Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 3
Open Access

Low Oxygen Concentration Enhances Chondrogenesis and Secretome Production in Mesenchymal Stem Cells Cultured in a Three-Dimentional Decellularized Bovine Cartilage Scaffolds

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Cite this paper as: Kukuh Dwiputra Hernugrahanto, Naufaldy Rifqiaulia Noerda, Jifaldi Afrian Maharaja Dinda Sedar, Lukas Widhiyanto, Dwikora Novembri Utomo, Djoko Santoso (2024) Low Oxygen Concentration Enhances Chondrogenesis and Secretome Production in Mesenchymal Stem Cells Cultured in a Three-Dimentional Decellularized Bovine Cartilage Scaffolds. *Frontiers in Health Informatics, 13* (3), 486-496

Abstract

Introduction: *Stem cells hold promise for tissue repair due to their secretome, which is influenced by the microenvironment. To improve cartilage regeneration, a three-dimensional (3D) natural cartilage scaffold system has been developed to create a more chondrogenic secretome. As human cartilage is avascular, a hypoxic environment may better mimic in vivo conditions. This study investigates if oxygen levels (normoxic vs. hypoxic) affect MSC chondrogenic potential and secretome composition in this 3D scaffold.* ***Methods:*** *This study used a randomized time series design to investigate how oxygen levels affect the transformation of MSCs into cartilage cells within a 3D cartilage scaffold. A control group (C) was grown in a standard medium. Two experimental groups, P1 and P2, were cultured n a 3D cartilage scaffold under normal (21%) and hypoxic (5%) oxygen concentration. respectively. The hypothesis was that a hypoxic environment would promote superior chondrogenic differentiation. Key markers were evaluated at specific time points using immunohistochemistry and enzyme-linked assay.* ***Results:*** *In the 3D cultures, the cells exposed to low oxygen (hypoxic) generally showed higher expression of markers related to cartilage formation (SOX-9, RUNX-2, Coll-II, and aggrecan) compared to those in normal oxygen levels (normoxic). Additionally, the hypoxic group had consistently higher levels of several growth factors important for tissue development and repair (BMP-2, BMP-7, TGF-β3, IGF-1, and FGF-2) throughout the study.* ***Conclusion:*** *Hypoxic conditions in 3D culture led to increased expression of all analyzed chondrogenic markers in mesenchymal stem cells, suggesting that a low-oxygen environment effectively promotes chondrocyte proliferation and differentiation.*

Keywords: *Stem Cells, Three-Dimensional, Culture Technique, Secretome, Cartilage.*

INTRODUCTION

Cartilage damage and loss, whether due to injury, disease, or aging, trigger subchondral bone remodeling and lead to osteoarthritis (OA).[1] As cartilage has limited regenerative capacity and current treatments offer limited success, new therapies are needed to slow OA progression[2–4]. Mesenchymal stem cells (MSCs) hold promises

for cartilage repair due to their ability to self-renew and differentiate into chondrocytes[5]. However, challenges like immune rejection, tumor risk, cost, and infection transmission hinder their clinical application[6,7]. Recent research suggests that MSCs' therapeutic effects primarily stem from secreted paracrine factors, collectively known as the secretome, rather than direct cell engraftment. This discovery opens new avenues for OA treatment by focusing on isolating and utilizing individual secretome components, thus bypassing the limitations of direct stem cell transplantation[6,8].

To maximize cartilage regeneration, the secretome composition should be optimized to be more chondrogenic, enriched with proteins crucial for cartilage formation like aggrecan, type II collagen, TGF-β, IGF-1, FGF-2, and BMP-2,7. The microenvironment, including chemical and physical stimuli, significantly influences the secretome's properties. In particular, the culture medium and oxygen concentration that mimic the natural microenvironment might play pivotal roles in producing a secretome with desired characteristics[9,10]. Three-dimensional (3D) culture systems offer advantages over traditional 2D cultures by mimicking the natural tissue environment, enhancing cell growth, proliferation, and self-renewal, while preventing premature differentiation. This approach facilitates better molecular exchange, nutrient uptake, gas exchange, and waste removal, mirroring in vivo conditions[9,11]. To replicate the in vivo environment more accurately, a hypoxic (low oxygen) condition is preferred, as it aligns with the avascular nature of human cartilage. Hypoxia can induce essential transcription factors for chondrogenesis, enhance stem cell proliferation and multipotency, and promote chondrocyte marker expression (e.g., collagen type II, aggrecan, SOX-9), ultimately leading to increased extracellular matrix synthesis. Both in vitro and in vivo studies have demonstrated improved chondrocyte proliferation and differentiation under hypoxic conditions in 3D scaffolds. However, the specific effects of oxygen concentration on the chondrogenic potential of 3D cultures remain unexplored[12,13].

By optimizing both culture media and oxygen concentration, we aim to create an ideal environment for chondrogenic differentiation. This study investigates whether difference in oxygen levels (normoxic vs. hypoxic) influence MSC chondrogenic potential and the composition of their secreted secretomes within a 3D cartilage scaffold.

