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Article

A comparison study in the expansion of human bone marrow mesenchymal stem cells

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SUMMARY

Mesenchymal stem cells (MSCs) have been intensively studied for their properties in several clinical conditions. They can be obtained from several sources including bone marrow, Wharton's jelly in the umbilical cord, and adipose tissue. MSCs exhibit fibroblast-like morphology and possess significant ability to differentiate into multiple collective tissue cells in vitro. Conventionally, fetal bovine serum (FBS) is the most common supplement for the expansion of MSCs in vitro. However, the use of FBS is not desirable due to its intrinsic drawbacks including source, xenogeneic antigens, and xenogeneic microbes or viruses that can majorly impact the clinical value of MSCs. Therefore, a reliable human-compatible source with minimal manipulations and ethical concerns could be an attractive alternative to circumvent these problems. In this study, we have explored the suitability of using human umbilical cord blood-derived serum (CS) to grow MSCs in vitro. We have elucidated its effects in terms of morphology, population density, and doubling time at every passage as well as the pluripotency markers at late passages in comparison to FBS. Our findings demonstrate the merit of using CS to support cell growth and expansion in vitro.

INTRODUCTION

Mesenchymal stem cells (MSCs) have recently gained significant relevance in the field of regenerative medicine (1). They are plastic adherent, fibroblast-like in shape, express CD90, CD71, CD105 markers, and can differentiate into certain connective tissue cells in vitro. They are multipotent cells that display self-renewal capacity and are obtained from multiple organs, including bone marrow, Wharton's jelly in the umbilical cord, limbal tissue of the eye, adipose tissue, and dental pulp (2–5). Their salient features include immunomodulatory properties which could be exerted via direct contact or paracrine effects (6, 7). They are known to home to inflammatory sites during tissue injury and promote healing and tissue remodeling (8–10). MSCs suppress activated T cells, thereby restoring homeostasis in the body after an infection or injury has resolved (11). MSCs can also be transplanted across immunological barriers (12, 13). In turn, these attributes make them an attractive candidate for treating several clinical conditions as an "off the shelf" biological product (14). Furthermore, their wound-healing capacity and medicinal applicability stem from their abilities to generate structural repair via cellular differentiation, behavior as immune-modulatory agents, and secretion of growth factors that drive neovascularization and re-epithelialization, and mobilize resident stem cells (15, 16). Consequently, large-scale production of MSCs would require an optimum balance of cell culture reagents, plasticware, and its consequent cost effectiveness.

Fetal bovine serum (FBS) is the most used media supplement for in vitro MSC cultivation (16). Although it is very effective, inherent drawbacks include its source, xenogeneic antigens, and xenogeneic microbes or viruses that could majorly impact its clinical value (17). An attractive alternative from a reliable human source with minimal manipulations and ethical concerns is umbilical cord blood serum (CS). CS can be considered a xenogeneic-contamination-free alternative to FBS in the in vitro growth of MSCs, as demonstrated by Caseiro et al. (15). The absence of animal pathogens in CS further bolsters the consideration of CS as an alternative to FBS (18).

We hypothesized that CS-enriched media would have higher potency in comparison to FBS with respect to four parameters: morphology, cell number, population doubling time (PDT), and pluripotent markers. The pluripotency markers studied in this paper were *Oct4, Sox2, and Nanog.* These three transcription factors play the key roles in inducing and maintaining pluripotency in stem cells and were measured via semi-quantitative polymerase chain reaction (PCR) amplification of the target genes (19).

This investigation aimed to provide insight into choosing an appropriate option for growing bone marrow mesenchymal stem cells (BM-MSCs). Our study showed that BM-MSCs grown in CS-enriched media were more suitable for long-term proliferation than those grown in FBS media.

RESULTS

We aimed to compare the morphology, cell number, PDT, and pluripotency marker expression of BM-MSCs grown in 10% CS-enriched media to those in 10% FBS-enriched media. Across all parameters, CS was attested to be more effective in facilitating BM-MSC growth than FBS.

Morphology of BM-MSCs

Inverted light microscope images showed a difference in the phenotype between the two groups (10% FBS and 10% CS). A subpopulation of BM-MSCs grown in 10% FBS appeared to have normal adherent elongated spindle-shaped (fusiform) with fibroblast-like morphology. A second subpopulation exhibited a polygonal-like phenotype up to passage 6, and at later passages exhibited enlarged, flattened, and irregularly shaped morphology (**Figure 1, A-C**).

