BIOMATERIAL HYDROGEL SUPPORTS THE 3D CULTURE SYSTEM FOR THE MAINTENANCE OF STEMNESS IN HUMAN MESENCHYMAL STEM CELLS

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การพัฒนาระบบการเพาะเลี้ยงแบบสามมิติโดยใช้ไฮโดรเจล เพื่อรักษาคุณสมบัติความเป็นเซลล์ต้นกำเนิดของเซลล์ต้นกำเนิดชนิดมีเซนไคน์

นายอมร แพ่งจันทึก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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อมร แพ่งจันทึก: การพัฒนาระบบการเพาะเลี้ยงแบบสามมิติโดยใช้ไฮโดรเจลเพื่อรักษาคุณสมบัติความเป็นเซลล์ต้นกำเนิดของเซลล์ต้นกำเนิดชนิดมีเซนไคน์ (BIOMATERIAL HYDROGEL SUPPORTS THE 3D CULTURE SYSTEM FOR THE MAINTENANCE OF STEMNESS IN HUMAN MESENCHYMAL STEM CELLSFFECT) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.ปริญญา น้อยสา, 65 หน้า.

คำสำคัญ: วัสดุทางชีวภาพ/ไฮโดรเจล/การเพาะเลี้ยงเซลล์แบบสามมิติ/เซลล์ต้นกำเนิดของเซลล์ต้นกำเนิดชนิดมีเซนไคน์/การคงคุณสมบัติความเป็นเซลล์ต้นกำเนิด/การสร้างหลอดเลือด

ระบบการเพาะเลี้ยงเซลล์แบบสามมิติถูกนำมาใช้เพื่อตรวจสอบเซลล์บำบัดและการซ่อมแซมเนื้อเยื่อ เนื่องจากสามารถเลียนแบบการเจริญเติบโตกับเซลล์ในร่างกายได้ ในการศึกษาครั้งนี้เพื่อพัฒนาวัสดุชีวภาพจากธรรมชาติ ได้แก่ อัลจิเนต กรดไฮยาลูโรนิก และเจลาติน เพื่อเลียนแบบการสร้างสภาพแวดล้อมนอกเซลล์ของเซลล์ต้นกำเนิดชนิดมีเซนไคน์แบบสามมิติและเพื่อเพิ่มประสิทธิภาพการแบ่งตัวเพิ่มจำนวนของเซลล์ในไฮโดรเจลแบบสามมิติ อัลจิเนต-กรดไฮยาลูโรนิกไฮโดรเจลอัลแบบสามมิติถูกสร้างขึ้นสำหรับใช้เป็นระบบการเพาะเลี้ยงเซลล์ต้นกำเนิดชนิดมีเซนไคน์ของมนุษย์และการตรวจสอบถูกวิเคราะห์โดยการเพิ่มจำนวนเซลล์ ความมีชีวิตของเซลล์ การรักษาคุณสมบัติความเป็นเซลล์ต้นกำเนิด การกระตุ้นของเทโลเมียร์ และการสร้างเส้นเลือดใหม่เปรียบเทียบกับการเพาะเลี้ยงเซลล์แบบสองมิติ และทำการตรวจสอบคุณสมบัติของไฮโดรเจล การเพิ่มจำนวนเซลล์ การย้อมสีเซลล์ที่มีชีวิตและไม่มีชีวิต การแสดงออกของยีน ความยาวสัมพัทธ์ของเทโลเมียร์ และการย้อมอิมมูโนฟลูออเรสเซนซ์ ผลการวิจัยพบว่าอัลจิเนต- กรดไฮยาลูโรนิกไฮโดรเจลแบบสามมิติเพิ่มการเพิ่มจำนวนเซลล์อย่างมีนัยสำคัญ เซลล์เจริญเติบโตเป็นเซลล์ทรงกลมที่เพาะเลี้ยงอย่างต่อเนื่องและมีอัตราการรอดชีวิตสูงถึงร้อยละ 77.36 หลังจากการเพาะเลี้ยงเป็นเวลา 14 วัน นอกจากนี้ แอลจิเนต-กรดไฮยาลูโรนิกไฮโดรเจลอัลแบบสามมิติยังเพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับการคงคุณสมบัติความเป็นเซลล์ต้นกำเนิด (*Oct-4*, *NANOG*, *SOX2* และ *SIRT1*), ยีนที่เกี่ยวข้องกับการเพิ่มจำนวนเซลล์ (*Ki67*), ยีนกระตุ้นการเจริญเติบโตของเนื้อเยื่อ (*YAP* และ *TAZ*) และกระตุ้นกิจกรรมของเทโลเมียร์ในการเพาะเลี้ยงเป็นเวลา 14 วัน นอกจากนี้ การกระตุ้นการสร้างหลอดเลือดของเซลล์ต้นกำเนิดชนิดมีเซนไคน์แบบสามมิติถูกควบคุมโดยการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างหลอดเลือดใหม่ (*VEGF* และ *VCAM-1*) เมื่อเปรียบเทียบกับการเพาะเลี้ยงเซลล์แบบสองมิติ ข้อมูลเหล่านี้บ่งชี้ว่าให้เห็นว่าอัลจิเนต-กรดไฮยาลูโรนิกไฮโดรเจลแบบสามมิติ สามารถทำหน้าที่เป็นตัวพาที่มีแนวโน้มในการรักษาและคงคุณสมบัติความเป็นเซลล์ต้นกำเนิดและใช้ตรวจสอบเกี่ยวกับวิศวกรรมเนื้อเยื่อต่างๆ

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา อมร แพ่งจันทึก

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา

AMORN PANGJANTUK: BIOMATERIAL HYDROGEL SUPPORTS THE 3D CULTURE SYSTEM FOR THE MAINTENANCE OF STEMNESS IN HUMAN MESENCHYMAL STEM CELLS. THESIS ADVISOR: ASSOC. PROF. PARINYA NOISA, Ph.D., 65 PP.

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The three-dimensional (3D) cell culture system is being employed more frequently to investigate cell engineering and tissue repair due to its close mimicry of *in vivo* cells. In this study, we developed natural biomaterials, including hyaluronic acid, alginate, and gelatin, to mimic the creation of a 3D MSCs (Mesenchymal Stem Cells) extracellular environment and selected hydrogels with high proliferation capacity for 3D MSC culture. The hydrogels were created for human mesenchymal stem cells (MSCs) culture, and an investigation was conducted into the effects on cell viability and proliferation, stemness properties, telomere activity, and angiogenesis activation compared to the 2D monolayer culture. Hydrogel characterization, cell proliferation, live/dead staining, gene expression, telomere relative length, and immunofluorescence staining were examined. The results showed that 3D alginate-hyaluronic acid (AL-HA) hydrogels increased cell proliferation, and the cells were grown as cellular spheroids with continuous culture and presented a high survival rate of 77.36% during the culture period of 14 days. Furthermore, the 3D alginate-hyaluronic acid (AL-HA) hydrogels increased the expression of stemness-related genes (*Oct-4*, *NANOG*, *SOX2*, and *SIRT1*), tissue growth genes (*YAP* and *TAZ*), and cell proliferation genes (*Ki67*) and the telomere activity after culture for 14 days. Moreover, the angiogenesis activation of the 3D MSCs was enhanced, as indicated by the upregulation of the angiogenesis related gene (*VEGF* and *VCAM-1*) compared to the 2D monolayer culture. Altogether, these data suggested that the 3D alginate-hyaluronic acid (AL-HA) hydrogels could serve as a promising carrier for maintenance stem cell properties and might be suitable for various tissue engineering proposals.

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LIST OF ABBREVIATIONS

%	=	Percent
°C	=	Degree Celsius
V	=	Volume
L	=	Liter
G	=	Gram
µg	=	Microgram
Nm	=	Nanometer
µm	=	Micrometer
Min	=	Minute
S	=	Second
ml	=	Milliliter
AL	=	Alginate
HA	=	Hyaluronic acid
GE	=	Gelatin
2D	=	Two-dimensional
3D	=	Three-dimensional
PCR	=	Polymerase chain reaction
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
cDNA	=	Complementary DNA
Eq	=	Equation

LIST OF ABBREVIATIONS (Continued)

Ct	=	Cycle threshold
mRNA	=	Messenger RNA
SIRT1	=	Sirtuin1

CHAPTER 1
INTRODUCTION

1.1 Significance of the research

Cell therapy using human mesenchymal stem cells (MSCs) has indeed been extensively studied in the fields of tissue engineering and regenerative medicine (Brown et al., 2019). MSCs have demonstrated therapeutic potential in the treatment of a wide range of disorders, including musculoskeletal deviations, osteoarthritis, and neurological disorders (Centeno et al., 2015). MSCs are a type of adult stem cell that can be isolated from various sources, such as bone marrow, umbilical cord blood, and adipose tissue. MSCs exhibited the ability to self-renew and differentiate into various cell types, including, osteocytes, chondrocytes, cardiomyocytes, and adipocytes (Kangari et al., 2020). MSCs have been shown to encourage tissue repair by reducing inflammation and angiogenesis(Lee et al., 2016). Additionally, they can also secrete factors that enhance cell function and stimulate endogenous repair mechanisms (Gnecchi et al., 2016). Due to limited cell survival, poor cell engraftment, and a lack of site-specificity, stem cell-based treatment is frequently futile (Noronha et al., 2019) (Choe et al., 2018). Therefore, it is imperative to develop methods and efficient cell culture systems to enhance cell survival and function by creating a specific environment that is as close as possible to in vivo.

For cell culture investigations, traditional two-dimensional (2D) cell culture models have been adopted because of their simplicity and convenience. Cells are grown as a monolayer on a flat surface, typically a plastic flask or dish (Kapałczyńska et al., 2018). While 2D culture has been widely adopted due to its simplicity and convenience, it has limitations when it comes to mimicking the complex cellular environment found in vivo. Cell attachment occurs on only one side of the cell, and cell-cell interactions primarily happen at the perimeter of the monolayer, leading to a lack of cell complexity and limited cell-matrix interactions (Jensen and Teng, 2020).

In contrast, 3D cell culture models provide a more physiologically relevant environment for cells by allowing them to grow in three dimensions, mimicking the spatial arrangement, cell-cell communication, and cell-matrix interactions found in vivo (Foglietta et al., 2020; Lv et al., 2017). In a 3D culture system, cells are encapsulated within a hydrogel or scaffold that provides structural support and mimics the characteristics of the extracellular matrix (ECM) (Jiang et al., 2019). The hydrogel can be designed to have similar mechanical properties and biochemical cues as the native tissue ECM, enabling better cell-matrix interactions (Munoz-Robles et al., 2020). Thus, 3D cell culture is an alternative to cell studies and research because it is consistent and close to in vivo.

Hydrogels are commonly employed as scaffolds for cell therapy, drug delivery, and tissue engineering because of their adaptability, biocompatibility, and ease of production (Zhang et al., 2018). Hydrogels with high hydrophilicity are biocompatible and can imitate natural tissues (Ghasemiyeh and Mohammadi-Samani, 2019). Hydrogels have been frequently used to create artificial ECM for in vitro studies of cellular function (Nicolas et al., 2020). One of the most used biomaterials is alginate, which comes from brown algae and is a natural heteropolysaccharide. Because of its biocompatibility, nontoxicity, and low immunogenicity, alginate has been used in a wide range of tissue engineering applications (Li et al., 2018). Alginate is shown to enhance cell viability, migration, and bone regeneration in vitro (Ho et al., 2016). Another natural hydrogel biomaterial that is a component of the extracellular matrix and cartilage matrix is hyaluronic acid (HA), a linear polyanionic polysaccharide consisting of N-acetyl-glucosamine (Pérez et al., 2021). Hyaluronic acid has a broad range of biological properties that are linked to angiogenesis, anti-inflammatory action, and chondrocyte differentiation (Ren et al., 2021). And gelatin, which is formed by the hydrolysis of collagen protein. gelatin biomaterials, which exhibit cross-linking and non-immunogenicity (Pires et al., 2023). These biomaterials enhanced the function of supporting cell attachment and cell retention in various cell types (Mehdizadeh Omrani et al., 2021; Bello et al., 2020). The choice of biomaterials as carriers for cell therapy strategies targeted at tissue regeneration has mostly been influenced by their extracellular matrix-like properties. Therefore, the natural biomaterials (alginate, hyaluronic acid, and gelatin) are excellent materials for the development of a 3D

extracellular microenvironment for investigating the functions of cell survival and MSC stemness.

In this present study, we design and develop natural biomaterials, including alginate hyaluronic acid, and gelatin, to mimic the creation of 3D MSCs extracellular microenvironment and optimize cell proliferation in 3D hydrogel MSC culture. Then, we selected the high proliferation of 3D hydrogel to investigate the proliferation capacity and survival of MSCs in 3D hydrogels, which will be verified in comparison with the 2D monolayer culture. Finally, to verify whether 3D hydrogels can act as a potential effective way to maintain pluripotency, exhibit telomere activity, and activate angiogenesis, the expression of stemness-related genes, CD MSC-surface genes, cell proliferation genes, pro-angiogenesis genes, and telomere length activity on MSCs in the 3D hydrogels will be evaluated compared to the 2D cell culture conditions.

1.2 Research objectives

1.2.1 To design a biomaterial hydrogel (hyaluronic acid, alginate, and gelatin) for optimizing cell proliferation in 3D hydrogel MSC culture.

1.2.2 To study the physical and mechanical properties of the hydrogel used as a 3D MSC culture.

1.2.3 To investigate the effective cellular activities of 3D hydrogels to enhance survival and maintain stemness of MSCs.

