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OPTIMIZING CULTURE CONDITIONS OF HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS (hUC-MSCs) BY ADJUSTING THE VOLUME OF XENO-FREE SUPPLEMENT SERUM

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SUMMARY

Mesenchymal stem cells (MSCs) have become an effective tool for treating immune-related diseases due to their multilineage potential and immunomodulatory capabilities. However, a high cell dose is frequently essential for stem cell infusion in clinical practice. Therefore, it is necessary to produce sufficient quantities of MSCs while ensuring cell quality for clinical application in humans. To be able to use stem cells in patients requires a more rigorous captive procedure than using a xeno-free medium that does not contain substances derived from the hypothetical allergenic regime. Therefore, current cell culture procedures substitute xeno-free culture media with added supplement serum for the traditional DMEM media with bovine fetal serum (FBS). This switch increased the production cost immensely and made it difficult to produce MSCs on an industrial scale. In this study, we optimized the condition of MSCs' cultures by adjusting the amount of the supplement serum usage to reduce production costs for industrial manufacturing. This is the first study to claim that reducing the amount of xeno-free supplement serum had no effect on the quality of hUC-MSCs isolated from Vietnamese children's umbilical cords.

Keywords: FBS, hUC-MSCs, optimization, supplement serum, xeno-free.

INTRODUCTION

MSCs are adult stem cells that possess self-renewal capacity and exhibit multilineage differentiation. MSCs are isolated from a variety of tissues, such as umbilical cord, bone marrow, adipose tissue, with the ability to differentiate into various cell types, such as osteoblasts, chondrocytes, and adipocytes, depending on the ambient culture conditions. Due to their multilineage potential and immunomodulatory properties, MSCs have become an effective tool for treating

immune-related disease (Pittenger *et al*., 2019) and regenerative medicine. However, in order for regenerative therapy to be effective, each patient required several million cells per kilogram of body weight for each transplantation or local administration (Česen Mazič *et al*., 2018). Therefore, industrial production of MSCs needs to not only meet the required quantity for clinical application but also maintain the quality of the cells. MSCs' self-renewal and differentiation capacity is influenced by a number of factors; one important factor is culture conditions such as pH levels or oxygen concentration (Nikolits *et al*., 2021). Previously, MSC culture media consisted of DMEM high glucose supplement and Fetal Bovine Serum (FBS). For industrial production, xeno-free media with supplement serum StemMacs iPS-Brew XF are chosen as the alternate culture media for the isolated hUC-MSCs because FBS is animal-derived and unsuitable for therapeutic use in humans (Chelladurai *et al., 2021;* Sallée *et al.,* 2014). However, the expense of the xeno-free media is excessively high and has greatly increased the cost of production, as has the high number of cells needed for each patient. In order to lower the cost of cell expansion without interfering with the differentiation capacity and quality of the cells, we optimized the concentration of supplement serum utilized during MSC cell culture in this work.

MATERIALS AND METHOD

hUC-MSCs isolation

Human umbilical cord mesenchymal stem cells (hUC-MSCs) were obtained from umbilical cords collected at the Vietnam Children's Hospital. Isolation and cell culture procedures were carried out according to a protocol (Smith *et al.*, 2017). hUC-MSCs from the 3rd passage were seeded into an 8-well plate, at an initial density of 73000 cells per well.

Xeno-free media preparation and cell expansion

StemMACS iPS-Brew XF media was divided into 6 different 50 ml tubes, each tube was added with different amount of supplement serum, starting from 0 % to 0.02 %, 0.06 %, 0.1 %, 0.14 % (recommended concentration) and 0.18 %. After the supplement serum was added, the culture medium was thoroughly mixed before being added to each well. The cells were monitored and checked every 2 days until the 9th. Cells were placed in a CO_2 incubator at 37^{o}C, 5 % CO_2 and 90 % humidity, and media were changed every 48 hours over the course of the experiment. The viable cell count was measured using a hemocytometer every 48 hours.

hUC-MSCs differentiation

After 9 days, the cells were differentiated into adipocytes using the StemMACS Adipo Diff Media, following the provided protocol from the manufacturer.

RESULTS AND DISCUSSION

Isolation of hMSC from umbilical cords

HPV, HCV, HBV, HIV, endotoxin, and *Mycoplasma* were used to test before and after hMSC isolation. All tests turned out negative (data not shown).

Fig. 1 shows that after 9 days of incubation, the cell proliferation rate in the 0.06% to 0.1% supplement group increased significantly. Meanwhile, in the hUC-MSC cell culture added with a 0.1 percent supplement, the cell confluence at day 9

reached nearly 90 percent, almost the same as the group with the recommended supplement concentration. Over the course of the experiment, the group with lower levels of supplement showed little change in cell proliferation. The proliferated cells were also able to maintain the spindle-shape morphology of normal MSCs. These findings were supported by the quantitative data presented in Figure 2.

