Original Research Article

Isolation, Characterization, and Genetic Stability Study of Human Umbilical Cord-Derived Mesenchymal Stem Cells for Clinical Research

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ABSTRACT :

Background*: Human umbilical cord mesenchymal stem cells (hUCMSCs) represent a valuable and versatile cell type derived from the umbilical cord tissue of newborns. These cells exhibit diverse characteristics, including multipotent differentiation potential and low immunogenicity. hUCMSCs hold promise for clinical applications, yet concerns persist regarding their chromosomal stability. This study employs short tandem repeat (STR) analysis to assess genomic integrity. By focusing on chromosomal stability, the study aims to enhance confidence in the clinical viability of hUCMSCs, contributing to their safe and effective use in regenerative medicine.*

Methods*: hUCMSCs were extracted from healthy newborn umbilical cords using tissue block adherence and enzyme digestion. The third generation of hUCMSCs undergoes biological characterization and quality checks, including examinations of cell morphology, viability, growth curves, surface markers, cell cycle, and multi-lineage differentiation potential.*

Results*: hUCMSCs exhibit mesenchymal stem cell characteristics, with robust growth and high viability. Positive surface markers are expressed at rates exceeding 95%, while negative markers are ≤2%. Cell karyotyping analysis, STR spectrum identification, and chromosomal microarray analysis confirm genetic stability. Microbial testing shows the absence of contaminants, and immunological studies demonstrate the immunomodulatory capabilities of hUCMSCs. Specific residues, such as trypsin, were not detected.*

Conclusion*: hUCMSCs produced under strict GLP and GMP conditions meet the quality guidelines for clinical application in various stem cell therapies. The findings support the clinical application of hUCMSCs in various medical contexts.*

Keywords: human umbilical cord mesenchymal stem cells, Wharton´s jelly stem cells, umbilical cord blood and genetic stability

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1.0 Introduction

The umbilical cord, a vital conduit between the fetal embryo and the mother's placenta, represents a unique and valuable biological resource [1]. The recycling of healthy newborn umbilical cords holds paramount significance as a precious biological resource. Moreover, within the umbilical cord tissue, specifically in Wharton's jelly, an extraordinary reservoir of stem cells exists in human umbilical cord mesenchymal stem cells (hUCMSCs) [2]. These hUCMSCs, isolated from Wharton's jelly, exhibit biological characteristics like stromal cells and bone marrow-derived MSCs. Notably, hUCMSCs display traits closer to primitive cells, demonstrating robust proliferation and differentiation capabilities [3]. Through In vitro models, these cells can differentiate into osteoblasts, adipocytes, chondrocytes, nerve-like cells, and other stem cell types. Beyond their regenerative potential, hUCMSCs possess immunosuppressive functions and support hematopoiesis [4].

Unlike other tissue-derived MSCs, the isolation process of UCMSCs is notably straightforward. The hUCMSCs express high levels of positive surface markers such as CD29, HLA-DR, CD73, CD90, CD105, and CD166, while showing minimal expression of endothelial and hematopoietic stem cell markers [5]. The potential applications of hUCMSCs extend to multiple medical conditions, such as ischemic heart disease, heart failure, cerebral infarction, liver fibrosis, pulmonary fibrosis, renal function injury, diabetes, Parkinson's syndrome, senile dementia, osteoarthrosis, autoimmune diseases, tissue trauma repair, and ageing [6]. The ability of MSCs to secrete functional exosomes and vascular endothelial growth

factor (VEGF) demonstrates their pivotal role in angiogenesis, myocardial precursor cell proliferation, and resistance to myocardial fibrosis [7]. Beyond these applications, MSCs exhibit promise in alleviating movement disorders, cerebellar atrophy, and interventions in ataxia models [8].

The present study aimed to comprehensively investigate the preparation of hUCMSCs for clinical applications, emphasizing their biological characteristics and standardized quality inspection. The objectives are to prepare hUCMSC, identify key biological characteristics, determine their genetic stability, and conduct quality inspections. This study seeks to contribute valuable insights to the field of regenerative medicine, providing a foundation for the safe and effective clinical application of hUCMSCs in diverse medical conditions.

2.0 Materials and Method

The following instrumentations, reagents, and kits were employed to ensure precision, reproducibility, and reliability of results:

Electron Microscope, Inverted Optical Microscope, and Inverted Phase Contrast Microscope (Olympus, Japan). PCR Instrument, Fluorescence Quantification PCR Instrument (Bio Molecular Systems, Australia), ABI 3730xl Genetic Analyzer (Applied biosystem, USA), UV-Visible Spectrophotometer (Agilent Technologies, USA). Flow Cytometry (BD Biosciences, USA). Cell Counter (Invitrogen, USA). ELISA Plate Reader (Agilent Technologies, USA).

Cell Culture Media and Reagents: Clinical Grade DMEM/F12 Complete Serum-Free Medium (Gibco, USA), Complete Serum-Free Medium (Stem Cell Technologies, CA), Complete Serum-Free Medium (Life Technologies, USA), Fetal Bovine Serum (Gibco, USA), Human Platelet Lysate Collagenase II, Collagenase IV, Hyaluronidase DNA Enzyme (Sigma, USA), Trypsin-EDTA (Invitrogen, USA),High-Quality Fetal Bovine Serum (USA Gibco), TrypLE/EDTA (Gibco, USA) Trypan Blue Staining Solution (Gibco, USA) Penicillin and Streptomycin Solution (Gibco, USA), Cell Culture Grade Dimethyl Sulfoxide (Sigma, USA), Calcium-Free Phosphate Buffer DPBS (Hyclone, USA), Phosphate Buffer PBS (Sinopharm, China), Adipogenic, Chondrogenic, and Osteogenic Induction Media; 3-Isobutyl-1-Methylxanthine (IBMX), L-Glutamine, β-Sodium Glycerophosphate, Dexamethasone, Ascorbic Acid, Insulin, Indomethacin (Sigma, USA), Alizarin Red (Schmid, Germany), Oil Red (Sinopharm, China), Alamar Blue (Life Technologies, USA), Phycoerythrin (PE) for Characteristic Surface Markers of Mesenchymal Stem Cells (CD14-PE, CD45-PE, CD34-PE, CD29-PE, CD90-PE, HLA-DR, CD105, Anti-human IgG (BD, Biosciences USA).

Informed Consent

Before sample collection, the parent(s) of the donor were provided with informed consent, clearly understanding the purpose, procedures, and potential implications of the donation. The consent process ensures transparency and respect for the autonomy of the donor's parent(s).

Sample Origin and Donor Criteria

The umbilical cords were obtained from a healthy pregnant woman who delivered by (caesarean section) at full-term pregnancy. The study was conducted per the Declaration of Helsinki. The Donors must meet specific health criteria, ensuring the absence of congenital disorders, genetic diseases, autoimmune diseases, infectious diseases, or malignant tumour diseases. Additionally, donors undergo thorough health inspections, including negative results for HBV antigen, anti-HCV antibody, anti-HIV antibody, anti-Treponema pallidum antibody, mycoplasma, and anti-CMV antibodies.

2.1 Collection and Isolation of hUCMSCs

It should be noted that the cells used for analysis in this study were manufactured in a Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) facilities.

2.1.1 Collection of Neonatal Umbilical Cord (NUC) Sample

The hUCMSCs collection was strictly adhering to aseptic operation standards consistent with routine obstetrics and gynaecology care following the previously established protocols [9], with a slight modification.

2.1.2 Pretreatment Method for Neonatal Umbilical Cord (NUC) Samples

In a clean bench or biosafety cabinet, the NUC was withdrawn using sterile surgical forceps and placed in a sterile petri dish. The outer epithelium is gently removed along the longitudinal axis with a sterile surgical blade and ophthalmic forceps. The inner layer was then excised, revealing the umbilical cord blood vessels surrounded by Wharton's jelly. The cleansed sample was meticulously cut into 1 cm segments with surgical scissors, and the remaining arteries and veins were removed.

Wharton's jelly was preserved and sliced into 1mm x 3mm pieces. These fragments were promptly placed into 15ml centrifuge tubes with serum-free culture medium MSCs, each containing approximately 1g of the sample for subsequent use. The above method was conducted per a previously established protocol [10].