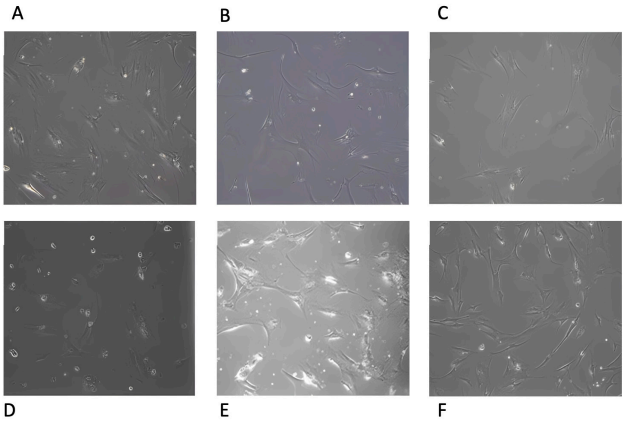

Figure 1: Morphology of BM-MSCs grown in CS-enriched media was more normal than those grown in FBS-enriched media. Images were obtained by a camera mounted on the microscope connected to ImageJ software. (A-C) Morphology of BM-MSCs grown in 10% FBS. (A) The appearance of the cells as long spindle shaped with fibroblast-like morphology at passage 5. (B) A subpopulation of cells exhibiting polygonal morphology at passage 5. (C) The appearance of the cells as flattened and irregularly shaped at passage 8. (D-F) Morphology of BM-MSCs grown in 10% CS. (D) Cells with spindle-shaped morphology at passage 5. (E) Cells seen growing as colonies at passage 5. (F) Reduction in the number of cells growing as colonies at passage 8.

In comparison, BM-MSCs grown in 10% CS showed spindle-shaped morphology and grew as colonies in early passages (up to passage 6) but lost their colony-forming states at later passages and grew in a non-uniform manner (**Figure 1, D-E**).

Cell Number and Population Doubling Time over Multiple Passages

The cell number of the group supplemented with 10% CS was remarkably higher when compared to the group supplemented with 10% FBS at all passages ranging from 5 to 8 (**Figure 2a**). Similarly, the population doubling time was found to be notably shorter in the 10% CS group than in the 10% FBS group, thereby indicating an enhanced proliferation

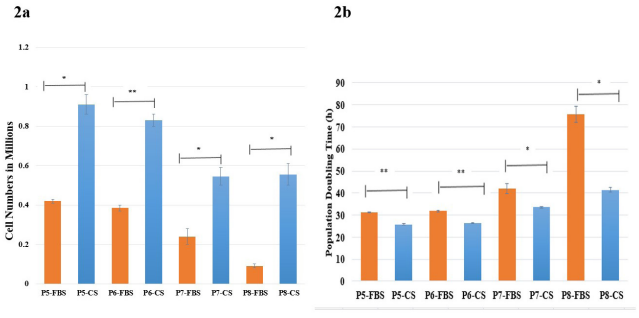

Figure 2: Morphology of BM-MSCs grown in CS-enriched media was more normal than those grown in FBS-enriched media. a) Cell numbers at each passage (5 to 8) for both groups (10% FBS and 10% CS). b) Population doubling time of BM-MSCs grown in 10% FBS and 10% CS-enriched media at each passage (5 to 8). The data is presented as mean ± standard deviation. In the graph, degrees of statistical significance have been denoted by $*p < 0.05$ and $**p < 0.01$ when comparing BM-MSCs grown in 10% FBS-enriched media to those in 10% CS. The overall *p*-value (Bonferroni) ≈ 0.0043 < 0.012 (corrected α-value).

of BM-MSCs when cultured in 10% CS (**Figure 2b**) (*p*-value (Bonferroni) ≈ 0.0043 < 0.012 (corrected α-value)).

Stem Cell Pluripotent Marker Analysis

We compared the pluripotent marker profile of BM-MSCs grown in 10% CS- and 10% FBS-enriched media for passages 5 and 6. We observed an increase in the expression of *Sox2* in the 10% CS group in comparison to the 10% FBS group at both passages (**Figure 3**). However, a similar expression of *Oct4* and *Nanog* was seen between these groups, thereby indicating the efficacy of CS as comparable to that of FBS. The determination of the expression of pluripotency markers (*Oct4*, *Sox2*, and *Nanog*) for passage 7 and 8 of the 10% FBS group was not included in the analysis because of the low number of cells obtained. Interestingly, the 10% CS group showed the presence of *Oct4* and *Sox2* in late passages (7 and 8), but we did not detect any expression of *Nanog*. From the obtained results, it is clear that BM-MSCs grown in CS-enriched media showed a relatively higher ability for long-term proliferation in culture before becoming senescent.