MATERIALS AND METHODS

Study Design

This experimental study investigated the impact of oxygen concentration on the chondrogenic differentiation of mesenchymal stem cells (MSCs) in a randomized time series design with a control group. The control group (C) was grown in standard 2D conditions. Two experimental groups were established: P1, cultured in a 3D decellularized bovine cartilage scaffold (DBCS) under normoxic conditions (21% oxygen), and P2, cultured in the same 3D scaffold but under hypoxic conditions (5% oxygen). A 5% oxygen concentration was chosen as it is considered optimal for promoting chondrocyte differentiation[8,13,14]. We hypothesized that the hypoxic environment (P2) would enhance chondrogenic differentiation compared to both the control and normoxic groups. Chondrogenic differentiation was evaluated by assessing the expression of key markers by immunohistochemistry evaluation and enzyme-linked assay (SOX-9, RUNX-2, type-II collagen, aggrecan, TGF-β1, TGF-β3, IGF-1, FGF-2, BMP-2, and BMP-7) at specific time points (days 2, 7, 14, and 21).

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) derived from adipose tissue were obtained from the infrapatellar fat pad (IFP) of eight healthy donors aged 20-45, who had provided informed consent and tested negative for infectious diseases (TORCH, hepatitis B/C, HIV, COVID-19). IFP-derived MSCs were selected due to their ease of isolation, high proliferation rate, and superior chondrogenic potential compared to other MSC sources[15,16]. Individuals with infections, inflammation, or degenerative tissue conditions were excluded. The isolated MSCs were expanded in culture until reaching a minimum of 2 x 10^7 cells and were characterized based on the International Society for Cellular Therapy criteria, exhibiting the presence of CD73, CD90, and CD105 surface markers, while lacking CD14, CD34, CD45, and HLA-DR[17].

Culture Media

This study utilized two primary types of media: (1) conventional cell culture media containing Dulbecco's Modified Eagle medium (DMEM), penicillin, and streptomycin (Gibco, UK) to provide essential nutrients, and (2) a three-dimensional (3D) decellularized bovine cartilage scaffold (DBCS) sourced from certified slaughterhouses, processed according to international tissue bank standards as described in previous studies[18,19]. The DBCS, with a 2 cm diameter, 1 cm thickness, and 100-130 micrometer porosity, served as the 3D culture medium. Cell cultures were maintained under two conditions: hypoxia (5% O2, 5% CO2) and normoxia (21% O2, 5% CO2), both at 37 °C with saturation humidity. Scanning electron microscopy (Figure 1a & b) was used to confirm the successful integration and infiltration of MSCs within the 3D scaffold. Alcian blue staining (Figure 1c) was employed to visualize and confirm the presence of glycosaminoglycans (GAGs), a major component of the cartilage extracellular matrix, within the 3D culture.