2.1.3 Isolation of hUCMSCs

The present study employed single-enzyme digestion methods (using type I collagenase, type II collagenase, or type IV collagenase), double-enzyme digestion methods (involving collagenase + trypsin or Type II collagenase + hyaluronidase), and three-enzyme digestion (involving collagenase + hyaluronidase + trypsin) as per the previously protocol outlined by [11] with slight modifications.

2.1.4 The Preparation of Clinical Application Research Grade hUCMSCs

The preparation of clinical grade hUCMSCs for research applications adhered to a systematic method following established standards [12]:

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Briefly, the hUCMSCs were revived and cultured from working bank cells. The cell confluency was monitored using an inverted microscope to ensure optimal growth and health of the hUCMSCs. Cells were digested and collected using a previously outlined procedure [12].
The cell density of hUCMSCs was carefully adjusted to a concentration of $1x10^{7}$ cells/ml to $2x10^{7}$ cells/ml using physiological saline or compound electrolyte injection. A volume ratio of 0.1% human serum albumin was added dropwise to a sterile syringe, finalizing the clinical application research-grade hUCMSCs preparation. The samples were refrigerated at 4°C and infused within 6 hours, ensuring the stability and viability of the hUCMSCs for clinical applications.

2.2 Cell Viability

The cell viability analysis using the Nexcelom Cellometer Mini was conducted following a standardized protocol [12].

2.3 The Detection and Analysis of hUCMSCs Biological Characteristics

2.3.1 Morphological Observation

The hUCMSCs were examined using an inverted microscope to analyse their morphology and growth patterns. The growth activity detection was determined using cell counting to measure hUCMSC proliferation and growth activity. The survival rate detection was determined using the trypan blue staining method in an automatic cell counter to assess viable cells and determine the survival rate [13].

2.3.2 Flow Cytometry

Flow cytometry was employed to analyse the hUCMSCs cell cycle, determining DNA content with fluorescence intensity and Mod Fit LT4.1 software. The phenotypic and surface marker detection was conducted in adherence to a previous study [14], utilizing flow cytometry for the detection and analysis of cell surface antigen molecules based on established standards.

2.3.3 Cell Karyotype Analysis

Cell karyotype analysis was evaluated through the chromosomal number, shape, and structure of randomly selected metaphase cells and assessed polyploidy incidence using karyotype analysis [15].

2.3.4 DNA Fingerprint Identification

The DNA fingerprint identification was identified by extracting the DNA using the STRAtlas method, amplified 20 different STR and sex determination loci, and analysed results with the ABI 3730xl genetic analyser and GeneMapper 3.2 software [16]. Species source and cross-contamination were determined using ATCC and DSMZ databases.

2.3.5 Chromosomal Microarray Analysis

Chromosomal microarray analysis was conducted to analyse for hUCMSCS using chromosomal microarray analysis. the differentiation ability detection was assessed differentiation ability through histochemical staining and morphological identification of specific cell types. The fluorescence quantification PCR was used for detecting genes associated with adipogenesis, osteogenesis, and chondrogenesis [17].

2.3.6 Multi-lineage differentiation potential of hUCMSCs

The study assessed the multi-lineage differentiation potential of hUCMSCs through adipogenic, osteogenic, and chondrogenic pathways [18]. Adipogenic differentiation was evaluated using Oil Red O staining, osteogenic differentiation with Alizarin Red staining, and chondrogenic differentiation through Alcian blue staining. These staining techniques enabled the visualization and qualitative assessment of adipocytes, osteocytes, and chondrocytes, respectively, demonstrating the versatile and tri-lineage differentiation capacity of hUCMSCs.

2.4 Contaminant Screening for High-Risk Agents

The detection of microorganisms was conducted using the previously established protocols [19] with slight modifications.

2.4.1 Mycobacteria Detection

Mycobacteria detection was performed using the Middlebrook 7H11 agar solid medium [20]. Adhering to the "Tuberculosis Laboratory Inspection Regulations" standard, cultures were considered negative if no colony growth was observed within a week.

2.4.2 Bacterial and Fungal Detection

Membrane filtration was employed for bacteria identification and culture detection for fungal identification. Membrane filtration allowed for speedy bacterial aggregation, while the culture detection method was used for fungal detection [21].

2.4.3 Mycoplasma Analysis

The hUCMSCs were cultivated in tiny culture flasks, smears were prepared and examined for mycoplasma using DNA fluorescent labelling [22]. The process involved staining with a fluorescent dye, washing, and observation under a fluorescence microscope.

2.4.4 Detection of Other Toxin Markers

Using the Limulus reagent gel method for endotoxin testing, the presence or absence of retroviruses through reverse transcriptase activity, infectivity testing, and electron microscope inspection were determined [23]. Various methods, including colloidal gold, fluorescent antibody, and fluorescence quantification PCR, were used to investigate human viruses. The Western blot method was employed to detect residues (trypsin).

2.5 Immunoregulatory Assays

The carboxyfluorescein succinimidyl ester (CFSE) dyeing method for fluorescence-labelled flow cytometry was used to detect lymphocyte proliferation [24]. CFSE dye allowed for the evaluation of proliferation inhibition by monitoring fluorescence intensity drop during cell division.

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2.5.1 Flow Cytometry Detection of Lymphocyte Subpopulations

The present study employed flow cytometry to identify and quantify specific lymphocyte subsets, namely TH1 (T-helper 1), TH17 (T-helper 17), and Treg (regulatory T cells). Flow cytometry allows for the precise identification and quantification of distinct lymphocyte subpopulations based on the expression of specific cell surface markers associated with TH1, TH17, and Treg cells [25]. The utilization of flow cytometry in this context provides valuable insights into the immune profile of the studied samples, contributing to a comprehensive understanding of the immune response and regulatory mechanisms involved in the immune system.

2.5.2 ELISA for Lymphokine Testing

Applied Enzyme-Linked Immunosorbent Assay (ELISA) technology was used to analyse lymphokines in a distinct immunoassay, providing insights into lymphokine levels present in the samples, and offering a quantitative assessment of these signalling molecules. By employing ELISA technology, the study aimed to elucidate and quantify the presence of lymphokines, contributing valuable information to the understanding of immune responses and related biological processes.

2.6 Statistical analysis

All data were expressed as mean ± standard deviation (SD). Significance differences were calculated using one-way ANOVA, and $p < 0.05$ was considered statistically significant.

3.0 Results

3.1 The Biological Characteristics and Identification of hUCMSCs

3.1.1 Morphological Characteristics of hUCMSCs

As depicted in **Figure 1**, hUCMSCs at passage 2 were observed under an inverted microscope at a magnification of 100 times (x100), displaying long spindle shapes with high refraction and organized in a conventional spiral form (**Figure 1A**). **Figure 1B** represents the first 3 days post-passage, showcasing classic adherent growth features. The identified morphological properties align with the normal cell morphology of human mesenchymal stem cells.