DISCUSSION

In this investigation, we explored the suitability of CS to grow MSCs in comparison to FBS. We elucidated the comparative effects of the sera in terms of morphology, population density, and doubling time at every passage, and pluripotency markers at late passages. We concluded that there was a statistically significant increases in the cell number of BM-MSCs grown in 10% CS in comparison to 10% FBS over multiple passages ranging from 5 to 8, affirming our initial hypothesis.

Morphology played a significant role in qualifying the differences between the cells grown in FBS and those in CS as it serves as the first determining factor in identifying MSCs. While cells grown in CS retained their fibroblast-like morphology until passage 6, FBS-enriched cells lost this characteristic morphology and expressed an irregular shape, which indirectly indicated the noteworthy loss of media quality compared to CS due to healthier growth.

The PDT value of BM-MSCs grown in 10% FBS was higher than those grown in 10% CS, thereby indicating an increased proliferation rate of the latter. Increased PDT emphasizes that CS could be used as an alternative for ex vivo expansion in vitro. We also determined the expression of pluripotent markers in both these groups. Pluripotent genes

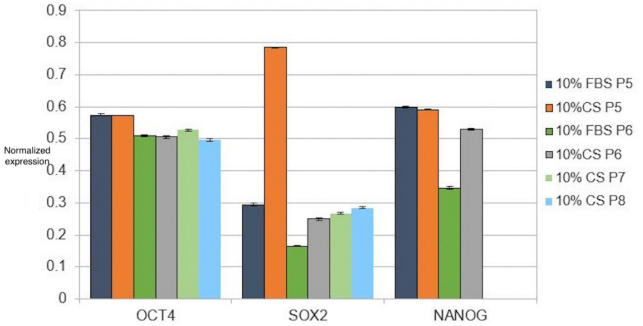

Figure 3: Expression of pluripotent genes analyzed by RT-PCR. Gene expression of pluripotent markers (*Oct 4*, *Sox 2*, and *Nanog*) for 10% CS and 10% FBS group at each passage. The data are presented as mean ± standard deviation.

(*Oct4*, *Sox2*, and *Nanog*) were expressed in a similar fashion in the 10% FBS and 10% CS groups at passages 5 and 6. A slight increase in *Sox2* expression seen in the 10% CS group. Another prominent finding was that *Oct4* and *Sox2* were expressed at later passages (7 and 8) in the group supplemented with 10% CS, though *Nanog* expression was not detected. A number of factors could explain the loss of *Nanog* gene expression, including prolonged culture time and aging of the cells, less cell contact for appropriate cell signaling, or loss of plastic adherence.

The expression of pluripotent markers was not analyzed at later passages in the 10% FBS group due to insufficient cell quantity. Considering the role *Oct4* and *Sox2* play in conferring MSCs with higher expansion and amplifying their intrinsic self-renewal properties (20), our results suggest that CS bears an increased ability to prolong MSC proliferation in culture compared to FBS.

Studies growing swine marrow cells in either FBS or CS have demonstrated that CS can effectively support cell growth and is comparable to FBS (21, 23). Cells showed more proliferation when grown in CS and were phenotypically similar to those grown in FBS (23). The study demonstrated that cord serum can effectively be a replacement for FBS to culture MSC for clinical purposes (20). In a study designed to compare CS of the four blood types with FBS on growth patterns with placenta derived MSCs, the authors observed that fold expansion and population doubling time significantly increased with Cord blood Serum when compared to FBS in long-term cultivation of MSCs (22). The generation time for the CS-supplemented cells was significantly reduced, thereby implying that human cord blood serum is more suitable for rapid ex vivo expansion. A similar study with CS had demonstrated the suitability of using CS to grow human placenta derived MSCs in comparison to FBS. CS-supplemented MSCs maintained their self-renewal capacity, differentiation capacity, and displayed normal karyotype (23, 24). In the context of the aforementioned studies, our results corroborate and add further evidence to the superiority of CS over FBS as a viable cell culture media supplement.

CS could be an attractive medium in which to grow MSCs, and our study has effectively demonstrated its merit. We could potentially follow up on our findings by determining the differentiation potential of BM-MSCs grown in 10% FBS and 10% CS into mature connective tissue cells. Furthermore, in light of MSCs grown in serum-free medium (SFM) that are functional and can be expanded to produce large numbers for clinical use (17), future studies could potentially compare and clarify the efficacy of SFM in comparison with CS.

