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Comparison of Animal Serum-Free Media Culture for cGMP-Compliant Mesenchymal Stem Cell Expansion

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) are multipotent stromal cells distinguished by their ability to self-renew, their potential for multilineage differentiation, and their strong immunomodulatory characteristics. The recent in vitro expansion of MSCs primarily relies on culture media that is supplemented with fetal bovine serum (FBS). Although FBS is effective in enhancing the proliferation of MSCs and ensuring their survival, its use poses several notable challenges, particularly in clinical environments. **Methods:** This study seeks to fill this important gap by conducting a comprehensive comparison of various commercially available animal source-serum free media formulations against traditional FBS-supplemented media for the expansion of umbilical cord-derived mesenchymal stem cells (UC-MSCs). The morphology and density of the cells were examined using an inverted microscope. The cell surface of UC-MSCs was analysed through flow cytometry. The evaluation of cytokines released by UC-MSCs was carried out using ELISA. **Result:** The conditioned medium obtained from UC-MSCs cultured in HPL shows increased levels of exosomes and anti-inflammatory cytokines. Moreover, the UC-MSCs cultured in HPL-supplemented medium maintained a normal morphology and exhibited expression of UC-MSC surface markers exceeding 95%. Additionally, HPL enhanced the proliferation of UC-MSCs to eight times the cell number on the day of seeding. **Conclusion:** Media enriched with HPL presents considerable potential for future applications within the pharmaceutical sector.

Keywords: UC-MSCs, culture medium, animal source-serum free, pharmaceutical industry.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells characterized by self-renewal capacity, multilineage differentiation potential, and potent immunomodulatory properties[1]. These unique attributes have positioned MSCs as a highly promising therapeutic tool in regenerative medicine for a wide range of degenerative diseases, including autoimmune diseases, tissue repair, and organ transplantation[2]. However, the implementation of MSC-based therapies from the laboratory to clinical practice requires the large-scale production of clinically compliant and functionally potent cells[3].

The recent in vitro expansion of mesenchymal stem cells (MSCs) is largely dependent on culture media that is enriched with fetal bovine serum (FBS)[1,4]. While FBS is effective in promoting the proliferation of MSCs and ensuring their viability, its application presents several significant

challenges, especially in clinical settings. Issues related to xenogeneic immune responses in patients, the potential for zoonotic pathogen transmission, and the variability in FBS composition between production batches create substantial barriers to regulatory approval and therapeutic consistency[4,5]. These challenges have driven extensive research into alternative culture systems that either eliminate or greatly minimize the dependence on animal-derived components.

Animal serum-free media (ASFMs) have become a prominent solution to tackle the issues related to FBS. These formulations, which are chemically defined or xeno-free, are designed to offer a safer, more reliable, and scalable platform for the expansion of MSCs, thus aiding in their clinical application[6,8]. Nevertheless, the extensive range of commercially available SFMs and the differing responses of MSCs derived from various tissue sources (such as bone marrow, adipose tissue, and umbilical cord) to these media formulations highlight the necessity for systematic comparative studies. Further research is still needed to identify the optimal SFM that ensures robust expansion while preserving MSC potency and safety. This study aims to address this critical gap by performing an extensive comparison of different commercially available serum-free media formulations against traditional FBS-supplemented media for the expansion of umbilical cord-derived mesenchymal stem cells (UC-MSCs). The evaluation of key parameters includes morphology, surface markers, and cytokine secretion of UC-MSCs.

MATERIALS AND METHODS

Study Design

UC-MSCs were cultured in 6-well plates at a seeding density of approximately 5×10^4 cells per well, using complete culture medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 until they reached 70–90% confluency.

Culture of UC-MSCs

Four cryotubes of UC-MSCs passage 6 were thawed and cultured in four different flasks. Each culture flask was supplemented with a distinct type of serum, specifically Fetal Bovine Serum (FBS), Human Platelet Lysate (HPL) (PLTGold Millcreek Life Sciences, Rochester, USA), CellCor™ MSC CD AOF (XCELL, Korea), and LONZA MSCBM™ (ThermoFisher, USA), respectively. Subsequently, UC-MSCs were incubated at 5% CO_2 and 37°C for 5 days. The culture medium was changed on the third day. Cell morphology and confluence were observed after 24 and 120 hours after thawing. After reaching 85% confluence, the UC-MSCs conditioned medium was collected, and cells were harvested with TrypLe (Gibco, USA). The cell number was counted using Bio-Rad TC20 automated cell counter (Bio-Rad, USA).

Flow Cytometric Assay Validates UC-MSC Surface Markers

The surface marker expression of UC-MSCs cultured in FBS containing media (FBS group) and UC-MSCs cultured in SFMs (SFMs group) were analysed using a flow cytometer (BD FACSLyrics, USA). Monoclonal antibodies against CD14- phycoerythrin (PE, BD Biosciences, San Diego, CA, USA), CD34-PE, CD45-PE, CD73-PE, CD90-PE, CD105-PE (PE, BD Biosciences, San Diego, USA), HLA-DR-PE (PE, BD Biosciences, San Diego, USA) as manufacturer protocols. In

addition, the exosome level within the conditioned medium was also analysed using flowcytometry (BD FACSLyrics, USA).