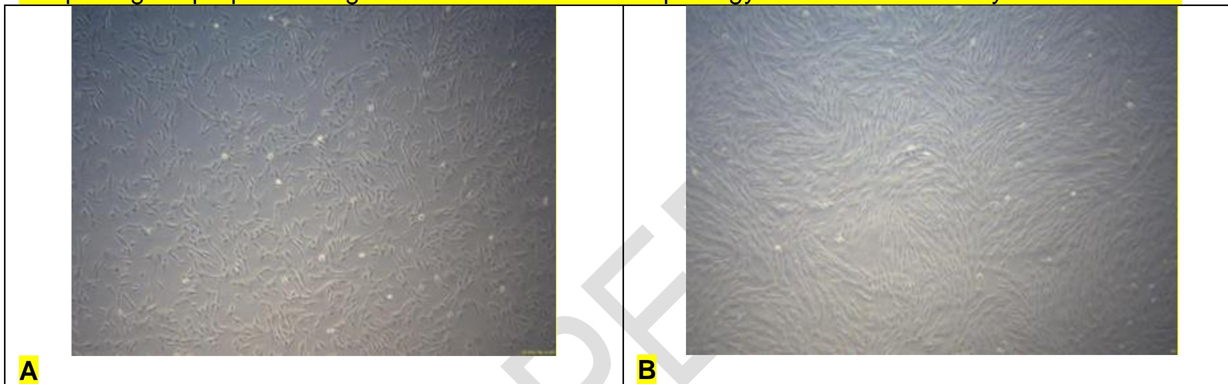

Figure 1A-B. The morphology of hUCMSCs under an inverted microscope (**A**. Day 2, **B** Day 3, all magnifications x100)

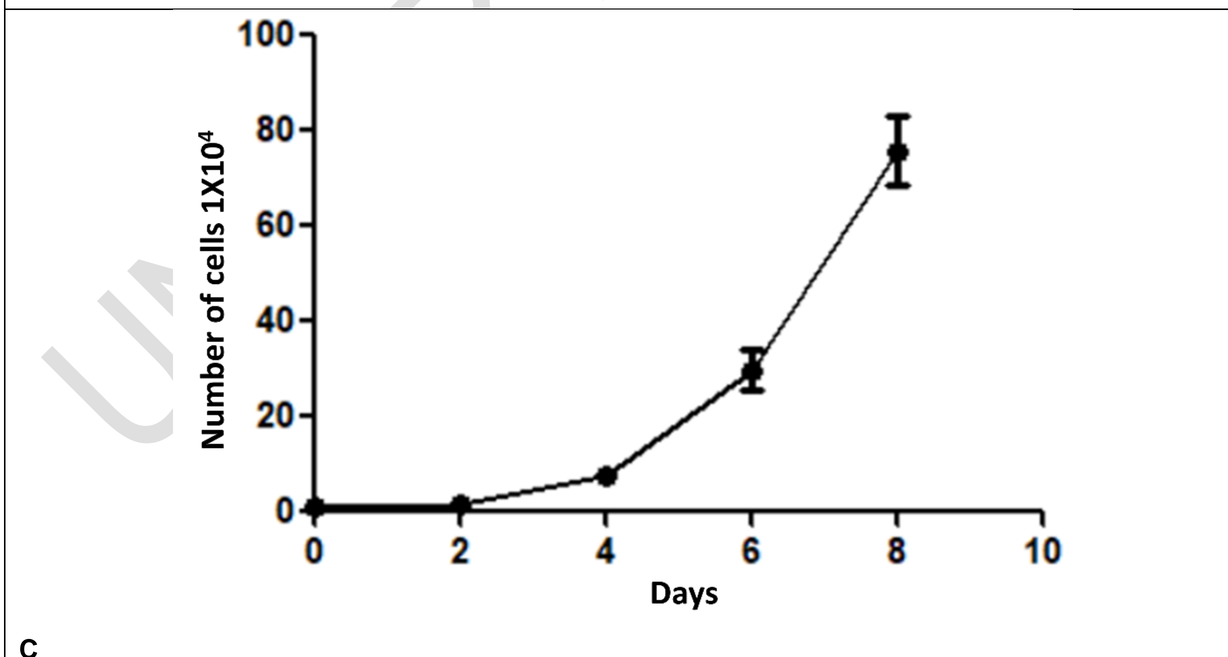

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Figure 1C. Cell growth curve of hUCMSCs
The viability of P5 hUCMSCs was assessed by trypsin digesting and harvesting them during the logarithmic growth phase (exceeding 70% confluence). Resuspended in DPBS, a portion was subjected to trypan blue staining, and an automatic cell counter (Nexcelom Cellometer Mini) was employed for cell detection. The observed cell viability was 99.2%. The results are given in **Table 1**.

3.1.2 Cell Growth Curve and Cell Proliferation Doubling Time

3.1.3 Cell Viability of hUCMSCs

The hUCMSCs were cultured in a 6-well plate as described in the methodology section. Daily, cells were extracted from each well, and their total count was determined, forming a growth curve for hUCMSCs. The in vitro-produced hUCMSCs were counted, revealing a doubling time of 24.5 hours, which is consistent with the proliferation rate of typical human mesenchymal stem cells [26]. The viability is shown in **Table 1** and **Figure 1C**.

Table 1. Counting and survival rate detection results of hUCMSCs before and after cryopreservation

Pre cryopreservation							
Sample	**Count Type**	**Cell Number**	**Dilution Factor**	**Concentration (Cells/ML)**	**Result Type**	**Result**	**Average Value (μm)**
P5 hUCMSCs	Number of living cells	496	2	2956000	Living cells	99.2%	17.9
P5 hUCMSCs	Dead cells	4	2	24000	dead cells	0.8%	11.6
P5 hUCMSCs	Total cell number	500	2	2980000	total cells	100%	17.7
Post-cryopreservation and revival							
P5 hUCMSCs	Number of living cells	401	2	2384000	Living cells	92.6%	17.9
P5 hUCMSCs	Dead cells	32	2	196000	dead cells	7.4%	11.6
P5 hUCMSCs	Total cell number	433	2	2580000	total cells	100%	17.7

The average value column in **Table 1** represents the mean value of cells in diameter.

3.1.4 Cell Cycle Detection and Analysis of hUCMSCs

The 5th generation hUCMSCs were cultivated until reaching the logarithmic growth phase with a confluency range of 70% to 90%. Subsequently, the cells were frozen, and stained, and the fluorescence intensity of the staining was evaluated using flow cytometry. The cell cycle was analyzed by assessing DNA content with ModFit LT4.1, revealing that 82.74% of hUCMSCs were in the G0/G1 phase, as depicted in **Figure 2A**.

3.1.5 Detection of Cell Phenotype and Surface Characteristic Markers

hUCMSCs were subjected to flow cytometry analysis to examine and identify cell phenotype and surface marker antigen molecules, including CD73, CD90, CD105, CD34, CD45, CD14, and HLA-DR. **Figure 2B** showed that the hUCMSCs employed in this test had a positive expression rate mean greater than 98% for CD73, CD90, and CD105, while the positive expression rate for CD34, CD14, CD45, CD19, and HLA-DR was ≦0.2%. These outcomes are altogether consistent with previous research [27]. Positive expression was determined to be ≥95% for CD73, CD90, and CD105, and ≤2% for CD34, CD45, CD14, CD19, and HLA-DR. The results are given in **Figure 2B**.

