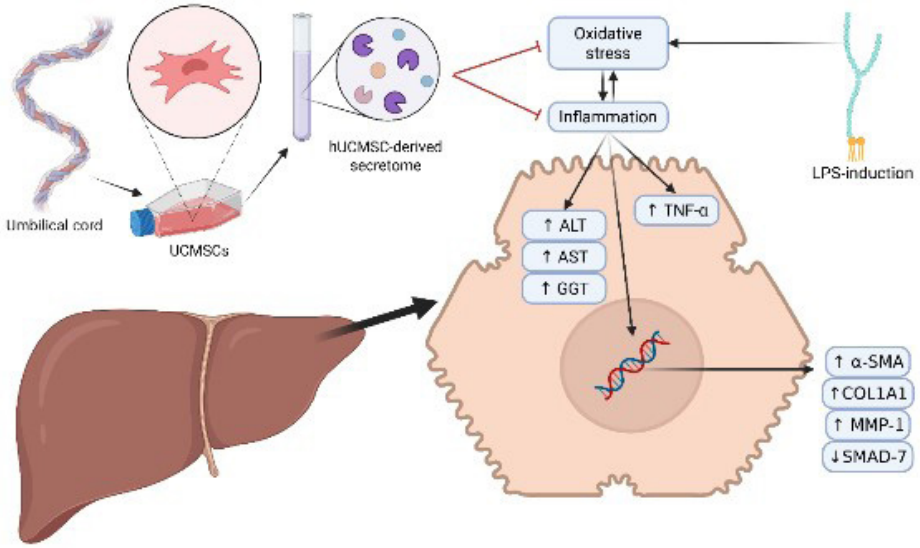


Figure 4. Proposed mechanism of hUCMSC-derived secretome in LPS-induced HepG2 cells
Black arrows indicate inflammatory and fibrotic pathways activated by LPS stimulation, while red arrows indicate the inhibitory effects of hUCMSC-derived secretome in suppressing inflammation and fibrosis-related signaling.
hUCMSC: Human umbilical cord mesenchymal stem cell; LPS: Lipopolysaccharide; MMP: Matrix metalloproteinase-1; α-SMA: α-Smooth muscle actin; COL1A1: Collagen type I alpha 1; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-Glutamyl transferase; TNF-α: Tumor necrosis factor-alpha

extracellular matrices (ECM) have been widely utilized as regenerative scaffolds because they preserve tissue-specific biochemical, mechanical, and structural cues that facilitate cellular engraftment and tissue regeneration (35). Similarly, the regenerative effects of hUCMSC-Sec are predominantly mediated through paracrine mechanisms that modulate the cellular microenvironment, thereby promoting tissue repair and functional restoration without direct cell replacement.

TNF-α, including macrophages and Kupffer cells, is a crucial pro-inflammatory cytokine produced by immune cells, in response to various stimuli. Inflammation is marked by elevated TNF-α, resulting from the NF-κB signaling system activation, which governs the gene expressions associated with inflammation and apoptosis (36). Increased TNF-α levels can significantly impact gene expression related to LF, including α-SMA, SMAD-7, COL1A1, and MMP-1. An increase in α-SMA expression indicates the activation of fibroblast into myofibroblasts, which contributes to the development of LF (37, 38). TNF-α activation promotes the expression of COL1A1 and MMP-1 genes, which exacerbates LF conditions by disrupting the balance between collagen synthesis and degradation. Conversely, elevated TNF-α levels can suppress SMAD-7 gene expression, leading to increased TGF-β signaling pathway, thereby enhancing collagen production, leading to fibrosis. Treatment with hUCMSC-Sec at a concentration of 4.17% effectively reduced TNF-α protein levels (Figure 2D1-D2), increased SMAD-7 gene expression (Figure 3B), and down regulated the expression of α-SMA, COL1A1, and MMP-1 (Figures 3A and 3C-D).

