Finding Preferred Food for Mesenchymal Stem Cells: A Media Comparison

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Abstract

Mesenchymal stem cells (MSCs) have been an advantageous choice for regenerative medicine, as result of their differentiation capabilities, proliferation potential, and ethically non-controversial character. Culturing these MSCs and ensuring that they maintain the MSC phenotype is critical for use in experiments that are reproducible. However, one element that can greatly affect the MSC phenotype over time is the media formulation in which MSCs are cultured. With the global stem cell market expected to reach \$15 Billion by 2025, several stem cell and cell therapies companies have developed a multitude of different media formulations for MSCs to encourage MSC proliferation while maintaining MSC phenotype. Each company offers products at different price points based on the raw materials used and the manufacturing practices. In this study, we highlight four different media formulations from different companies. The findings from this study may help researchers in the selection of media for their MSC cultures in future studies.

INTRODUCTION

Stem Cell Culture

Since the discovery of mesenchymal stem cells (MSCs) there have been continued advances to extract these cells from different tissues. MSCs have been procured from tissues such bone marrow, adipose, and umbilical cords.¹ The multipotency of MSCs give them the ability to differentiate into many different lineages when provided the correct environment.² MSCs reside in a delicate environment where physical features such as cell substrate, temperature, oxygen, pH, fluid flow, and media formulation can directly affect MSC health, behavior, and function if modified.³ Exploiting the physical features of the MSC environment is one way in which to direct MSC differentiation.⁴ However, to maintain the MSC phenotype, physical features must be maintained and carefully monitored. One source of variation can stem directly from the media formulation used to grow MSCs in, as most media formulations are proprietary. Hence, selection of MSC media is crucial in ensuring the reproducibility and validity of experiments that utilize MSCs.

Different Types of Stem Cell Media

There are a variety of different media formulations produced by multiple companies to choose from to culture MSCs. Several different factors should be considered when selecting a media formulation, such as cost, shelf life, consistency, and ability to maintain stemness. The composition of the media can substantially affect the aforementioned factors. Some of the major distinguishing factors between media formulations include the use of a serum such as Fetal Bovine Serum (FBS), reduced serum formulations, non-serum formulations, and xeno-free formulations. FBS containing media is commonly used for economic purposes, but the use of FBS is somewhat contentious due to the variability of growth factors, steroids, and other nutrients found in FBS that vary from lot to lot⁵. Xeno-free media formulations mitigate the variability found in serum-based media formulations, and maintain MSC phenotype, but are not as advantageous economically.⁶ While most media formulations are capable of providing cells with appropriate nutrients to maintain viability, it is not clear whether different media formulations are maintaining MSC phenotype or contributing MSC differentiation. Maintaining MSC phenotype is important for ensuring that the MSCs used in experiments are actually MSCs.

Mesenchymal Stem Cell Identification

For a cell to be classified as an MSC, it must meet the minimal criteria set forth by the International Society for Cellular Therapy in 2006.⁷ The minimal criteria established includes the ability to adhere to plastic in standard culture conditions, expression of CD73, CD90, and CD105, while lacking the expression of CD45, CD34, CD14, and human leukocyte antigen DR (HLA-DR). Lastly, they must still be able to differentiate to osteoblasts, adipocytes, and chondroblasts in standard *in vitro* differentiation conditions. In addition to the minimal criteria set forth there are more positive makers that others have been looking into that appear to be common to MSCs such as CD29 and CD44.⁸⁻¹⁰

METHODS

MSC Harvest and Cell Culture

Human Wharton's Jelly Cells (HWJCs) were isolated from a single human umbilical cord (female, single-birth, full-term) according to our previous published methods.¹¹ Briefly, the cord was washed vigorously with 2% antibiotic-antimycotic (AA) solution in sterile phosphate buffered saline (PBS) (Life Technologies, Carlsbad, CA), and ends of the cords were removed. The cord was sectioned into 3-cm increments, and then washed twice with PBS. Next the blood vessels were removed, and the cord segments were washed again to remove waste. Using a scalpel, the tissue was physically degraded by mincing until it reached a "pulp-like" consistency. Digestion media consisting of 0.2% (w/v) collagenase type II (Worthington-Biochem, Lakewood, NJ), 1% penicillin-streptomycin (Pen-Strep) in Dulbecco's Modified Medium (DMEM) (Life Technologies) was added to the dish. The dish was covered and placed in the incubator at 37°C and 5% CO₂ on an orbital shaker for 4 hrs at 50 rpm. The dish was then removed from the incubator and diluted 4:1 with sterile PBS in 50-mL conical tubes. The conical tubes were centrifuged at approximately 500 x g for 10 min followed by aspiration of the supernatant. The cells were resuspended in complete medium consisting of 10% Fetal Bovine Serum qualified (FBSq) (Life Technologies) and 1% Pen-Strep in DMEM. Cells were seeded into T-75 flasks at 7,000 cells/ cm².

