Enhancement of Chondrogenesis in Hypoxic Precondition Culture: A Systematic Review

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Introduction

Cartilage has many important roles to support the normal joint function. It provides the gliding between two bone surfaces and as shock-absorber [1]. Cartilage is incapable to repair and regenerate once it is damaged [2]. Treatment of choice in cartilage damage varies from pharmacologic therapy to surgery. The latest surgical therapy methods are marrow stimulating technique, osteochondral transplantation, and autologous chondrocyte implantation [2], [3], [4]. Cell-based therapy is currently used to treat focal cartilage defects [5].

Stem-cell-based therapy has been used as an additional treatment because of its self-renewal properties, differentiation potentials, and immune-modulatory activities [6]. Stem cells can generate more fibrous cartilage to construct its biomechanical property and have higher durability [4]. After a 6-month follow-up, hyaline cartilage was regenerated after Mesenchymal Stem Cells (MSCs) were injected into the joint [7].

Stem cell culture is done to collect enough cells for transplant process. The previous researches did not consider the oxygen level condition in stem cell culture. Room air oxygen level (21%) is widely used to culture stem cells [8], [9]. Normally, cartilage is relatively avascular with a range of 1–7% of oxygen concentration [9]. Hypoxic state can induce sex-determining region Y-box9 (SOX9) expression that is an important transcription factor for chondrogenesis [9]. Stem cell proliferation and multipotency are maintained in hypoxic condition [5], [9], [10], [11]. Hypoxic state also increases extra-cellular matrix synthesis by chondrocytes [9].

However, there is still not enough data to confirm the level of hypoxic condition medium for stem cell culture. A systematic review from existing preclinical studies is needed to consider the safety and efficacy, and to guide future studies. The main purpose of this review is to summarize the in vitro studies regarding the hypoxic level of stem cell culture as a treatment for any cartilage damages.
Methods

Eligibility criteria
The inclusion criteria for this review consist of the following:
- Study design: Controlled laboratory study (in vitro study)
- Study group: Stem cell isolation from human or animals
- Interventions: Any application of hypoxic level condition to the study groups
- Outcomes: Main outcomes were any chondrogenic marker, cell size, and gene expression
- Language: English.
Non-English studies, duplicates, review articles, and irrelevant articles were excluded from the study.

Literature search and study selection
A comprehensive search was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [12]. The search was conducted from 9 sources including PubMed (MEDLINE), OVID, EMBASE, the Cochrane Library, Scopus, Web of Science, Science Direct, Wiley Online Library, Google Scholar, and bibliography of selected articles on July 1, 2020. The date range was restricted to all studies conducted through July 1, 2020. The term (“culture”) AND (“stem cell” OR “mesenchymal stem cell” OR “MSC”) AND (“hypoxic” OR “hypoxia”) AND (“cartilage” OR “chondro*”) was used as the search keyword.

Two authors (R.S. and S.R.) independently screened the title and abstracts for eligibility by reading the full texts, therefore using it to apply the inclusion and exclusion criteria. Additional searches were done to further include studies mentioned in the reference lists. Discussion was done to resolve any disagreements between the two authors.

Methodological quality assessment and risk of bias
ROBIS systematic review tool was used to assess the methodological quality of the included

Figure 1: Flow chart of study process selection
**Table 1: In vitro study result**

