BMEG 4999 – Thesis II, 2024-2025

Role of Hypoxic Conditions in Cartilage Tissue Engineering

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Abstract

Cartilage damage is a common ailment that can be caused by many factors. Due to the avascular nature of cartilage, its natural regeneration capacity is limited. Moreover, the phenotypic destabilisation into hypertrophy induced by trauma can lead to cartilage degradation, which harms joint articulation. Cartilage repair techniques, particularly *in vitro* cartilage culturing, are under heavy research; however, phenotypic instability is a significant challenge for such techniques. In previous studies, hypoxic conditions are suggested for incubation to mimic the *in vivo* environment of cartilage more closely. This study aims to further investigate the culturing technique by comparing the chondrogenic performance of mesenchymal stem cells (MSCs) between normoxia (5% O_2) and hypoxia (21% O_2). Samples are collected every seven days to examine the progress via microscopic observations, quantitative polymerase chain reaction (qPCR), and immunohistochemistry (IHC). While quantitative results have yet to be obtained, preliminary microscopic observations support the claim that hypoxia can enhance chondrogenesis. An improvement in cartilage culturing techniques can reduce the necessity of using factor and hormone cocktails for phenotypic control, simplifying the culturing process drastically.

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Table of Contents

1 Introduction

1.1 Background Information

Cartilage damage is a common symptom of trauma, arthritis, and joint injuries [1]. Osteoarthritis (OA) is the most common form of arthritis that affects 7% (~500 million) of the global population [2]. Ageing is strongly correlated to a decrease in cartilage elastic modulus [3], and therefore, one of the major risk factors of OA. Roughly 13% of women and 10% of men aged 60 or above suffer from symptomatic knee OA [4].

However, the regeneration capacity of cartilage is very low due to its lack of vasculature, neural connection, lymphatic system, and progenitor cells [5]. Cartilage tissue can be classified into elastic cartilage, hyaline cartilage, and fibrocartilage. Hyaline cartilage is rich in type II collagen and can be found in joints to aid articulation due to its smooth nature and ability to resist compressive loads [6]. After cartilage injuries, especially those of high severity, fibrocartilage scar tissue can form during the healing process as a compensatory mechanism; however, fibrocartilage is a tough material, has inferior biomechanical properties compared to hyaline cartilage, and can remodel the extra-cellular matrix (ECM) into one unsuitable for joint articulation [6, 7, 8, 9]. Hence, cartilage damage can easily lead to cartilage breakdown and progress into other diseases like OA [7].

Notably, many cartilage tissue damages are not yet reversible, and current non-surgical treatments can only reduce pain and alleviate the symptoms [10]. Current cartilage injury treatments fail to produce satisfactory therapeutic outcomes, and cartilage repair techniques often possess undesirable traits or exhibit side effects, including immunogenicity, allergenicity, and invasiveness [11].

1.2 Literature Review

As can be observed, there is a medical need for cartilage regeneration. The *in vitro* culturing of cartilage tissue has been a research focus. Mesenchymal stem cells (MSCs) have the potential to differentiate into chondrocytes and can produce ECM molecules that are important for cartilage function [11], making them a popular choice for cartilage tissue culturing.

However, there are still significant challenges that make cartilage repair difficult. Conventional culturing techniques can sparsely control the phenotype of the chondrocytes and often face the loss of the hyaline cartilage phenotype [12, 13]. Notably, there are currently 3D culturing techniques, both with and without scaffolds, under research [1, 14, 15, 16], with some suggesting chondrocytes cultured using a hydrogel scaffold can be injected intra-articularly as a treatment [1]. However, these culturing techniques are often complicated, utilising many added factors to maintain phenotypic stability.

1.3 Study Overview

This study aims to investigate the effectiveness and feasibility of using hypoxia-mimetic agent $CoCl_2$ to induce the differentiation of MSCs into the desired phenotype, namely hyaline cartilage. The study compares the degree of hyaline cartilage differentiation between normoxic and hypoxic (100μM $CoCl_2$) incubation via microscopic observation and quantitative polymerase chain reaction (qPCR).

2 Theory

2.1 In Vivo Hypoxia

Due to the avascular nature of cartilage, it has a native oxygen concentration of 2% to 7% [11].

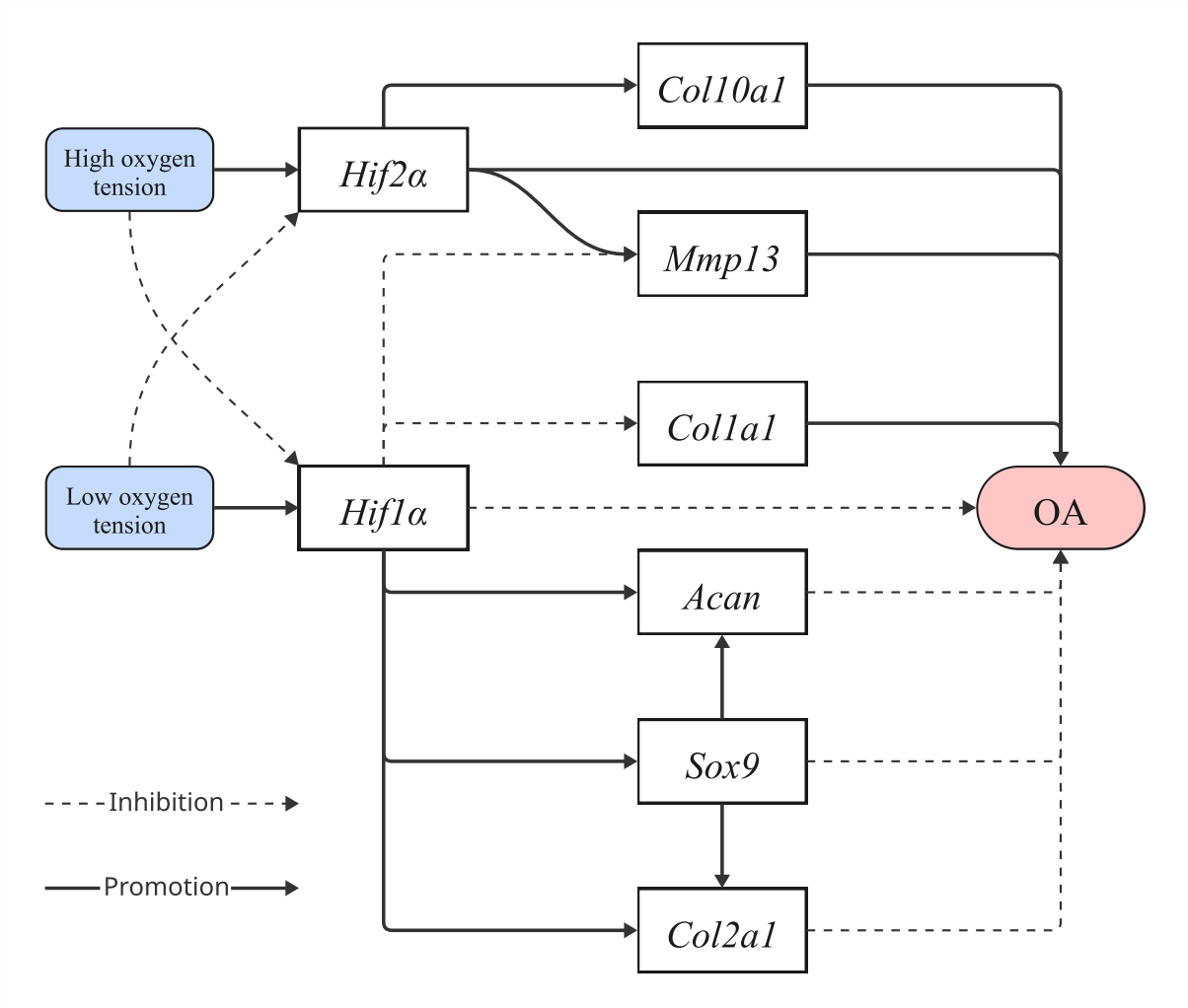

Figure 1: Relationships between related genes/proteins and conditions

Under normoxia, hypoxia-inducible factor-1α (HIF-1α) is rapidly degraded via prolyl hydroxylation and proteasomal degradation [17] by oxygen-dependent prolyl (P4Hs) and arginyl hydroxylases (FIH) [18], giving it a half-life of only 5 to 10 minutes [19]. However, under hypoxia, the hydroxylases are inhibited [18, 20], leading to the stabilisation of HIF-1α.

