GROWTH CAPACITY OF MESENCHYMAL STEM CELLS IN SERUM-DEPLETED MEDIA

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Abstract. Mesenchymal stem cell (MSC) therapy is a promising therapy with a wide range of applications. However, the reliability of this therapy remains uncertain. Synchronizing MSCs before their application is a crucial strategy to align the cells in the same phase of the cell cycle and improve the therapy's reliability. Starving MSCs in a serum-depleted medium can promote synchronization, although it may also adversely impact the physiological function of the cells. This study aims to investigate the impact of serum starvation on the growth of MSCs. Human MSCs isolated from umbilical cord were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS). The cells were then incubated with low-glucose α-MEM or high-glucose DMEM with or without FBS for three days. Images of ten distinct areas were captured, and the covered areas were quantified using image analysis software. The difference between groups was calculated with student's t-test. Results showed a significant decrease in MSC growth in α-MEM compared to α-MEM+FBS ($p<0.0001$) and in DMEM compared to DMEM+FBS ($p<0.001$). There is no difference in cell growth between α-MEM+FBS and DMEM+FBS ($p=0.323$) or α-MEM and DMEM ($p=0.496$). These findings showed that serum-depleted medium halts the growth capacity of MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) are a type of multipotent stem cell with the potential to be used in cancer therapy and inflammatory diseases [1]. MSCs can differentiate into various tissues and secrete extracellular vesicles called secretomes. The secretome contains growth factors, cytokines, active metabolites, and RNA that can be utilized for therapy. The content of the secretome can be determined by examining the molecular composition in its culture medium [2,3]. The secretome of MSCs has immunomodulatory properties and promotes the growth of surrounding cells due to the presence of cytokines and growth factors such as IL-6, IL-8, and TGF-β [4].

Evidence suggests that the secretome produced by MSCs has the ability to repair damaged tissues. Extracellular vesicles from MSCs have been shown to enhance tendon repair in a rat model of Achilles tendon trauma [5]. Another study demonstrated that the secretome from

MSCs has a bone-repairing effect in a rat model of bone growth defect [6]. This repair capability is further supported by in vitro research showing that the secretome activates the PI3K/Akt and FAK/ERK1/2 pathways, which enhance the proliferation and migration of fibroblasts, keratinocytes, and epithelial cells, thereby improving wound healing [7]. Clinical trials have shown that the secretome has a potency to repair tissues and anti-inflammatory effects, making it suitable to be applied for wound healing, cardiovascular, neurodegenerative, and inflammatory diseases. However, clinical trials using the secretome from MSCs have yielded varied results, with some studies showing no successful therapy with the secretome in humans. This discrepancy is due to the differences in cell cycle among individual cells, resulting in variations in secretome secretion and composition in different studies [8,9]. Initial conditioning is needed to standardize the cell cycle in the population of MSCs used, resulting in a more uniform secretome and improving the effectiveness and reliability of the therapy.

One of the conditions that can alter the physiological processes of cells is starvation, a condition of energy deficiency due to a lack of carbohydrates, amino acids, and/or fats for a certain period of time [10,11]. Evidences show that starvation in cells activates autophagy, protein degradation, stress response, and inhibits proliferation. These physiological changes are important for cell survival and maintaining homeostasis [12–14].

Starvation is a common treatment in cellular research. It can alter the physiological processes in cells and is frequently used to study metabolic pathways under stress conditions [10,15]. A study showed that starvation increases the activity of extracellular vesicles and alters the composition of exosomes in stem cells, potentially enhancing the therapeutic value of MSCs [16]. Starvation can stimulate the maturation process of stem cells as it promotes oxidative phosphorylation, the changes of which have an impact on cell differentiation and maturation [17]. Furthermore, the starvation condition induces cells into a quiescent phase, a phase where cell growth does not occur. Unlike senescence, quiescent cells can re-enter the cell cycle with appropriate induction. Researchers utilize this method for cell synchronization, ensuring that cultured cells have similar conditions at the same time [18]. However, prolonged starvation can potentially affect the physiological function of cells and even induce apoptosis and cell death [19].

The influence of starvation on the physiology of MSCs and the molecular composition of extracellular vesicles is not fully understood. Further exploration of optimal starvation methods is required to induce synchronization in MSCs without adverse physiological changes. Therefore, research on the effects of starvation on MSC physiology, particularly MSC growth, is necessary.