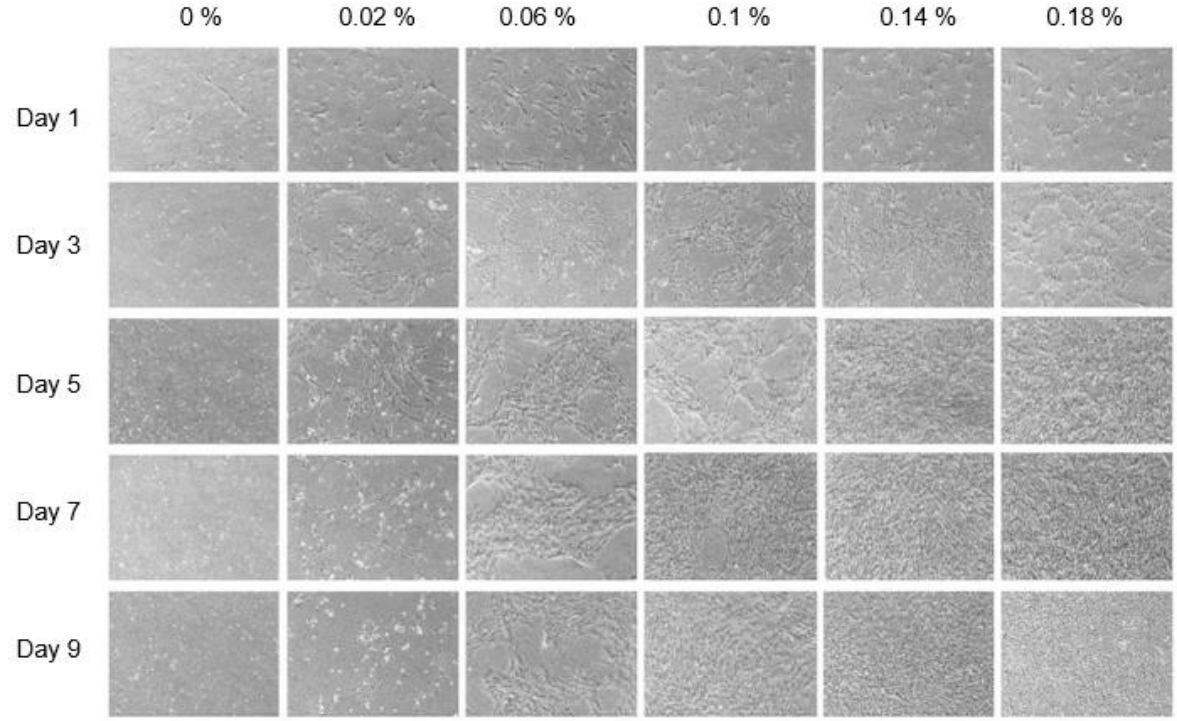

Figure 1. Image of hUC-MSCs cultured in StemMacs media with varying levels of supplements hUC-MSCs at the 3rd passage were cultured in 6 different concentrations of supplement, from left to right, in the order of 0 % to 0.02 %, 0.06 %, 0.1 %, 0.14 %, and 0.18 %, with the 0.14 % being the recommended concentration from the manufacturer. The cells were placed in an incubator at 37^{O}C, 5 % CO_2 and 90 % humidity and changed media every 48 hrs over the course of 9 days.

Cell culture quality under low supplemented conditions

In the 0.1 percent supplemented cell culture, the proliferation rate increased gradually from day 3 until the cell count peaked on day 9, with the number of viable cells equal to the recommended concentration from the manufacturer. The morphology of the cell in the 0.1 percent group recorded on day 7 showed that it was not affected by the decrease in supplement serum and still maintained a regular spindle shape. A similar pattern can be seen in the 0.06 percent supplement group; however, the proliferation rate in this group was slower compared to the percent group, and till day 9, the cell confluence was only at 70 percent.

Adipogenic and osteoblastic differentiation of hUC-MSCs implied that the morphology of the cells after differentiation using StemMACS AdipoDiff Media still retained the naturally long spindle-shape for both the normal adipocytes and osteoblast cells. A decrease in the supplement serum didn't

have any effect on the morphology of the differentiated hUC-MSC cells.

Overall, xeno-free media with a 0.06–0.1% supplement showed the most promising results for hUC-MSC proliferation without compromising the cell proliferation rate or the quality of the cells.