HWJCs were maintained through one passage, and cryogenically preserved in 1-mL cryotubes in liquid nitrogen at a concentration of 1x10⁶ cells/cryotube, according our previously published protocols.¹¹ When needed for experiments, HWJCs were thawed cultured to passage 4 in Gibco MesenPro RS Media (Cat. No. 12746012; Life Technologies). Cells were passaged once reaching 70% confluency.

At passage 5, HWJCS were plated in T-75 flasks at density of 7,000 cells/cm². HWJCs were cultured in one of the following media formulations: Group (1) Complete Medium, Group (2) MesenPro RS Media (Life Technologies), Group (3) Mesenchymal Stem Cell Growth Medium (MSCGM) Bullet Kit (Cat. No. PT3001; Lonza, Basel, Switzerland); Group (4) MesenCult ACF Plus Medium (Cat. No. 5445, Stemcell Technologies, Vancouver, Canada) Group (5) PRIME-XV MSC Expansion XSFM (Cat. No. 91149, Fujifilm Irvine Scientific, Santa Ana, CA). PBS was used as a negative control and was designated as Group (6). All media were changed every 48 hours. When a sub-set of HWJCs reached 70% they were passaged using trypsin with 0.5% EDTA (Life Technologies) and plated in 24-well plates at a density of 7,000 cells/cm² using the same media they were cultured in for passage 5.

Viability

When the HWJCs reached 70% confluency they were trypsinized and Trypan Blue was used to asses cell viability. Cells were imaged using a Nikon Eclipse TsR2-FL inverted epi-fluorescent microscope with a DS-Ri2 camera attached (Nikon Instruments Inc., Melville, NY). Three random fields of view were imaged for each sample. FIJI open-source image processing software was used to count cells in each field of view. The total number of blue cells in each field of view was divided by the number of total cells in the field of view. That result was multiplied by 100, and subtracted from 100 to determine the percent viability.

Immunocytochemistry

The remaining HWJCs were seeded at a density of 7,000 cells/cm² in 6-well plates on a square glass cover slip coated with 5 ng/cm² of human fibronectin using the same media formulations that cells were previously cultured in. The HWJCs were cultured for 10 days in 2 mL media with changes occurring every 48 hours. On day 10, all media was aspirated off and cells were washed twice with PBS. Afterward, cell samples were fixed for ten minutes in 1 mL of 0.1% saponin and

4% paraformaldehyde in Hanks buffered salt solution (HBSS) (VWR, Radnor, PA). Samples were washed twice with 0.1% saponin in HBSS (HBSS:S), and then 5 mL of HBSS was added to each well. Plates were sealed with parafilm and covered in aluminum foil and stored in a 4°C refrigerator until ready for immunohistochemistry.

Immunocytochemistry was then performed; the samples were rinsed with ice-cold HBSS:S for 5 minutes in the dark. Afterward, cells were blocked and permeabilized in 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO), 6% Normal Donkey Serum (Life Technologies) in HBSS:S for 2 hours in the dark. Samples were washed with HBSS:S two times for 5 minutes each in the dark. The samples were then incubated with primary antibodies overnight in the dark at 4°C in a humidified chamber (See **Table 1** and **Table 2**). The next day the samples were washed three times with HBSS:S for 5 minutes each in the dark. Then the secondary antibodies were added to the samples and incubated for 2 hours in the dark at room temperature on an orbital shaker (See **Table 3**). The samples were washed three times with HBSS:S and then two times with HBSS in the dark before a drop of ProLong Gold Antifade Reagent with DAPI (Life Technologies) was added to the substrate side of the coverslip. Coverslips were mounted on SuperFrost glass slides. Slides were imaged with the previously used Nikon inverted epi-fluorescent microscope and camera, using the acquisition software Nikon Elements Basic Research (Nikon Instruments Inc.)

Statistics

All numbers are expressed as means with standard deviations. A two-tailed two-way analysis of variance with a post hoc Tukey's test was run on all samples to determine statistical significance. Statistical parameters were set at a desired detection difference of 60%, a power of $1 - \beta = 0.80$ and a significance level of $\alpha = 0.05$.