2. Methods

2.1. Research Design

This is an experimental study with a post-test-only group design with a control in each experiment. The experiments were conducted at the Research Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman.

2.2. Materials and Methods

The sample used in this study consisted of mesenchymal stem cells (MSCs) obtained from umbilical cords provided by the Stem Cell and Cancer Institute-Kalbe (SCI-Kalbe). The materials used in the research included a 6 cm culture dish (Iwaki), DMEM (Sigma), α-MEM (Sigma), fetal bovine serum (FBS; Sigma), phosphate-buffered saline (PBS; ABclonal), and trypsin-EDTA (Elabscience).

2.3. Cell Culture and Starvation

The cultivation of MSCs was performed in vitro using α-MEM supplemented with 10% fetal bovine serum (FBS) and incubated in a 5% CO2. The cells were subcultured 1-2 times until the MSCs were healthy and grew well. For the intervention, cells were cultured in four 6 cm culture dishes with 120,000 cells per culture dish. After incubation for 24 hours, the cells were washed with PBS twice, and the media were replaced with the following treatment media: DMEM + FBS, α-MEM + FBS, DMEM without FBS, or α-MEM without FBS (**Table 1**).

Table 1. Treatment groups and media composition.

Group	Media	Composition		
		Glucose	Glutamine	Serum
Complete media with high glucose	DMEM + 10% FBS	High	High	High
Complete media with low glucose	α-MEM + 10% FBS	Low	High	High
Serum-depleted media with high glucose	DMEM	High	High	None
Serum-depleted media with low glucose	α-MEM	Low	High	None

2.4. Growth Evaluation

Following the replacement of the treatment media, the subsequent step involved observing the alterations in cell growth. The rate of cell growth was assessed by capturing images at a magnification of 100x using a camera, with 10 fields of view captured for each camera. Images were captured every 24 hours over a 4-day period until the MSCs reached full confluence in the complete medium. Subsequently, the coverage of the fields of view was analyzed using ImageJ software, and a graph was generated. A statistical analysis using the t-test was conducted to determine the significance between the groups.

3. Results And Discussion

The evaluation of MSC growth demonstrated that MSCs can grow in α-MEM or DMEM media in the presence or absence of FBS. MSCs cultured in serum-rich media reached almost confluence by day 3 (Figure 1). The surface coverage area was determined using ImageJ software. All groups exhibited similar coverage areas on day 0, with an average of 38.37±6.54% in α-MEM+FBS, 37.39±6.25% in α-MEM, 35.76±2.48% in DMEM+FBS, and 39.71±3.78% in DMEM (ANOVA; p=0.502). On day 3, cell growth was observed in all media, with the coverage percentages as follows: 97.93±1.73% in α-MEM+FBS, 46.79±2.28% in α-MEM, 94.65±2.28% in DMEM+FBS, and 44.57±4.58% in DMEM (Figure 2). These findings suggest that culture media without serum can still support the growth of MSCs, although at a slower rate compared to media with FBS.

Our results showed that the serum starvation group exhibited a decrease in surface coverage area compared to the control group that was supplemented with serum (**Figure 1**). The analysis result with student's t-test revealed a significant decrease in coverage area in α-MEM compared to α-MEM+FBS ($p<0.0001$) and in DMEM compared to DMEM+FBS ($p<0.001$). These findings suggest that serum starvation inhibits the growth of MSCs, regardless of their glucose concentration. On the other hand, no significant difference in coverage area was observed between α-MEM+FBS and DMEM+FBS ($p=0.323$) or α-MEM and DMEM ($p=0.496$),

suggesting MSCs can grow equally well in media with low glucose α-MEM or high glucose DMEM (**Figure 2**).