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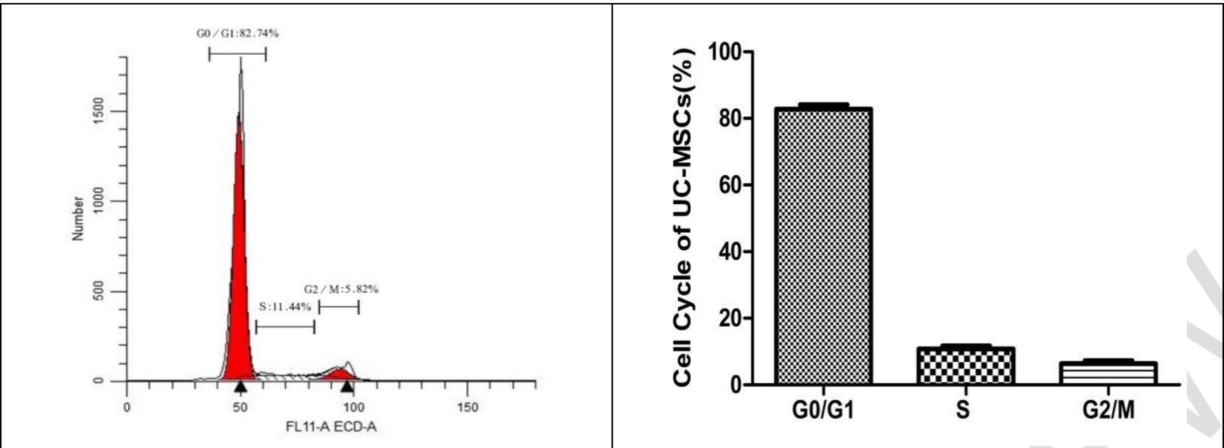
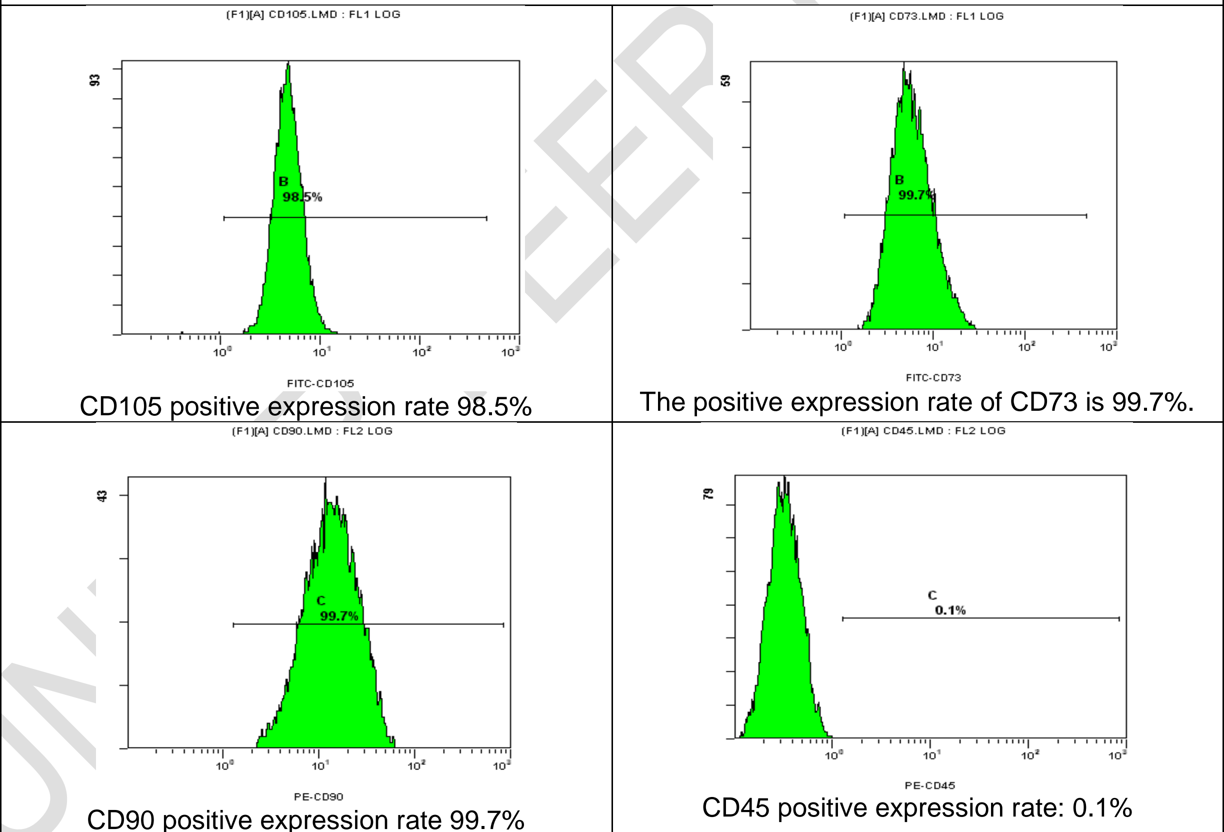


Figure 2A. Results of cell cycle flow cytometry detection and analysis of hUCMSCs reveals that 82.74% of hUCMSCs were in the G0/G1 phase



CD105 positive expression rate 98.5%

The positive expression rate of CD73 is 99.7%.

CD90 positive expression rate 99.7%

CD45 positive expression rate: 0.1%

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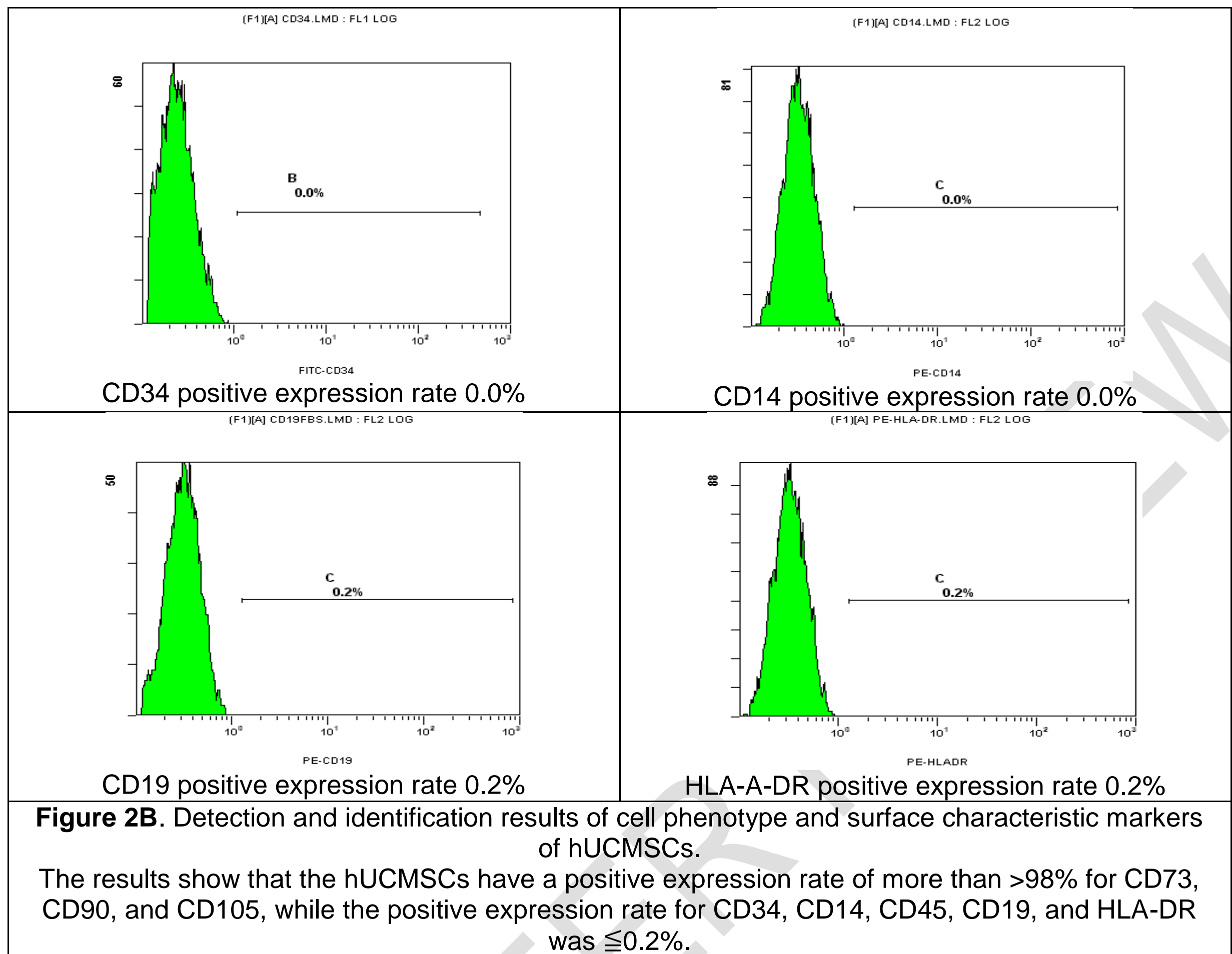

Figure 2B. Detection and identification results of cell phenotype and surface characteristic markers of hUCMSCs.
The results show that the hUCMSCs have a positive expression rate of more than >98% for CD73, CD90, and CD105, while the positive expression rate for CD34, CD14, CD45, CD19, and HLA-DR was ≦0.2%.

3.1.6hUCMSCs In vitro Cell Differentiation Ability

The hUCMSCs can differentiate into adipocytes, osteoblasts, and chondrocytes. In the present study, this differentiation ability was confirmed through staining methods (Oil Red, Alizarin Red, and Alcian blue) (**Figure 3A)**.

