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# In Vitro Cultivation of 3D Umbilical Cord MSC Spheroid Model for Chondrogenic Differentiation

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**Abstract:** Cartilage is a tissue that specifically difficult to heal when injured due to its avascular, aneural, and alymphatic nature, in addition to the limited ability of cells to migrate in the tissue for repair. Human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) possess promising chondrogenic potential, particularly when cultured in three-dimensional (3D) systems that better mimic the native tissue environment. It is important to note that the current study is limited to an in vitro setting, and no in vivo experiments were conducted. This work aimed to evaluate the ability of hUC-MSCs to undergo chondrogenic differentiation over time using a 3D spheroid culture system. hUC-MSCs were isolated from umbilical cords of two donors, expanded to passage 5, and seeded into ultra-low attachment well plates with chondrogenic differentiation medium. Spheroids were incubated for 14 and 21 days. Chondrogenesis was assessed by crystal violet staining, and the crystal violet-positive area was quantified using ImageJ software. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. hUC-MSCs successfully formed compact spheroids and exhibited progressive matrix accumulation throughout the differentiation period. Compared with baseline (day 0), the crystal violet-positive area increased significantly on day 14 ($36.33 \pm 5.13$, $p<0.01$) and further increased on day 21 ($51.67 \pm 2.08$), with significant differences compared with both day 0 ($p<0.01$) and day 14 ($p<0.01$). These findings demonstrate progressive matrix-associated staining during chondrogenic differentiation. Three-dimensional spheroid culture of hUC-MSCs provides a simple and reproducible in vitro model for investigating the temporal progression of chondrogenic differentiation and may support future studies in cartilage tissue engineering.

**Keywords:** 3D spheroid culture, chondrogenic d ifferentiation, MSCs, umbilical cord.

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## Introduction

Cartilage is a tissue that specifically difficult to heal when injured due to its avascular, aneural, and alymphatic nature, in addition to the limited ability of cells to migrate in the tissue for repair (Zhao et al., 2022). Several treatment efforts for cartilage injury have been carried out using nonoperative treatments, intrinsic repair (fibrocartilage) stimulations, autologous

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chondrocyte implantation (ACI), allograft transplantation, periosteal transfer, and the newer strategies using tissue engineering (TE) (Kwon et al., 2019; Hafezi et al., 2021; Zhou et al., 2023). However, these treatments still have many shortcomings. For example, for ACI procedure the chondrocyte has limited life-span following implantation, because it potentially de-differentiate throughout culture proliferation, ensuing in downregulating of the chondrogenic marker expressions (Y. Liu, Shah, et al., 2021). Furthermore, patients strictly restricted with near-ideal surgical conditions and still have to endure two distinct surgeries and an extended recovery period, potential of surgical infection, thus increasing financial cost (Martín et al., 2019; Chimutengwende-Gordon et al., 2020). Therefore, there is a need to develop a treatment for long-term repair and/or regeneration for cartilage injury.

Mesenchymal stem cells (MSCs), which can be isolated from various human tissue, are multipotent cells with low immunogenicity. The sources of MSCs, such as umbilical cord, nervous tissue, adipose tissue, and amniotic fluid can be isolated and used for various therapeutic applications (Harrell et al., 2020; Shang et al., 2021). Known to have significant self-renewal and multi-lineage differentiate properties (Liu, Li, et al., 2021), umbilical cord MSCs (UC-MSCs) can be easily cultured and have non-invasive procedure compared from other source of MSCs (Shaikh et al., 2022). This ability has encouraging using UC-MSCs for treatment various injuries such as peripheral injury, cartilage injury and spinal cord injury. (L. Huang et al., 2021; Wang et al., 2022). In addition, MSCs are expressing various surface markers, including CD90, CD105, CD73 and slightly/not expressing CD19, CD45, CD34, CD56, CD14, dan HLA-DR (Zha et al., 2021).