During the initial stages of chondrogenesis, HIF-1α is required for MSC condensation, chondrocyte proliferation, and ECM synthesis [20] due to its ability to regulates genes associated with cartilage anabolism and chondrocyte differentiation, primarily the master transcriptional regulator of chondrogenesis *Sox9* [21, 22]. The downstream targets of *Sox9* include *Col2a* and *Acan*, which are the major components in cartilage ECM [22]. The existence of HIF-1α stabilises the desired articular hyaline cartilage phenotype [22].

Importantly, HIF-1α is not the sole affecter of chondrogenesis and ECM composition. If oxygen tension increases, the relative levels of *Hif2α* expression increases [20], triggering hypertrophic differentiation from its upregulation of osteoplastic *Col10a* and catabolic genes like ECM-destroying *Mmp13*, which are also observable in OA cases [23].

The maintenance of ECM and subsequently the cartilage phenotype is thus a delicate balance between the effects of HIF-1α and HIF-2α, which are primarily affected by the oxygen tension. A hypoxic condition is, therefore, essential for chondrogenesis.

2.2 In Vitro Hypoxia

In *in vitro* culture of cartilage cells, hypoxic incubators are generally used to emulate the *in vivo* environment to promote differentiation. Hypoxic incubations at both 2% O_2 [24] and 5% O_2 [25] have been found to facilitate chondrogenic differentiation.

However, hypoxia-mimetic agents are also used in laboratories due to their low cost and stability compared to hypoxic incubation [18]. Cobalt (II) chloride ($CoCl_2$) is a common agent that mimics hypoxia by inhibiting HIF-P4Hs and FIH by occupying their binding sites and blocking HIf-1α degradation [18].

Different concentrations of $CoCl_2$ have been used for different cell types to induce the intended effects. For MSC, a study investigated the cytotoxicity of $CoCl_2$ at concentrations ranging from 50μM to 400μM by culturing them in a $CoCl_2$ culture medium for 48 hours, and it found that

100μM of $CoCl_2$ gave the highest cell viability in addition to a four-fold increase of HIF-1α expression [18]. Therefore, 100μM was chosen to be the $CoCl_2$ concentration used for this experiment.

2.3 Chondrogenesis Evaluation

As mentioned briefly in *1.3 Study Overview* above, qPCR will be used to evaluate the degree of chondrogenesis in the cultures. Cells are harvested after 14 days for evaluation, where the expression levels of *Hif1α*, *Col1a1*, *Col2a1*, *Col10a1*, *Acan*, *Sox9*, and *Mmp13* will be tested with qPCR. These chondrogenic gene markers can be used to compare the chondrogenesis between the normoxic and hypoxic cultures. Their involvement in chondrogenesis and their expected observation results are detailed in *Table 1* below. Please also see *Figure 1* above for the regulatory relationships between these genes.

Table 1: The chondrogenic involvement and expected observation results of the chondrogenic gene markers.

Gene	Chondrogenic Involvement	Expectation
Hif1α	Regulates chondrogenic differentiation	Higher in hypoxia
Col1a1	Type I collagen	Lower in hypoxia
Col2a1	Type II collagen: major cartilage component	Higher in hypoxia
Col10a1	Type X collagen	Lower in hypoxia
Acan	Aggrecan: major cartilage component	Higher in hypoxia
Sox9	Maintains cartilage homeostasis	Higher in hypoxia
Mmp13	Degrades type II collagen	Lower in hypoxia

3 Materials and Methods

3.1 Thawing of MSCs in T75 flask

P4 MSCs were retrieved from liquid nitrogen and thawed in a T75 flask. 20mL of growth medium (*Table 3*) was used for this step. The medium was changed every two days. The MSCs were allowed to grow at 37℃ with 5% CO_2 until confluency with ~8.4×10^6 cells.

3.2 Culturing of MSCs in Well Plate

The cells were then trypsinised using 2mL of 0.25% Trypsin EDTA and transferred evenly into a 12-well plate to allow growth and recovery. 1.5mL of growth medium (*Table 3*) was used per well in this step. The medium was changed every two days. The MSCs were allowed to grow at 37℃ with 5% CO_2 until confluency with ~0.5×10^6 per well.

3.3 Chondrogenesis of MSCs in Well Plate

The medium was changed to a chondrogenic medium to induce differentiation. 1.5mL of chondrogenic medium (*Table 4*) was used per well in this step. In half of the wells, $CoCl_2$ was added at 100μM as the experimental hypoxic group with the other half as the normoxic control group. The medium was changed every two days. The MSCs were allowed to differentiate at 37℃ with 5% CO_2 for 14 days.

3.4 Fixation and Staining of Differentiated MSCs

For each group, two wells were allocated for fixation and staining. 10% formalin solution was added to each well for 30 minutes to fixate the cells. Alcian Blue Cartilage Stain Kit, pH 1.0 (*Beijing Solarbio Science & Technology Co.,Ltd.*) was used to stain the cells according to manufacturer's instructions.

3.5 RNA Extraction of Differentiated MSCs

For each group, four wells were allocated for RNA extraction. FastPure Cell/Tissue Total RNA Isolation Kit V2 (*Vazyme Biotech Co., Ltd.*) was used to extract the RNA according to

manufacturer's instructions. For two of the wells in each group, the digested samples were combined before the washing and extraction steps. 50μL of ddH_2O per sample was used for elution.

3.6 qPCR of Extracted RNA

After RNA extraction, the concentration and purity of the RNA was tested with a nanodrop spectrophotometer. RNA samples measuring the highest purities and concentrations were chosen. cDNA of the RNA was then synthesised with Hifair Ⅲ 1^{st} Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) kit (*Yeasen*) according to the manufacturer's instructions. qPCR was then done with QuantStudio 3 (*Thermo Fisher Scientific Inc.*) using the Hieff qPCR SYBR Green Master Mix (No Rox) (*Yeasen*) according to the manufacturer's instructions. Three samples were taken per group per gene marker for qPCR.

4 Results and Discussion

4.1 Culturing of MSCs

The thawing step as described in *3.1* above took nine days to reach confluency, while the initial culturing step as described in *3.2* above took five days to reach confluency. The thawing step in trial 4 took much longer than that in trial 3 (five days) due to poor cell health and viability. The culturing steps were otherwise not noteworthy.

The microscopic images obtained can be reviewed in *7.2 Culturing Microscopic Images* below.

4.2 Alcian Blue Staining

The staining process appeared to not have been successful. Even at a low magnification (4×), little to no blue colour can be observed (See *Figure 7* below).

There are several possible reasons for this result.

The first possible reason is insufficient staining. There was insufficient staining solution left in the kit when the staining was done, and therefore the amount of staining solution use had to be reduced. Staining time was increased to compensate for this change, although to little avail. This was likely to be the major reason for the unsuccessful staining, although not likely to be the sole reason. Compared to the trial before where the staining solution was sufficient, an uneven staining was also obtained although the colour was more prominent (See *Figure 6* below).

The second possible reason is the insufficient expression of proteoglycans. Since the kit available was a pH 1.0 kit, the major stained species are the sulphate groups found in the mucins and proteoglycans of the cartilage ECM [26]. However, as will be discussed in *0*

qPCR below, the chondrogenic differentiation appeared to be insufficient, which could explain the reduced collagen formation and the weaker staining.

The third possible reason is that the microscope does not have sufficient magnification. Typically, the magnification level used for this type of observation in typical research is around ~80×. Even at the maximum magnification (40×), the image was still not clear enough and not large enough to observe the internal structures of the cells clearly. This led to the inability to interpret the results properly.