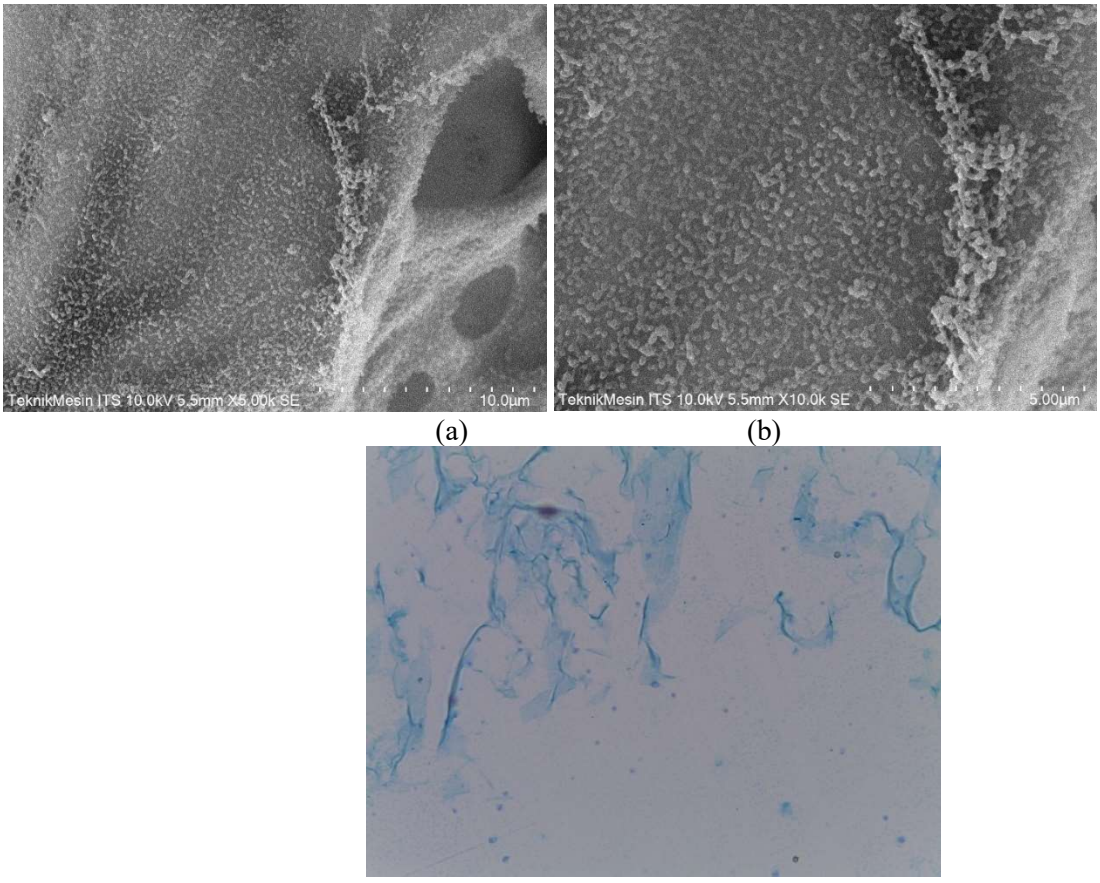

Figure 1. Scanning electron microscopy images revealed that cells not only adhered to the surface of the 3D medium but also infiltrated its porous structure, as shown at 5,000x (a) and 10,000x (b) magnification. Additionally, Alcian blue staining (c) confirmed the presence of glycosaminoglycans (GAGs), essential components of the cartilage extracellular matrix, within the 3D culture.

Secretome Processing and Extraction

Upon reaching 80% cell confluence, the secretome was harvested under controlled conditions. It was transferred to a 50 ml dialysis tubing membrane, sealed, and immersed in cold PBS solution within a beaker. The beaker

was subjected to continuous stirring overnight using a magnetic stirrer until the secretome's color faded. Subsequently, the dialysis tube was opened, and the secretome was filtered through a 0.22-micron filter. The resulting filtrate was collected in 50 ml conical tubes, sealed, and stored at -20°C for further analysis.

Immunohistochemistry Evaluation

Immunohistochemical analysis was employed to evaluate the expression of chondrogenic markers, including transcription factors SOX-9 and RUNX-2, and extracellular matrix components type-II collagen and aggrecan. Cells were deparaffinized, rehydrated, and subjected to antigen retrieval steps. Endogenous peroxidase activity was blocked, followed by incubation with specific monoclonal antibodies (Sigma, USA) against the target proteins. Slides were then assessed blindly by two independent observers using a light microscope (Nikon H600L with digital camera DS Fi2 200mp and Nikon Image System software). Positive staining was visualized as brown-colored cytosol (Figure 2). Quantification of marker expression was performed using a modified immunoreactive score (IRS) that considered both staining intensity and the proportion of positive cells (Table 1)[20].

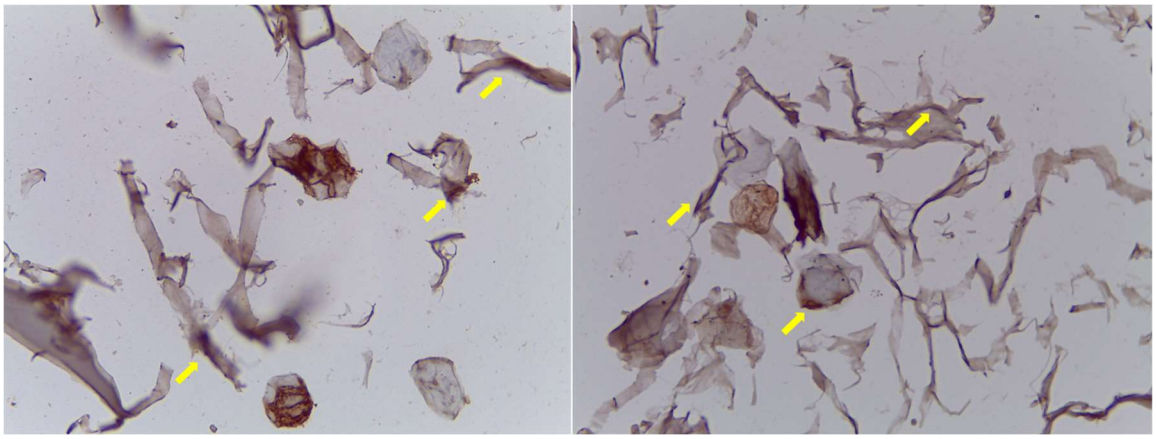

Figure 2. Brown-colored cytosol (arrow) indicates positive expression of the monoclonal antibody.