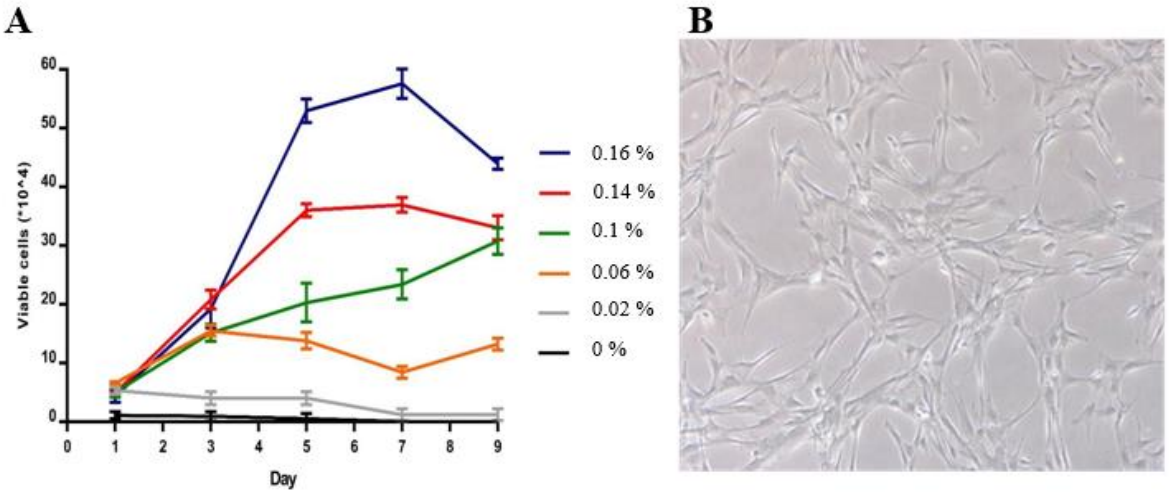

Figure 2. Total number of viable hUC-MSC records during the experiment. **A.** The number of viable cells in each group, counted every 48 hours using the hemocytometers. **B**. Morphology of MSCs in groups with a 0.1 percent supplement added at day 7 of the experiment.

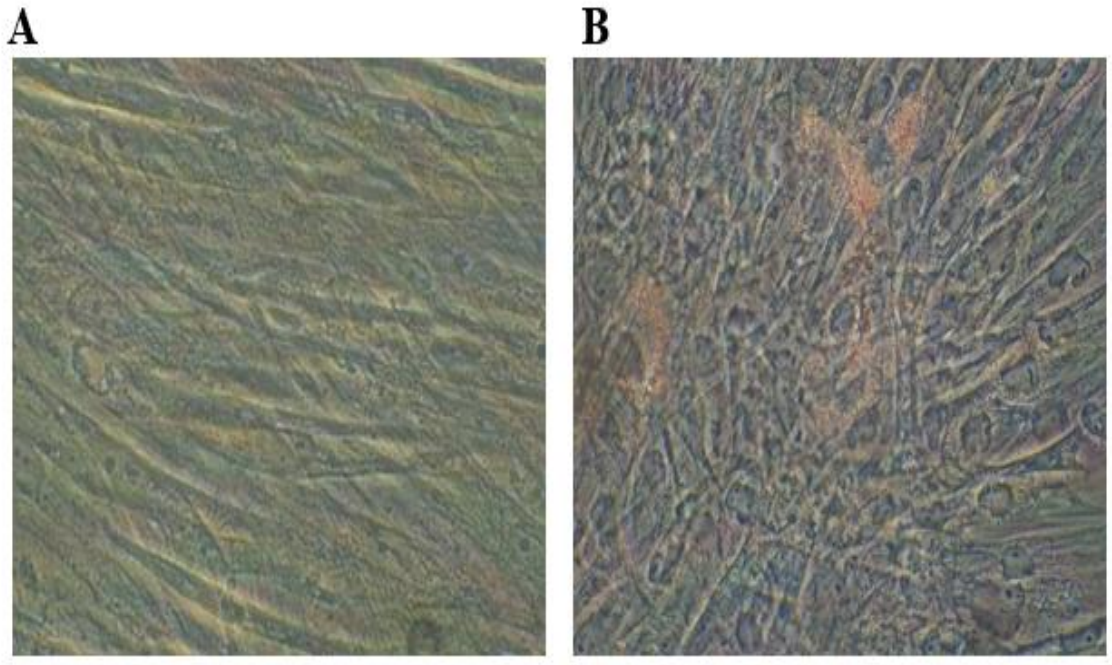

Figure 3. The adipogenic differentiation capacity of hUC-MSCs by Oil-O-Red Staining. A. The control sample of undifferentiated hUC-MSCs.B. Indication of the successful differentiation of hUC-MSCs into adipocytes (pictures taken under 40x magnifications, respectively).