Chondrogenesis as an effort to repair cartilage injury has been widely carried out using MSCs. Previous study reported that chondrogenesis were employed by bone marrow MSCs (BM-MSCs) because they were able to differentiate into 3 lineage, including osteogenic, chondrogenic and adipogenic lineage (Wang et al., 2022). However, the utilization of BM-MSCs as a resource of adult MSCs has its limit such as limited intrinsic self-healing capacity, low provision of regenerated chondrocytes and poor matrix productivity to the injured site (Mardones et al., 2020; Xia et al., 2022). In cartilage differentiation, UC-MSCs provide several features, such as synthesis of aggrecan, SOX-9, and type II collagen (Kangari et al., 2020). Furthermore, UC-MSCs also express chemokines, cytokines and other growth factors at levels similar to those cartilage (Russo et al., 2022).

The therapeutic potential of UC-MSCs in its application demands large-scale production to guarantee its availability and affordability. Conventional culture of MSCs still used 2-dimensional (2D) surface-treated containers to facilitate cell attachment, such as a disk or flask. The 2D culture is suitable for performing functional test because of its short time of culture formation, low-cost maintenance and commercially available test and media culture (Kapałczyńska et al., 2018). However the use of 2D culture, specifically on MSC differentiation, is expensive and not suitable for up-scale cell production (Mizukami et al., 2018). Three-dimensional (3D) culture system application is more promising method for scaling up cell production, and has flexibility to maintain dissolved oxygen, thus increasing hydrodynamic stress for cells (Nurhayati et al., 2021). On the other hand, 3D cultures also preserving cell morphology and way of divisions, have proper interaction of cell and cell-extracellular environment and not changing expression of genes, splicing, topology and biochemistry of cells as *in vivo* (Biju et al., 2023; D'Imprima et al., 2023; Farouk et al., 2023).

Previous studies have demonstrated that 3D culture systems offer a more physiologically relevant environment than traditional 2D cultures. For example, Huang et al. (2020) developed a microfluidic hanging-drop system for mesenchymal stem cells, which stabilized droplet formation and maintained stem cell phenotype, highlighting the potential of 3D culture to mimic in vivo conditions (Huang et al., 2020). Similarly, Imamura et al. (2015) showed that dense 3D multicellular spheroids in breast cancer cells better recapitulate features of tumors in vivo, including hypoxia, dormancy, and anti-apoptotic behavior, compared with 2D cultures. These findings indicate that 3D cultures improve in vitro modeling, though most prior studies focused on system optimization or non-cartilage tissues. In contrast, our study evaluates chondrogenic differentiation of hUC-MSC spheroids over 14 and 21 days, using progressive matrix accumulation as a quantitative marker. Previous studies have demonstrated the potential of 3D MSC spheroids. However, comparative observations describing the temporal progression of matrix formation during spheroid culture remain limited, particularly in hUC-MSC-derived spheroids under a simple ultra-low attachment culture system. Therefore, this study aimed to characterize the temporal changes in crystal violet-positive matrix formation during chondrogenic differentiation of hUC-MSC spheroids at defined culture time points.

In this study, evaluate the chondrogenic potential of hUC-MSCs by culturing them as 3D spheroids and assessing their progression toward chondrocyte-like morphology over defined time points.

## Materials and Methods

### Research design

The experimental laboratory study was conducted at the Stem Cell and Cancer Research (SCCR) facility in Semarang, Indonesia, from December 2024 to January 2025. Ethics approval with number No:328/UN18.F7/ETIK/2022 issued by the ethics committee of Fakultas Kedokteran, University of Mataram, Indonesia.

### Isolation and expansion of hUC-MSCs

Umbilical cords (UCs) were collected immediately after full-term delivery from healthy donors after obtaining written informed consent. The isolation of UC-MSCs was carried out following previously established protocols (Liu et al., 2021). The UC-MSCs were cultivated in a flask with DMEM (Sigma-Aldrich, Louis St, MO), supplemented with 1.5% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco™ Invitrogen, NY, USA), 10% FBS (Gibco™ Invitrogen, NY, USA), and 0.25% Amphotericin B (Gibco™ Invitrogen, NY, USA). The cells were incubated at 37°C and 5% CO2, with the culture medium replenished every three days. Once the cells had attained 80% confluence, they were transferred to a fresh flask. UC-MSCs from the 5th passage were used for subsequent experiments.