Table 1. Semi-quantitative immunoreactive score (IRS) is the result of multiplication between percentage of positive cells (A) and color intensity (B)

A	B
Score 0: No positive cell	Score 0: No color observed
Score 1: Positive cells <10%	Score 1: Low color intensity
Score 2: Positive cells 11-50%	Score 2: Intermediate color intensity
Score 3: Positive cells 51-80%	Score 3: Strong color intensity
Score 4: Positive cells >80%	

Enzyme-linked Immunoassay Evaluation

Specific proteins (TGF-β1, TGF-β3, IGF-1, FGF-2, BMP-2, and BMP-7) within the secretome were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Sigma, USA). Diluted capture antibodies were coated onto wells and incubated overnight. Following washes, samples were added and incubated to allow for antigen binding. Next, streptavidin-HRP conjugate was added, binding to the captured antigens. The addition of substrate solution generated a color signal, the intensity of which was proportional to protein concentration. Absorbance at 450 nm was measured, and results were calculated using a log-log plot or 4-parameter curve fit.

Data Collection and Statistical Analysis

Collected data underwent statistical analysis, assessing normality and homogeneity. For normally distributed

data, two-way ANOVA was employed to evaluate the impact of each variable, followed by post hoc tests to identify specific group differences. Non-parametric data were analyzed using the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. To maintain rigor and reliability, all experiments were conducted in a standardized laboratory with proven expertise in scaffold production and stem cell handling. Experienced researchers blinded to group assignments performed assessments using immunohistochemistry and ELISA, ensuring objectivity. Data collection was meticulously controlled to minimize potential biases.

RESULT & DISCUSSION

Evaluation of transcription factors and extracellular matrix components

In the normoxic 3D decellularized bovine cartilage scaffold, the final SOX-9 expression was slightly lower than in the hypoxic scaffold (8.23 vs. 8.99). SOX-9 expression generally increased over time, except in the normoxic group on day 14 where a slight decrease was observed (7.79 from 7.85). RUNX-2 expression was similar between the normoxic and hypoxic groups, though the hypoxic group consistently showed higher levels. Both groups demonstrated increasing Coll-II expression throughout the experiment, with higher levels observed in the hypoxic scaffold, except on day 7 (8.99 vs 8.5). The highest Coll-II expression was found in the hypoxic group on day 21 (10.88 vs. 9.85). Aggrecan levels also increased over time in both groups, with the hypoxic group showing higher levels at the final observation (10.89 vs. 10.34) (Table 2). Post-hoc analysis revealed significantly higher levels of the transcription factor SOX-9 and type-II collagen matrix in the hypoxic group compared to both the normoxic and control groups (Table 3).

Table 2. Immunohistochemistry evaluation of transcription factors and extracellular matrix components

		Control (C)	Normoxic, 3D medium (P1)	Hypoxic, 3D medium (P2)	p
SOX-9	**Day 2**	5.61	5.56	5.54	0.000
	Day 7	6.69	7.85	8.01	
	Day 14	6.64	7.79	8.57	
	Day 21	6.94	8.23	8.99	
RUNX-2	**Day 2**	3.46	3.61	3.64	0.000
	Day 7	3.54	3.64	3.64	
	Day 14	3.47	3.53	3.60	
	Day 21	3.57	3.52	3.58	
Coll-2	**Day 2**	5.61	6.64	6.94	0.000
	Day 7	7.62	8.99	8.50	
	Day 14	7.83	9.56	10.67	
	Day 21	7.86	9.85	10.88	
Aggrecan	**Day 2**	7.07	7.89	8.21	0.000
	Day 7	7.88	9.24	9.44	
	Day 14	9.07	9.83	10.13	
	Day 21	9.89	10.34	10.89	

Table 3. Post-hoc Analysis for Immunohistochemistry Evaluation

	Control (C) (Mean ± SD)	Normoxic, 3D medium (P1) (Mean ± SD)	Hypoxic, 3D medium (P2) (Mean ± SD)
SOX-9	6.467 ± 0.516^{a}	7.359 ± 1.066^{b}	7.775 ± 1.353^{c}
RUNX-2	3.510 ± 0.060^{a}	3.573 ± 0.064^{b}	3.616 ± 0.042^{b}
Coll-2	7.231 ± 0.951^{a}	8.759 ± 1.280^{b}	9.249 ± 1.645^{c}
Aggrecan	8.479 ± 1.099^{a}	9.324 ± 0.925^{b}	9.666 ± 0.997^{b}

^{a,b,c} *Different letter labels assigned to groups in a statistical analysis indicate that those groups have significantly*

different results from each other

Evaluation of secretome compositions

BMP-7 levels fluctuated only in the normoxic group, increasing initially but slightly decreasing by day 21. In contrast, the hypoxic group showed a consistent increase in BMP-7 throughout the experiment. Both normoxic and hypoxic groups exhibited increasing BMP-2 levels, with the hypoxic group consistently showing higher levels from the start. TGF-β1 levels remained relatively stable across all observations, with the hypoxic group showing slightly higher levels at the beginning and end of the study. TGF-β3 levels increased until day 14 in the normoxic group before slightly decreasing. The hypoxic group consistently demonstrated an increasing trend in TGF-β3 levels, surpassing the normoxic group after the initial time point. Both groups showed a progressive increase in IGF-1 levels. The hypoxic group consistently displayed higher IGF-1 levels compared to the normoxic group, reaching the highest level (11.033) at the end of the experiment. FGF-2 levels peaked in the normoxic group on day 14. However, the hypoxic group showed a consistent increase in FGF-2 levels throughout the experiment and consistently had higher levels compared to the normoxic group (Table 4). Post-hoc analysis of secretome compositions revealed that the hypoxic group exhibited significantly elevated levels of BMP-2, BMP-7, TGF-β1, and FGF-2 compared to both the control and normoxic groups (Table 5).