Mesenchymal stem cells (MSCs) exhibit immunomodulatory effects of proinflammatory and antiinflammatory cytokines through secretion. Their secretome comprises various anti-inflammatory cytokines, including Neurotrophin-3 (NT-3), TNF-β1, Ciliary Neurotrophic Factor (CNTF), IL-18 Binding Protein (I-18BP), and Interleukin-13 (IL-13). Furthermore, cytokines such as IL10, IL12p70, IL17E, IL27, and IL1 family factors have been recognized. MSCs release proinflammatory cytokines TGF-β such as IL-1β, IL-6, IL-8, and IL-9 (39, 40). The hUCMSC-Sec comprises anti-inflammatory cytokines and GF, notably Hepatocyte Growth Factor (HGF) and TGF-β, which have been shown to inhibit TNF-α levels (27). A study by Widowati *et al.* reported that hUCMSC-Sec secretes indole amine 2,3 dioxygenase (IDO), a key enzyme with potential for anti-inflammatory therapy (18). Furthermore, previous studies demonstrated that hUCMSC-Sec can down-regulate the expression of key components of the TGF-β1/SMAD pathway, including COLI, COLIII, TGF-β1, SMAD-2, and SMAD-3, indicating its potential role in anti-fibrotic mechanisms (41, 42).

Based on the integrated biochemical and molecular findings, hUCMSC-derived secretome is proposed to exert hepatoprotective effects primarily through immunomodulatory mechanisms (Figure 4). LPS exposure induces inflammatory signaling in HepG2 cells, leading to elevated TNF-α levels and subsequent activation of fibrosis-related pathways, as reflected by increased α-SMA, COL1A1, and MMP-1 expression. Treatment with hUCMSC-secretome attenuates this inflammatory response by reducing TNF-α levels, thereby limiting inflammation-driven fibrotic activation. Concurrent up-regulation of SMAD-7 suggests inhibition of profibrotic signaling, contributing to the suppression of extracellular matrix remodeling. Through coordinated regulation of inflammatory mediators and fibrosis-associated gene expression, hUCMSC-secretome preserves hepatocellular integrity and mitigates inflammation-associated fibrotic responses in LPS-induced HepG2 cells.

These findings highlight the potential of hUCMSC-derived secretome as a promising acellular therapeutic approach for modulating liver inflammation and early-stage fibrotic responses. Compared to whole-cell MSC therapies, secretome-based interventions may offer improved safety profiles by minimizing risks associated with immune rejection and tumorigenicity. In addition, the scalability of secretome production and its suitability for pharmaceutical formulation support its potential application in regenerative

medicine. Nevertheless, this study is limited by its *in vitro* design, which may not fully recapitulate the complexity of liver pathology *in vivo*. Therefore, further studies are required to investigate the pharmacokinetics, optimal dosing, and safety of hUCMSC-secretome in relevant animal models, as well as to explore its efficacy in clinical settings.

Conclusion

Treatment with hUCMSC-Sec showed significant hepatoprotective effects in LPS-induced HepG2 cells. This was evidenced by its ability to reduce both the expression and activity of key biomarkers associated with liver damage, including decreased levels of ALT, AST, GGT, and TNF-α. In addition, hUCMSC-Sec down-regulated the gene expression of α-SMA, MMP-1, and COL1A1, while up-regulating SMAD-7 gene expression. These findings suggest the potential of hUCMSC-Sec in mitigating liver inflammation and fibrosis. However, this study was limited to an *in vitro* LPS-induced HepG2 model, which may not fully represent the complexity of liver pathology *in vivo*. Therefore, further studies are required to validate these findings in relevant animal models, optimize dosing and delivery strategies, and assess the safety and therapeutic efficacy of hUCMSC-Sec for potential clinical translation.

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Authors' Contributions

W W helped conceive the study, acquire funding, and supervise. P O provided methodology and resources. MV contributed by validation and formal analysis. DN T and AP helped draft the manuscript, review, and edit. LW N contributed to data curation and investigation. I R performed data curation and provided resources. A A helped with methodology and investigation. D P performed formal analysis and visualization. R A provided software analysis and data curation. DS H performed software analysis and validation. P R contributed by review, editing, and visualization. KS A performed project administration and supervision. SD R contributed by review and editing.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

The authors declare the use of generative AI tools (such as QuillBot and ChatGPT by OpenAI) solely for the purpose of enhancing language clarity and correcting grammar in this manuscript. AI was not involved in generating content or interpreting data. The authors accept full responsibility

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