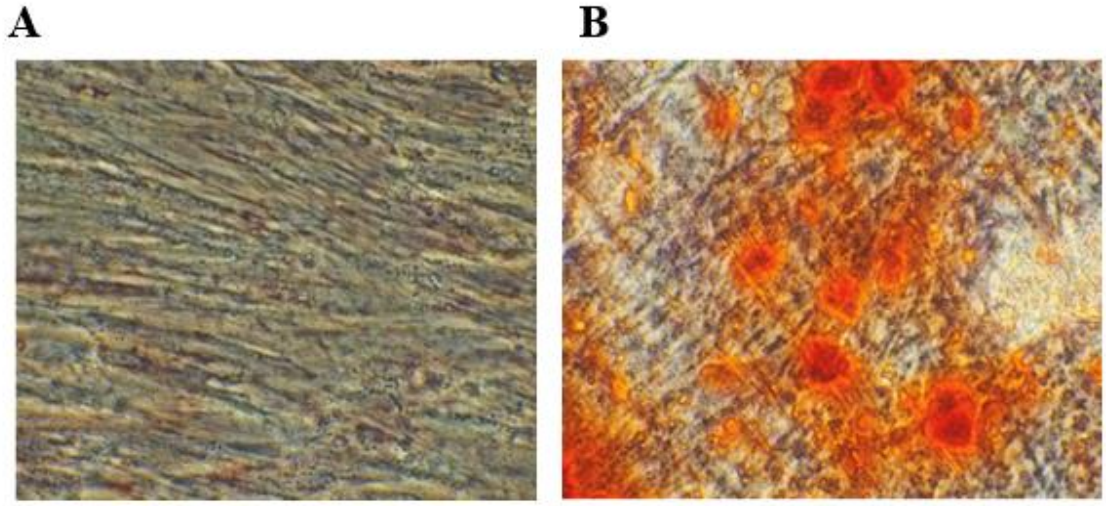

Figure 4. Osteoblastic differentiation of hUC-MSCs using Oil-O-Red Staining. **A.** The control sample of undifferentiated hUC-MSCs; **B**. Indication of the successful differentiation of hUC-MSCs to osteoblasts (pictures taken under 40x magnifications, respectively).

MSCs are a type of multipotent stem cells found in adult tissues and are capable of exerting immunomodulatory effects and promoting tissue regeneration under certain conditions (Patel, Shah, Srivastava 2013). Thus, they have become the forthcoming candidates in regenerative medicine (Cheung *et al.,* 2020; Yang *et al*., 2021). Umbilical cord mesenchymal stem cells possess the same characteristics as MSCs, such as high self-renewal and differentiation potential, as well as immunoregulatory properties and low immunogenicity (Shang *et al*., 2021). In the past, MSCs were cultured in DMEM high glucose media with Fetal Bovine Serum (FBS), a highly rich supplement containing a cocktail of cell attachment proteins, growth factors, and other important biomolecules (Pilgrim *et al.,* 2022). However, since FBS has an animal origin, there is a strong concern regarding contamination with xenogeneic compounds and microbiological contaminants. This may cause cross-specific or zoonotic transmission of unknown pathogens related to the exposure of cells in culture (Tsai *et al.,* 2020). Moreover, the quality of FBS varied between batches, which can cause inconsistency in the growth and quality of MSCs, making them unsuitable for therapeutic application. Another potential alternative is StemMACS iPS-Brew XF, a xeno and animal component-free medium that will eliminate all of the above issues (Cimino *et al.*, 2017). However, since the price of xeno-free media is costly, the production of cell mass using xeno-free media can drive up the cost of the treatment and make it difficult for industrial application. Hence, in this study, we optimized the cell culture process by decreasing the concentration of supplemented xeno-free media in an attempt to minimize the production cost. The cell proliferation rates in the cell culture cultivated with a 0.06–0.1% supplement were indifferent as compared to the group with the recommended concentration. The cell morphology in both groups remained as

usual. According to the findings, the initial reduction in serum supplement concentration had no long-term effects on cell quality or a significant difference in cell proliferation. This study implies that reducing the amount of supplement from the recommended amount to 0.06-0.1% in culture media may be an economical solution to reduce the price of stem cell therapy and regenerative medicine.

CONCLUSION

Using xeno-free expansion media for MSC cell culture in clinical research is one of the optimal methods to ensure the best quality and quantity of the cells. To the best of our knowledge, this is the first study to show that the reduction of the media supplement had no effect on the quality of hUC-MSC isolated from Vietnamese children's umbilical cords. The method is known as being cost-efficient. Thus, the study suggested that minimizing supplement serum was able to solve this problem while ensuring cell culture quality.

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