A future suggestion would be to try to incorporate a counter staining step to increase contrast. For this, Nuclear Fast Red is a common option. Another approach would be to use a fluorescent dye. Not longer is this approach more sensitive, it also allows the precise quantification of expression levels using an imager for data analysis.

4.3 RNA Extraction

The concentrations and purities of the extracted samples are as follows.

Table 2: Concentrations and purities of the extracted RNA samples.

Sample	Concentration (ng/µL)	A260/A280	A260/A230
Norm-1	0.74	1.67	0.09
Norm-2	1.28	1.80	0.18
Norm-3*	***1.92****	***2.09****	***3.30****
Hypo-1*	***1.98****	***2.98****	***3.09****
Hypo-2	1.70	1.57	0.58
Hypo-3	1.58	3.37	0.01

The RNA concentrations of the samples were very low, which indicated poor extraction or significant RNA degradation between the extraction and measurement. Similarly low

concentrations were also obtained in the trial before, and extra precautions and attention were taken for this trial but to little avail, which suggested the latter might be a more significant factor for this result.

A large variation can be seen in the A260/A280 and A260/A230 values, with most far from the ideal value of 2.0 [27]. This suggested the samples were contaminated, although it was more likely that the variations originated from measurement errors due to the low sample concentrations.

For the cDNA synthesis and subsequent qPCR steps, the measured values were treated as accurate, and the samples with the highest concentration and purity from each group (as marked in *Table 2*) were chosen to be used.

A future recommendation would be to put even more emphasis in laboratory precautions, to try to use another RNA extraction kit, and to perform the RNA extraction in the same laboratory as the spectrophotometer and PCR thermocyclers to minimise the chance of degradation and contamination. More cells could also be cultured at the same time to increase the sample size, and more or all wells could be combined into one RNA extraction sample to increase the extracted concentration.

4.4 qPCR

The qPCR results are as below. Normalisation was done with the average of all Cq values in the qPCR run as the baseline. Note that some values were empty as marked undetermined by the qPCR machine. The P-values were calculated using a one-tailed t-test, based on the expectations given in *Table 1* above.

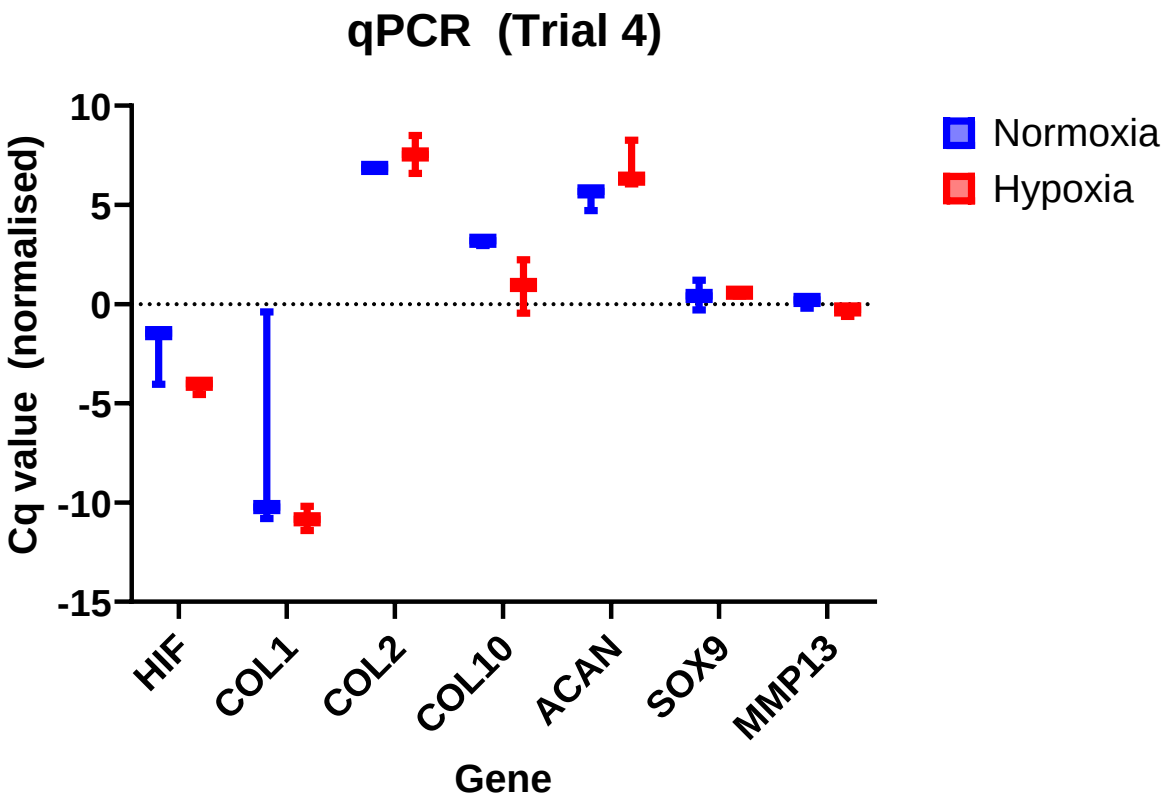

Figure 2: qPCR results of trial 4

As seen in *Figure 2* above, there exists a large variance in some of the samples, possibly due to errors from the poor RNA extraction quality. There is a noticeable difference ($p = 0.054$) for the *Hif1α* expression levels between the groups, with $\Delta\Delta\text{Cq} = -1.88$. It translates into $\text{RQ} = 2^{-\Delta\Delta\text{Cq}} = 3.69$ times higher *Hif1α* levels in the hypoxia group than the control, which is considerably less than the 4.4-fold difference obtained by Teti et al. [18]. This result suggests that the MSCs in my experiments were less responsive to $CoCl_2$ than expected, which led to

the emulated oxygen tension to be higher than expected or insufficiently different from the control group. This explanation is corroborated by the insignificant difference of *Sox9* expression levels, which is the direct effector of *Hif1a*. Other gene markers similarly also produced insignificant differences ($0.053 \leq p \leq 0.392$) between the groups. Curiously, the expression levels of *Col10a1* appeared to be significantly higher ($p = 0.024$) in the hypoxia group than the control, which is opposite to the initial expectations.

From the results, it appears that the MSCs did not go through sufficient chondrogenic differentiation, leading to insignificant differences between the groups. The apparent differences observed in the Cq values are likely only due to experimental and measurement errors or random variances in expression levels instead of due to the hypoxic conditions.

The possibility of RNA degradation must also be considered, as mentioned in *4.3 RNA Extraction* above, which might have skewed the results. Note that the results for trial 3 illustrated similar issues, which suggests this is a consistent issue. The detailed results for trial 3 and 4 and the combined results can be found in *7.4 qPCR Results* below.

A future recommendation would be to include the use of multiple reference housekeeping genes in the qPCR run for normalization purposes instead of relying on only the average values of the samples. More samples (>5) could also be used per gene/group pair such that the problem of undetermined Cq values could be mitigated and that the statistical tests could produce more powerful results.

5 Conclusion

Although the results obtained from this experiment did not generate a statistically significant result that aligned with the initial expectations, it provided valuable feedback as to what can be improved in the future should more trials of this experiment be performed.

However, based on the claim of similar studies, it is suggested that future *in vitro* cartilage culturing can be done under hypoxia or with $CoCl_2$, as doing so can effectively control the phenotype of the cartilage tissue without the necessity for complex factor and hormone cocktails. This can prove to be an improvement to current cartilage engineering techniques and reduce a significant difficulty in cartilage repair, namely phenotypic instability.

6 References

[1] M. Liu, X. Zeng, C. Ma, H. Yi, Z. Ali, X. Mou, S. Li, Y. Deng and N. He, "Injectable Hydrogels for Cartilage and Bone Tissue Engineering," *Bone Research,* vol. 5, p. 17014, 30 May 2017.

[2] D. J. Hunter, L. March and M. Chew, "Osteoarthritis in 2020 and beyond: a Lancet Commission," *The Lancet,* vol. 396, no. 10264, pp. 1711-1712, 28 November 2020.