MATERIALS AND METHODS

Culturing of Bone Marrow Mesenchymal Stem Cells

Human BM-MSCs were commercially obtained from Lonza. BM-MSCs at passage 4 were expanded in two different culture conditions: Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco) and 10% CS alongside 1% Antibiotic Antimycotic solution (HiMedia) under standard cell culture conditions (5% CO2 and 37°C). The cells were grown in plastic tissue culture dishes. The culture media was replenished every 3–4 days, and once the cells reached 70-80% confluency, they were harvested with 0.25% trypsin-EDTA (HiMedia) and reseeded at a density of 1000 cells/ cm² in a 6-well plate for further expansion. For all of our experiments, we used cells of passages ranging from 5 to 8.

Preparation of CS

CS was collected from the umbilical cord following delivery after obtaining written informed consent from the mothers. The blood was tested for markers of infectious agents (Syphilis, HIV, HCV, and Hepatitis B) before processing. CS was prepared in our laboratory according to the standard operating procedure (25, 26).

Determination of Cell Number, Population Doubling Time, and Morphology Over Multiple Passages

For the proliferation experiment, passages ranging from 5 to 8 in both groups (10% CS and 10% FBS) were used. The protocol for each subculture was as follows: BM-MSCs were cultured in a 6-well plate at a density of 1000 cells/cm², and the culture media was changed every 3-4 days. The cells were passaged at 70-80% confluency, which took an average of 7 days for passages 5 and 6, 8 days for passage 7, and 10 days for passage 8. Cell numbers were determined post-trypsinization and counted manually using the trypan blue exclusion method. The PDT at each passage was calculated as (14):

$$PDT = (t\text{-}t_0) * \log 2 / (\log N_H - \log N_0),$$

where $(t\text{-}t_0)$ is the culture time in hours, N_H is the number of harvested cells, and N_O is the initial cell number.

All morphological images were obtained by using inverted bright field microscope.

RNA Extraction and cDNA synthesis

Total RNA from the cells of each group (10% CS and 10% FBS) and at each passage were extracted using Nucleospin RNA Isolation Kit (Takara) according to the manufacturer's protocol. The concentration (A260) and purity of the RNA (A260/A280) extracted from each group and at each passage were determined using a spectrophotometer (Thermo Scientific). Two thousand ng of each RNA sample was used for cDNA synthesis using a Genei RT-PCR kit following the manufacturer's instructions.

Semi-Quantitative Polymerase Chain Reaction (RT-PCR)

Thirty ng of each cDNA samples was used to amplify pluripotency genes (*Oct4, Sox2, and Nanog*). PCR was set up using 6 µL Jumpstart mix (Sigma), 1 pmol (1 µL) of appropriate forward and reverse primer for each gene (Bioserve, **Table 1**). The remaining volume for a 20 µL PCR reaction was obtained using molecular grade water. The thermal cycling PCR conditions consisted of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 52°C (*Nanog*), 53.5°C (*Sox2*), 57.5°C (*Oct4*), and 61.5°C (G3PDH) for 30 seconds, and extension at 72°C for 1 minute with a final extension at 72°C for 10 minutes. The PCR products obtained were resolved on a 2% agarose gel and were observed under a UV-transilluminator (Biobee Tech). The target gene expression was analyzed and quantified using ImageJ software and the results were normalized as a ratio to G3DPH.

Statistical Analysis

All data obtained were from a minimum of three independent experiments. Given the comparison of the two

Gene	Primer Sequences
G3PDH	F- GGTCGGAGTCAACGGATTTGGTCG R- CCTCCGACGCCTGCTTCACCAC
NANOG	F- GCTGGTTGCCTCATGTTATTATGC R- CCATGGAGGAAGGAAGAGGAGAGA
SOX2	F- GCCGAGTGGAAACTTTTGTCG R- GCAGCGTGTACTTATCCTTCTT
OCT4	F-GACAACAATGAGAACCTTCAGGAGA R-TTCTGGCGCCGGTTACAGAACCA

Table 1: PCR primer sequences. The primer sequences used in PCR were taken from previously published work (27–30).

experimental parameters – cell numbers and population doubling time – between FBS and CS, one-tailed unpaired t-tests for all cell passages were conducted. The t-tests were then followed by Bonferroni post-hoc tests to determine the corrected a level. All statistical tests were conducted using Microsoft Excel.

Graphically, the degree of statistical significance of the groups in comparison were represented by $*p < 0.05$ and $**p < 0.01$.

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