### 3D chondrogenic differentiation of hUC-MSCs

We subsequently performed the chondrogenic differentiation examination of MSCs at the 3rd passage. UC-MSCs were cultivated in MesenCult™ chondrogenic differentiation basal medium, respectively, enriched with each supplement (Stem Cell Technologies, Singapore), 1% penicillin, 1% L-glutamine, and 0.25% amphotericin B (Gibco) using ultra-low adherent well plates (Stem Cell Technologies, Singapore), incubated at 37°C and 5% $CO_2$. The medium was changed every three days. After 21 days of incubation, the pellets were subjected to violet staining (Sigma-Aldrich, Louis St, MO).

### Crystal violet staining

Crystal violet staining of the 3D cell culture was performed following to the previous established protocol (Feoktistova et al., 2016). Briefly, the cells were rinsed with Phosphate-Buffered Saline (PBS; Sigma-Aldrich, Louis St, MO) and then exposed to 50 µl of 0.5% of crystal violet solution (Sigma-Aldrich, Louis St, MO). After 10 min of staining, excess crystal violet was removed, and the spheroids were observed under an inverted microscope (Zeiss Primovert, Jena, Germany). The crystal violet-positive area was quantified using ImageJ software and expressed as the percentage of the stained area.

### Data Analysis

All statistical analyses were carried out with SPSS version 24 (IBM, NY, USA). All data were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. The values are reported as mean ± SD. P-values $<0.05$ were considered statistically significant.

## Result and Discussion

### The characteristics of hUC-MSCs

To determine the characteristics of hUC-MSCs, we examined the cell morphology with an inverted microscope. The isolated cells displayed characteristics of spindle-shaped and fibroblast-like cells, as shown in Figure 1.

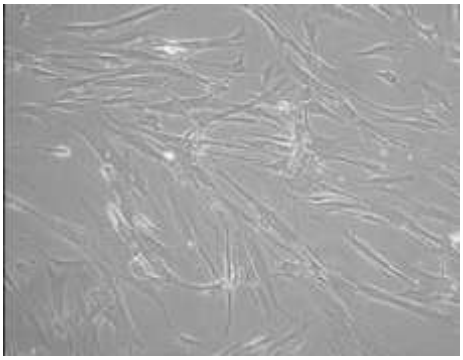

**Figure 1.** The characteristic of isolated UC-MSCs. At 3rd passage, UC-MSCs showed a spindle-shaped and fibroblast-like characteristics (200× magnification).

### 3D Chondrogenic Differentiation of hUC-MSCs

We used chondrogenic differentiation analysis to confirm hUC-MSC differentiation capabilities. hUC-MSCs grew in an ultra-low adherent well plate for 14 and 21 days. Following incubation, hUC-MSCs developed into spheroids and then differentiated into chondrocytes at day 14, characterized by the deposition of collagen, shown in Figure 2A, marked as blue colour. The spheroid shape was getting bigger on day 21 with an expanding blue colour as shown in Figure 2B, indicating the larger formation of spheroid chondrocytes. Compared with baseline (day 0), Figure 2 shows the crystal violet-positive area increased significantly on day 14 ($36.33 \pm 5.13$; $p<0.01$). The stained area continued to increase on day 21 ($51.67 \pm 2.08$), with significant differences observed compared with both baseline ($p<0.01$) and day 14 ($p<0.01$), indicating progressive extracellular matrix formation during chondrogenic differentiation.