[3] A. E. Peters, R. Akhtar, E. J. Comerford and K. T. Bates, "The effect of ageing and osteoarthritis on the mechanical properties of cartilage and bone in the human knee joint," *Scientific Reports,* vol. 8, p. 5931, 12 April 2018.

[4] B. Heidari, "Knee osteoarthritis prevalence, risk factors, pathogenesis and features: Part I," *Caspian Journal of Internal Medicine,* vol. 2, no. 2, pp. 205-212, Spring 2011.

[5] C. A. Vilela, C. Correia, J. M. Oliveira, R. A. Sousa, J. Espregueira-Mendes and R. L. Reis, "Cartilage Repair Using Hydrogels: A Critical Review of in Vivo Experimental Designs," *ACS Biomaterials Science & Engineering,* vol. 1, no. 9, pp. 726-739, 13 August 2015.

[6] J. C. Sherwood, J. Bertrand, S. E. Eldridge and F. Dell'Accio, "Cellular and Molecular Mechanisms of Cartilage Damage and Repair," *Drug Discovery Today,* vol. 19, no. 8, pp. 1172-1177, August 2014.

[7] J. Li, H. Jiang, Z. Lv, Z. Sun, C. Cheng, G. Tan, M. Wang, A. Liu, H. Sun, H. Guo, F. Chen, Z. Liu, Y. Fei, Y. Liu, R. Wu, X. Xu, W. Yan, Q. Jiang and D. Shi, "Articular Fibrocartilage-Targeted Therapy by Microtubule Stabilization," *Science Advances,* vol. 8, no. 46, p. eabn8420, 18 November 2022.

[8] M. Benjamin and E. J. Evans, "Fibrocartilage," *Journal of Anatomy,* vol. 171, pp. 1-15, August 1990.

[9] London Knee Clinic, “Articular Cartilage Damage,” 2014. [Online]. Available: https://londonkneeclinic.com/knee-problems/articular-cartilage-damage. [Accessed 30 November 2024].

[10] R. E. Miller, J. A. Block and A.-M. Malfait, “What is new in pain modification in osteoarthritis?,” *Rheumatology (Oxford),* vol. 57, no. suppl_4, pp. iv99-iv107, May 2018.

[11] H. Le, W. Xu, X. Zhuang, F. Chang, Y. Wang and J. Ding, “Mesenchymal Stem Cells for Cartilage Regeneration,” *Journal of Tissue Engineering,* vol. 11, 26 August 2020.

[12] A. R. Armiento, M. J. Stoddart, M. Alini and D. Eglin, “Biomaterials for Articular Cartilage Tissue Engineering: Learning from BIology,” *Acta Biomaterilia,* vol. 65, pp. 1-20, January 2018.

[13] C. Chung and J. A. Burdick, “Engineering Cartilage Tissue,” *Advanced Drug Delivery Reviews,* vol. 60, no. 2, pp. 243-262, 14 January 2008.

[14] J. Hendriks, J. Riesle and C. A. van Blitterswijk, “Co-culture in Cartilage Tissue Engineering,” *Journal of Tissue Engineering and Regenerative Medicine,* vol. 1, no. 3, pp. 161-243, 17 April 2007.

[15] J. Riesle, A. P. Hollander, R. Langer, L. E. Freed and G. Vunjak-Novakovic, “Collagen in Tissue-Engineerd Cartilage: Types, Structure, and Crosslinks,” *Journal of Cellular Biochemistry,* vol. 71, no. 3, pp. 313-327, 12 December 1998.

[16] D. J. Huey, J. C. Hu and K. A. Athanasiou, “Unlike Bone, Cartilage Regeneration Remains Elusive,” *Science,* vol. 338, no. 6109, pp. 917-921, 16 November 2012.

[17] R. Amarilio, S. V. Viukov, A. Sharir, I. Eshkar-Oren, R. S. Johnson and E. Zelzer, “HIF1α Regulation of Sox9 is Necessary to Maintain Differentiation of Hypoxic Prechondrogenic Cells during Early Skeletogenesis,” *Development,* vol. 134, no. 21, pp. 3917-3928, 1 November 2007.

[18] G. Teti, S. Focaroli, V. Salvatore, E. Mazzotti, L. Ingra, A. Mazzotti and M. Falconi, “The Hypoxia-Mimetic Agent Cobalt Chloride Differently Affects Human Mesenchymal Stem Cells in Their Chondrogenic Potential,” *Stem Cells International,* p. 3237253, 13 March 2018.

[19] S. Salceda and J. Caro, “Hypoxia-inducible Factor 1α (HIF-1α) Protein Is Rapidly Degraded by the Ubiquitin-Proteasome System under Normoxic Conditions: ITS STABILIZATION BY HYPOXIA DEPENDS ON REDOX-INDUCED CHANGES,” *Journal of Biological Chemistry,* vol. 272, no. 36, pp. 22642-22647, 5 September 1997.

[20] M. Y. Koh and G. Powis, “Passing the baton: the HIF switch,” *Trends in Biochemical Sciences,* vol. 37, no. 9, pp. 364-372, 18 July 2012.

[21] M. B. Goldring and S. R. Goldring, “Articular Cartilage and Subchondral Bone in the Pathogenesis of Osteoarthritis,” *Annals of the New York Academy of Sciences,* vol. 1192, no. 1, pp. 230-237, 5 April 2010.

[22] D. K. Taheem, G. Jell and E. Gentleman, “Hypoxia Inducible Factor-1α in Osteochondral Tissue Engineering,” *Tissue Engineering Part B: Reviews,* vol. 26, no. 2, pp. 105-115, 16 April 2020.

[23] S. Yang, J. Kim, J.-H. Ryu, H. Oh, C.-H. Chun, B. J. Kim, B. H. Min and J.-S. Chun, “Hypoxia-inducible factor-2α is a catabolic regulator of osteoarthritic cartilage destruction,” *Nature Medicine,* vol. 16, no. 6, pp. 687-693, 23 May 2010.

[24] B. D. Markway, G.-K. Tan, G. Brooke, J. E. Hudson, J. J. Cooper-White and M. R. Doran, “Enhanced Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells in Low Oxygen Environment Micropellet Cultures,” *Cell Transplantation,* vol. 19, no. 1, pp. 29-42, 1 January 2010.

[25] C. Merceron, C. Vinatier, S. Portron, M. Masson, J. Amiaud, L. Guigand, Y. Chérel, P. Weiss and J. Guicheux, “Differential Effects of Hypoxia on Osteochondrogenic Potential of Human Adipose-Derived Stem Cells,” *American Journal of Physiology - Cell Physiology,* vol. 298, no. 2, pp. C355-C364, February 2010.

[26] C. Layton and J. D. Bancroft, “Carbohydrates,” in *Bancroft's Theory and Practice of Histological Techniques*, 8th ed., Elsevier, 2019, pp. 176-197.

[27] Thermo Fisher Scientific, “260/280 and 260/230 Ratios,” [Online]. Available: https://dna.uga.edu/wp-content/uploads/sites/51/2019/02/Note-on-the-260_280-and-260_230-Ratios.pdf. [Accessed 13 April 2025].

7 Appendix

7.1 Medium Components

Table 3: Components of the growth medium and their functions.

Component	Ratios	Function
α-MEM or low DMEM	(Base)	Mammalian cell culture base
FBS	10%	Provides hormone factors for cell proliferation and growth
P/S/F (anti-anti)	1×	Antimicrobials to prevent contamination
FGF2	1 ng/mL	Modulates proliferation and differentiation

Table 4: Components of the chondrogenic medium and their functions.