RESULTS

Cell Viability

When the cells reached 70% confluency the cell viability was determined using three images of suspended cells with Trypan Blue for each flask (**FIG. 1**). This was done to ensure that live cells were transferred to the well plates. Upon inspection of the images there appears to be no substantial difference between the flasks. This is confirmed when statistical analysis comparing average cell viability between the flasks was performed, there was no significant difference in viability.

<u>Immunocytochemistry</u>

To verify MSC phenotype of the cells, immunocytochemistry was performed after the cells were fixed at day 10 (**FIG. 2**). Analysis of the cells was performed using FIJI to determine the expression of markers. Markers CD29 and 90 showed no significant difference in expression between all of the groups (**FIG. 3C, 3D**). For marker CD44 there was statistically significant greater expression when comparing group 1 vs. group 6 (71%, 95% Confidence Interval (CI) 26.33-115.8, P<0.05), group 2 vs. group 5 (57.5%, 95% CI 12.72-102.3, P<0.05), group 2 vs. group 6 (94%, CI 49,22-138.8, P<0.05), group 3 vs. group 6 (56.5%, CI 11.72-101.3, P<0.05), and group 4 vs. group 6 (76.5%, CI 31.72-121.3, P<0.05). CD45 exhibited a statistically significant greater expression in group 1 vs group 6 (84.5%, CI 32.52-136.5, P<0.05), group 2 vs group 6 (100%, CI 48.02-152, P<0.05), group 3 vs group 6 (76.5%, CI 24.52-128.5, P<0.05), and group 4 vs group 6 (79.5%, CI 27.52-131.5, P<0.05). Statistics were not run on CD105 due to a low sample size (n=1).

Price Point Comparison

Depending on the formulation of media and fabrication methods, the price of cell culture media can vary greatly. For example, xeno-free media formulations typically carry a higher expense than non-xeno-free formulations. Media that is manufactured and certified in a good manufacturing practice (GMP) facility will typically cost more to produce but has the certification of being made in a GMP facility for pre-clinical and early clinical studies. The listed prices for the media formulations we analyzed are listed in **Table 4**.

DISCUSSION

HWJCs were isolated from the umbilical cord due to their availability and similarity to bone marrow MSCs and adipose MSCs. ¹² HWJCs can be identified using the same methods to identify MSCs from the bone marrow and adipose tissue. In our experiments, HWJCs were imaged at days 2,4, and 6 to assess confluency for trypsinization (**FIG. 4**). At day 2 when the first images were captured for confluency, group 3 cells had a different confluency at 25% confluency. Whereas, all the cells in the other groups exhibited similar confluency rates of 10%. Images captured at day 4 showed that group 3 cells exhibited 60% confluency, group 5 cells had 40% confluency, while groups 1, 2 and 4 cells exhibited a confluency of 10%. Finally, at day 6. group cells had confluency rate of 95%, group 5 was 60%, group 1 was 50%, and groups 2 and 4 remained at 10-15% confluency. All cells in each group reached at least 70% confluency before they were passaged.

Cell viability was assessed via Trypan Blue staining to identify dead and dying cells. All groups displayed similar viabilities and were transferred into their designated 6-well plates, which suggest that all media formulations did not hinder cell viability.

MSC phenotype was assessed by identifying CD markers consistent with MSC phenotype. Cell surface markers CD29, CD44, CD90, and CD105, and CD45 were identified using immunocytochemistry. Interestingly, all cells expressed the hematopoietic surface marker CD45. which is normally negative in MSCs. The hyaluronic acid receptor, CD44, is a prominent MSC marker that was positively expressed by all samples, although, to varying degrees.¹³ No significant differences in CD44 expression were detected between samples. Hyaluronic acid is an abundant extracellular matrix component found in connective tissues such as cartilage, bone, and neuro tissues, and is important for cell migration. Integrin beta-1 is another MSC surface marker known as CD29, which associates with integrin alpha 1 and integrin alpha 2 to interact with collagen in the extracellular matrix of connective tissues.¹⁴ While CD29 was positively identified in all samples, its expression was the least in group 5. Of particular note, is the lack of expression of the MSC surface marker in groups 1 and 2, and CD 105 expression is absent from groups 2 and 4. Taken into account that all the samples expressed CD45, this may suggest that the HWJCs may be undergoing de-differentiation. However, this is not necessarily indicative of any one media formulation. It would be interesting to run this experiment again with other MSCs to see if similar results are observed. All medial formulations performed similarly with no significant differences in surface marker expression, or viability. The only difference of note is the proliferation rate seen in cells from group 3, which could be a result of a difference in plating density, and not necessarily an attribute of the media formulation.