1.2.4 To establish the MSC culture to induce angiogenesis activation by using a 3D hydrogel.

1.3 Research hypothesis

It is hypothesized in this study to design and develop biomaterials (alginate, hyaluronic acid, and gelatin) to imitate the formation of 3D MSCs in their extracellular environment and then, we selected the highest proliferation of 3D hydrogel to investigate the survival, maintenance of stem cell pluripotency, telomere activity, and induction of angiogenesis of MSCs because the 3D hydrogel determines the stem cell fate and supports adhesion to the surrounding matrix. Therefore, it can enhance cell-cell communication and cell-matrix interaction. The results indicate that 3D hydrogels

can support cell proliferation and cell viability during the continuous culture of MSCs. The 3D hydrogels increased the proliferation capacity and cell by enhancing the proliferation gene (*Ki67*) and tissue growth genes (*YAP* and *TAZ*) and the maintenance of stemness in MSCs is supported by upregulating the gene and protein expression of MSC stemness-related genes (*SOX2*, *OCT4*, *NANOG*, and *SIRT1*). The telomere activity shown by the increased expression of the hTERT gene, the relative telomere length (*T/S ratio*), and the angiogenesis activation will be determined by increasing the expression of the pro-angiogenesis gene in the 3D hydrogel systems and comparing them to the cells in 2D culture.

1.4 Scope of thesis

The aim of this research is to develop natural biomaterials, including hyaluronic acid, alginate, and gelatin, to mimic the development of 3D MSC extracellular matrix and enhance cell proliferation in 3D hydrogel MSC culture. Natural biomaterials such as hyaluronic acid, alginate, and gelatin are being developed in this study. Then, we chose a 3D hydrogel with a high rate of proliferation to examine MSC survival and proliferation, which was validated in contrast to a 2D monolayer culture. Finally, the expression of stemness-related genes, CD MSC-surface genes, cell proliferation genes, pro-angiogenesis genes, and telomere length activity on MSCs was assessed in comparison to the 2D cell culture conditions to determine whether 3D hydrogels can act as a potential effective way to maintain pluripotency, exhibit telomere activity, and activate angiogenesis.

1.5 Expected results

This study attempted to create a 3D system for growing mesenchymal stem cells, supported their ability to proliferate, and demonstrated high MSC survival rates in long-term cell culture. MSCs' stemness characteristics, proliferation, and telomere activity were improved in the 3D AL/HA hydrogel systems. After that, in comparison to 2D cell culture settings, the 3D AL/HA hydrogels improved the ability to regenerate. This suggests that the 3D AL/HA culture environment promoted stemness maintenance and angiogenesis activation, potentially enhancing MSCs' capacity for tissue regeneration.

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CHAPTER 2
LITERATURE REVIEW

2.1 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are a type of adult stem cell that can be isolated from various sources, including umbilical cord blood, bone marrow, and adipose tissue. MSCs have gained significant attention in regenerative medicine due to their unique characteristics and therapeutic potential. The key features and functions of MSCs are self-renewal and differentiation (Dorronsoro et al., 2021). MSCs possess the ability to self-renew, meaning they can undergo numerous cell divisions while maintaining their undifferentiated state (Ferreira-Faria et al., 2022). Additionally, MSCs have the capacity to differentiate into multiple cell types of mesenchymal origin, including osteocytes (bone cells), neurocytes (nerve cells), chondrocytes (cartilage cells), cardiomyocytes (heart muscle cells), and adipocytes (fat cells) (Arjmand et al., 2019; Chen, Lai et al., 2019). This multilineage differentiation potential makes MSCs valuable for tissue regeneration and repair. MSCs have been shown to contribute to tissue repair processes through several mechanisms. MSCs can migrate to the site of injury or inflammation due to their homing abilities, allowing them to reach the damaged tissue (Markov et al., 2021). MSCs possess immunomodulatory properties, which means they can modulate the immune response by reducing inflammation and promoting immune tolerance (Wu et al., 2020). This anti-inflammatory effect can create a favorable environment for tissue repair (Arabpour et al., 2021). Moreover, MSCs can secrete various factors that stimulate angiogenesis, the formation of new blood vessels, which is essential for supplying oxygen and nutrients to the regenerating tissue (Shineh et al., 2023).

2.2 The application of MSCs

MSCs have significant potential for various applications in regenerative medicine, tissue engineering, toxicology screening and disease models.

2.2.1 Regenerative medicine

MSCs can differentiate into various cell types and can be utilized for tissue repair and regeneration. By guiding the differentiation of stem cells into specific cell lineages, they can be used to generate functional cells for transplantation, such as cardiomyocytes for heart regeneration or neurons for nervous system repair (Siehler et al., 2021). Stem cells can also be used to develop engineered tissues and organs in the laboratory, which can potentially overcome the limitations of donor organ availability (Goulart, 2022).

2.2.2 Tissue engineering applications

MSCs can be combined with scaffolds or hydrogels to create tissue constructs in the laboratory. These constructs can then be implanted into the body to replace or repair damaged tissues. MSCs can interact with the scaffold or hydrogel, proliferate, and differentiate into specific cell types, leading to the formation of functional tissue (Taheri et al., 2023).

2.2.3 Disease models

Stem cells, including induced pluripotent stem cells (iPSCs), can be derived from patients with specific diseases (Kim et al., 2022). These patient-specific stem cells can then be differentiated into disease-relevant cell types to create in vitro disease models. These models provide a valuable platform for studying disease mechanisms, identifying therapeutic targets, and testing the efficacy of potential drugs. They can also help in personalized medicine by allowing the development of patient-specific treatment approaches (Hu and Lazar, 2022).

2.2.4 Toxicology screening

MSCs and their differentiated progeny can be used for toxicology screening to assess the safety and potential toxic effects of drugs, chemicals, and environmental

compounds. By exposing stem cells or specific cell types derived from them to various compounds, researchers can evaluate their impact on cell viability, functionality, and potential adverse effects. This enables the identification of potential toxicants and the development of safer drugs and chemicals (Bhonde et al., 2021).

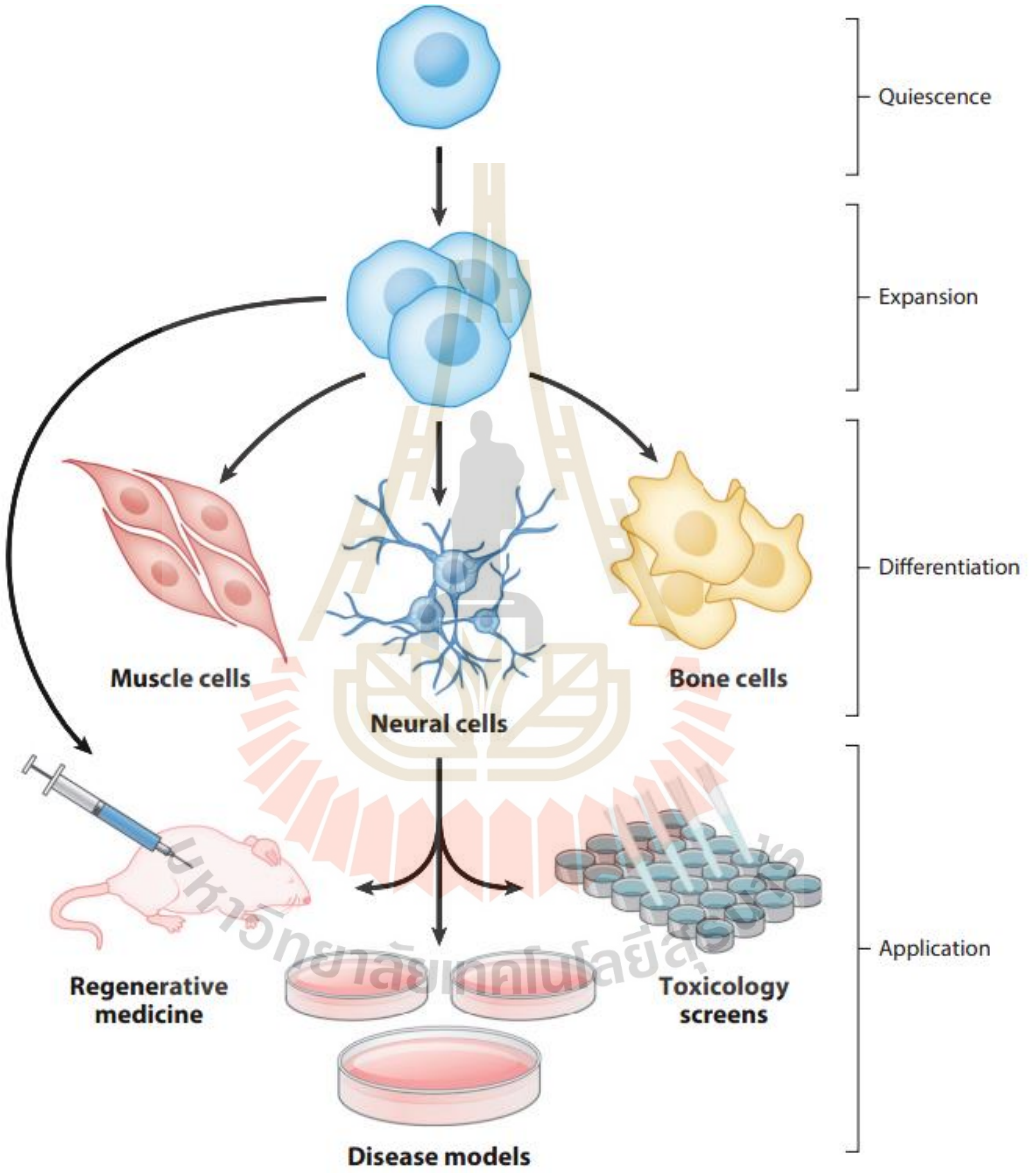

Figure 2.1 The roles and applications of Mesenchymal stem cells (MSCs) (Madl et al., 2018).

2.3 MSC-based therapies

The clinical applications of MSC cell-based therapies have showed promise, particularly for the treatment of illnesses and injuries that are resistant to conventional chemotherapy (Zhuang et al., 2021). MSCs research has explored the use of cells to treat a wide range of conditions, including cardiovascular disease, neurological abnormalities, musculoskeletal deformities, and osteoarthritis (Jovic et al., 2022) (Alizadeh et al., 2019). The ability of MSCs to differentiate into various cell types in response to appropriate signaling cues and their capacity to integrate into host tissues enable them to play a unique role in the development of functional cells and tissues (Xia et al., 2018). The ability of MSCs to regenerate tissue has created new opportunities for therapeutic interventions.

In addition to having the ability to differentiate, MSCs release a wide range of bioactive chemicals and growth factors that are essential for cell survival, tissue regeneration, and controlling the local microenvironment (Dong et al., 2020). These paracrine processes cause the production of cytokines, chemokines, growth factors, and extracellular vesicles, which can encourage angiogenesis (the creation of new blood vessels), decrease inflammation, activate endogenous tissue repair systems, and improve tissue regeneration (Dabrowska et al., 2019). It is evident that these paracrine processes contribute to the therapeutic effectiveness of several MSC-based therapies. The transplanted MSCs influence the surrounding cells and tissues rather than directly replacing diseased or defective cells, which promotes enhanced angiogenesis, tissue remodeling, and functional recovery (Ahangar et al., 2020). The possibility for substantial tissue regeneration without the necessity for intensive engraftment of the donated cells is one benefit of this through this mechanism of action.

MSC-based therapies have been researched in the field of cardiovascular disease to improve cardiac function following myocardial infarction (heart attack). The secreted substances by the transplanted MSCs may encourage the development of new blood vessels, prevent the formation of scar tissue, and enhance the contractile activity of the heart (Huang and Li, 2008). Like this, MSCs in neurological disorders can release neurotrophic substances that promote endogenous repair mechanisms and maintain neuronal survival (Andrzejewska et al., 2021) The affected area. These transplanted

cells can differentiate into the desired cell types and contribute to tissue regeneration and repair. Moreover, they can release paracrine factors that promote angiogenesis, reduce inflammation, and stimulate the local microenvironment to support tissue healing and osteoarthritis, a degenerative joint disease, has also been a major focus of MSC research. In osteoarthritis, the cartilage that cushions the joints progressively breaks down, leading to pain, stiffness, and loss of joint function. MSC-based therapies offer a potential approach to regenerate damaged cartilage and alleviate the symptoms of osteoarthritis (Toh et al., 2017). Overall, MSC-based treatments have shown promise in leveraging the regenerative potential of MSCs and their paracrine effects to enhance tissue repair and functional recovery. However, while MSC-based therapies for treatment of disease show promise, further research is needed to optimize the protocols, standardize the procedures, and ensure their long-term safety and effectiveness. Clinical trials to evaluate the efficacy and safety of these therapies.

2.4 Angiogenesis

2.4.1 Angiogenesis process

Angiogenesis plays a significant role in various physiological processes, including embryogenesis, organ growth, and wound healing. It is a vital process in the development and maintenance of tissues and organs. Angiogenesis is essential for supplying oxygen and nutrients to the healing area during wound healing and tissue restoration. To regenerate and repair tissue, immune cells, growth factors, and other critical chemicals must be delivered. This is made possible by the development of new blood vessels. The removal of waste and poisons from the healing site is another benefit of angiogenesis (Ismail et al., 2021)

The process of angiogenesis includes several steps, including the activation and proliferation of endothelial cells (the cells lining blood vessels), the breakdown of the extracellular matrix that already exists, the migration of endothelial cells to form new vessels, and the development of a functional vascular network. (Guo and Mo, 2021)The creation of blood vessels is balanced and tightly controlled by several pro- and anti-angiogenic elements.

In pathological conditions such as cancer, abnormal angiogenesis can occur, leading to the formation of excessive and disorganized blood vessels. This abnormal angiogenesis plays a critical role in tumor growth and metastasis by supplying the tumor with nutrients and facilitating its spread to other parts of the body (Huang et al., 2021). Developing therapeutic interventions to promote wound healing, tissue regeneration and treat diseases related to angiogenesis, such as cancer and cardiovascular disorders.

2.4.2 Angiogenesis and MSCs

Angiogenesis plays a crucial role in various physiological and pathological processes, including tissue repair and the growth of solid tumors. MSCs have been found to secrete a range of bioactive molecules that can stimulate angiogenesis and facilitate the formation of new blood vessels (Kastana et al., 2019). The key aspects of MSCs and angiogenesis including, paracrine factors, extracellular vesicles, and immune modulation.