Component	Ratios	Function
DMEM (w/ Na-Pyruvate)	(Base)	Mammalian cell culture base
P/S/F (anti-anti)	1×	Antimicrobials to prevent contamination
ITS+	10 µg/mL	Basal medium supplement
Proline	40 µg/mL	Enhances mitochondrial clearance and cell metabolism
Dexamethasone	0.1 µM	Decreases cell proliferation but increases ALP activity and collagen synthesis
Ascorbate	50 µg/mL	Protects esterified and non-esterified unsaturated fatty acids from peroxidation
TGF-β1	10 ng/mL	Regulates cell growth and differentiation
$CoCl_2$ (hypoxia group only)	100 µM	Mimics hypoxia conditions

7.2 Culturing Microscopic Images

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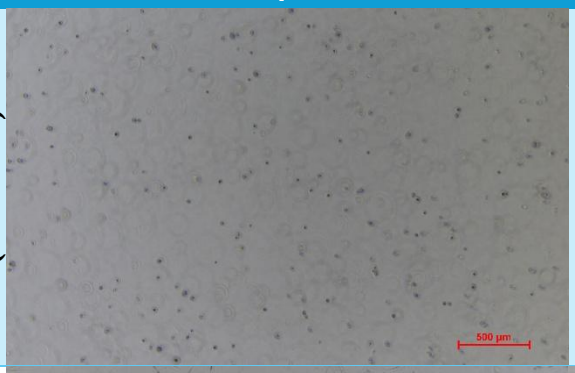
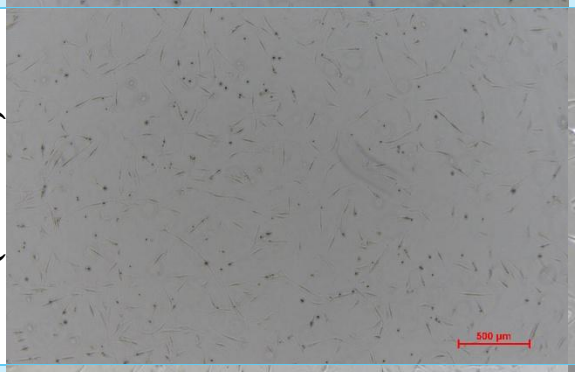
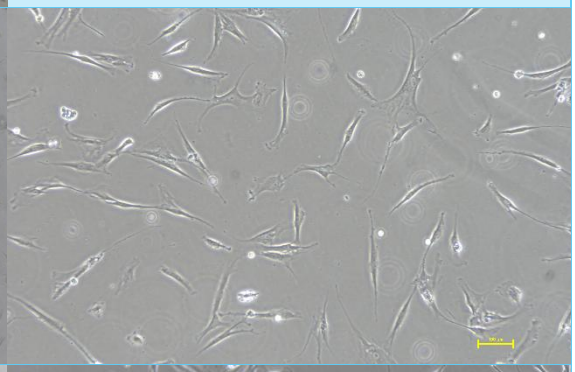
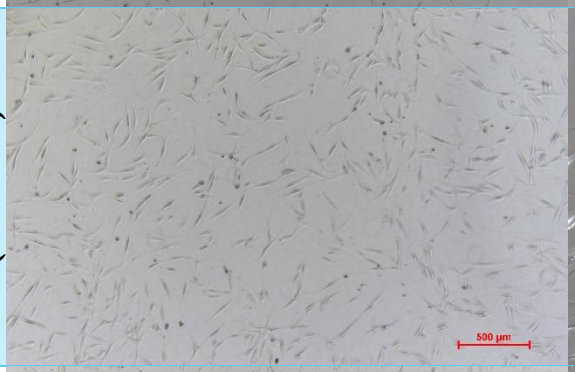
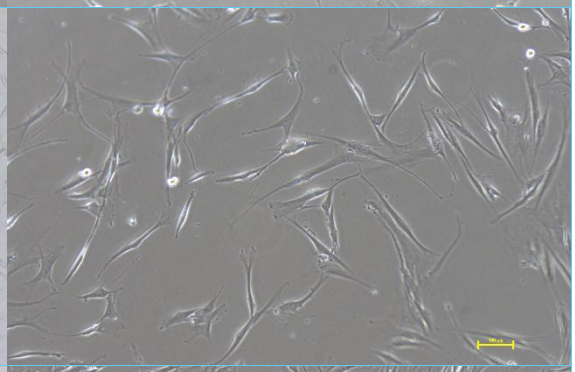
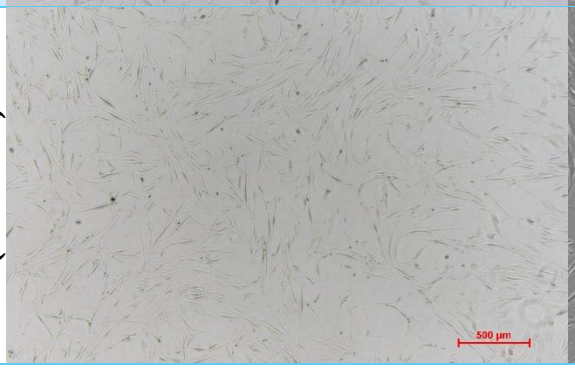
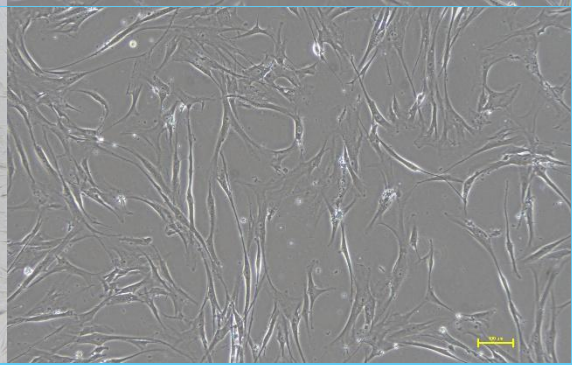
Day 1 (12-3-2025)		/
Day 3 (14-3-2025)		
Day 5 (16-3-2025)		
Day 7 (18-3-2025)		