In addition to performance analysis of media formulations, we compared the price points at which vendors sold their media formulations. Media formulations that contained animal components such as serum, had lower price points per milliliter than media formulations that were free of animal components. The most expensive media formulation was the PRIME-XV MSC Expansion XSFM by Fujifilm Irvine Scientific, which is in part due to the fact that the media is GMP certified. The GMP certification is advantageous for clinical experiments.

CONCLUSION

Cell viability was uninhibited by all tested media formulations. While, expression of CD markers varied between formulations, there is no evidence that media formulation negatively affects MSC phenotype. However, it would be prudent to test media formulations on cells before performing live experiments to ensure that media performance does not negatively affect experiments.

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Tables and Figures

Table 1. Primary Antibody							
Primary Antihody	1º BLOCK	Ab Betrievel	Secondary Antibody				
Antibody	Dilution	Retrieval					
CD44	1:200	Yes	Donkey-Anti Mouse Alexa Fluor 488				
CD45	1:1,000	Yes	Donkey-Anti Rabbit Alexa Fluor 555				
CD90	1:1,000	Yes	Donkey-Anti Mouse Alexa Fluor 488				
CD29	1:500	Yes	Donkey-Anti Rabbit Alexa Fluor 555				
CD105	1:1,000	Yes	Donkey-Anti Mouse Alexa Fluor 488				

Table 2. 6-well Plate Layout Labeling						
DAPI	DAPI	DAPI				
CD45	CD29					
CD44	CD90	CD105				
DAPI	DAPI	DAPI				
CD45	CD29	AF 555 Ctrl				
CD44	CD90	AF 488 Ctrl				

Table 3. Dyes and 2° Antibody								
Stain	Dilution	Target	Excitation (nm) [Filter]	Emission (nm) [Filter]				
DAPI	1:50	Nucleus	358 [DAPI]	461 [DAPI]				
Alexa Fluor 555	1:500	Primary Antibody	555 [TRITC]	584 [TRICT]				
Alexa Fluor	1:1,500	Primary Antibody	488 [FITC]	525 [FITC]				
488								

Table 4. Media Price Comparison								
Group #	1	2	3	4	5			
Media	HWJC Media (DMEM/10%FBS /1% Pen_Strep) [Control]	MesenPro RS Media	Mesenchymal Stem Cell Growth Medium (MSCGM)	MesenCult ACF Plus Medium	PRIME-XV MSC Expansion XSFM			
Vendor	ThermoFisher	ThermoFisher Scientific	Lonza	Stemcell Technologies	Fujifilm Irvine Scientific			
Location	Carlsbad, CA	Carlsbad, CA	Basel, Switzerland	Vancouver, Canada	Santa Ana, CA			
Cat. No.	11885084/10437 028/15140122	12746012	PT3001	5445	91149			
Quantity	500 mL	500 mL	500 mL	500 mL	250 mL			
Price	\$83.84	\$192.00	\$153.00	\$349.00	\$179.00			
Price/mL	\$0.17	\$0.39	\$0.31	\$0.70	\$0.72			

Prices are reflective of values published on vendor websites on June 1st, 2019.



Figure 1. Cell Viability at 70% confluency.

(A) Average cell viability was determined by taking the average of 3 different images from each plate, when the cells were transferred to 6-well plates. (B) Images were taken in random areas of the cell suspension; the most representative image was selected for the figure.



Figure 2. Immunocytochemistry Analysis of MSC Markers and DAPI.

Fluorescence microscopy images of HWJCs cultured in different medias. DAPI was used to stain the nuclei blue, Alexa Fluor 555 was used to stain CD29, and CD45 red, and Alexa Fluor 488 stained CD44, 90, and 105 green.



Figure 3. HWJC Characterization.

Percentage of cells identified as positive using FIJI after fluorescence microscopy, Marker CD45 a hematopoietic marker was present in all groups except for the control group 6. This suggests possibly dedifferentiation within the groups. CD44, 29, 90, and 105 are common markers for MSCS. CD44 and 29 had expression in all of the groups, while CD90 and 105 were only expressed in a few of the groups.



Figure 4. Cell Confluency.

Bright field microscopy of HWJCs during growth in T-75 flasks. Images were obtained at two-day intervals up to 6 days. Cells were then transferred to well plates once 70% confluency was obtained.