Paracrine Factors: MSCs secrete a variety of growth factors, cytokines, and chemokines that can influence the behavior of endothelial cells, which line the inner surface of blood vessels (Li et al., 2022). These paracrine factors include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and angiopoietin-1 (Ang-1), among others. These factors promote endothelial cell proliferation, migration, and the formation of tube-like structures. In addition to these factors, MSCs secrete various other cytokines and chemokines, such as interleukins (IL-6, IL-8), tumor necrosis factor-alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1). These factors contribute to the regulation of endothelial cell behavior by promoting cell recruitment, proliferation, and migration, and by modulating the local microenvironment. Overall, the paracrine factors secreted by MSCs play a vital role in promoting endothelial cell activities necessary for angiogenesis, including proliferation, migration, and tube formation, thereby facilitating the formation of new blood vessels and tissue repair.

Extracellular Vesicles (EV): MSCs release extracellular vesicles, including exosomes and macrovesicles, which carry various bioactive molecules, including proteins, nucleic acids, and lipids. These extracellular vesicles can be taken up by

proteins, nucleic acids, and lipids. These extracellular vesicles can be taken up by endothelial cells and deliver their cargo, thereby modulating angiogenic processes (Lucotti et al., 2022).They can transfer specific miRNAs (microRNAs) that regulate gene expression in recipient cells, promoting angiogenesis. These EVs play a crucial role in intercellular communication by transferring various bioactive molecules, such as proteins, lipids, nucleic acids (including miRNAs), and growth factors, to recipient cells. In the context of angiogenesis, MSC-derived EVs have been found to be enriched in angiogenic factors, including cytokines, chemokines, and growth factors that promote blood vessel formation. When these EVs are taken up by recipient endothelial cells, they initiate proangiogenic signaling pathways, stimulating the expression of angiogenic factors, and promoting the formation of new blood vessels. Hypoxia, or low oxygen conditions, is a potent inducer of angiogenesis. In response to hypoxia, MSCs release EVs that possess an increased angiogenic potency (Jensen and Teng, 2020). These hypoxic MSC-derived EVs can activate specific signaling pathways within recipient endothelial cells, leading to the upregulation of angiogenic factors and the promotion of angiogenesis in the surrounding tissue (Figure 2.2). Overall, the transfer of MSC-derived EV cargo to recipient endothelial cells through paracrine signaling is an essential mechanism by which MSCs contribute to angiogenesis and tissue repair processes. The cargo carried by these EVs, including angiogenic factors, miRNAs, and lipids, modulates the behavior of endothelial cells and supports the formation of new blood vessels, ultimately aiding in tissue regeneration and repair.

Immune Modulation: MSCs possess immunomodulatory properties, and their interaction with immune cells can indirectly influence angiogenesis. By modulating the immune response and reducing inflammation, MSCs create an environment that is conducive to angiogenesis. (Chandra, Mankuzhy, & Sharma G, 2022). Inflammatory factors in the microenvironment can inhibit angiogenesis and impede the formation of new blood vessels. MSCs can suppress inflammation by secreting anti-inflammatory cytokines and modulating the activity of immune cells (R. Li et al., 2022). MSCs can interact with immune cells, such as macrophages and T cells, and modulate their activity. MSCs can induce a shift in macrophages from a pro-inflammatory (M1) phenotype to an anti-inflammatory (M2) phenotype, which further reduces inflammation and promotes tissue repair. Moreover, MSCs can inhibit the proliferation

and activation of T cells, leading to immune suppression and reduced inflammation. In addition, MSCs have been shown to promote the differentiation and expansion of regulatory T cells, which are key players in immune tolerance and prevent excessive inflammation, thereby creating a favorable environment for angiogenesis. By modulating the immune response and reducing inflammation, MSCs indirectly promote angiogenesis (Chandra et al., 2022). The reduction of inflammatory factors and the establishment of an anti-inflammatory environment help remove inhibitory signals that would otherwise impede the formation of new blood vessels. Consequently, the immunomodulatory properties of MSCs contribute to tissue repair and regeneration by creating a supportive environment for angiogenesis to occur.

Based on these mechanisms, MSCs have been investigated in preclinical and clinical studies for their potential to promote angiogenesis in various contexts. This includes applications in tissue engineering, wound healing, ischemic diseases, and regenerative medicine (Huang et al., 2020). The ability of MSCs to secrete pro-angiogenic factors and modulate the local microenvironment makes them attractive candidates for therapeutic approaches aimed at enhancing angiogenesis and tissue repair.

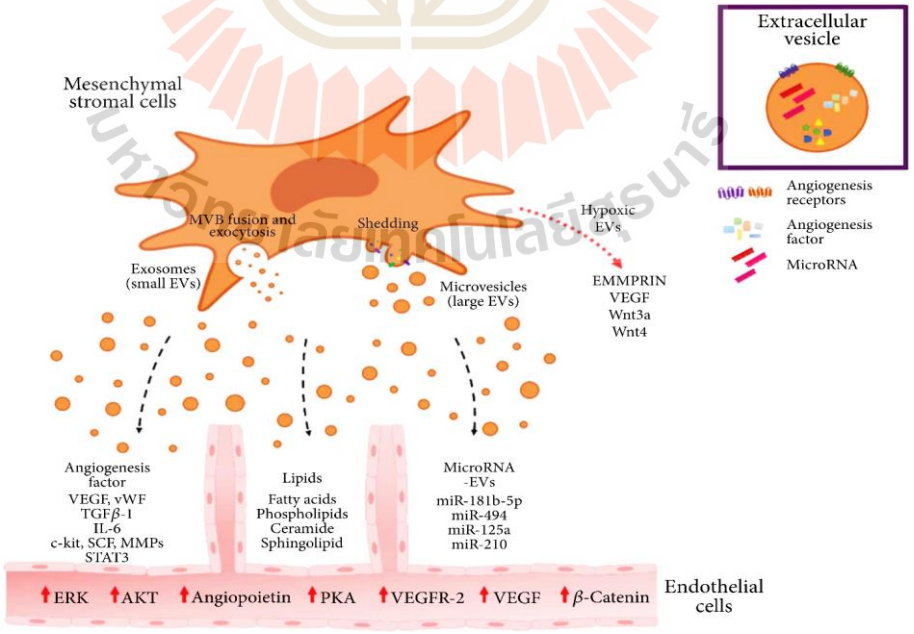

Figure 2.2 MSC paracrine activity during angiogenesis through Extracellular Vesicles (EV) (Maacha et al., 2020).

2.5 The role of telomeres in MSCs

A chromosome's end contains a stretch of repeating DNA sequences known as the telomere. The ends of chromosomes are shielded by telomeres (Smith et al., 2020). They serve to protect the integrity of chromosomes during DNA replication and prevent degradation or fusion with neighboring chromosomes. During each round of cell division, the DNA replication machinery is unable to fully replicate the telomeric DNA at the end of chromosomes, resulting in a gradual shortening of the telomeres. This is due to the "end replication problem" where the lagging strand of DNA is unable to replicate the very end of the chromosome. Telomeres act as sacrificial sequences, protecting the essential genetic material within the chromosome. In the context of MSCs, which are adult stem cells, telomere shortening occurs with each round of cell division as they undergo proliferation and expansion (Libertini et al., 2021). This progressive shortening of telomeres is a natural part of the aging process. As telomeres shorten beyond a critical threshold, they can no longer ensure chromosomal stability and proper DNA replication. When the telomeres become critically short, cell cycle arrest mechanisms, including cellular senescence, are triggered. Senescence refers to a state of permanent growth arrest where cells can no longer divide and function properly. In the case of MSCs, senescence can lead to a decline in their regenerative potential and functional properties (Roger et al., 2021).

Understanding telomere dynamics and the impact of telomere shortening on MSCs is important in the context of aging, regenerative medicine, and tissue engineering. Strategies to mitigate telomere shortening and maintain telomere length, such as telomerase activation or epigenetic modifications, are being investigated to enhance the regenerative potential and longevity of MSCs.

2.6 2D cell culture conditions

2D cell culture systems, where cells are grown on flat surfaces such as plastic dishes, have limitations and do not fully replicate the complex three-dimensional (3D) environment found in the human body. Cells grown in 2D culture systems do not accurately mimic the in vivo conditions and interactions that occur within a living organism (Zoetemelk et al., 2019). In the human body, cells exist in a 3D environment,

surrounded by other cells, extracellular matrix (ECM), and biochemical gradients. These factors play crucial roles in cell behavior, function, and response to external stimuli, which cannot be fully replicated in a 2D culture. Inadequate cell-cell and cell-ECM interaction. In 2D culture, cells lack the ability to interact with neighboring cells and ECM in a physiologically relevant manner(Rodrigues, Heinrich, Teixeira, & Prakash, 2021). Cell-cell interactions are essential for processes like cell signaling, cell migration, and tissue organization. Similarly, cell-ECM interactions are crucial for cell adhesion, migration, and differentiation (Yamada and Sixt, 2019). These interactions are significantly different in 2D cultures compared to the complex 3D architecture found in vivo.

The distorted morphology and behavior of 2D culture may exhibit altered morphology and behavior compared to their counterparts in a 3D environment. Cells in 2D culture tend to spread and adopt a flattened morphology, which can impact their gene expression, signaling pathways, and functional characteristics (Chang et al., 2021). These changes can limit the ability to accurately study cell behavior and responses in a physiological context and the limited nutrient and waste exchange: In standard 2D culture, cells consume nutrients and excrete waste, which can lead to issues with nutrient depletion and waste accumulation over time. These limitations can affect cell viability, function, and the ability to maintain a physiologically relevant microenvironment.

To address the limitations of 2D culture, researchers have developed 3D cell culture models, such as scaffold-based systems, organoids, and tissue-engineered constructs. These 3D culture systems aim to replicate the complex architecture and interactions found in vivo, providing a more representative environment for studying cell behavior, tissue development, disease modeling, and drug screening (Silvani et al., 2019). By moving towards 3D culture models, researchers can better understand the biology of cells and tissues in a more physiologically relevant context, leading to improved translational research and potential therapeutic applications.

2.7 3D cell culture conditions

3D culture systems offer several advantages over traditional 2D culture when it comes to mimicking in vivo cell environments. 3D cell shown the resemblance to in vivo cells. Cells grown in 3D culture systems more closely resemble their counterparts in vivo in terms of appearance and molecular control (Mazzocchi et al., 2019). The 3D environment allows cells to adopt more native-like morphologies and cell-cell interactions, which can influence various cellular processes. 3D culture adds a new dimension to cell adhesion, where cells interact with the surrounding extracellular matrix (ECM) or scaffold material (Afewerki et al., 2019). This interaction can affect integrin ligation, cell contraction, and intracellular signaling pathways, influencing cell behavior and function.

In 3D culture models, cells can be encapsulated within biomaterials, grown as cellular spheroids, or generated on 3D scaffold materials. These formats enable cells to form complex 3D structures and mimic tissue-like environments, allowing for more physiologically relevant studies. Various biomaterials are used to create 3D culture systems, including poly (lactic-co-glycolic acid) (PLGA), alginate, collagen, chitosan, and many others (Park et al., 2021) (Kang et al., 2020). These materials can provide structural support, mimic the ECM, and affect cellular behavior. Different biomaterials may have specific effects on MSC migration, proliferation, and differentiation, providing a tool for modulating cellular responses in 3D culture. Finally, the 3D cell enhanced cell-cell and cell-ECM interactions. 3D culture models allow for improved cell-cell and cell-ECM interactions compared to 2D culture (Ma et al., 2018). This is important as these interactions influence cellular processes, including cell signaling, gene expression, proliferation, migration, and differentiation. By providing a more realistic environment, 3D cultures systems better reflect the in vivo conditions.

By utilizing 3D culture systems, researchers can gain a deeper understanding of cell behavior, tissue development, disease mechanisms, and drug responses. These models are valuable for studying MSCs and their behavior in a more physiologically relevant context, allowing for better predictions of their behavior in vivo and enhancing the development of regenerative medicine strategies.

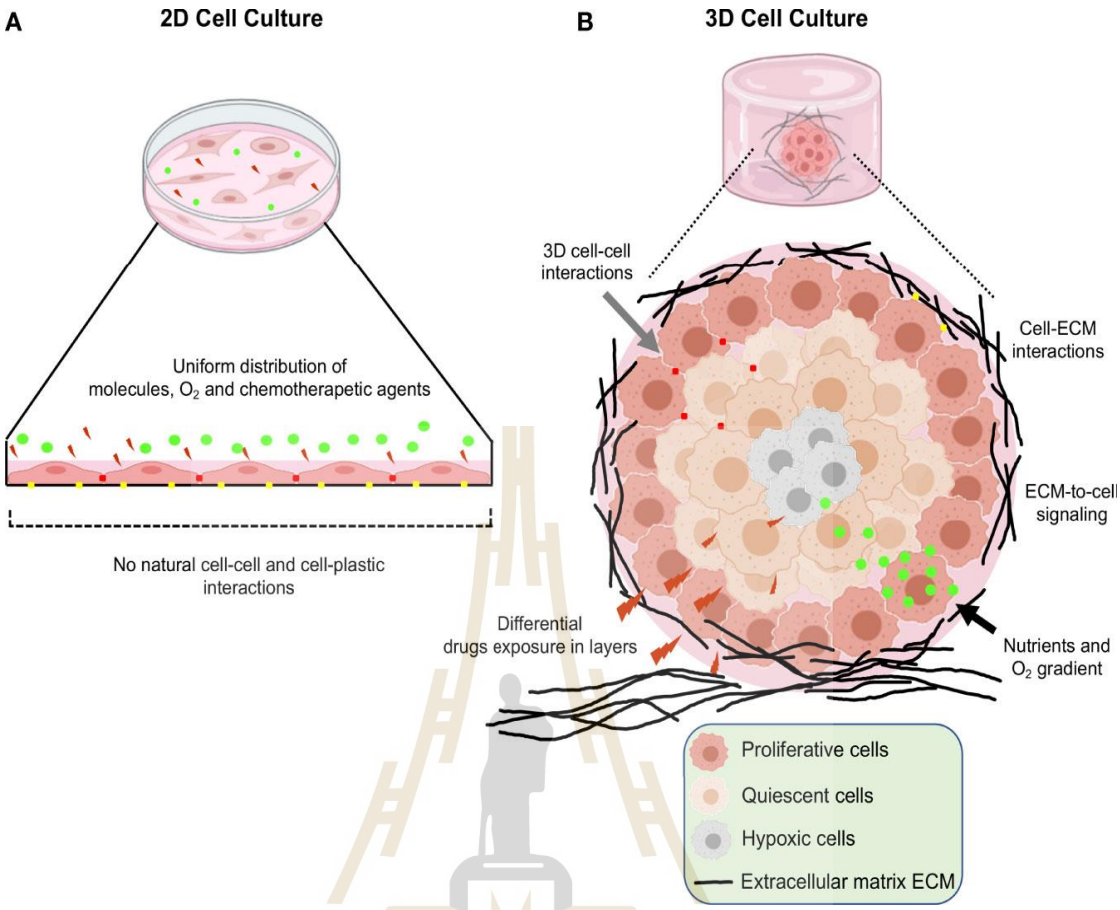

Figure 2.3 The main differences between 2D and 3D cell cultures (Vera et al., 2020).