Table 4. ELISA evaluation of secretome composition

		Control (C)	Normoxic, 3D medium (P1)	Hypoxic, 3D medium (P2)	p
BMP-2	**Day 2**	2.173	2.496	2.779	0.000
	Day 7	2.426	2.712	2.920	
	Day 14	2.465	2.998	3.192	
	Day 21	2.572	3.083	3.223	
BMP-7	**Day 2**	3.775	3.087	3.385	0.000
	Day 7	3.877	3.987	4.104	
	Day 14	3.888	4.276	4.887	
	Day 21	3.756	4.161	4.896	
TGF-β1	**Day 2**	0.652	0.692	0.735	0.000
	Day 7	0.688	0.923	0.925	
	Day 14	0.701	0.946	0.984	
	Day 21	0.721	0.945	0.995	
TGF-β3	**Day 2**	0.458	0.378	0.364	0.000
	Day 7	0.473	0.498	0.512	
	Day 14	0.466	0.554	0.593	
	Day 21	0.461	0.544	0.598	
IGF-1	**Day 2**	7.325	6.892	7.042	0.000
	Day 7	7.218	8.759	8.981	
	Day 14	7.211	9.288	10.189	
	Day 21	7.198	9.926	11.033	
FGF-2	**Day 2**	3.448	3.094	3.911	0.000
	Day 7	3.922	4.115	4.929	
	Day 14	3.892	4.891	5.184	
	Day 21	3.876	4.875	5.199	

Table 5. Post-hoc Analysis for ELISA Evaluation

	Control (C)	Normoxic, 3D medium (P1)	Hypoxic, 3D medium (P2)

	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)
BMP-2	2.408 ± 0.148[a]	2.822 ± 0.236[b]	3.028 ± 0.188[c]
BMP-7	3.824 ± 0.062[a]	3.877 ± 0.474[a]	4.317 ± 0.635[b]
TGF-β1	0.690 ± 0.026[a]	0.876 ± 0.108[b]	0.909 ± 0.105[c]
TGF-β3	0.464 ± 0.006[a]	0.493 ± 0.070[b]	0.516 ± 0.095[b]
IGF-1	7.237 ± 0.051[a]	8.715 ± 1.145[b]	9.311 ± 1.518[b]
FGF-2	3.784 ± 0.197[a]	4.243 ± 0.743[b]	4.805 ± 0.534[c]

[a,b,c] *Different letter labels assigned to groups in a statistical analysis indicate that those groups have significantly different results from each other*

The hypoxic (5% O2) condition within the 3D decellularized bovine cartilage scaffold aimed to mimic the articular cartilage environment. Knee cartilage chondrocytes reside in varying oxygen levels, ranging from 1% to 10%, with higher oxygen concentrations in the superficial layers. Hyaline cartilage receives oxygen and nutrients through diffusion from synovial fluid[21].

Cytokines and growth factors play crucial roles in chondrogenesis. BMPs and TGF-βs are essential for tissue homeostasis and collagen production. They activate signaling pathways involving SMAD and MAPKs, such as TAK1 and ERK[22]. BMPs induce cartilage synthesis and suppress catabolic cytokine activity, with BMP-7 specifically reducing articular cartilage degradation in osteoarthritis[23]. TGF-βs promote extracellular matrix (ECM) synthesis and the expression of SOX-9, aggrecan, and collagen type II (Coll-II) by activating the SMAD2/3 phosphorylation pathway[24].

In our study, BMP-7 levels consistently increased in the hypoxic group, reaching 4.896 on day 21, while fluctuating in the normoxic group. BMP-2 levels were consistently higher in the hypoxic group from day 2 (2.779 vs. 2.496) to day 21 (3.223 vs. 3.082). TGF-β1 levels remained stable across observations, with the hypoxic group showing slightly higher levels at the beginning and end. TGF-β3 levels steadily increased in the hypoxic group, while initially increasing and then slightly decreasing in the normoxic group.

The impact of hypoxia on MSC differentiation and proliferation remains unclear, particularly in 3D cultures. Studies have shown conflicting results, with some suggesting that hypoxia may either hinder terminal differentiation and hypertrophy[25] or have no significant effect compared to normoxia[26]. Further investigation is needed, especially in 3D cultures with various scaffold materials.