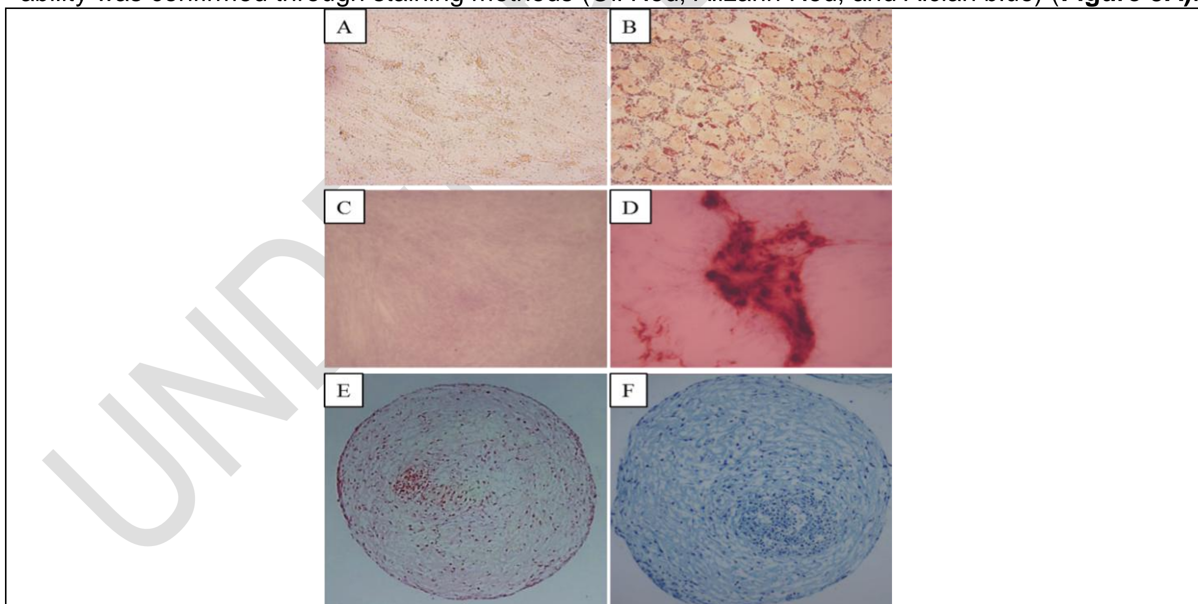

Figure 3A. Three staining results of hUCMSCs multilineage differentiation potential
The first row of pictures is oil red of adipogenic differentiation dyeing: (**A**) Negative control; (**B**) Intracellular red lipid droplets show adipogenic differentiation. The second row of pictures is alizarin red staining of osteogenic differentiation; (**C**) Negative control; (**D**) Red calcium deposits show osteoblastic differentiation. The third row of images shows characteristic staining for chondrogenic

differentiation; (**E**) Negative control; (**F**) Alcian blue staining showed that the microspheres showed a cartilage-like structure surrounded by a large amount of cartilage matrix

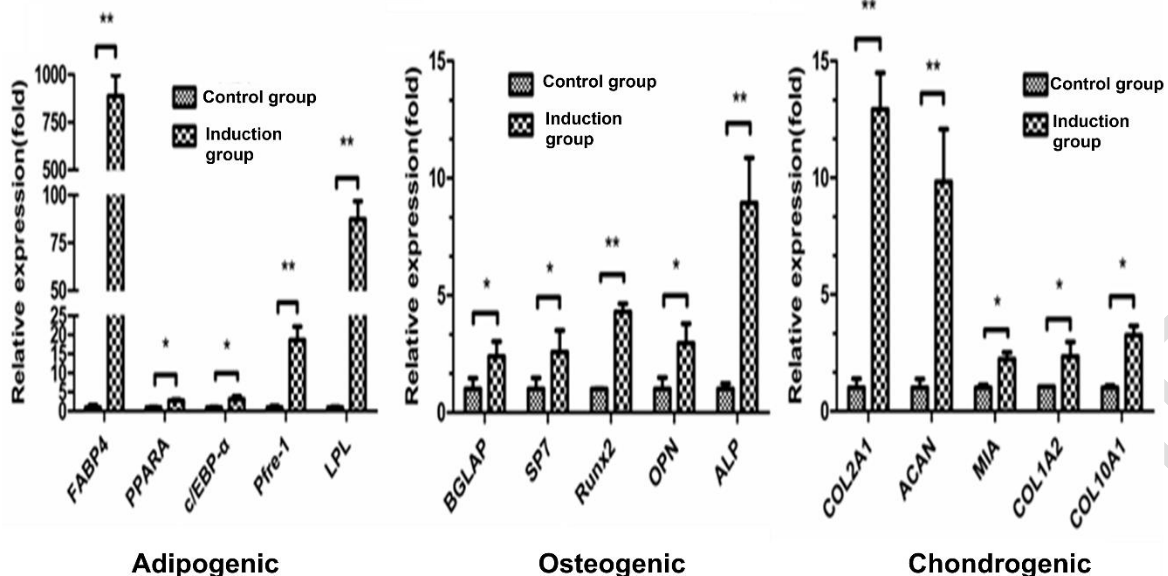

Figure 3B. Results of detection of adipogenic, osteogenic and chondrogenic differentiation potentials of hUCMSCs.
After inducing and differentiating hUCMSCs in vitro in adipogenic, osteogenic, and chondrogenic differentiation systems. The expression levels of adipogenic, osteogenic, and chondrogenic genes were found to be significantly higher than in the control group. Statistically significant differences were observed (*: P<0.05) and highly significant differences (**: P<0.01).

3.1.7 The In Vitro Identification of hUCMSCs Characteristic Genes Related to The Ability to Induce Differentiation

Fluorescence quantification PCR was employed to assess the expression of specific genes associated with cell differentiation types in induced hUCMSCs. FABP4 for adipogenic differentiation, Runx2 for osteogenic differentiation, and COL2A1 for chondrogenic differentiation were analyzed (**Figure 3B**). The results indicated that, compared to the control group, the expression levels of all detected characteristic genes related to cell differentiation types were significantly higher after culturing hUCMSCs in adipogenic, osteogenic, and chondrogenic induction systems (P<0.05).

3.1.8 Karyotype Analysis and Identification

In this study, hUCMSCs underwent cell passage and purification and randomly selected 1000 metaphase cells were evaluated for chromosomal number, morphology, and structure, while an additional 500 metaphase cells were examined for polyploidy. At least 50 photomicrographs of metaphase cells were taken for karyotype analysis. Results indicated that the number of chromosomally atypical cells in the hUCMSCs sample was low (**Table 2**), affirming the genetic stability of hUCMSCs [28].

Table 2. Chromosome count analysis of hUCMSCs (1000 metaphase cells)

Chromosomal Analysis	**The Standard Limit of The Number of Chromosomally Abnormal Cells**	**Detected Number of Abnormal Chromosomal Cells in The Sample**
Chromatids and chromosome breaks	47	17
Abnormal Structure	17	7
Hyperdiploid	8	6
Hypodiploid	180	95
Polyploid	30	10
Crude Count of 500 Metaphase Cells		

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Polyploid	17	10

Note: More than one metaphase cell 53 chromosomes are a polyploid.

The G-banding technique was used to analyze the karyotypes of hUCMSCs. This involves staining chromosomes with G-band dye, followed by microscopic examination and CCD imaging for karyotype analysis (**Figure 4**). Analytical results demonstrate no aberrant changes in the chromosomal structure of hUCMSCs, maintaining a normal diploid karyotype consistent with typical hUCMSCs [29].