2.8 Hydrogels

Hydrogels have emerged as versatile scaffolds for various applications in cell therapy, drug delivery, and tissue engineering (Jacob et al., 2021). Hydrogels can be easily tailored to meet specific requirements due to their tunable properties. They can Adaptability and ease of production by designed with desired mechanical strength, porosity, and degradation rates (Riley et al., 2019). The ease of production allows for efficient customization and scalability for different applications. Hydrogels offer biocompatibility, meaning they are well-tolerated by cells and tissues. They can mimic the native extracellular matrix (ECM) in terms of softness, flexibility, and high-water content. These properties allow for enhanced cell viability, proliferation, and retention within the hydrogel scaffold (Toh and Loh, 2014).

Hydrogels are highly hydrated and permeable, facilitating the exchange of nutrients, oxygen, and cellular metabolites with the surrounding environment (Akther et al., 2020). This is crucial for the survival and proper functioning of cells encapsulated within the hydrogel. The efficient transport of essential molecules supports cell viability and functionality. Hydrogels offer a wide range of tunable properties, including hydration and stiffness. These properties can be adjusted to mimic the specific tissue characteristics and create an optimal microenvironment for cells. By tuning the hydrogel properties, researchers can better control cell behavior, differentiation, and tissue formation. Hydrogels can be formulated as injectable materials, allowing for minimally invasive delivery to a specific target site (Torgersen et al., 2013). This is particularly useful for cell therapy and regenerative medicine applications, as it enables precise and localized administration of cells encapsulated within the hydrogel. The injectable format provides a 3D microenvironment for cells and supports their survival, proliferation, and tissue integration at the target site (Liu et al., 2014).

Overall, hydrogels have numerous advantages in mimicking tissue properties and providing a suitable microenvironment for cells. Their adaptability, biocompatibility, tunable properties, and injectable format make them well-suited for a range of applications in cell therapy, drug delivery, and tissue engineering.

2.9 Biomaterial for hydrogels

2.9.1 Alginate (AL)

Alginate is a natural polysaccharide that has been extensively used in tissue engineering and stem cell encapsulation due to its unique properties. Alginate can form hydrogels through noncovalent crosslinking, typically using bivalent metal ions such as calcium ions (Li et al., 2021). This allows for the generation of hydrogel scaffolds with temporary support for cells, including mesenchymal stem cells (MSCs). The crosslinking process is relatively simple and does not require organic solvents or hazardous chemicals. Alginate hydrogels have a hydrophilic nature and high-water content, providing a favorable environment for cell encapsulation and growth (Aguero et al., 2021). The hydrogel's porosity and pore interconnectivity facilitate the exchange of oxygen, nutrients, and waste products between the encapsulated cells and the surrounding medium (Tang et al., 2020).

Alginate hydrogels are biocompatible and do not elicit significant immune responses, making them suitable for cell encapsulation and tissue engineering applications. Additionally, alginate hydrogels can be tailored by modifying their composition or incorporating other bioactive components to enhance their performance and cell adhesion properties (Zhu et al., 2022). Alginate has been widely used to create microbeads or microcapsules for encapsulating cells, including MSCs. The encapsulation process helps protect the cells, provide a controlled microenvironment, and allow for controlled release of bioactive factors (Boi et al., 2020). These alginate-based microencapsulation approaches have been utilized for stem cell therapy, drug delivery, and in vitro models.

Alginate and its derivatives have been extensively studied and continue to show promise in various biomedical applications, particularly in stem cell encapsulation and tissue engineering. Their biocompatibility, ease of gelation, porosity, and ability to incorporate bioactive cues make alginate hydrogels attractive for creating artificial ECMs and supporting cell-based therapies.

2.9.2 Hyaluronic acid (HA)

Hyaluronic acid (HA) has gained significant attention in the field of tissue engineering and regeneration due to its biocompatibility and resemblance to the native extracellular matrix (ECM). HA is a naturally occurring polysaccharide and one of the major components of the ECM in various tissues (Buckley et al., 2022). It is widely distributed in connective, epithelial, and neural tissues, making it an attractive biomaterial for tissue engineering applications. HA-based hydrogels provide a favorable microenvironment for stem cell cultures. They can support stem cell adhesion, proliferation, and differentiation due to their resemblance to the native ECM (Fang et al., 2021). HA hydrogels can be formulated with specific physical and chemical cues to enhance stem cell behavior and guide tissue regeneration.

The hyaluronic acid enhanced the osteogenesis in MSCs, confirmed by the previous studies shown that HA-based hydrogels, when combined with mesenchymal stem cells (MSCs), promote osteogenesis (bone tissue formation). The addition of bone morphogenetic protein-2 (BMP-2) to HA hydrogels with MSCs has been found to enhance the expression of osteocalcin, a marker of osteogenic differentiation, and

promote bone tissue regeneration in vivo (Zheng et al., 2022). The design of HA-based hydrogels for stem cell transplantation emphasizes the incorporation of bioactive signals within the hydrogel. Photo-crosslinked hybrid HA/gelatin hydrogels have been developed to provide a bioactive signal that influences the cellular functions of encapsulated cells. These signals can regulate stem cell behavior, including their proliferation, differentiation, and tissue regeneration potential (Zhang et al., 2019).

Overall, HA-based biomaterials and hydrogels offer several advantages for stem cell cultures and tissue engineering applications. Their resemblance to the native ECM, support for stem cell functions, and ability to incorporate bioactive signals make them promising platforms for regenerative medicine and transplantation therapies.

2.9.3 Gelatin (GE)

Gelatin, derived from the hydrolysis of collagen protein, has gained significant attention in tissue engineering and regenerative medicine due to its unique properties. Gelatin biomaterials can be cross-linked to enhance their mechanical properties and stability (Dieterle et al., 2022). The cross-linking process improves the function of supporting cell attachment and cell retention in various cell types. Additionally, gelatin exhibits non-immunogenic properties, meaning it does not elicit significant immune responses, making it suitable for biomedical applications. Gelatin hydrogels closely resemble the natural microenvironments in vivo, making them valuable tools for studying the interactions between the extracellular matrix and cells (Dai et al., 2022). Their properties, such as biocompatibility, cell attachment patterns, and degradability, contribute to creating a conducive environment for cell growth and tissue regeneration.

Gelatin has excellent biocompatibility and low antigenicity, making it suitable for various biomedical applications (Ndlovu et al., 2021). The digestion process during gelatin production reduces its antigenicity, resulting in a low inflammatory response when used in vivo (Singh et al., 2019). This characteristic broadens its range of applications in the pharmaceutical and biomedical industries. Photo-cross-linking of gelatin methacrylate (GelMA) has become a widely utilized method for forming gelatin hydrogels (Krishnamoorthy et al., 2019). This approach allows for rapid gelation, strong mechanical properties, and ease of use in applications such as micropatterning and 3D

printing. GelMA hydrogels offer versatility and control in creating complex structures for tissue engineering and drug delivery systems.

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CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Hyaluronic acid (40583) Low molecular weight (MW) 8,000-15,000 Da, sodium alginate (A0682), gelatin (G1890), resazurin sodium salt (R7017), and 4,6-Diamidino-2-phenylindole (DAPI) (F6057) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 1X MEM Alpha modification (AMEM) and L-glutamine was purchased from HyClone (HyClone, Logan, UT). Nonessential amino acids (NEAA), Fetal bovine serum (FBS), 1X trypsin TrypLE™ Express 1X, and penicillin-streptomycin are purchased from Gibco (Gibco, CA, USA). Calcein-AM and propidium iodide (PI) were purchased from Life Technologies Inc. (Carlsbad, CA). Antibodies anti-CD73, CD90, CD105, and vascular endothelial growth factor (VEGF) were purchased from Merck (Merck KGaA, Darmstadt, Germany), and antibodies anti-phospho-SIRT1 were purchased from Affinity Biosciences (Cincinnati, USA).

3.2 Methods

3.2.1 MSCs culture

I Wellness Co., Ltd. (Mueng, Nakhon Ratchasima, Thailand) provided Mesenchymal Stem Cells (MSCs) under ethically acceptable terms. MSCs were cultured in complete medium with MEM Alpha modification (AMEM) medium, 10% (v/v) FBS, 1% NEAA, 1% (v/v) L-glutamine, and 1% (v/v) penicillin–streptomycin. Cells were incubated at 37°C in a humidified incubator atmosphere with 5% CO_2. The media for 2D and 3D culture conditions were changed every 3 days. All experiments used cells from passage 3.

3.2.2 Hydrogel preparation

To create a 1 weight percent hydrogel aqueous solution that is sterile sterilized 0.20 μm, alginate, hyaluronic acid, and gelatin were dissolved in sterile deionized (DI) water while being stirred at 37°C. Photo-crosslinking by UV radiation for 20 minutes. To create porous white foam, products were lyophilized for two days and then kept at 20°C for later usage. The mixtures were all incubated at 37 °C to create hydrogels, and all the samples were given 30 minutes to fully gelatinize. In every experiment, three weight percent gels were employed.

3.2.3 Cell seeding and 3D encapsulation of MSCs in the hydrogels

MSCs were seeded onto different biomaterial hydrogels for 3D encapsulation in a 24-well plate. Cells were encapsulated in hydrogels as single cells (2 x 106 cells/ml). Following that, 2D cell culture conditions were seeded into a 24-well plate (50,000 cells/well) in medium. The 2D cell culture and 3D hydrogels will be used to investigate the proliferation, cell survival, maintenance stemness, and angiogenesis activation of MSCs.

3.2.4 Scanning electron microscopy (SEM) image analysis

The structure and porosity of the hydrogels after the freeze-drying process were observed using a scanning electron microscope. Before being examined using a scanning electron microscope (FIB-SEM, Carl Zeiss), the sample was coated with a thick layer of gold using a sputter coating device (Sputter Coater Leica EM ACE600, Leica Microsystems (SEA) Pte Ltd).

3.2.5 Mechanical properties of the hydrogels

The mechanical properties of hydrogels after freeze-drying as described by (Yusof, Jaswir, Jamal, & Jami, 2019) and determined by Texture profile analysis (TPA) methods using TA.XTplus100C Texture Analyzer (Texture Technologies Corp. and Stable Micro System, Ltd. Hamilton, MA). The samples were analyzed with two sequential compression tests with a 2 mm diameter cylinder probe. The TPA test parameters used were a pre-test speed of 5 mm/sec, a test speed of 0.2 mm/sec, and a post-test speed of 5.0 mm/sec, and the strain rate was 50% for analyzing the

hardness, springiness, gumminess, and adhesiveness. Three repeated measurements for each of the hydrogels.

3.2.6 Swelling ratio measurement

The hydrogels were incubated with PBS at 37 °C, weighed, and removed at the times specified. The swelling ratios were determined by comparing the weight of swollen gels (Ws) to freeze-dried gels (Wd) at various time intervals, using the following formula: The swelling ratio (%) = (Ws – Wd)/Wd. Each measurement was carried out three times.

3.2.7 The design biomaterial hydrogels by cell proliferation assay

The proliferation of MSCs was measured by the resazurin assay, as previously described (Chaicharoenaudomrung et al., 2019). MSCs were cultured with 2D cell culture and 3D hydrogels in 24-well plates. The 3D biomaterial hydrogel includes 1% alginate (Al), 1% hyaluronic acid (HA) with a low molecular weight (MW) 8,000-15,000 Da, 1% gelatin (GE), 1:1 Al-HA, 1:1 Al-GE, 1:1 HA-GE, and 1:1:1 Al-HA-GE. MSCs were washed with PBS (phosphate buffered saline) and added the resazurin solution (25 µg resazurin/1 ml complete medium), which was incubated at 37°C for 20 minutes. The relative fluorescence unit (RFU) of the resazurin is determined using a fluorescence microplate reader (Varioskan, Thermo Fisher Scientific, Waltham, MA) after being transferred to a 96-well plate. The excitation wavelength is 530 nm, and the emission wavelength is 590 nm. The media for 2D and 3D culture conditions were changed every 3 days and cultured for 16 days.

3.2.8 Live/Dead straining

The vitality of MSCs in the 3D HA-Al hydrogels was evaluated using live/dead cell fluorescent double staining. MSCs in the HA-Al hydrogels were incubated with calcein AM (2 µM) and propidium iodide (4 µM) diluted in serum-free medium for 20 minutes at room temperature after being cultured for 3, 7, and 14 days. The living cells exhibit green fluorescence (calcein AM), and the dead cells exhibit red fluorescence (propidium iodide). The live/dead images in the HA-Al hydrogels were observed under a fluorescence microscope (ZOE™ Fluorescent Cell Imager, Bio-Rad

Laboratories, Hercules, CA) and the percentage of live and dead cells measured using the ImageJ program (magnification of 100×).