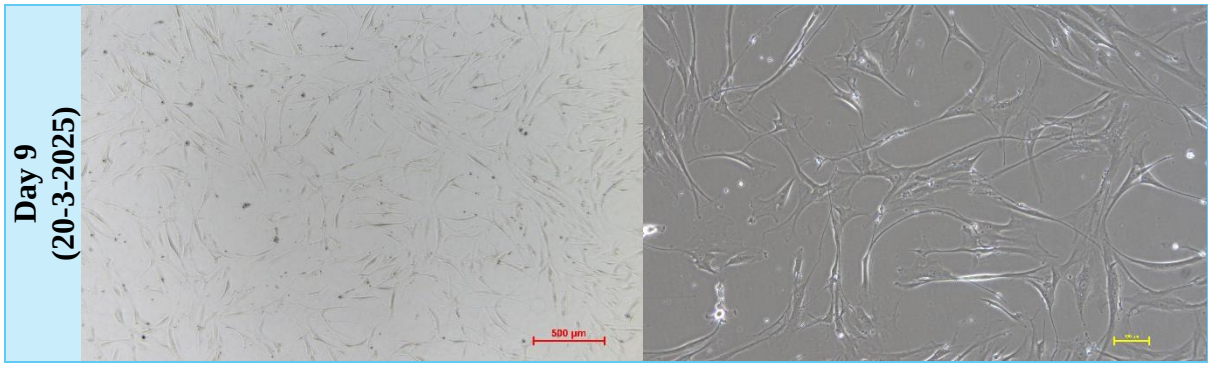


Figure 3: Thawing of MSCs in T75 flask

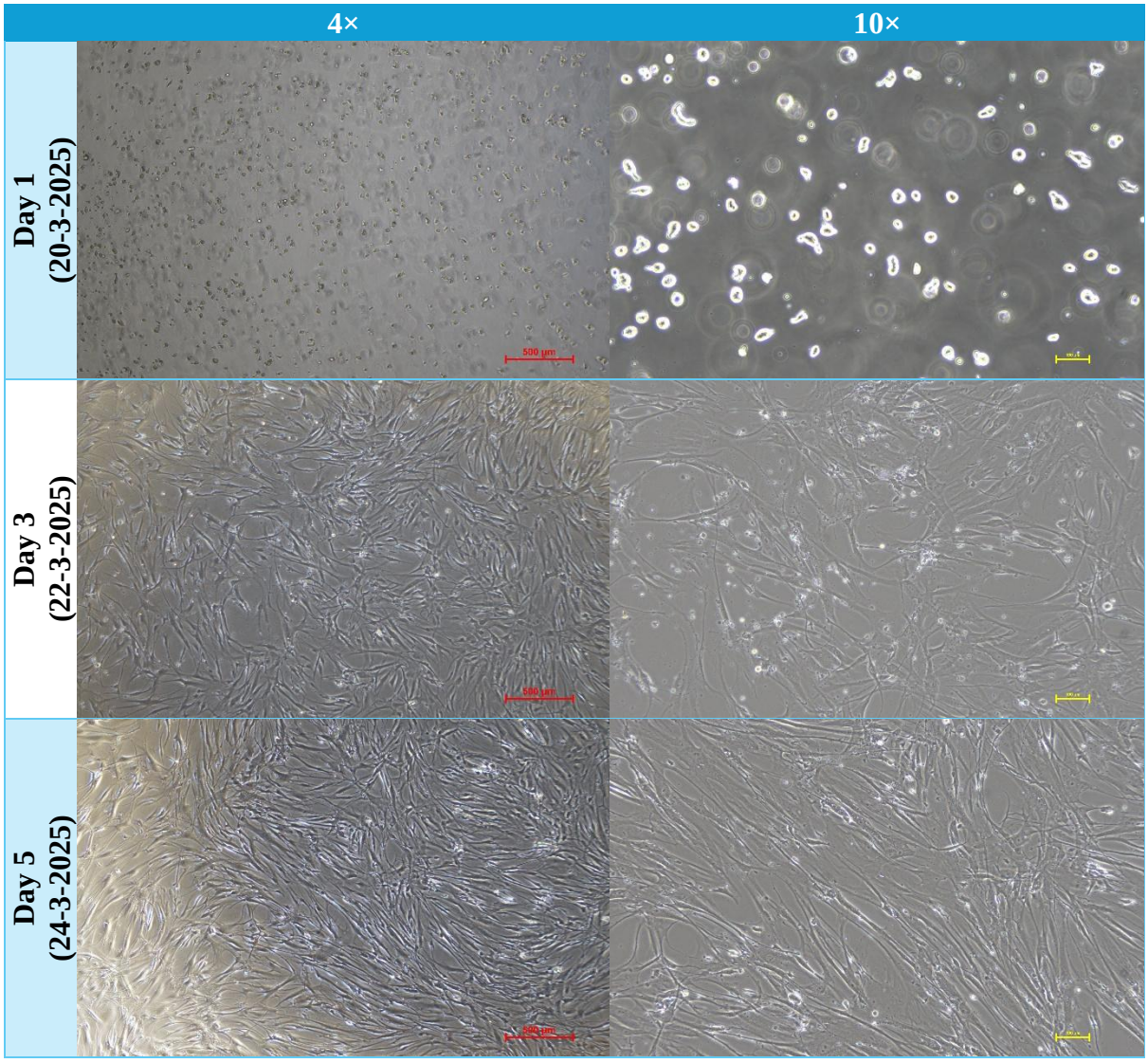


Figure 4: Culturing of MSCs in well plate

Normoxia (10×)
Hypoxia (10×)
Day 1 (24-3-2025)
Day 3 (26-3-2025)
Day 5 (28-3-2025)
Day 7 (30-3-2025)
Day 9 (1-4-2025)