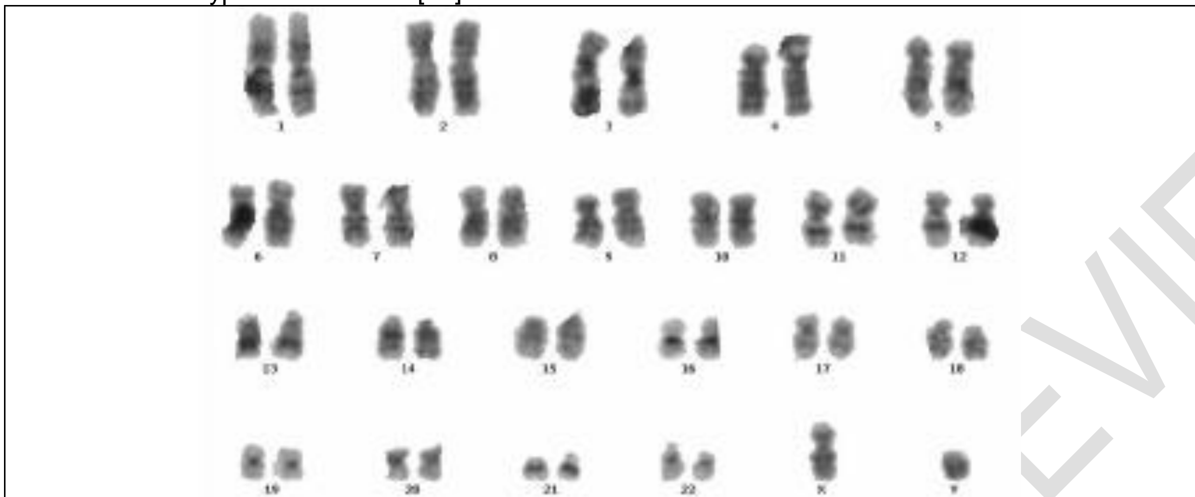

Figure 4A. The standard karyotype of the hUCMSCs passage cell line of the test product

3.1.9 STR Analysis and Cell DNA Fingerprint Identification

To analyze the chromosomal DNA of hUCMSCs, restriction endonucleases were employed to digest the DNA, leading to the separation of DNA fragments with varying sizes. Subsequently, the repeat sequence was hybridized with a nucleic acid probe based on the consensus sequence. This process allowed us to obtain band patterns of DNA fragments specific to hUCMSCs (**Table 3**).

Table 3. Genotyping results of STR loci and Amelogenin loci of hUCMSCs

STR Analysis and cells DNA fingerprint identification Used hUCMSCs The test results		
Marker	**Allele 1**	**Allele 2**
D19S433	14	16.2
D5S818	11	12
D21S11	31.2	32.2
D18S51	13	15
D6S1043	18	18
AMEL	x	x
D3S1358	16	16
D13S317	9	11
D7S820	11	12
D16S539	9	11
CSF1PO	10	11
Penta D	9	12

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D2S441	10	11
wxya	14	18
D8S1179	11	12
TPOX	10	11
Penta E	11	17
TH01	7	9
D12S391	19	20
D2S1338	19	23
FGA	20	24

Short Tandem Repeat gene sequences (STRs), also known as microsatellites, are repetitive DNA sequences present in eukaryotic genomes [30]. In this study, DNA was extracted and 20 STR loci along with sex identification loci were amplified using the Microreader TM21 ID System. The amplified products undergo analysis with the ABI 3730xl genetic analyser, and the results are assessed using GeneMapper3.2 software. These outcomes were then compared to databases (ATCC and DSMZ) to determine the species origin of the cells and identify any cross-contamination. Genotyping results of hUCMSCs at various STR loci, including the Amelogenin locus, reveal no tri-allelic phenomenon, contamination, or cross-contamination with other human cell types [31].

3.1.10 hUCMSCs Gene Stability

The genetic stability of hUCMSCs was evaluated by assessing both cell chromosome microarray detection and chromosome karyotype detection simultaneously. In the genome-wide scan study, hUCMSCs exhibited no clinically significant gene copy number deletions, duplications, or extensive regions of homozygosity, ensuring gene stability [32]. Chromosome karyotype analysis further confirmed a normal diploid karyotype (46, XY) for the examined hUCMSCs, free from chromosome abnormalities.

3.2 Quality Analysis

3.2.1 Bacteria, Fungi and Mycobacteria Analysis

To ensure the quality and safety of hUCMSCs products, rigorous testing was conducted. Distinct tests were carried out to identify bacteria, fungi, and mycobacteria in hUCMSCs.

For Bacterial and Fungal Detection in hUCMSCs, the membrane filtering method was utilized with both negative and positive control groups established. For mycobacteria, the Middlebrook 7H10 Plate culture method was employed. **Table 4** presents the results of the bacteria, fungi, and mycobacteria examinations for hUCMSCs, affirming the safety (<0.5 EU) and quality status of the product.

Table 4. Bacterial, fungal and mycobacteria examination results in hUCMSCs injection

Test sample	**Media for Bacteria and Fungi Examination**	**Culture temperature (℃)**	**Culture time (days)**	**Test result**
hUCMSCs	Thioglycollate medium	25	14	Negative
		35	14	Negative
	Tryptone soy broth	25	14	Negative
Negative control	Thioglycollate medium	35	14	Negative
	Tryptone soy broth	25	14	Negative
Positive control *Staphylococcus aureus* (CMCC (B) 26003)	Thioglycollate fluid medium	35	14	Positive
	Tryptone soy broth	25	14	Positive

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Mycobacteria Analysis			
Test sample	**Culture temperature (℃)**	**Culture time (days)**	**Result**
hUCMSCs	37	59	Negative
Negative control	37	59	Negative
Positive control *Mycobacterium phlei* (CGMCC4.1180)	37	59	Positive

3.2.2 Mycoplasma Analysis
A comprehensive test for mycoplasma contamination was conducted. This examination employed two methods: cultivation and DNA fluorescence staining. **Tables 5-6** present the results of the mycoplasma test for hUCMSCs, providing a comprehensive assessment of the product's quality and safety.

Table 5. Mycoplasma detection results in hUCMSCs injection

Sample	Cultivation type	Medium type	Culture temperature (℃)	Observation time (days)	Result
hUCMSCs	Primary culture	solid medium	37	21	Negative
		liquid medium		21	Negative
	Subculture	solid medium		21	Negative
		liquid medium		21	Negative

Table 6. hUCMSCs of DNA Fluorescent staining test results

Samples	Culture Time	Result
hUCMSCs	7 days	Negative
negative control	7 days	Negative
positive control	3 days	Positive

3.2.3 Bacterial Endotoxin Analysis
The LAL (Limulus Amebocyte Lysate) test method was employed to identify bacterial endotoxin [33]. The inspection findings indicated negative results (<0.5 EU) for all bacterial endotoxin tests in the hUCMSCs (**Table 7**).

Table 7. hUCMSCs endotoxin test results

Serial Number	Cells	Limulus Reagent Method	
		observation time	**Result**
1	hUCMSCs	60minute	Negative

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2	negative control	60minute	Negative
3	positive control	60minute	Positive

3.2.4 Virus Contamination Analysis
The present study assessed the internal and external viral contamination in hUCMSCs using two methods: cell morphology observation and hemadsorption test. The results obtained are presented in **Table 8**.

Table 8. Observation results of cell culture morphology of hUCMSCs

Samples	**Culture Time (Days)**	**Result**
hUCMSCs	15	normal cell morphology
Positive control (influenza virus)	2-5	CPE
Note: The CPE Indicates abnormal cell morphology and cell pathological changes.		
Cell culture hemadsorption test results of hUCMSCs		
Test Sample	**0.2%~0.5%Adsorption time of chicken and guinea pig erythrocyte suspension (minutes)**	**Result**
hUCMSCs	30	Negative
Positive control (influenza virus)	30	Positive

Furthermore, the in vivo inoculation method of chicken embryos was used for the inspection of internal and external viral contamination in hUCMSCs [34]. **Table 9** depicts the findings of this study.

Table 9. Inspection results of chicken embryos inoculated with hUCMSCs

Age of embryo (Days)	**No. of inoculated chicken embryos**	**Inoculation Site**	**Sample Inoculation Volume (mL/Pieces)**	**Observation Days**	**Survival Rate**
9~11	10pieces	Allantoic cavity	0.2	4	≥80% (Negative hemagglutination test)
5~7	10pieces	Yolk sac	0.5	5	≥80%

Additionally, a retrovirus test was performed using the reverse transcriptase activity assay (TM-PERT technique), infectivity test, and a visual examination with a transmission electron microscope. The positive control used was mouse myeloma cell Sp2/0-Ag14, and the negative control was the PG-4 (S+L-) cell. **Table 10** displays the findings.