Our findings align with a previous study using TGF-β medium and PLLA scaffolds, where higher levels of Coll-II and SOX-9 were observed under 2% oxygen compared to normoxia after 21 days of culture[27]. Hypoxia-mediated signaling has been implicated in osteogenic differentiation through the regulation of TWIST, a transcription factor impacting cell differentiation, on RUNX-2. RUNX-2, a master regulator of skeletogenesis, is crucial for osteoblast proliferation and maturation[28]. Another study showed that hypoxia favored osteogenic differentiation in periodontal ligament stem cells, with significantly increased RUNX-2 expression on day 3 compared to normoxia, although it declined by day 9[29]. Our study also found higher RUNX-2 levels in the hypoxic group throughout all observations.

An experiment with 3% oxygen revealed increased Coll-II levels from day 3, while normoxia induced Coll-II only from day 14[30]. Chondrospheroids also showed better Coll-II expression under hypoxia on days 7, 14, and 21[31]. However, a study using collagen sponges found no significant differences in SOX-9 levels between hypoxic and normoxic conditions on days 7, 14, and 21[32]. Our study observed increasing Coll-II levels in both groups, with higher levels in the hypoxic group, peaking on day 21 (10.88 vs. 9.85).
Aggrecan levels also increased in both groups, with higher levels in the hypoxic group (10.89 vs. 10.34). This aligns with a previous study showing that 5% oxygen in pellet cultures of human adipose tissue-derived stromal cells increased Aggrecan, Coll-I, and SOX-9 expression while decreasing Coll-X compared to normoxia, suggesting that 5% oxygen in vitro promotes chondrogenesis[33].

IGF-1 is a key signaling molecule for cartilage maintenance and regeneration, crucial in cartilage engineering[34]. A study using preconditioned serum found that IGF-1 and TGF-β1 were approximately twofold higher in hypoxia serum compared to normoxia[35]. Similarly, our study observed higher IGF-1 levels in the hypoxic group, reaching 11.033. However, both groups showed an increasing trend in IGF-1.

FGF-2 promotes human bone marrow stromal cell proliferation without altering the low oxygen response. FGF-2, along with hypoxia, has been reported to stimulate multipotency and regeneration of these cells[36]. In our study, FGF-2 levels progressively increased in the hypoxic culture, with consistently higher levels compared to normoxia. While some studies suggest a positive impact of hypoxia on cell proliferation, especially in monolayer cultures[37–39], the overall impact of hypoxia on mesenchymal stem cell differentiation and proliferation remains debated. Monolayer cultures may be more susceptible to consistent oxygen levels across all cells. However, in 3D cultures, oxygen pressure can vary greatly between the scaffold surface and interior, depending on the scaffold type and culture technique[3]. One study suggested that hypoxia enhances chondrocyte proliferation and prevents hypertrophy in human umbilical cord blood mesenchymal stem cells, maintaining a stable chondrocyte phenotype[32].

Another study showed that low oxygen alone might not promote Coll-I and SOX-9 expression and even decreased Coll-I after 21 days. However, the addition of TGF-β under hypoxia significantly increased Coll-II levels compared to normoxia[3]. Combining hypoxia with HIF-2α also enhanced chondrogenesis in adipose-derived mesenchymal stem cells, as evidenced by increased expression of Col2a1, aggrecan, and Sox9[40].

CONCLUSION

Under hypoxic conditions within a 3D culture, mesenchymal stem cells exhibited increased expression of all analyzed transcription factors, extracellular matrix components, and secretome substances compared to normoxic conditions. This suggests that a low-oxygen environment in 3D culture effectively simulates the natural articular cartilage niche, fostering chondrocyte proliferation and differentiation.

Ethical Approval

Approval by the Medical Research Ethical Committee, Dr Soetomo General Academic Hospital, Surabaya, Indonesia (Certificate number: 0076/KEPK/IX/2020)

Acknowledgement

We would like to thank Heri Suroto and Annas Prasetyo Adi from the Tissue Bank and Regenerative Medicine, Dr Soetomo General Academic Hospital, Surabaya, Indonesia

Authors' Contributions

KD Hernugrahanto: Conceived and designed the study, performed experiments, analyzed data, wrote the manuscript

NR Noerda: Performed experiments, analyzed data, wrote the manuscript

JAMD Sedar: Performed experiments, analyzed data, wrote the manuscript

L Widhiyanto: Contributed to study design, analyzed data, reviewed and edited the manuscript.