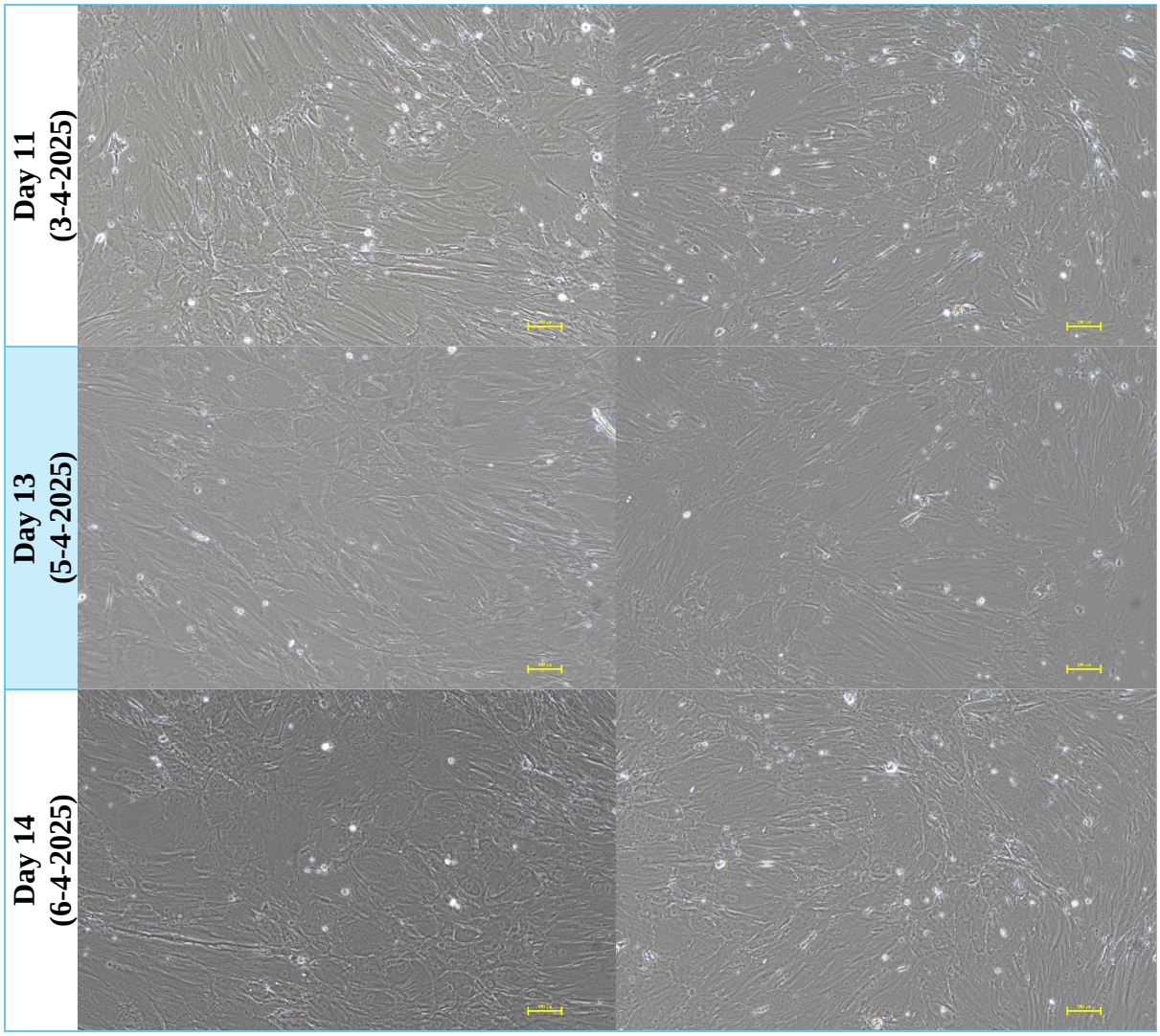


Figure 5: Chondrogenesis of MSCs in well plate

7.3 Alcian Blue Staining Images

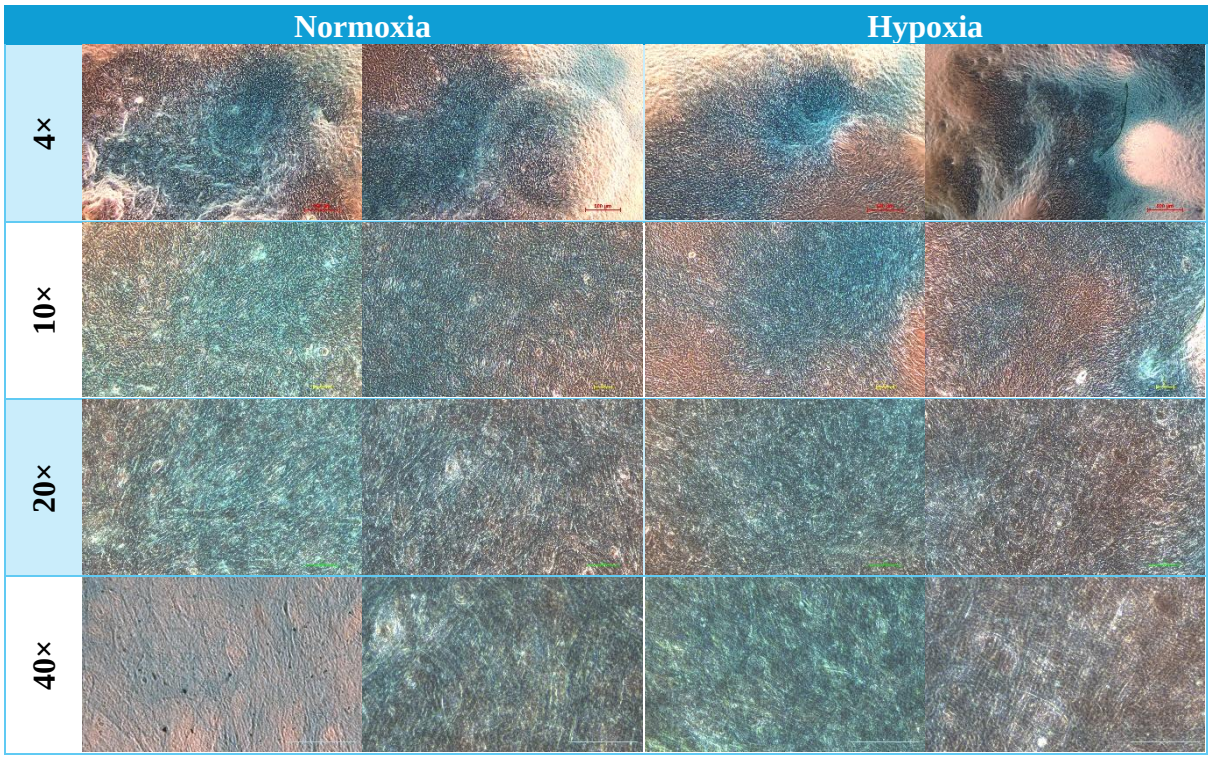


Figure 6: Alcian blue staining of MSCs (Trial 3)

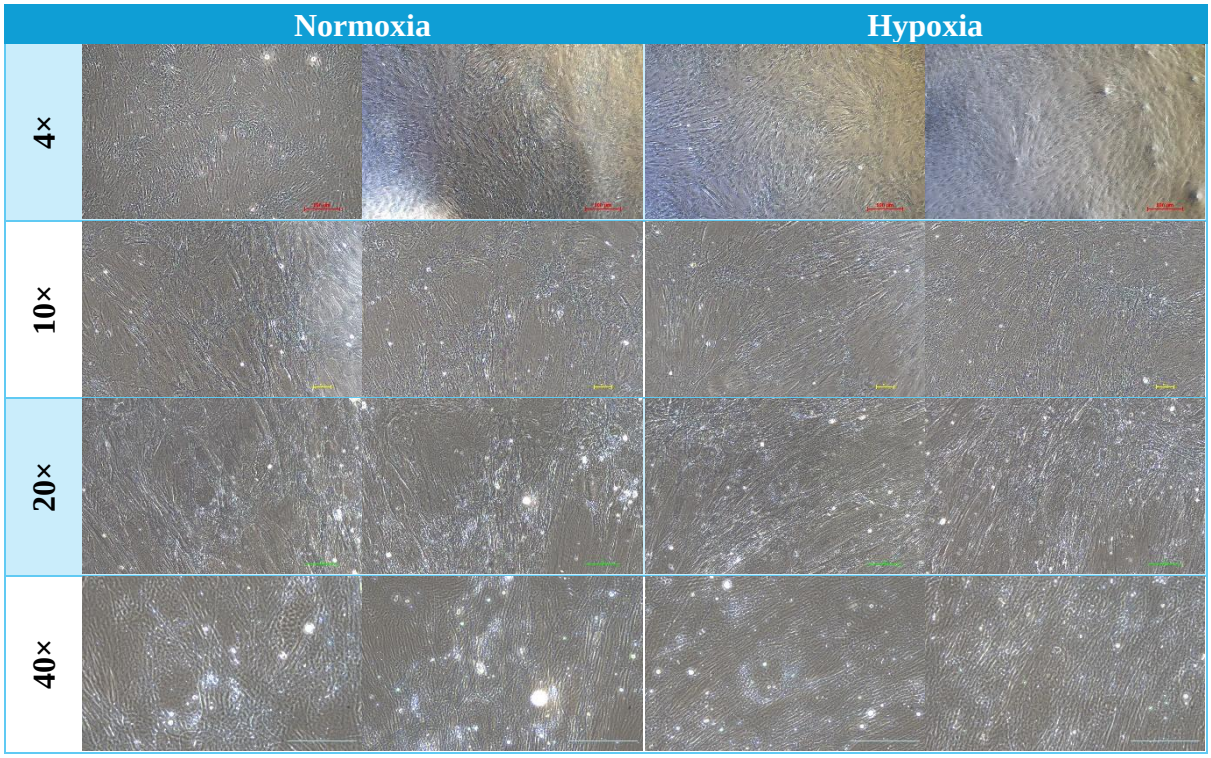


Figure 7: Alcian blue staining of MSCs (Trial 4)

7.4 qPCR Results

Table 5: qPCR results of trial 3

Gene	Normalised Cq												Mean		SD		P-value
	NORM						HYPO						NORM	HYPO	NORM	HYPO	
Hif1α	-1.62	-1.88	-5.24	-1.23	-3.67	-	-	-1.61	-0.70	-1.36	-0.45	-1.46	-2.73	-1.11	1.69	0.51	0.037
Col1a1	-7.53	-8.85	-8.72	-10.3	-11.5	-12.1	-8.20	-8.77	-9.26	-10.1	-10.2	-15.0	-9.82	-10.3	1.75	2.46	0.368
Col2a1	+10.5	+10.1	+11.9	+6.98	+8.50	-	+9.95	+10.0	9.90	-	-	+7.05	+9.59	+9.24	1.90	1.46	0.384
Col10a1				+2.78	+2.41	+2.79				+3.35	+0.22	+1.45	+2.66	+1.67	0.22	1.57	0.171
Acan				+5.55	+3.62	+4.12				+4.00	+4.97	+4.47	+4.43	+4.48	1.00	0.49	0.471
Sox9				+0.99	+1.73	+1.31				-0.47	+1.33	-0.52	+1.34	+0.11	0.37	1.06	0.065
Mmp13				+2.13	+4.15	+1.71				+1.01	+1.38	+0.20	+2.66	+0.86	1.30	0.60	0.048

Table 6: qPCR results of trial 4

Gene	Normalised Cq						Mean		SD		P-value
	NORM			HYPO			NORM	HYPO	NORM	HYPO	
Hif1α	-1.46	-4.04	-1.31	-4.56	-4.02	-3.89	-2.27	-4.15	1.54	0.36	0.054
Col1a1	-0.40	-10.8	-10.2	-11.4	-10.8	-10.2	-7.14	-10.8	5.85	0.61	0.170
Col2a1	-	-	+6.87	+8.50	-	+6.59	+6.87	+7.54	1.35	1.35	-
Col10a1	+3.19	+3.22	+2.95	+0.96	+2.24	-0.46	+3.12	+0.91	0.15	1.35	0.024
Acan	+4.71	+5.68	+5.73	+6.06	+8.26	+6.32	+5.37	+6.88	0.57	1.2	0.061
Sox9	-0.30	+1.20	+0.41	+0.42	+0.71	+0.57	+0.44	+0.57	0.75	0.15	0.392
Mmp13	0.21	+0.32	-0.21	-0.62	-0.14	-0.26	+0.11	-0.34	0.28	0.25	0.053