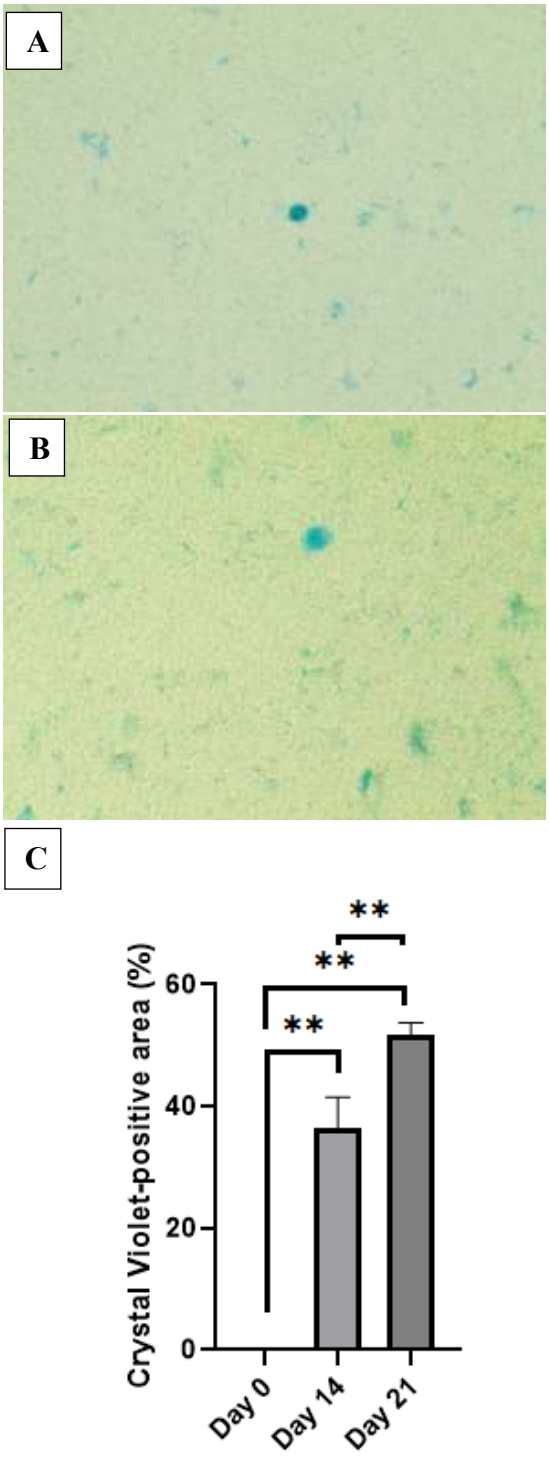


**Figure 2.** Spheroid UC-MSCs differentiation into chondrocytes. UC-MSCs spheroid could differentiate into chondrocytes at day 14 (A) and 21 (B) which marker as blue colour (200× magnification). (C) The graph illustrates the percentage of cartilage that marked as crystal violet-positive areas, highlighting significant differences between days. **p<0.01.

**Discussion**

Perfect culture model should mimic a microenvironment specific to the physiology or pathophysiology of a particular tissue. This environment should facilitate cell proliferation, aggregation, and differentiation (Mebarki et al., 2021). Therefore, 3D culture provides such an environment. In this study, we successfully established hUC-MSCs 3D spheroids culture for chondrogenesis.

The source of MSCs used in cartilage injury repair efforts holds important values (Huang et al., 2022). The use of umbilical cords derived by MSCs possesses longer passage usage than other sources of MSCs, such as BM-MSCs. The senescence of BM-MSCs occurs sooner than that of UC-MSCs (Yea et al., 2023). Significantly, UC-MSCs have a better ability for expansion than BM-MSCs. While BM-MSCs can constantly expand in less than 10 passages, UC-MSCs may actively expand up to 20 passages before their proliferative potential declines (Meesuk et al., 2022). Thus, we use UC-MSCs derived from donor at fifth passage. One of the characteristics of MSCs that are suitable for clinical use requires to have ability to adhere to plastic when cultivated in a standard treatment dish (Chouw et al., 2022). We discover that our hUC-MSCs fit that criteria (Figure 1). For immunomodulatory activities, hUC-MSCs also has expressing higher concentration of TGF-$\beta$2, HGF, IL-8, and IL-10 than of that BM-MSCs (Kim et al., 2018). To sustain the advantages of hUC-MSCs, it is crucial to utilize a culture method that mimics in vivo conditions.