ELISA for Analysis of Cytokine released by UC-MSCs

After the fifth day of culture, the conditioned media of UC-MSCs from each experiment group was collected. The cytokines level released by UC-MSCs were analysed using Enzyme-Linked Immunosorbent Assay (ELISA) (Elabscience®, USA) as manufacturer protocols.

RESULT AND DISCUSSION

Comparison of MSCs Morphological and Confluence

The morphology and confluence of UC-MSCs was observed under a confocal microscope at 100x magnification.

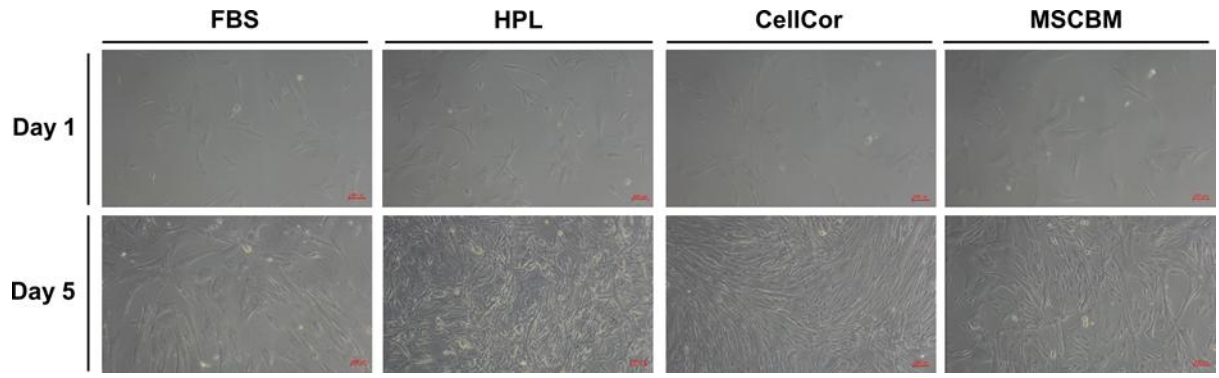

Figure 1. The morphology of MSCs which were cultured in four different mediums (100x magnification). The morphology was observed on the 24 and 120 hours after thawing. FBS: Fetal Bovine Serum, HPL: Human Platelet Lysate.

Comparison of UC-MSC Density

The UC-MSCs number which was cultured on each T75 flask was $5x10^6$ cells. There was a difference in aspect of cell number which was harvested on the fifth day of incubation (Table 1).

Table 1. The cell number of UC-MSCs which were cultured and harvested (cell/flask)

	FBS	***HPL***	***CellCor***	***MSCMBM***
Seeding	5×10^5	5×10^5	5×10^5	5×10^5
Harvesting	6.84×10^5	4.25×10^6	5.23×10^6	1.3×10^6

Comparison of UC-MSC Surfaces Markers

The analysis of surface marker of UC-MSCs cultured on four different mediums was performed using BD FACSLyrics. UC-MSCs which where cultured in four different mediums expressing more than 95% of UC-MSCs surface markers (CD90, CD73, CD44, and CD105) (Table 2).

Table 2. The percentage of UC-MSCs surface marker after being cultured in different serum

	FBS	***HPL***	***CellCor***	***MSCBM***
CD90	100.0%	100.0%	100.0%	99.9%
CD73	100.0%	100.0%	100.0%	100.0%
CD44	100.0%	100.0%	100.0%	100.0%
CD105	99.1%	99.0%	98.8%	98.6%
Lin	0.27%	0.04%	0.00%	0.42%

Comparison of Conditional Medium Cytokine Expression

The exosome and cytokines level within conditioned medium of UC-MSCs was analysed using ELISA.

Table 3. The cytokine released by MSCs which were cultured in different serums-containing media

	FBS	***HPL***	***CellCor***	***MSCBM***
Exosome (ng/mL)	200	Can't be determined	Can't be determined	120
TNF-a (pg/mL)	22.18	768.06	70.24	17.82
IL-6 (pg/mL)	145.77	83.38	70.24	131.58
VEGF-a (pg/mL)	72.5	3526.82	Below LOD	82.64
SDF-1 (pg/mL)	6904.53	2995.09	Below LOD	6285.66

DISCUSSION

There were no differences in the cell morphology of UC-MSCs cultured in medium containing four different types of serum both after 24 and 120 hours (Figure 1). Although the cell density of each group was no different on the first 24 hours of culture, UC-MSCs cultured in medium containing HPL and CellCor were denser than those cultured in medium supplemented with FBS and MSCBM on the fifth day (Figure 1). The initial Seeding Number was standardized at $5x10^5$ cells/flask

for the four culture conditions. UC-MSCs cultured in medium supplemented with FBS gave in the highest expansion fold (13.68-fold). In addition, there was 2.60; 8.50; and 10.46-fold of UC-MSCs number which was cultured in medium supplemented with MSCMBM, HPL, and CellCor, respectively (Table 1).