DN Utomo: Conceived and designed the study, supervised the project, analyzed data, reviewed and edited the manuscript

D Santoso: Conceived and designed the study, supervised the project, analyzed data, reviewed and edited the manuscript

REFERENCES

1. An X, Wang J, Xu K, Zhao RC, Su J. Perspectives on Osteoarthritis Treatment with Mesenchymal Stem Cells and Radix Achyranthis Bidentatae. Vol. 15, Aging and Disease. International Society on Aging and Disease; 2024. p. 1029–45.
2. Zubillaga V, Alonso-varona A, Fernandes SCM, Salaberria AM, Palomares T. Adipose-derived mesenchymal stem cell chondrospheroids cultured in hypoxia and a 3D porous chitosan/chitin nanocrystal scaffold as a platform for cartilage tissue engineering. Int J Mol Sci. 2020;21.
3. Theodoridis K, Aggelidou E, Manthou ME, Kritis A. Hypoxia Promotes Cartilage Regeneration in Cell-Seeded 3D-Printed Bioscaffolds Cultured with a Bespoke 3D Culture Device. Int J Mol Sci. 2023;24.
4. Schmitz C, Alt C, Pearce DA, Furia JP, Maffulli N, Alt EU. Methodological Flaws in Meta-Analyses of Clinical Studies on the Management of Knee Osteoarthritis with Stem Cells: A Systematic Review. 2022; 11:965. Available from: https://doi.org/10.3390/cells.https://doi.org/10.3390/cells11060965https://www.mdpi.com/journal/cells
5. Thoene M, Bejer-Olenska E, Wojtkiewicz J. The Current State of Osteoarthritis Treatment Options Using Stem Cells for Regenerative Therapy: A Review. Vol. 24, International Journal of Molecular Sciences. Multidisciplinary Digital Publishing Institute (MDPI); 2023.
6. Zubillaga V, Alonso-varona A, Fernandes S, Salaberria A, Palomares T. Adipose-derived mesenchymal stem cell chondrospheroids cultured in hypoxia and a 3D porous chitosan/chitin nanocrystal scaffold as a platform for cartilage tissue engineering. Int J Mol Sci. 2020;21.
7. Vinod E, Johnson NN, Kumar S, Amirtham SM, James JV, Livingston A, et al. Migratory chondroprogenitors retain superior intrinsic chondrogenic potential for regenerative cartilage repair as compared to human fibronectin derived chondroprogenitors. Sci Rep. 2021; 11:1–13.
8. Rhatomy S, Setyawan R, Romulo MA. Enhancement of chondrogenesis in hypoxic precondition culture: A systematic review. Open Access Maced J Med Sci. 2021; 9:492–504.
9. Samvelyan H, Hughes D, Stevens C, Staines K. Models of Osteoarthritis: Relevance and New Insights. Calcif Tissue Int. 2021; 109:492–504.
10. Zhu C, Wu W, Qu X. Mesenchymal stem cells in osteoarthritis therapy: a review [Internet]. Vol. 13, Am J Transl Res. 2021. Available from: www.ajtr.org
11. Hwang N, Im S, Wu P, Bichara D, Zhao X, Randolph M. Chondrogenic priming adipose-mesenchymal stem cells for cartilage tissue regeneration. Pharmacol Res. 2011; 28:1395–405.
12. Co C, Vickaryous M, Koch T. Membrane culture and reduced oxygen tension enhances cartilage matrix formation from equine cord blood mesenchymal stromal cells invitro. Osteoarthritis Cartilage. 2014; 22:472–80.
13. Edward M, Mahyudin F, Manyakori DBP. the Effect of Culture Techniques of Hypoxic Stem Cell Secretome on the Number of Growth Factor Tgf-ß, Bmp-2, Vegf. (JOINTS) Journal Orthopaedi and Traumatology Surabaya. 2022; 11:5–9.
14. Martinez-Armenta C, Suarez-Ahedo C, Olivos-Meza A, Camacho-Rea MC, Martínez-Gómez LE, Jimenez-Gutierrez GE, et al. The Critical Role of Hypoxia in the Re-Differentiation of Human Articular Chondrocytes. Cells. 2022;11.
15. Koh YG, Choi YJJ. Infrapatellar fat pad-derived mesenchymal stem cell therapy for knee osteoarthritis. Knee. 2012; 19:902–7.