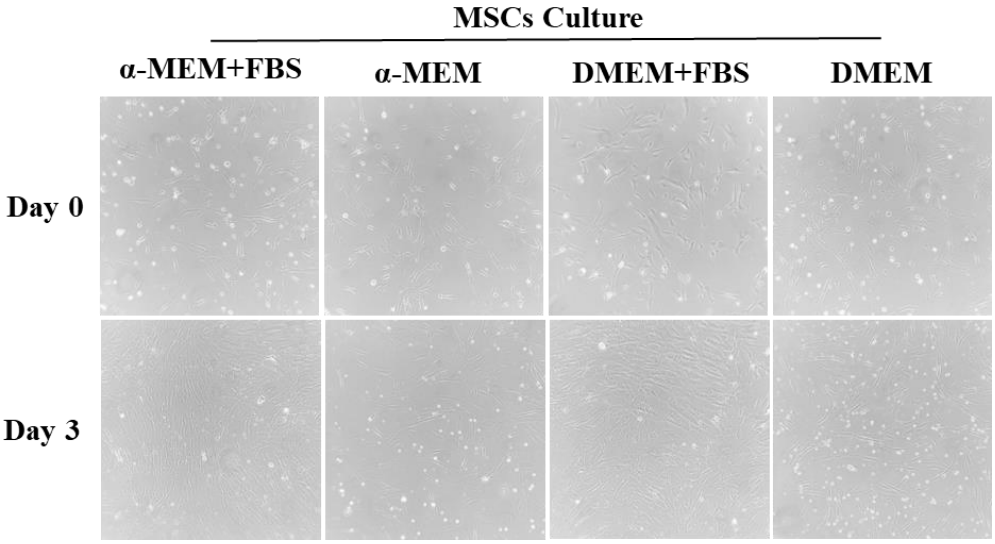

Figure 1. MSC density in media with or without serum. The MSCs were cultured in 6 cm dishes with an initial seeding of 120,000 cells. The MSCs were observed and captured under an inverted light microscope. Day 0 represents 24 hours after subculturing the cells, and day 3 represents three days after day 0.

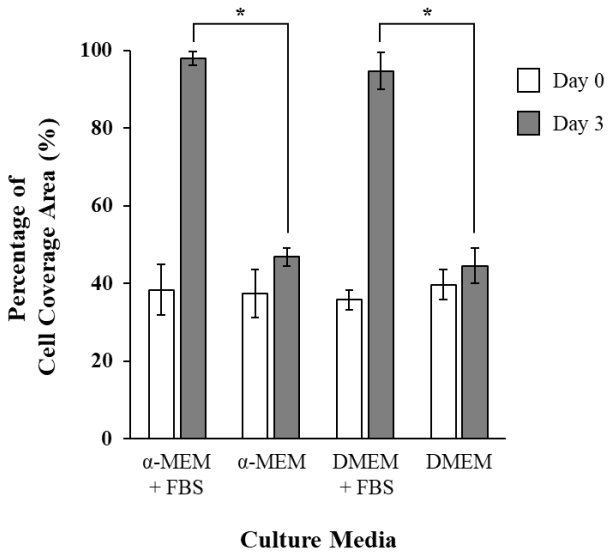

Figure 2. Quantification of the percentage of cell coverage area for MSCs cultured in media with or without serum. Ten field areas were randomly taken from each group, and the coverage area was calculated as the percentage of cell coverage relative to the total field area. Each bar represents the mean percentage of cell coverage area, and error bars represent the standard deviation. Statistical analysis was performed using student's t-test. * $p<0.001$.

Our findings suggest that serum deprivation had a negative impact on the proliferative capacity of MSCs. Serum plays a crucial role in providing essential nutrients, growth factors, and hormones that are necessary for cell growth and proliferation. The presence

of FBS in the complete media used provided a rich source of growth factors, amino acids, and other nutrients, which supported robust cell growth. In contrast, the starvation group was deprived of these essential components, resulting in a less favorable environment for cell proliferation [20].

It is important to note that the inhibitory effect of serum starvation on cell growth observed in this study was relatively modest. This suggests that MSCs may have some degree of adaptability and resilience to survive and grow under unfavorable conditions. Furthermore, reduced growth capacity of MSCs in serum-depleted media implies their entry into the quiescence phase of the cell cycle [21]. However, prolonged serum deprivation or other forms of nutrient deprivation may have more pronounced effects on the growth and viability of MSCs [19]. Further studies are warranted to elucidate the underlying mechanisms involved in the response of MSCs to serum deprivation.

4. Conclusion

Serum starvation resulted in less dense cell growth and decreased surface coverage area in mesenchymal stem cells (MSCs).

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