Prominent challenge in the implementation of MSCs technology pertains to the intricate process of effectuating successful engraftment at the designated therapeutic site. This difficulty can be attributed to several factors, such as insufficient growth factors, nutrient inadequacies and suboptimal oxygen conditions which hinder survivability of the cell (Peck et al., 2021). Immunological responses, limited engraftment capabilities and age-related influences on cell function also key challenges of successfully applying MSCs (Li et al., 2020). 3D spheroid cultures may overcome these challenges.

Deng et al., (Deng et al., 2021). successfully engrafting 3D human placenta-derived MSCs (3D-HPMSCs) to attenuate spinal cord injury in mice and retained the advantageous properties in secretion, minimizing lesion cavity and inhibiting inflammation. Therefore, the utilization of 3D spheroids culture in cartilage injury repair is considerably promising. In this study we successfully induced the differentiation of spheroids hUC-MSCs into chondrocytes (Figure 2). Chondrocytes, marks as blue colour forming in day 14 (Figure 2A) and day 21 (Figure 2B), this in accordance

with BM-MSCs usually undergo chondrogenic formation within 2-3 weeks (Solchaga et al., 2011).

Our findings demonstrate a progressive increase in the blue-stained area, indicative of cartilage production, with significant enhancements observed on day 14 and a marked peak by day 21 (Figure 2C). The largest cartilage area on day 21 highlights that the experimental conditions effectively promote cartilage regeneration over time. This trend aligns with previous studies emphasizing the critical role of sustained extracellular matrix (ECM) formation and prolonged cellular activity in cartilage repair (Deng et al., 2021). The observed peak in cartilage formation by day 21 likely reflects the activation of key signaling pathways involved in chondrogenesis and ECM synthesis. These pathways may facilitate the differentiation of progenitor cells and enhance the production of cartilage-specific components such as proteoglycans and collagen. Additionally, adhesion molecules, including integrins and cadherins, likely play an integral role in this process by mediating cell-matrix and cell-cell interactions critical for ECM organization and cartilage stability.

Adhesion and differentiation, particularly cadherin and integrin, have a direct role in the formation of multicellular spheroids (Ryu et al., 2019). During spheroid formation, initially individual cell within the suspension aggregate, giving rise to cell spheroids with loose adhesion. As a result, the extracellular matrix, along with the binding of peripheral cell surfaces to integrins, aids the first cell aggregation (LaFoya et al., 2018). Following this, E-cadherin strengthens adhesion in the initial cell aggregation occurs through the homophilic binding of cadherins on peripheral cells (Zhang et al., 2019). The β-catenin complex also plays a crucial role facilitating cellular signal transduction (J. Liu et al., 2022). Study shows both cadherin and integrin are enhances chondrogenesis (Dieterle et al., 2021; Ke et al., 2022). Therefore, using 3D spheroids culture method as chondrogenesis is promising in an efficient and cost-effective way. Limitations of this study included MSC characterization according to the ISCT minimal criteria, including immunophenotyping and trilineage differentiation assays, was not performed in the present study. Second, chondrogenic differentiation was evaluated using crystal violet staining, which reflects matrix-associated staining but is not specific for cartilage extracellular matrix. Future studies should include cartilage-specific histological and molecular markers to validate chondrogenesis more comprehensively.

## Conclusion

Chondrogenic differentiation of hUC-MSCs in a 3D spheroid culture system was successfully established. Progressive matrix accumulation was observed by day 14 and increased significantly by day 21. These findings suggest that sequential induction in a 3D spheroid culture system promotes extracellular matrix formation and may provide a useful in vitro platform for investigating the mechanisms underlying chondrogenesis.

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