Table 10. Determination results of reverse transcriptase activity of hUCMSCs

Samples	**SQ value (pU/ml)**
Standard 1	1.0×10^{12}

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Standard 2	1.0×10^{11}
Standard 3	1.0×10^{10}
Standard 4	1.0×10^{9}
Standard 5	1.0×10^{8}
Standard 6	1.0×10^{7}
Sp2/0-Ag14cell	1.68×10^{10}
hUCMSCs	—
PG-4(S+L-)cell	—
FP/RTP system control	—

Note: "—" is quantitative PCR test negative
No virus-like particles were found in the hUCMSCs culture similar to that of the negative control (PG-4(S+L-)cell), however, virus-like particles were observed in the positive control (Sp2/0-Ag14cell).

3.2.5 Analysis of Several Important Human Infectious Viruses

The analysis of significant human infectious viruses was conducted for several important human infectious viruses (HAV, HBV, HCV, CMV, HIV, and EBV) [35]. The data showed that all detection results of numerous key human infectious viruses in the hUCMSCs employed in this investigation were negative. **Table 11** displays the test results.

Table 11. Results of immunofluorescence antibody detection of hUCMSCs

Serial Number	Detect Virus Type	Test Sample Indicator Cells	Positive Control	Negative Control	Test Sample
1	Hepatitis A virus (HAV)	Huh7	/	Negative	Negative
2	Hepatitis B virus (HBV)	Huh7	Positive	Negative	Negative
3	Hepatitis C virus (HCV)	Huh7	Positive	Negative	Negative
4	human cytomegalovirus (CMV)	2BS	/	Negative	Negative
5	human immunodeficiency virus (HIV)	Jurkat	/	Negative	Negative
6	Epstein-Barr virus	293	Positive	Negative	Negative

The testing for the human papillomavirus (HPV) in hUCMSCs was done using a highly specific and sensitive fluorescent PCR method. All the hUCMSCs tested negative for 18 high-risk HPV strains (**Figure 5).**

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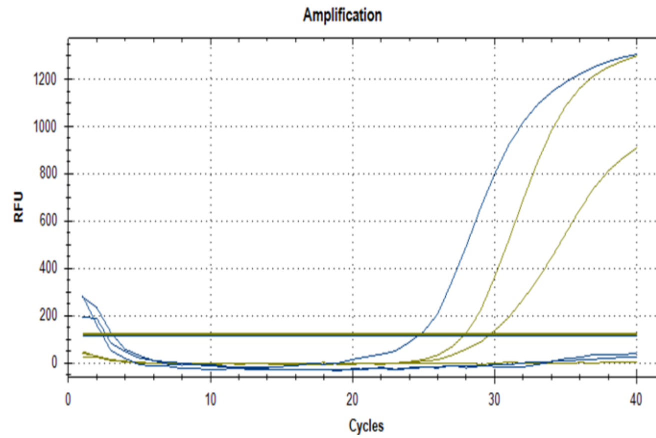

Figure 5. Human papillomavirus amplification curve

3.3 Analysis of Immune Response of hUCMSCs

3.3.1 The Proliferation Ability of Human Total Lymphocytes

hUCMSCs have been shown to have immunological regulatory roles [36]. A test was performed using the CFSE marker on PBMCs treated with mitomycin C and co-cultured with hUCMSCs to evaluate the immunomodulatory effect of hUCMSCs on the proliferative potential of human total lymphocytes. Flow cytometry was used to determine lymphocyte proliferation. The results showed that hUCMSCs suppressed the proliferation of human lymphocytes induced by PHA (phytohemagglutinin) considerably (P <0.001) (**Figure 6A).**

Figure 6A. Inhibition experiment of hUCMSCs on lymphocyte proliferation
Note: hUCMSCs were treated with CFSE-labeled PBMC mitomycin **C**. Flow cytometry was utilized to detect lymphocyte proliferation after co-culture. **A.** PBMCs Blank control group; **B**. PBMC+hUCMSCs co-cultivation, no PHA Stimulate; **C**. PBMC+PHA positive control group; **D**. PBMC+ hUCMSCs +PHA; e. lymphocyte proliferation index (PI) Compare. The cell proliferation index of each group was calculated using ModFit, and hUCMSCs stimulated lymphocyte proliferation was considerably inhibited (**P<0.001).

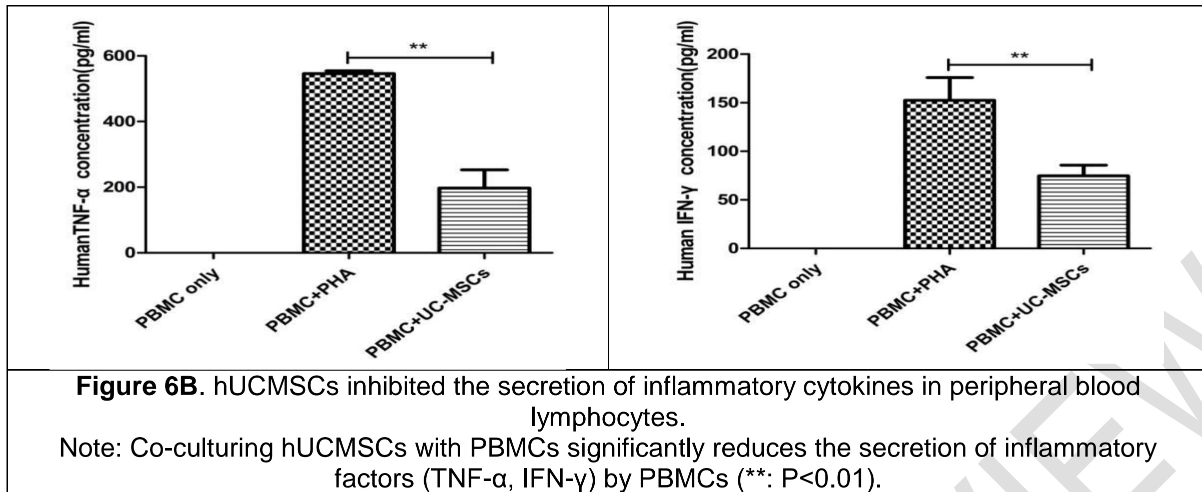

Figure 6B. hUCMSCs inhibited the secretion of inflammatory cytokines in peripheral blood lymphocytes.
Note: Co-culturing hUCMSCs with PBMCs significantly reduces the secretion of inflammatory factors (TNF-α, IFN-γ) by PBMCs (**: P<0.01).

3.3.2 The Regulation of T Cell Subsets

The ability of hUCMSCs to reduce the secretion of pro-inflammatory factors is a crucial aspect of their immune modulation [37]. In an in vitro setting, hUCMSCs were co-cultured with human PBMCs, and the impact on lymphocyte secretion of pro-inflammatory factors TNF-α and IFN-γ was assessed using ELISA. The findings indicated a significant reduction in TNF-α and IFN-γ production by PBMCs in the presence of hUCMSCs (P<0.01) (**Figure 6B**).

3.4 Trypsin Residues Analysis

The Coomassie brilliant blue detection method and the western blot method were employed to evaluate trypsin residue presence in the hUCMSCs culture media and other components. The absence of trypsin residue in the frozen cell supernatant indicated adherence to the required standards. Additionally, trypsin testing for porcine parvovirus in routinely used trypsin for in vitro culture and passage of hUCMSCs yielded negative results (**Figure 7**).