3.2.9 RNA extraction and qPCR analysis

To examine the mRNA expression of MSC stemness-related genes (SOX2, OCT4, NANOG, SIRT1, CD44, CD73, and CD90 genes), cell proliferation genes (Ki67, YAP, and TAZ genes), and angiogenesis-related genes (VCAN1 and VEGF genes) in 2D and 3D culture conditions. MSCs were seeded in hydrogel or plated into each well of a 24-well plate and cultured for 3, 7, and 14 days. Total RNA from MSCs was extracted using a NucleoSpin RNA Plus kit (Macherey-Nagel, Dueren, Germany). A ReverTra Ace®qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) was utilized to convert the total mRNA into cDNA. The QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) and qPCR BioSyGreen Mix Low-Rox (PCR Biosystems, UK) were used to conduct qPCR analysis. The following cycling conditions by 40 cycles, 95˚C for 30 s, 60˚C for 30 s, and 72˚C for 45 s. The PCR products underwent melting curve analysis by being heated at 60 ℃ for 60 s and 95 ℃ for 15 s. The primer sequences are described in Table 3.1 Using the 2−ΔΔCt method, relative mRNA expression was determined and normalized to the Ct of GAPDH (glyceraldehyde 3-phosphate dehydrogenase), a housekeeping gene, and the 2D culture condition.

Table 3.1 Sequences of the primers used for the Real-Time PCR analysis.

Gene	Forward	Reverse
SOX2	5'-GCGAACCATCTCTGTGGTCT-3'	5'-GGAAATTTGGGATCGAACAA-3'
OCT4	5'-CAGTGCCCGAAACCCACAC-3'	5'-GGAGACCCAGCAGCCTCAAA-3'
Nanog	5'-TAATAACCTTGGCTGCCGTCTCTG-3'	5'-GCCTCCCAATCCCAAACAATACGA-3'
Sirt1	5´-GAATACCTCCACCTGAGTTG-3´	5´-GGCGAGCATAAATACCATCC-3´
CD73	5´-GCCTGGGAGCTTACGATTTTG-3´	5´- TAGTGCCCTGGTACTGGTCG -3´
CD90	5'-ATCGCTCTCCTGCTAACAGTCT -3'	5'-CTCGTACTGGATGGGTGAAC-3'
CD105	5´-CTGCCGCTTTGCAGGTGTA-3´	5´- CATTGTGGGCAAGGTGCTATT-3´
hTERT	5'- GCCGATTGTGAACATGGACTACG-3'	5'-GCTCGTAGTTGAGCACGCTGAA-3'
Ki67	5'- CCTGTACGGCTAAAACATGGA-3'	5'-GCTGGCTCCTGTTCACGTA-3'
YAP	5'-CCTGCGTAGCCAGTTACCAA-3	5'-CCATCTCATCCACACTGT-3'
TAZ	5'-CTTGGATGTAGCCATGACCTT-3'	5'-TCAATCAAAACCAGGCAATG-3'
Telomere	5´-CGGTTTGTTTGGGTTTGGGTTTGG GTTTGGGTTTGGGTT-3´	5´-GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT-3´
36B4	5´-GGTTACCTCCGAAACTGAAGA-3´	5´-CCTTTCATATGCAGTACATTAGCC-3´
VEGF	5'-AGGGCAGAATCATCACGAAG-3'	5'-CACACAGGATGGCTTGAAGA-3'
VCAN1	5'-CACCCTCCACAGCCTCCGAA-3'	5'-CTATTCCGCCCCGTGTCAGC-3'
GAPDH	5'-CTCTGCTCCTCCTGTTCGAC-3'	5'-TTAAAAGCAGCCCTGGTGAC-3'

3.2.10 Telomere activity measurement by qPCR analysis

Total RNA was isolated from MSCs in 2D and 3D cell culture conditions at 3, 7, and 14 days using a NucleoSpin RNA Plus kit (Macherey-Nagel, Dueren, Germany). RNA was amplified into cDNA by using a ReverTra Ace®qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The human telomerase reverse transcriptase (hTERT) gene is used to determine the regulation of telomerase activity and maintain telomere length as previously described (Fathi et al., 2021). Telomere length analysis of human mesenchymal stem cells by quantitative PCR. The qPCR BioSyGreen Mix Low-Rox (PCR

Biosystems, UK) and QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) were used for all quantitative PCR reactions. The mRNA expression was examined and normalized to GAPDH, a housekeeping gene, using the 2−ΔΔCt method.

3.2.11 Telomere measurement analysis

MSCs were cultured in 2D and 3D culture conditions at 3, 7, and 14 days. DNA was extracted from MSCs with the genomic RNA extraction kit (Macherey-Nagel). The relative telomere length was measured using the qPCR amplification method as previously described (Hanson et al., 2021). The relative telomere/single copy gene (T/S) ratio produced by this approach is inversely correlated with the mean telomere length. Except for the oligonucleotide primers, the components of the T and S PCR reactions were identical. The SYBR Green PCR master mix (PCR Biosystems, UK) was used to conduct all qPCR analysis. The telomere repeats (Tel PCR) were amplified in one PCR reaction, and the 36B4 gene, a single copy gene used as a control, was amplified in the second reaction using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The primer sequences of the Tel and 36B4 genes are shown in Table 1. The following cycling conditions, Tel PCR, 30 cycles of 95˚C for 15 s and 56˚C for 1 min, and 36B4 PCR, 30 cycles of 95˚C for 15 s and 56˚C for 20 s. The average duplicate telomere over the average duplicate single-copy gene (T/S) ratio served as the unit of measurement for relative telomere length. All the telomere lengths that were shown were relative to the 2D culture condition.

3.2.12 Immunofluorescence straining

MSCs were cultured with 2D cells or 3D hydrogels in 24-well plates after being cultured for 7 days. The cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature, then washed three times with PBS. MSCs were incubated with blocking buffer solution (3% BSA and 0.5% Triton X-100 diluted with PBS) for 1 h. at 4℃. Cells were strained with a 1:500 dilution of anti-CD73, anti-CD90, anti-CD105, anti-SIRT1, and a 1:250 dilution of anti-VEGF antibodies and incubated at 4℃ overnight. The cells were washed with two times of washing buffer solution (0.5% Triton X-100 diluted with PBS) for 1 h. at 4℃ and incubated with secondary antibodies for 1 hour. After that, cells were washed with washing buffer and strained with DAPl to observe

the nucleus of the cell. The images were observed using a fluorescence microscope (ZOETM Fluorescent Cell Imager, BIO-RAD).

3.2.13 Statistical analysis

SPSS (version 16.0, SPSS Inc., USA) was used to conduct the statistical analysis. The data is displayed as mean SD values. Utilizing one-way ANOVA, multiple quantitative data sets were compared. The student's t-test was used to compare the two quantitative data sets. The level required for statistical significance was $p < 0.05$.

3.3 References

Fathi, E., Vandghanooni, S., Montazersaheb, S., Farahzadi, R. (2021). Mesenchymal stem cells promote caspase-3 expression of SH-SY5Y neuroblastoma cells via reducing telomerase activity and telomere length. Iranian journal of basic medical sciences, 24(11), 1583.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Results

4.1.1 The design biomaterial hydrogel for 3D culture MSCs

We developed the biomaterial hydrogels for 3D native ECM components in Human We developed the biomaterial hydrogels for 3D native ECM components in Human Mesenchymal stem cells (MSCs). First, we designed biomaterial hydrogels, including alginate (AL), hyaluronic acid (HA), and gelatin (GE), for optimizing cell proliferation in 3D hydrogel MSC culture. Then, we selected the high proliferation of 3D hydrogel to investigate the morphology and proliferation capacity in comparison to 2D cell cultures.

In this research, we design the biomaterial hydrogels (1% AL, 1% HA, 1% GE, 1:1 AL-HA, 1:1 AL-GE, 1:1 HA-GE, and 1:1:1 AL-HA-GE hydrogels) to be used for 3D MSC cultures. Cell proliferation of biomaterial hydrogels was assessed by the resazurin assay, which measured the relative fluorescence unit (RFU). The results indicated that the proliferation rate (RFU) of all hydrogel treatments increased with increasing culture time (days) (Figure 4.1 A). However, after being cultivated for 15 days, it was discovered that the AL/HA hydrogels had a higher proliferative capability than the various biomaterial hydrogels. Next, we compared the proliferation rates of 2D cell culture and 3D AL-HA hydrogels, cultivated for 15 days. The results showed that during the early stages of culture, the 2D cell conditions grew faster than the 3D AL-HA hydrogels during the 11-day culture period. And then, the proliferation rate of 2D cell conditions decreased. However, after 11 days of continuous culture, the proliferation capacity of 3D AL-HA hydrogels was rapidly increasing (Figure 4.1 B). The morphology of MSCs in the 2D monolayer cultures displays them as spindle-shaped flat cells, which is different from the cells in the 3D AL-HA hydrogels, which grew as cellular spheroids (the cellular aggregates) (Figure 4.1 C). These results indicate that 3D AL-HA hydrogels support cell proliferation and the continuous culture of Mesenchymal stem cells. Our results with

the AL-HA hydrogel showed a high proliferation capacity (RFU) compared with the various biomaterial hydrogels.

Thus, we create a 3D mimic microenvironment in MSCs using the AL-HA hydrogel to study cell survival, stemness maintenance, and angiogenesis activation, compared to 2D cell conditions.

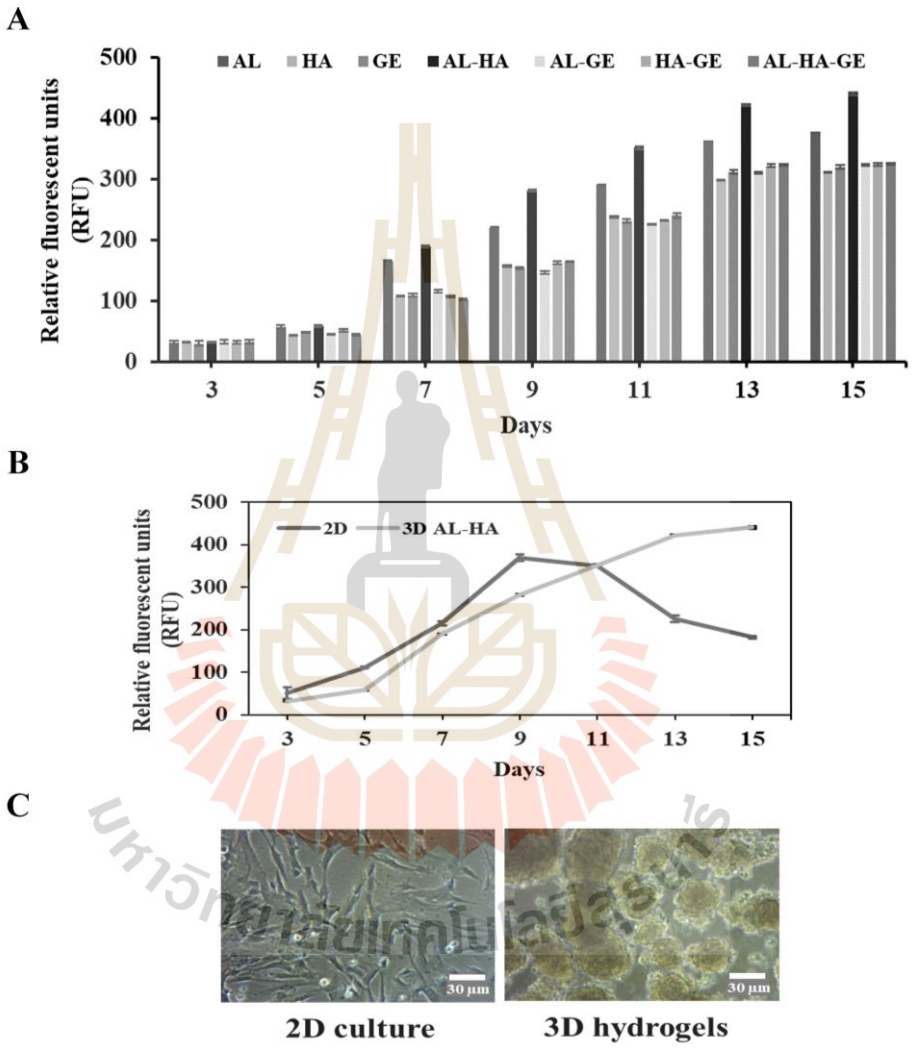

Figure 4.1 The cell proliferation and morphology of 3D MSCs in biomaterial hydrogels. (A) The proliferation capacity of MSCs in various biomaterial hydrogels (B) The proliferation rate of 2D and 3D AL/HA hydrogels during 15 days of culture, determined by resazurin assay and (C) The morphology of MSCs in 2D and 3D AL/HA hydrogels.

4.1.2 The characterization of hydrogels

We developed and examined the influence of AL and AL/HA on the morphological structure, swelling ratio, and mechanical characteristics of hydrogels. Images obtained by scanning electron microscopy (SEM) were used to examine the morphological structure of the AL and AL/HA hydrogels. The data suggested that the hydrogel matrix in both AL and AL/HA had a microporous structure (figure 4.2 A). The mechanical properties of AL and AL/HA hydrogels were determined by Texture profile analysis (TPA) methods. The results show that the AL/HA treatment exhibited significantly greater hardness, springiness, and gumminess when compared to the AL hydrogel. However, the adhesiveness of AL and AL/HA hydrogels showed no statistically significant difference (Figure 4.2 B-E). As for the swelling ratio, the water absorption of AL and AL/HA hydrogels increased the swelling ratio to 51% and 56% of their initial weight, respectively (Figure 4.2 F). The results confirmed that the AL/HA hydrogels exhibit porous morphology, increase the swelling ratio of their initial weight, confirm the effect of water absorption, and enhance their mechanical properties.