16. Maumus M, Manferdini C, Toupet K, Peyrafitte JA, Ferreira R, Facchini A, et al. Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis. Stem Cell Res. 2013; 11:834–44.
17. Hayrapetyan A, Jansen J, Van Den Beucken J. Signaling pathways involved in osteogenesis and their application for bone regenerative medicine. Tissue Eng Part B Rev. 2015; 21:75–87.
18. Widhiyanto L, Utomo DN, Perbowo AP, Hernugrahanto KD. Macroscopic and histologic evaluation of cartilage regeneration treated using xenogenic biodegradable porous sponge cartilage scaffold composite supplemented with allogenic adipose derived mesenchymal stem cells (ASCs) and secretome: An in vivo experiment. J Biomater Appl. 2020; 35:422–9.
19. Utomo DN, Mahyudin F, Wardhana TH, Purwati P, Brahmana F, Waya A, et al. Physicobiochemical Characteristics and Chondrogenic Differentiation of Bone Marrow Mesenchymal Stem Cells (hBM-MSCs in Biodegradable Porous Sponge Bovine Cartilage Scaffold. Int J Biomater. 2019;2019.
20. Remmele W, Stagner H. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe. 1987; 8:138–40.
21. Demoor M, Ollitrault D, Gomez-Leduc T, Bouyoucef M, Hervieu M, Fabre H, et al. Cartilage tissue engineering: Molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. Vol. 1840, Biochimica et Biophysica Acta - General Subjects. Elsevier B.V.; 2014. p. 2414–40.
22. Thielen NGM, Van der Kraan PM, Van Caam APM. TGFβ/BMP signaling pathway in cartilage homeostasis. Vol. 8, Cells. MDPI; 2019.
23. Badlani N, Oshima Y, Healey R, Coutts R, Amiel D. Use of Bone Morphogenic Protein-7 as a Treatment for Osteoarthritis. In: Clinical Orthopaedics and Related Research. Springer New York; 2009. p. 3221–9.
24. Coricor G, Serra R. TGF-β regulates phosphorylation and stabilization of Sox9 protein in chondrocytes through p38 and Smad dependent mechanisms. Sci Rep. 2016;6.
25. Gawlitta D, Van Rijen MHP, Schrijver EJM, Alblas J, Dhert WJA. Hypoxia impedes hypertrophic chondrogenesis of human multipotent stromal cells. Tissue Eng Part A. 2012; 18:1957–66.
26. Mwale F, Ciobanu I, Giannitsios D, Roughley P, Steffen T, Antoniou J. Effect of oxygen levels on proteoglycan synthesis by intervertebral disc cells. Spine (Phila Pa 1976). 2011;36.
27. Feng G, Jin X, Hu J, Ma H, Gupte MJ, Liu H, et al. Effects of hypoxias and scaffold architecture on rabbit mesenchymal stem cell differentiation towards a nucleus pulposus-like phenotype. Biomaterials. 2011; 32:8182–9.
28. Yang DC, Yang MH, Tsai CC, Huang TF, Chen YH, Hung SC. Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. PLoS One. 2011;6.
29. Xu Q, Liu Z, Guo L, Liu R, Li R, Chu X, et al. Hypoxia Mediates Runt-Related Transcription Factor 2 Expression via Induction of Vascular Endothelial Growth Factor in Periodontal Ligament Stem Cells. Mol Cells. 2019; 42:763–72.
30. Baumgartner L, Arnhold S, Brixius K, Addicks K, Bloch W. Human mesenchymal stem cells: Influence of oxygen pressure on proliferation and chondrogenic differentiation in fibrin glue in vitro. J Biomed Mater Res A. 2010; 93:930–40.

31. Zubillaga V, Alonso-varona A, Fernandes SCM, Salaberria AM, Palomares T. Adipose-derived mesenchymal stem cell chondrospheroids cultured in hypoxia and a 3D porous chitosan/chitin nanocrystal scaffold as a platform for cartilage tissue engineering. Int J Mol Sci. 2020;21.
32. Gómez-Leduc T, Desancé M, Hervieu M, Legendre F, Ollitrault D, de Vienne C, et al. Hypoxia is a critical parameter for chondrogenic differentiation of human umbilical cord blood mesenchymal stem cells in type I/III collagen sponges. Int J Mol Sci. 2017;18.
33. Munir S, Foldager CB, Lind M, Zachar V, Søballe K, Koch TG. Hypoxia enhances chondrogenic differentiation of human adipose tissue-derived stromal cells in scaffold-free and scaffold systems. Cell Tissue Res. 2014; 355:89–102.
34. Mullen LM, Best SM, Ghose S, Wardale J, Rushton N, Cameron RE. Bioactive IGF-1 release from collagen–GAG scaffold to enhance cartilage repair in vitro. J Mater Sci Mater Med. 2015; 26:1–8.
35. Jiang J, Altammar J, Cong X, Ramsauer L, Steinbacher V, Dornseifer U, et al. Hypoxia Preconditioned Serum (HPS) Promotes Proliferation and Chondrogenic Phenotype of Chondrocytes In Vitro. Int J Mol Sci. 2023;24.
36. Lee JS, Kim SK, Jung BJ, Choi SB, Choi EY, Kim CS. Enhancing proliferation and optimizing the culture condition for human bone marrow stromal cells using hypoxia and fibroblast growth factor-2. Stem Cell Res. 2018; 28:87–95.
37. Adesida AB, Mulet-Sierra A, Jomha NM. Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells [Internet]. Available from: http://stemcellres.com/content/3/2/
38. Shi S, Xie J, Zhong J, Lin S, Zhang T, Sun K, et al. Effects of low oxygen tension on gene profile of soluble growth factors in co-cultured adipose-derived stromal cells and chondrocytes. Cell Prolif. 2016; 49:341–51.
39. Kakudo N, Morimoto N, Ogawa T, Taketani S, Kusumoto K. Hypoxia enhances proliferation of human adipose-derived stem cells via HIF-1α activation. PLoS One. 2015;10.
40. Khan WS, Adesida AB, Hardingham TE. Hypoxic conditions increase hypoxia-inducible transcription factor 2α and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. Arthritis Res Ther. 2007;9.