As stated by the International Society for Cell Therapy (ISCT), the minimum criteria for defining MSCs were that the cells should express more than 95% of CD90, CD73, CD44 and CD 105 surface marker as well as the low percentage of hematopoietic stem cell markers[9,10]. After being cultured for five days, UC-MSCs were harvested, counted, and analysed the cell surface marker. In this study, all four-culture media (HPL, FBS, CellCor, and MSCBM) maintained the expected high purity and positive surface marker expression profile of UC-MSCs, the minimum criteria determined by ISCT (Table 2). In addition, the proportion of Lin-positive cells was notably low under all conditions, varying from 0.00% (CellCor) to 0.42% (MSCBM). These low percentages validate that the cell populations consisted of highly purified MSCs.

The conditioned medium was analysed for the concentration of exosomes and the several cytokines released by the UC-MSCs. The highest exosome concentration was found in UC-MSCs cultured in medium supplemented with FBS (22.18 ng/mL), followed by those cultured in medium supplemented with MSCBM (120 ng/mL) (Table 3). The exosome level within conditioned medium of UC-MSCs cultured in medium supplemented with HPL and CellCor was higher than the upper limit of instrument detection so that it cannot be determined.

Tumour necrosis factor (TNF)-α is central to the creation of an inflammatory microenvironment[11-13]. UC-MSCs cultured in medium supplemented with HPL released the highest concentration of TNF-α (768.06 pg/mL), while UC-MSCs cultured in medium supplemented with MSCBM had the lowest level of TNF-α (17.82 pg/mL) (Table 3). FBS induced the highest level of IL-6 released by UC-MSCs (145,77 pg/mL), followed closely by conditioned mediums of UC-MSCs supplemented with MSCBM (131.58 pg/mL) (Table 3). UC-MSCs supplemented with CellCor had the lowest level of IL-6 (70.24 pg/mL), suggesting minimal release of IL-6 in this condition. Interleukin-6 (IL-6) is a multifunctional cytokine that plays a vital role in modulating immune responses, regulating inflammation, and facilitating various physiological processes within the body[14-16]. Its diverse functions highlight its significance in health maintenance. Abnormal IL-6 levels are strongly linked to numerous diseases, establishing it as a critical target for research and therapy[17-20].

The highest level of VEGF-α was found in the conditioned medium of UC-MSCs supplemented FBS (3526.82 pg/mL), followed by MSCBM (82.64 pg/mL) (Table 3). The concentration of VEGF in the conditioned medium of UC-MSCs supplemented with HPL was 72.5 pg/mL, while its value was zero in the conditioned medium of UC-MSCs supplemented with CellCor. VEGF is vital in regenerative therapy as it facilitates angiogenesis, the process of forming new blood vessels, which is necessary for tissue repair and the delivery of oxygen and nutrients[21]. UC-MSCs release VEGF to boost the number of capillaries, aid in the proliferation and migration of endothelial cells, and improve the wound healing process by promoting neovascularization and the development of granulation tissue[22,23]. MSCs could also stimulate organ normal function by inducing angiogenesis through the secretion of VEGF, and macrophage colony-stimulating factor (MCF)[24].

SDF-1 also known as C-X-C motif chemokine 12 (CXCL12) is crucial for cell homing and repair of the transplanted UC-MSCs[25]. Thus, the appropriate level of SDF-1 plays an important role in UC-MSCs migration to the intended location. FBS and MSCBM induced the intensive release of SDF-1 (6904.53 pg/mL and 6285.66 pg/mL, respectively), followed by HPL (2995.09 pg/mL) (Table 3).

This study has several limitations that should be acknowledged. First, the analysis was limited to a short-term culture period of 120 hours, which may not fully capture the long-term effects of each medium on UC-MSC proliferation, differentiation potential, or senescence. Second, although exosome and cytokine levels were measured, functional assays to assess the biological activity of these secreted factors were not performed. Additionally, the quantification of exosomes was restricted by the detection limits of the instrument, particularly for the HPL and CellCor groups, which may have affected the comparative analysis. Lastly, while this study focused on surface marker expression and secretome profiling, other critical parameters such as gene expression, immunomodulatory potency, and in vivo functionality were not assessed. Future studies should incorporate these aspects to provide a more comprehensive evaluation of each culture supplement's suitability for clinical applications.

CONCLUSION

In conclusion, the conditioned medium derived from UC-MSCs cultured in HPL exhibits elevated levels of exosomes and anti-inflammatory cytokines. Additionally, the UC-MSCs cultured in medium supplemented with HPL maintained a normal morphology and demonstrated expression of UC-MSC surface markers more than 95%. Furthermore, HPL stimulated the proliferation of UC-MSCs eight-fold higher than cell number on the day of seeding. Therefore, medium enriched with HPL holds significant potential for future applications in the pharmaceutical industry

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Competing Interests

The authors declare that there is no conflict of interest.

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