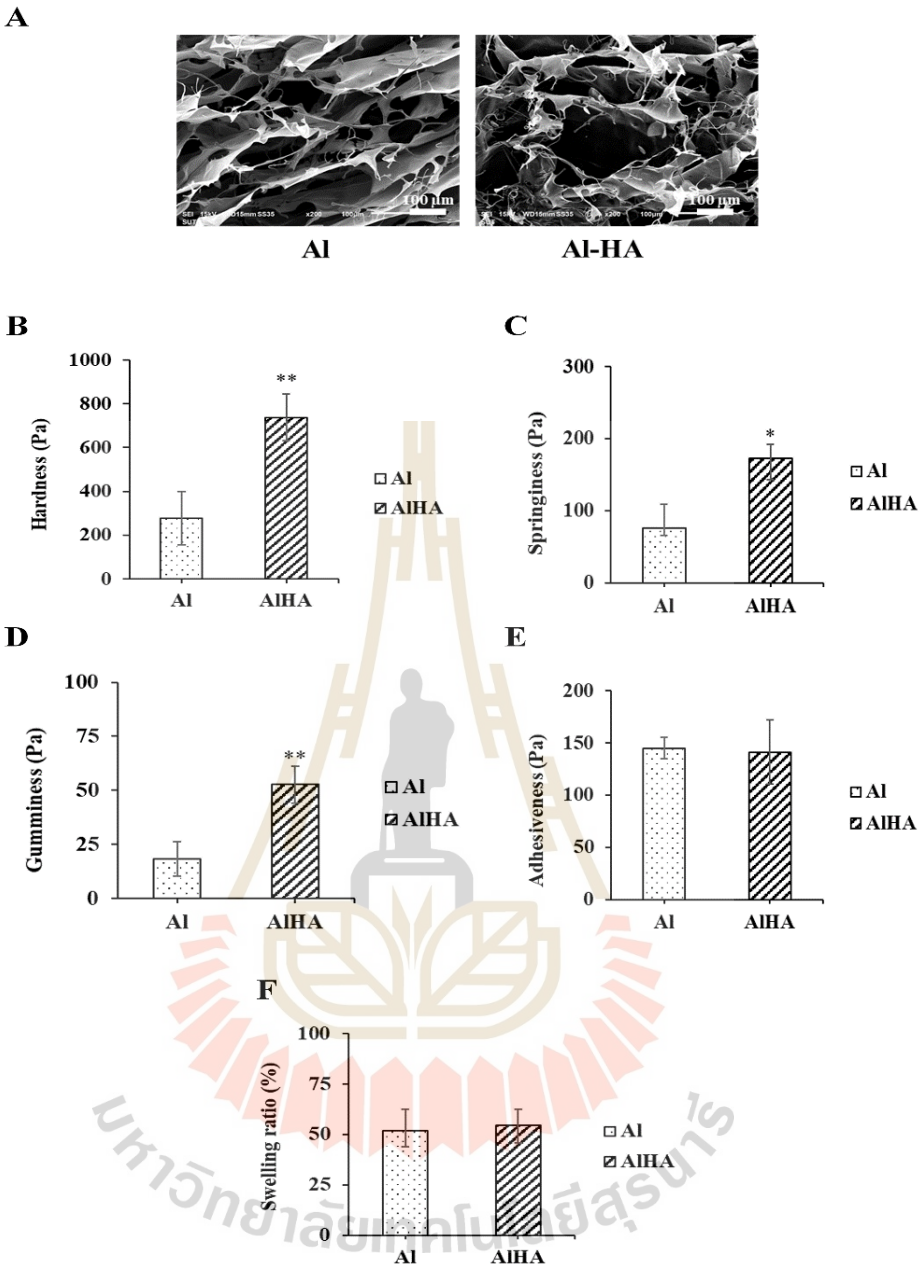

Figure 4.2 Characterization of alginate hydrogels. (A) SEM image of AL and AL/HA hydrogels. (B-E) The hardness, springiness, gumminess, and adhesiveness of AL and AL/HA hydrogels, measured by Texture profile analysis (TPA) methods and (F) the swelling ratio of AL and AL/HA hydrogels. alginate hydrogels. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

4.1.3 Live/dead of MSC cells in the 3D HA-Al hydrogels

MSCs were encapsulated in the HA-Al hydrogels (2 x 10^6 cells/ml), cultivated for 3, 7, and 14 days. Live/Dead staining (calcein AM/PI) was used to determine the UC-MSCs viability in the HA-Al hydrogels. The results showed high cell viability of MSCs after encapsulation in HA-Al hydrogels, most of the UC-MSCs survived (green) and only a few dead cells (red) occurred during the culture period. The percentage of live cells was presented as 83.11%, 80.26%, and 77.36% in the HA-Al hydrogels after being cultured at days 3, 7, and 14, respectively (Figure 4.3 A-B). Our results suggested that the HA-Al hydrogels may be a good strategy for supporting cell survival and 3D cell encapsulation of MSCs.

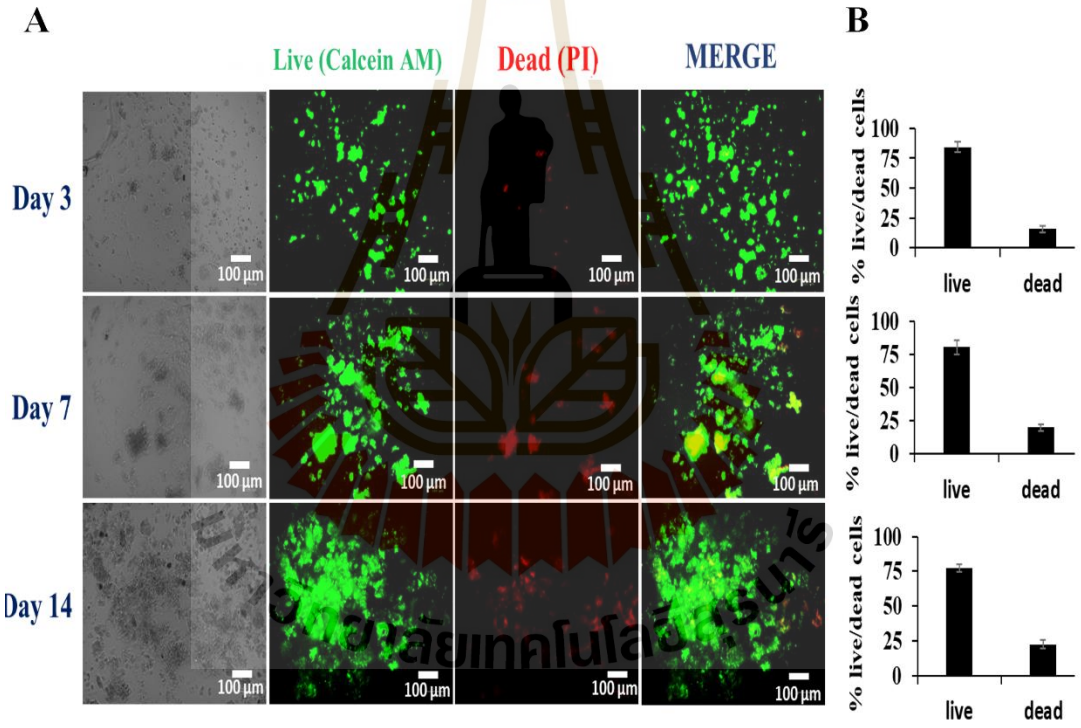

Figure 4.3 Cell viability of MSCs in alginate hydrogels at 3, 7, and 14 days of culture was determined by fluorescence to live/dead staining. (A-B) Living cells show green (calcein AM) fluorescence, and dead cells emit red (PI) fluorescence.

4.1.4 Effect of maintenance stemness, proliferation and telomere activity of MSC cells in 3D HA-Al hydrogels

To evaluate the expression of maintenance stemness, proliferation, and telomere measurement in the HA/Al hydrogel system of MSCs, were assessed by qPCR using the GAPDH gene as a reference gene and compared to the 2D cell culture conditions. The stemness properties of MSCs were determined by the MSC stemness-related genes (*SOX2, NANOG, OCT4,* and *SIRT1) and* CD MSC-related genes (*CD73, CD90* and *CD105*). Our findings showed that, after 14 days of cultivation, the stemness-related genes were significantly upregulated in the HA/Al hydrogel by 3.2, 5.2, 4.0, 3.1, 2.7, 2.8, and 3.3-fold, respectively, compared to 2D cell culture (Figure 4.4 – 4.5). Our results suggested that the 3D HA/Al hydrogels maintain the stemness of human mesenchymal stem cells.

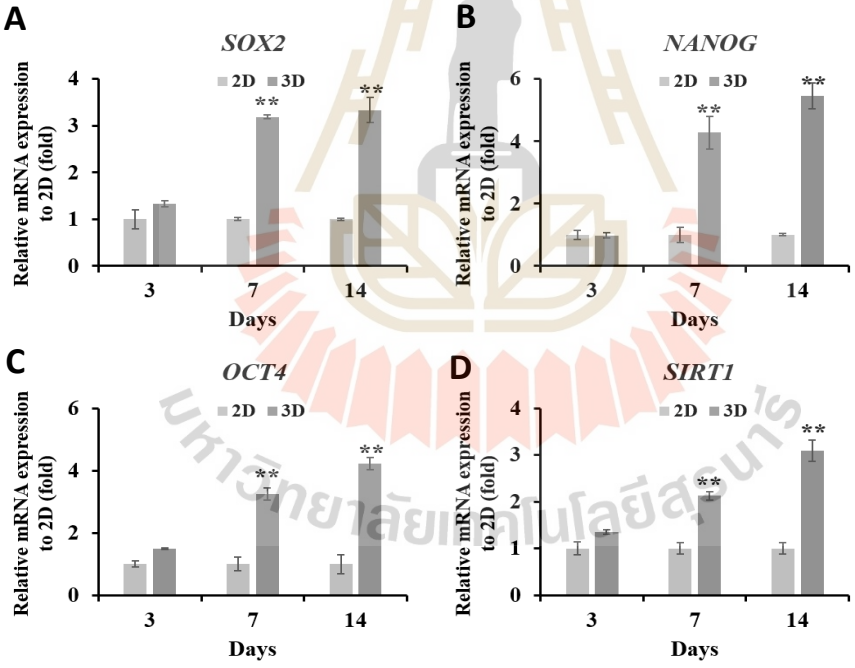

Figure 4.4 Effect of maintenance stemness on MSC cells in 3D HA-Al hydrogels. The mRNA expression of MSC stemness-related genes including, (A) SOX2, (B) NANOG, (C) OCT4 and (D) SIRT1 gene were examined by qPCR. GAPDH was used as a reference gene. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

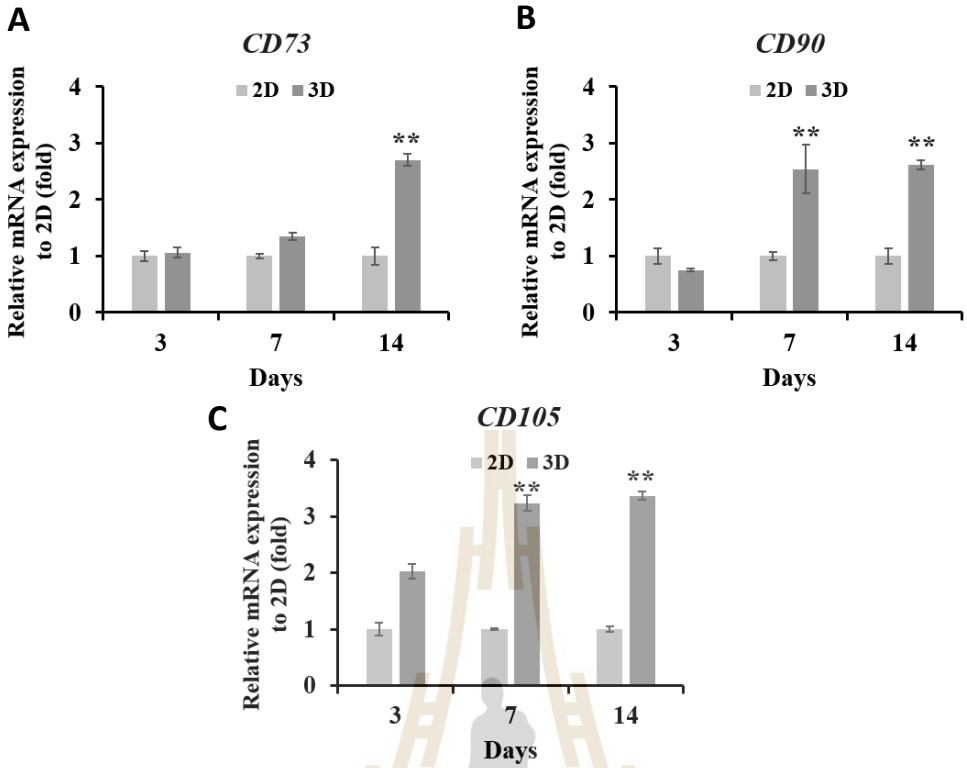

Figure 4.5 Effect of maintenance stemness on MSC cells in 3D HA-Al hydrogels. The mRNA expression of CD MSC stemness-related genes including, (A) CD73, (B) CD90 and (C) CD105 gene were examined by qPCR. GAPDH was used as a reference gene. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

The expression of tissue growth genes (*YAP* and *TAZ*) and cell proliferation genes (*Ki67*) and was determined upon the HA/Al hydrogels of MSCs. The findings indicate that after day 14, the HA/Al hydrogel significantly increased the YAP, TAZ, and Ki67 genes by 3.1, 4.0, and 2.48-folds, respectively, in comparison to the 2D culture (Figure 4.6). These findings imply that the 3D HA/Al hydrogels enhanced the proliferation of human mesenchymal stem cells.