Table 7: qPCR results of trial 3 & trial 4 combined

Gene	Normalised Cq		Mean		SD		P-value
	NORM	HYPO	NORM	HYPO	NORM	HYPO	
Hif1α	Please refer to *Table 5* and *Table 6* above		-2.56	-2.25	1.54	1.63	0.355
Col1a1			-8.93	-10.4	3.50	1.99	0.139
Col2a1			+9.14	+8.67	2.03	1.55	0.333
Col10a1			+2.89	+1.29	0.30	1.38	0.010
Acan			+4.90	+5.68	0.89	1.55	0.156
Sox9			+0.89	+0.34	0.73	0.72	0.108
Mmp13			+1.39	+0.26	1.63	0.78	0.079

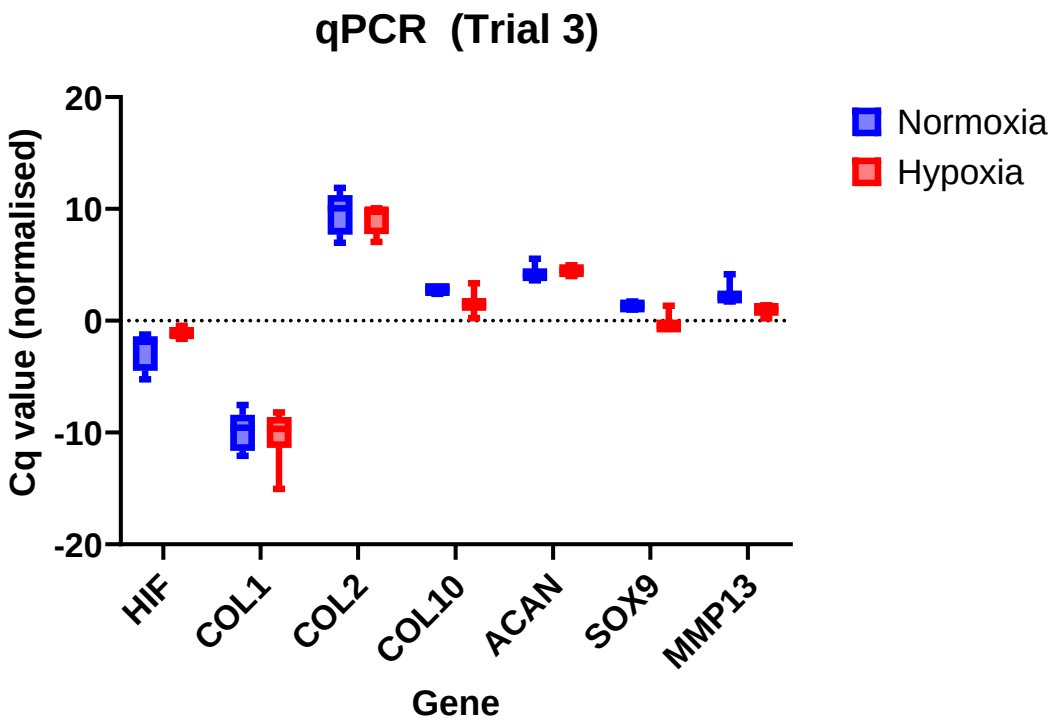
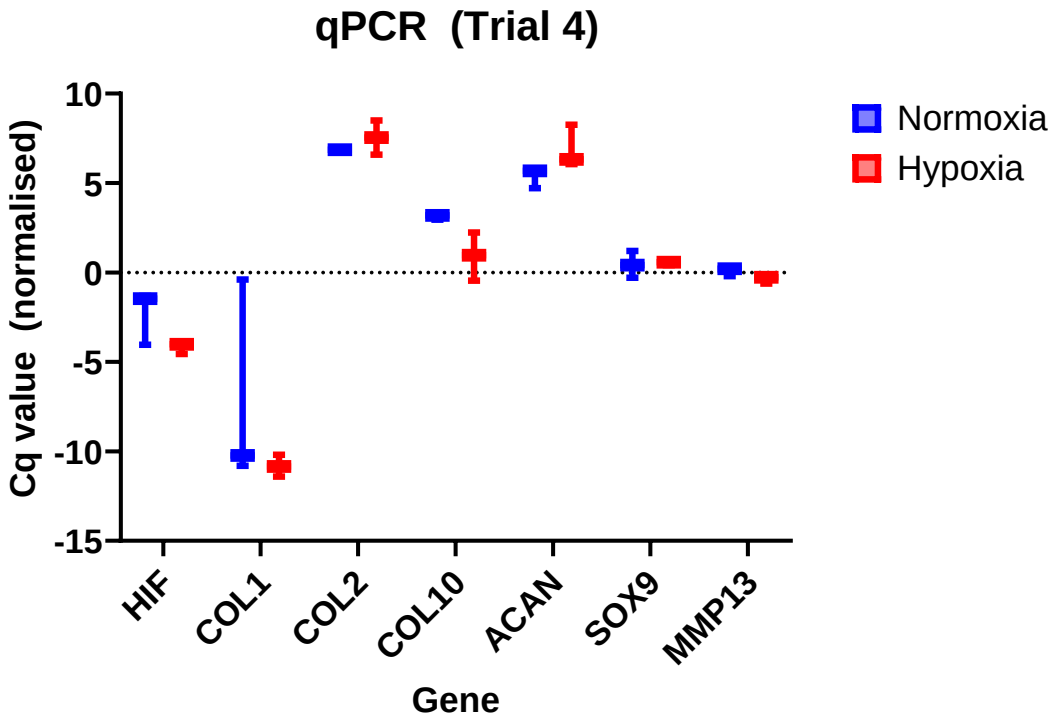


Figure 8: qPCR results of trial 3



***Error! Not a valid bookmark self-reference.**: qPCR results of trial 4*

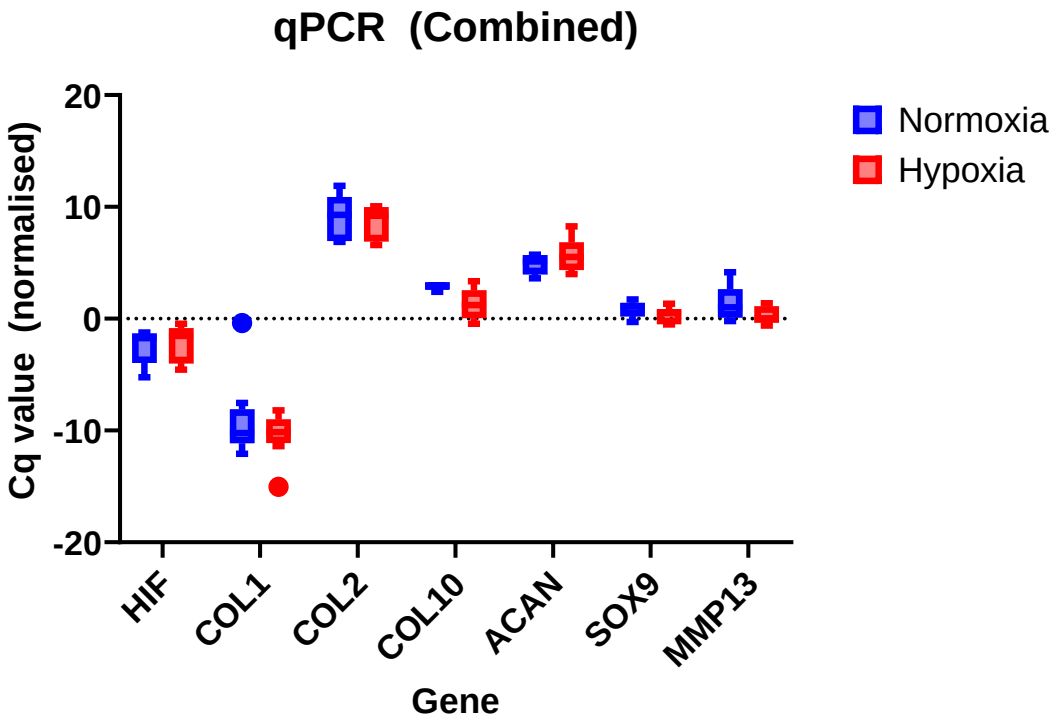


Figure 9: qPCR results of trials 3 and 4 combined