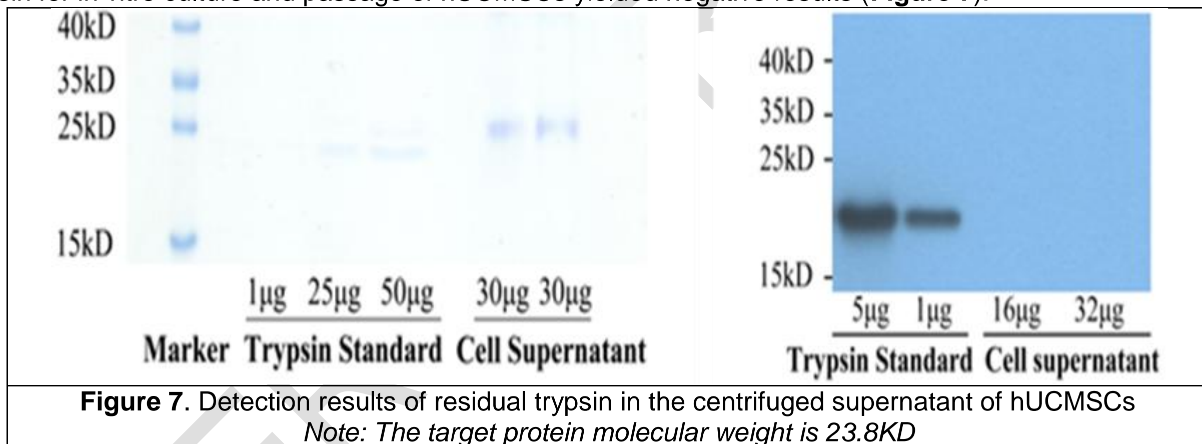

Figure 7. Detection results of residual trypsin in the centrifuged supernatant of hUCMSCs
Note: The target protein molecular weight is 23.8KD

4.0 Discussion

The generation of hUCMSCs involves extracting cells from the umbilical cord tissue of healthy newborns, particularly rich in Wharton's jelly. Theoretically, 1g of umbilical cord tissue should yield $1x10^7$ nucleated cells and $1x10^6$ hUCMSCs [38], however, current preparation technologies fall short. Improving these methods is crucial to enhance standardization and large-scale production of hUCMSCs for clinical applications.

In the past years, two main procedures have been employed for hUCMSCs extraction: the tissue block attachment approach, which yields low-purity hUCMSCs and poses challenges for large-scale manufacture, and the enzymatic digestion and isolation approach, which employs digestive enzymes to break down tissue, reducing culture time and increasing output and purity [39].

Achieving optimal separation effects in the umbilical cord enzyme digestion and separation procedure hinges on identifying suitable digestive enzymes with precise action duration and dosage. The present study examined cell morphology which is essential for the identification of hUCMSCs. Additionally, a cell growth curve was established which serves as a crucial method for assessing the absolute growth number, vitality, and biological properties of hUCMSCs during in vitro cultivation [40]. The findings of the present study highlighted that hUCMSCs exhibit a robust amplification ability, as evidenced by the cell growth curve and cell proliferation doubling time.

The cell survival rate is crucial for assessing the viability of these cells, the present study determined the percentage of living cells among the total examined using the Trypan blue staining method. Cell death in hUCMSCs can result from processes such as isolation from umbilical cord tissue, cell culture, digestion, collection, freezing, and revival. Assessing

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the cell survival rate is essential to identify any potential damage incurred during isolation and application. The present study measured the cell survival rate of hUCMSCs, revealing a survival rate of ≥95% before cryopreservation and ≥85% after recovery. These results agree with previously conducted research [41].
Cell cycle is a crucial aspect of understanding the biological traits of hUCMSCs. Analyzing the cell cycle of hUCMSCs, which consists of G1 (pre-DNA synthesis), S (DNA synthesis), G2 (post-DNA synthesis), and M (cell division) phases, provides valuable insights into their biological activity. Molecular changes characterize each of these phases, and detecting the cell cycle is essential for evaluating hUCMSCs' viability [42]. The present study employed flow cytometry to analyze the cell cycle of hUCMSCs, revealing a G0/G1 ratio of 75-85%.
The hUCMSCs represent a specific type of MSCs and their distinctive cell functions are closely tied to surface markers defining their fundamental characteristics [43]. In this study, flow cytometry was utilized to assess the cell phenotypic and surface markers of hUCMSCs. The results demonstrated the expression of positive markers (CD73, CD90, and CD105) with mean positive rates exceeding 98%. Conversely, negative markers (CD34, CD45, CD11b, CD19, and HLA-DR) exhibited very low or no expression, with positive rates less than or equal to 0.2%. Similar findings were made by Fonseca et al., 2023.
Chromosome karyotype analysis is a method used to examine metaphase chromosomes, involving the analysis and categorization of chromosomes based on various properties. [44]. In this study, chromosomal analysis, and identification of hUCMSCs revealed a stained karyotype consistent with 46, XX for females and 46, XY for males. The examination indicated a low count of chromosomally abnormal cells and no absence of clinically significant gene copy number deletions, duplications, or major homozygous events in MSCs [45].
Short Tandem Repeat (STR) gene loci serve as highly polymorphic markers found throughout the human genome [46]. They are essential for identifying human-derived stem cell lines, acting as unique cellular fingerprints [47]. The present study employed this analysis to identify the STR spectrum, confirming that the hUCMSCs cell line originated from a single source [48].
Chromosomal microarray analysis, a genome-wide screening technique was employed to identify chromosomal abnormalities in cells [49]. The findings of this study revealed no clinically relevant abnormalities such as gene copy number loss, duplication, or high homozygosity were detected in the chromosomes of hUCMSCs [50].
Evaluating the cells' multilineage differentiation capacity, specifically their ability to differentiate into osteocytes, adipocytes, and chondrocytes is of significant importance [51]. Using fluorescence-based PCR and differentiation kit procedures to assess the differentiation ability of hUCMSCs, the results demonstrated that hUCMSCs can indeed differentiate into adipogenic, osteogenic, and chondrogenic lineages, confirming the multipotent nature of hUCMSCs as noted in the previous study [52].
Microbial contamination in clinical injection medications can lead to allergies, infections, poisoning, and other complications [53]. A comprehensive test analysis was conducted to detect exogenous and associated viral agents, including bacteria, fungi, mycobacteria, mycoplasma, bacterial endotoxins, endogenous viral factors, retroviruses, and specific human viruses. The findings of this study demonstrated the absence of all the above-mentioned endotoxins ≤0.5 EU/ml [54], and no presence of trypsin porcine parvovirus. Retrovirus, endogenous viral factors, and specific human viruses (TP, HIV, HAV, HBV, HCV, HCMV, EBV, HPV) were also not detected. Additionally, negative findings were observed for 18 high-risk HPV. These comprehensive tests affirm the sterility and safety of the extracted hUCMSCs.
Immunological tests were conducted on hUCMSCs, specifically employing the lymphocyte proliferation inhibition assay, specific lymphocyte subsets (TH1/TH17/Treg) modulation test, and lymphokine detection test [55]. The results indicated a significant inhibition of lymphocyte proliferation by the hUCMSCs. The modulation of specific lymphocyte subsets (TH1/TH17/Treg) demonstrated that there is an increased proportion of Tregs in lymphocytes while decreasing the ratio of TH1 to TH17, suggesting a regulatory effect on immune responses [56]. Furthermore, the ELISA technique was used to assess lymphokine secretion. The outcomes revealed that the hUCMSC preparation possesses the ability to suppress the expression and secretion of TNF-α and IFN-γ, indicating an immunomodulatory capacity [57].
Besides, the analyses of trypsin levels in hUCMSC were conducted to determine the residual levels of additional added substances and the results were negative. Additional methods such as the cell culture system, immunofluorescence method, hemagglutination test, and aniline dye staining method were employed to determine the presence of porcine parvovirus. The results indicated the absence of porcine parvovirus in the trypsin preparation [58].

5.0 Conclusion

In the comprehensive examination of hUCMSCs, various assessments and analyses were conducted to elucidate their fundamental biological characteristics. The assessments covered a range of factors, including cell viability and growth activity assays, cell cycle detection, cell phenotype and surface characteristic marker detection, cell karyotype detection, cell STR and DNA fingerprint analysis, chromosomal microarray analysis, in vitro cell differentiation ability analysis, quality control analysis, as well as adhesive growth and morphological observation. The combined results from these analyses led to the conclusion that the hUCMSCs utilized in the experiment originated from a single source and exhibited the expected basic biological traits and qualities. This comprehensive evaluation provides a thorough understanding of the biological properties of hUCMSCs, ensuring their suitability for various scientific and clinical applications in line with established standards and guidelines.

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Ethical approval and consent to participate
Before sample collection, the parent(s) of the donor were provided with informed consent, clearly understanding the purpose, procedures, and potential implications of the donation. The consent process ensures transparency and respect for the autonomy of the donor's parent.
Consent for publication
Not applicable.

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