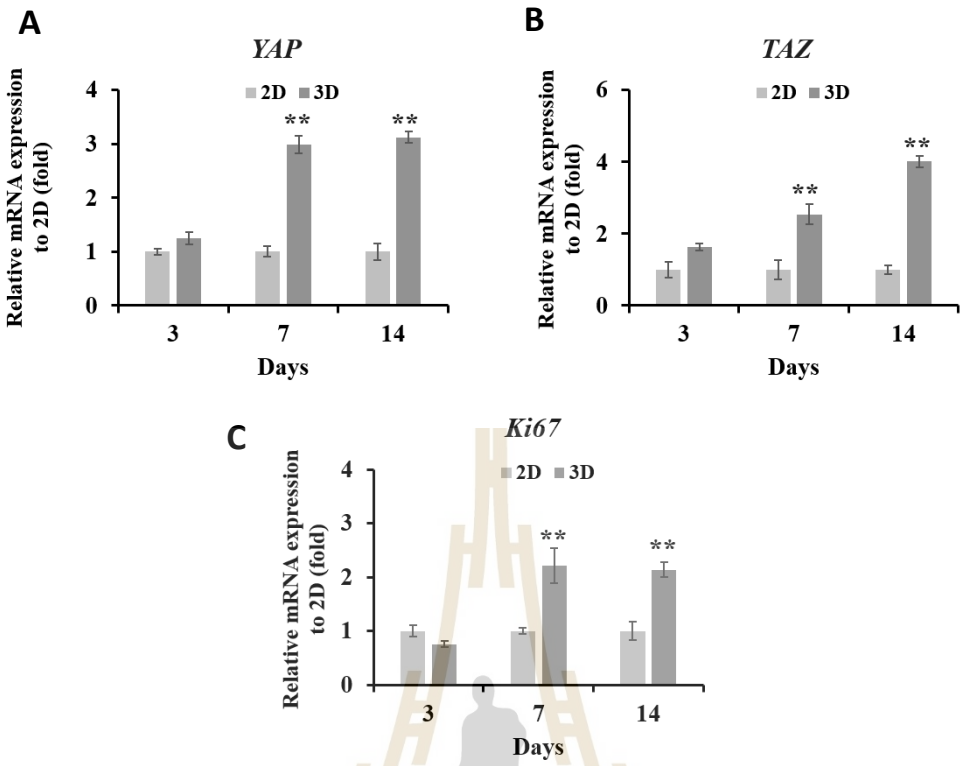

Figure 4.6 Effect of cell proliferation on MSC cells in 3D HA-Al hydrogels. (A-B) The mRNA expression of tissue growth genes (YAP and (TAZ) and (C) proliferation gene (Ki67) were examined by qPCR. GAPDH was used as a reference gene. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

To determine the telomere activity in 2D and 3D HA/Al hydrogel systems, hTERT (human telomerase reverse transcriptase) primers were used to analyze telomerase activity. The 36B4 gene was employed as a single-copy gene, and the TEL and 36B4 gene was used to determine the relative telomere length (*T/S ratio*). The results indicate that after day 14, the HA/Al hydrogel significantly increased the telomere activity (*hTERT*) and the relative telomere length (T/S ratio) by 3.4- and 2.48-folds, respectively, in comparison to the 2D culture (Figure 4.7). Our results suggested that the 3D HA/Al hydrogels increased the telomere activity of human mesenchymal stem cells.

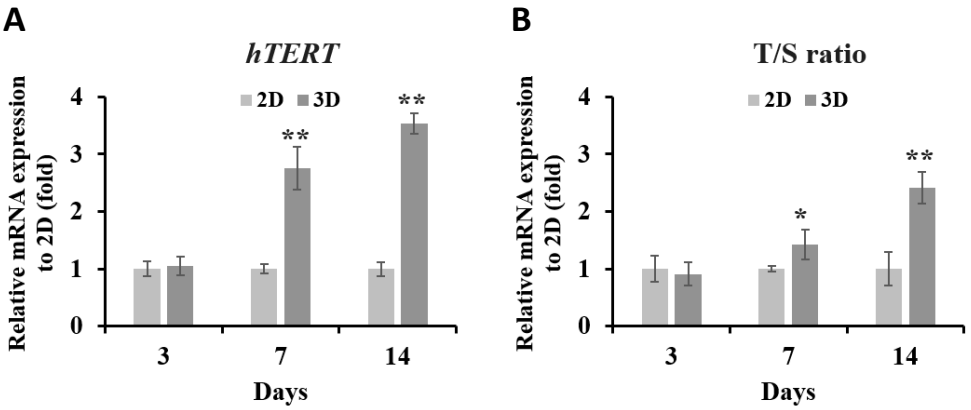

Figure 4.7 Effect of telomere activity on MSC cells in 3D HA-Al hydrogels (A) mRNA expression of telomerase gene (hTERT gene) and (B) the relative telomere length (T/S ratio) were examined by qPCR. GAPDH was used as a reference gene. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

4.1.5 Protein level of MSC stemness in the 3D HA/Al hydrogels

Our findings show that 3D HA/Al hydrogels increased the mRNA expression of MSC stemness-related genes, maintaining the stemness of human mesenchymal stem cells. The level of MSC stemness-related proteins (CD73, CD90, CD105, and SIRT1 proteins) in the 3D HA/Al hydrogel of MSCs was therefore evaluated by immunofluorescent labeling and compared to the 2D cell culture conditions to further our investigation. The findings show that at day 7, compared to 2D cell culture conditions, the fluorescent images in the 3D HA/Al hydrogels increased the fluorescence intensity of MSC stemness-related proteins (CD73, CD90, CD105, and SIRT1) (Figure 4.8 A-C). The overall results suggest that 3D HA/Al hydrogels enhanced the stemness properties of human mesenchymal stem cells.

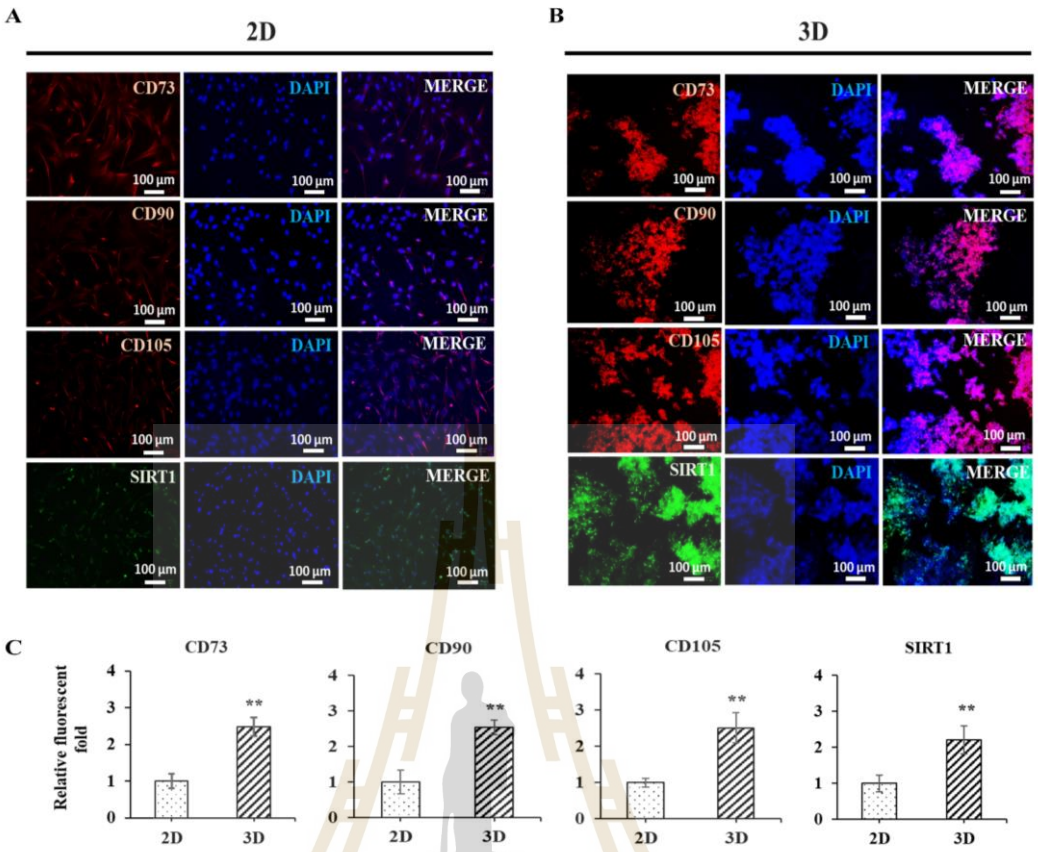

Figure 4.8 Immunofluorescent image of MSC stemness related protein (CD73, CD90, CD105, and SIRT) in (A) 2D and (B) 3D AL/HA hydrogels at 7 days and (C) the fluorescence intensity image of MSC stemness related protein in 2D and 3D AL/HA hydrogels. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

4.1.6 Effect of angiogenesis activation on MSC cells in 3D HA-Al hydrogels

To determine the mRNA expression of angiogenesis activation of 3D MSCs in HA/Al hydrogels, compared to 2D cell culture. The mRNA expression of angiogenesis-related genes, including Human vascular endothelial growth factor (VEGF) and Vascular cell adhesion molecule-1 (VCAM-1), was assessed by qPCR. The results show that when cells were cultured with AL/HA hydrogel, the mRNA expression of the VEGF and VCAM-1 genes increased by 3.6- and 3.9-fold, respectively, after 14 days of culture when compared to 2D monolayer culture (Figure 4.9 A-B).

Similarly, the expression of angiogenesis-related proteins (VEGF) was evaluated on MSCs in both culture conditions and was assessed by

immunofluorescence straining. The fluorescent images in the 3D HA/Al hydrogels increased the fluorescence intensity of angiogenesis-related proteins (VEGF) after 7 days of culture compared to 2D monolayer culture (Figure 4.9 C-D). These findings imply that the 3D HA/Al hydrogels enhanced the angiogenesis potential of Human mesenchymal stem cells.

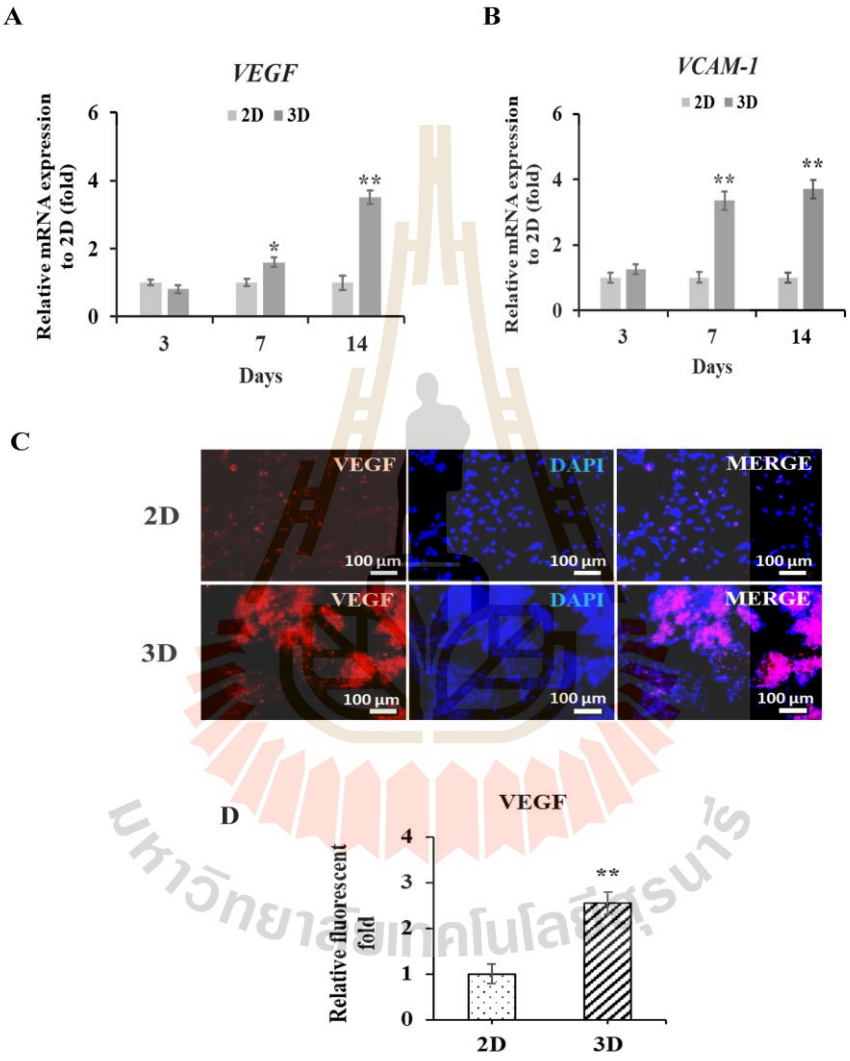

Figure 4.9 Effect of angiogenesis activation on MSC cells in 3D HA-Al hydrogels. The mRNA expression of angiogenesis-related genes including, (A) VEGF, and (B) VCAM-1 gene were examined by qPCR. GAPDH was used as a reference gene. (C) Immunofluorescent image of VEGF protein in 2D and 3D AL/HA hydrogels after culture for 7 days and (D) The fluorescence intensity image of VEGF protein in 2D and 3D AL/HA hydrogels. The values were presented as mean ± SD, n=3 (*$p < 0.05$ and **$p < 0.01$ versus control).

4.2 Discussion

In this research, we develop the biomaterial hydrogels for the 3D microenvironment of MSCs using the natural biopolymers (alginate, hyaluronic acid, and gelatin) to support cell survival, maintain pluripotency, increase telomere activity, and induce angiogenesis for enhanced regenerative capacity of MSCs. Our results designed and examined the proliferation of 3D biomaterial hydrogels (1% AL, 1% HA, 1% GE, 1:1 AL-HA, 1:1 AL-GE, 1:1 HA-GE, and 1:1:1 AL-HA-GE hydrogels) in MSCs. The results showed that the AL-HA hydrogels had a higher proliferation capability (relative fluorescence unit: RFU) than the various biomaterial hydrogels after being cultivated for 15 days and compared the proliferation capacity of 2D cell culture and 3D AL-HA hydrogels, cultivated for 15 days. The results showed that the 2D cell conditions grew faster than the 3D AL-HA hydrogels during the 11-day culture period, after that, the 2D cells grew steadily decreasing due to space limitation (disk, well), resulting in reduced cell-cell communication and cell-ECM interaction, and when the cells fill the culture area, they exhibit cell shrinkage and cell floating (cell death). Thus, cell proliferation and cell attachment decreased. However, for further cell growth, there must be subculture (passage cells) for cell expansion. Next, the proliferation capacity of 3D AL-HA hydrogels was rapidly increasing during 15 days of continuous culture, according to the morphology of MSCs in the 3D AL-HA hydrogels, which grew as cellular spheroids for support continuously grew, cellular aggregates developed, and the proliferation of mesenchymal stem cells (Figure 4.1 A-C).

Due to their biocompatibility, flexibility, and high-water content, biopolymer hydrogels have been applied in cell-based experiments and can serve as cues for cell attachment and proliferation (Xu et al., 2021). Previous studies encouraged the biomaterial's ability to promote proliferation, adhesion, and survival in a variety of cell types including, Pancreatic Cancer Cell, Olfactory-mesenchymal stem cells (OE-MSCs), embryonic stem cells (ESCs), Murine bone marrow-derived stem cells (BMSCs), and corneal epithelial cells (CECs) (Hamdy, 2023; Jaloux et al., 2022). The hydrogel, a 3D cross-linked hydrophilic polymer derived by brown sea algae, has been employed as a 3D scaffold for investigations in cell therapy, tissue engineering, and drug delivery, and because of its adaptability, biocompatibility, non-immunogenicity, and ability to maintain shape (Liu et al., 2023). Alginate is an enormous natural biomaterial for

regenerative medicine and tissue remodeling due to their non-toxicity, and simplicity of cross-linking. The alginate hydrogels exhibit cell viability, migration, proangiogenic growth factor secretion and bone regeneration in vitro (Cao et al., 2023). The other natural hydrogel biomaterial that is a promising ECM for cell regeneration is hyaluronic acid (HA), which as the component in the extracellular matrix (ECM) of cartilage (Vaca-González et al., 2023). The previous data showed that the hyaluronic acid enhance cell adhesion, anti-inflammations and induce osteogenic differentiation (Ma et al., 2021). And Gelatin, the biopolymer that can derive from hydrolysis of collagen, it plays a role function as cell retention, cell aggregation and wound healing processes (Lu et al., 2020). In this present study, we chose the natural biomaterials (alginate, hyaluronic acid, and gelatin) to mimic the creation of a 3D extracellular microenvironment that closely resembles that *in vivo*. Our data examined the design biomaterial hydrogel for 3D MSCs by cell proliferation analysis, the result indicates that the AL-HA hydrogel showed the high cell proliferation than the other biomaterial hydrogels for continuous culture of MSCs, according to the previous research, The 3D calcium (Ca)-alginate scaffolds enhanced high proliferation capacity the growth rate increased gradually, continuous culture during 21 days in human glioblastoma cells (Chaicharoenaudomrung et al., 2019). These results suggested that the biomaterial of alginate (AL) and AL-HA support cell proliferation in various cell types (Setayeshmehr et al., 2019) (Murugan and Parcha, 2021). The next step was to investigate the hydrogels properties of AL-HA hydrogels and apply them to the 3D mimic extracellular matrix (ECM) of MSCs, and then the survival and cell functions were examined and compared to 2D cell conditions.

The 3D biomaterials play a role in cell culture's efficiency and achievement, they function effectively and can be used as a scaffold to support cell growth and differentiation (Liu et al., 2021). Our results, we examined of AL and AL-HA hydrogels on the Morphological characteristics by SEM image, results indicate that both of AL and AL-HA showed the porous structure of the hydrogel matrix (Figure 4.2 A), according to the previous research, showed that the biomaterial hydrogels, including AL, AL-HA, chitosan-gelatin, pullulane-collagen hydrogels also exhibit the porous morphology in the matrix of hydrogels (Murugan and Parcha, 2021). The porous structure of the hydrogel matrix supports cell attachment, expansion, the ability to absorb water, the

retention of substances in large quantities, and the exchange of nutrients, oxygens and waste with the cells while maintaining their shape (Öfkeli et al., 2021). And then, the mechanical properties of AL/HA hydrogels were examined by Texture profile analysis (TPA) methods and in comparison, to the AL hydrogels. These results confirmed that the AL-HA hydrogels enhanced the hardness, springiness, and gumminess (Figure 4.2 B-E). The main variable influencing their morphology and function is the mechanical strength of the hydrogel employed for culture (Rizwan et al., 2021). Next, the swelling ratio measurement of AL/HA hydrogels was characterized and compared to the AL hydrogels. Our results confirm that the water absorption of AL/HA hydrogels (51%) is higher than that of AL hydrogels (56% of their initial weight) (Figure 4.2 F). Our data were consistent with the previous results of AL, HA, 2-Aminoethyl methacrylate (AEMA)–modified hyaluronic acid (HA), gelatin/alginate, and collagen/nanocrystalline cellulose based-hydrogels (Wang et al., 2023; H. Li et al., 2019). The swelling ratio is associated with water uptake and metabolite exchange, both of which are crucial for cell migration and proliferation (Abpeikar et al., 2022). The results confirmed that the AL/HA hydrogels exhibit porous morphology, increase the swelling ratio of their initial weight, confirm the effect of water absorption, and enhance their mechanical properties.

Biomaterial hydrogels are used in cell treatment to create a 3D cell environment that enhances cell survival and functionality (Park et al., 2021). Thus, using live/dead fluorescence staining, we investigated MSC survival in the 3D HA-Al hydrogels in this study. These investigations suggest that after 14 days of cultivation, this resulted in a high cell survival rate (77%) (Figure 4.3 A-B). Our findings demonstrate that the 3D AL/HA hydrogel promotes cell viability during MSCs' prolonged culture, according to the previous study of another biomaterial hydrogel that can exhibit high cell survival in various cell types, including the heparin-hyaluronic acid hydrogel, which showed a survival rate of human adipose-derived mesenchymal stem cells (ADSCs) above 95%, the methacrylate collagen hydrogel, which showed a survival rate of 75% in the human osteosarcoma cell line, the alginate/polycaprolactone hydrogels, which showed high cell survival as 82% in chondrocytes, and the AL/HA scaffolds enhance the survival rate above 80% in human Periodontal Ligament- and Gingiva-derived Mesenchymal Stem Cells (Choe et al., 2019; Lamaison et al., 2021). The biomaterial hydrogels,

especially the AL/HA in these studies, enhance cell survival. Consequently, they are highly suited for cell regeneration and biomedical applications in Human mesenchymal stem cells.

To study the effects of 3D HA-Al hydrogels on maintenance stemness, proliferation, and telomere activity in MSC cells, they were measured by qPCR analysis and compared the results to those obtained from 2D cell culture. The stemness properties exhibited to the ability of stem cells to self-renew and differentiate into various cell types (Wilson et al., 2020). The stemness-related genes (SOX2, OCT4, NANOG, and SIRT1) are transcription factors that play important roles in maintaining the pluripotent state of embryonic stem cells. These genes have been found to be expressed in various stem cell types, including mouse embryonic stem cells (mES cells), Bone marrow mesenchymal stem cells (BM-MSCs), and Adipose-derived stem cells (ASCs) (Fazaeli et al., 2021). Our studies have shown that stemness-related genes, including SOX2, OCT4, NANOG, and SIRT1, were upregulated in 3D HA/AL hydrogels. Additionally, the expression of MSC surface genes CD73, CD90, and CD105 on MSCs was enhanced in these 3D hydrogels compared to traditional 2D cell culture. This suggests that the 3D HA/Al hydrogels may provide a more favorable environment for the maintenance and differentiation of MSCs. The transcriptional expression of Ki67 that is present in cells during active phases of the cell cycle. Therefore, measuring the transcriptional expression of Ki67 can provide information about the proliferation rate of cells (Mrouj et al., 2021). Our results confirmed that the HA/Al hydrogel significantly increased the Ki67 gene in comparison to the 2D culture. According to the proliferation capacity of this study, the data exhibited 3D AL-HA hydrogels that grew faster than the 2D culture after 9 days of long-term culture, the proliferation is necessary for the expansion of MSC populations. Thus, these data imply that the AL/HA hydrogels provided a supportive environment for the growth and multiplication of 3D MSCs. In addition, the 3D MSCs in AL/HA were significantly higher expression levels of YAP and TAZ genes, according to the 3D biomaterial hydrogel to enhance the expressions of theses gene, the Fibronectin–Hyaluronic Acid Hydrogels increased the expression of YAP protein in MSCs, and the stiffness of Alg-Gle hydrogel-based bioink promote the gene expression of YAP and TAZ gene in mMSCs (Vermeulen et al., 2022). The YAP and TAZ genes are transcriptional co-activators that are downstream effectors of the Hippo

pathway, a signaling pathway that regulates tissue growth and organ size by controlling cell proliferation and apoptosis (Sun and Chi, 2021). Next, to compare the telomere activity in 2D and 3D HA/Al hydrogels measured by the hTERT gene expression. In our study, the hTERT gene were significantly upregulated in the HA/AL hydrogel systems. The hTERT gene plays a crucial role in telomerase activity, and the expression of this gene is strictly controlled in many cell types to preserve genomic stability and avoid cellular senescence (Taheri et al., 2022). Previous studies have confirmed that, when combined with the biomaterial hydrogels, telomerase-immortalized human adipose-derived stem cells (hASC/hTERT) showed advantageous properties for cell proliferation and differentiation (Luciano et al., 2022). Apart from that, the relative telomere length (T/S ratio) in HA/Al hydrogels exhibited higher than the 2D cell culture in our studies. The standard method for calculating telomere length is the T/S ratio, which relates the number of telomeres repeats to that of a single-copy gene, such as 36B4. Maintaining the integrity of chromosomal ends during cell division depends on telomere activity. According to our research, utilizing 3D HA/Al hydrogels enhanced the telomere activity of human mesenchymal stem cells.

In our studies, findings indicate that 3D HA/Al hydrogels increased the mRNA expression of MSC stemness-related genes and maintained the stemness of MSCs. We examined the level of MSC stemness-related proteins (CD73, CD90, CD105, and SIRT1) in the 3D hydrogel and compared it to 2D cell culture conditions. The results showed that the fluorescent images in the 3D HA/Al hydrogels demonstrated an enhanced fluorescence intensity of MSC stemness-related proteins compared to 2D cell culture. According to the previous result, the PEG (Polyethylene glycol)-based 3D printed hydrogels that exhibit the MSC stemness-related proteins (CD73, CD90 and CD105) higher fluorescence image than current 2D culture systems in MSCs for promote regenerative activity of keratinocytes and fibroblasts (Luciano et al., 2022). Mesenchymal stem cells' (MSCs) ability to regenerate tissue depends on the maintenance of their stemness, which is accomplished by increasing the expression of genes and proteins. Overall, results suggest that 3D HA/Al hydrogels improve the stemness properties of human mesenchymal stem cells. This discovery provides a potential method for preserving and improving the stemness of MSCs in 3D culture systems, which may have significant implications for tissue engineering and regenerative medicine.

Mesenchymal stem cells (MSCs) have been shown to exhibit regenerative capacity through various mechanisms, including angiogenesis, which is the process of new blood vessel formation from pre-existing blood vessels and is crucial for supplying nutrients and oxygen to cells within a tissue or organ (Barachini et al., 2023). Angiogenesis does play a significant role in enhancing cell survival, proliferation, and differentiation when creating a microenvironment using 3D biomaterial hydrogels. The various biomaterials hydrogels exhibited angiogenesis by increasing the expression of VEGF, a pro-angiogenic growth factor of MSCs, including, alginate-collagen (Sodium alginate/collagen hydrogel loaded with human umbilical cord mesenchymal stem cells promotes wound healing and skin remodeling), hyaluronic acid and gelatin-methacrylate hydrogels (Las Heras et al., 2022). In these studies, we determined the expression of angiogenesis-related genes (VEGF and VCAM-1 genes) of MSCs in 3D HA/Al hydrogels compared to 2D cell culture. The results exhibited higher gene expression of VEGF and VCAM-1 genes in the culture of HA/AL hydrogels. According to our data, the angiogenesis-related proteins (VEGF) showed higher fluorescence intensity in the 3D HA/Al hydrogels. These results demonstrate that the 3D HA/Al hydrogels promoted the potential for angiogenesis in human mesenchymal stem cells. Overall, we proved that the 3D HA/AL hydrogel systems support MSC viability, growth, and functions. According to our present research, 3D HA/AL hydrogels enhance maintenance pluripotency, raise telomere activity, and promote angiogenesis. It improves tissue engineering and cell regeneration depending on the responses to the implanted MSCs.

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CHAPTER 5
CONCLUSION

In summary, this research showed the development of a 3D Mesenchymal stem cell culture system using AL/HA acid hydrogels. Our data showed that this system mimicked the in vivo microenvironment, supported the proliferation capacity, and exhibited high survival in continuous cell culture of MSCs. In the 3D AL/HA hydrogel systems, Mesenchymal stem cells enhanced stemness properties, proliferation, and telomere activity compared with the 2D culture system. This suggests that the 3D AL/HA culture environment provided a more favorable condition for the maintenance of MSCs' characteristics and cell proliferation. Finally, the 3D AL/HA hydrogels enhanced the regenerative capacity through angiogenesis activation compared to the 2D cell culture conditions. This indicates that the 3D AL/HA culture environment facilitated the activation of angiogenesis and thus potentially enhanced the regenerative potential of MSCs. Our findings suggest that 3D AL/HA hydrogels can serve as a promising carrier for stem cell-based therapy and various tissue engineering applications.

VITAE

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