| Author          | Type of MSC (Donor)         | Donor characteristics                                      | Cells preparation                                                                                                                                                                                                 | Intervention to the main group | Control(s)                         | Duration | Scores/Results |
|-----------------|-----------------------------|------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|------------------------------------|----------|----------------|
| Aeda et al.     | BM-MSC (Human)              | Iliac crest of 6 donors                                    | α-MEM supplemented with 10% heat inactivated FBS, penicillin-streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate and 5 ng/ml bFGF or FGF2 at 37 °C with 5% CO₂ | Hypoxia (oxygen tension 3%)   | Normoxia (oxygen tension 21%) | 14 days; 21 days | 1. CFU; ig<cg |
| Bae et al.      | SDSC (Human)                | Synovium tissue from 5 female osteoarthritic patients      | LG-DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin at 37 °C with 5% CO₂                                                                                                        | Hypoxia (oxygen tension 5%)  | Normoxia (oxygen tension 21%) | 14 days and 21 days | 2. Gene Expression (ACAN, Col1, Col2, Col10, COMP) |
| Bomes et al.    | BM-MSC (Bovine)             | Iliac crest from 6 skeletally mature, female Suffolk sheep | α-MEM supplemented with 10% heat inactivated FBS, penicillin-streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate and 5 ng/ml bFGF or FGF2 at 37 °C with 5% CO₂ | Hypoxia (oxygen tension 3%)  | Normoxia (oxygen tension 21%) | 14 days             | 3. Extracellular matrix (GAG*) |
| Cicione et al.  | BM-MSC (Human)              | Three patients who underwent total hip replacement with mean age 64 year                                    | DMEM supplemented with 20% FBS and 1% penicillin/streptomycin with 5% CO₂                                                                                                                                        | Severe Hypoxia (oxygen tension 1%) | Normoxia (oxygen tension 21%) | 14 days             | 4. Cell scaffold construct size* |
| Duval et al.    | BM-MSC (Human)              | Iliac crest of adult donors (ages 54-75 y.o. with median 68 y.o.)                                           | α-MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 ng/ml FGF-2, and antibiotics                                                                                                                        | Hypoxia (oxygen tension 5%)  | Normoxia (oxygen tension 21%) | 7 days               | 4. Cell scaffold construct size* |
| Felka et al.    | BM-MSC (Human)              | Twenty-eight patient undergoing total hip replacement with age range from 45 to 83 y.o.                     | LG-DMEM with 5% human FFP, 10³/ml platelets, 2 mM glutamine, 1000 IU heparin sodium, 100 U/ml penicillin, and 100 ng/ml streptomycin at 37 °C with 5% CO₂ | Hypoxia (oxygen tension 2%)  | Normoxia (oxygen tension 21%) | 28 days            | 5. Expression of TGFβ: ig>cg |
| Gale et al.     | SM-MSC and BM-MSC (Horse)   | Synovium was harvested in standing horses or during arthroscopic procedure in 5 horses                      | N/A                                                                                                                                                                                                             | Hypoxia (oxygen tension 5%)  | Normoxia (oxygen tension 21%) | 28 days            | 6. mRNA expression (HIF-1α; HIF-2α*): ig<cg |
| Galeano et al.  | SM MSC and chondrocyte (Human) | Lipoaspiration was obtained from 3 consenting healthy donors Human primary chondrocytes were obtained from healthy donors undergoing amputation procedures for congenital limb deformity | Advanced MEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO₂                                                                                                                        | Hypoxia (oxygen tension 2%)  | Normoxia (oxygen tension 21%) | 14 days            | 6. mRNA expression (HIF-1α; HIF-2α*): ig<cg |

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Table 1: (Continued)

| Author                           | Type of MSC (Donor)               | Donor characteristics                                      | Cells preparation                                                                 | Intervention to the main group                  | Control(s)                        | Duration     | Scores/Results                                                                                     |
|----------------------------------|-----------------------------------|------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------|-----------------------------------|--------------|---------------------------------------------------------------------------------------------------|
| Gomez-Leduc et al. (2017)         | UCB-MSC (Human)                   | Samples collected from Obstetrics and Gynecology Unit with normal consent from mothers | LD-DMEM supplemented with 20% fetal calf serum, 10 M dexamethasone and incubated at 37°C in a humidified 5% CO₂ atmosphere | Hypoxia (oxygen tension <5%)                   | Normoxia (oxygen tension 21%) | 7, 14, 21 days | 1. Chondrogenesis occurs in the presence of BMP-2 and TGF-ß1: ig<cg 2. Gene Expression (Col1a1; Col2a1; Col10a1, MPP-13): ig<cg |
| Gong et al. (2017)                | MSC (Murine)                      | The murine mesenchymal cell line was purchased from ATCC (Mansassas, VA, USA) | DMEM supplemented with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere            | Hypoxia (oxygen tension 2%)                    | Normoxia (oxygen tension 21%) | 14 days      | 1. Gene Expression (Sox9²; Col1a1¹; Col2a1¹; ACAN¹): ig<cg 2. Reduction of mir-124: ig<cg |
| Henrionnet et al. (2017)          | BM-MSC (Human)                    | Six patients (3 men and 3 women) undergoing total hip arthroplasty with mean age 64.6±3 y.o. | LD-DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 1 mg/ml bFGF at 37°C in a humidified 5% CO₂ atmosphere | Hypoxia (oxygen tension 5%)                    | Normoxia (oxygen tension 21%) | 14, 28 days | 1. Continuous hypoxia (Col2a1¹; ACAN¹; Sox9²; COMP²): ig<cg 2. Gene Expression (VCAN¹; ALP¹; RUNX2²; BIGLAP²): ig<cg |
| Huang et al. (2017)               | Human articular chondrocyte; MMSC; co-culture hAC/ hMSC (Human) | Human primary chondrocytes were derived from healthy looking and full thickness cartilage, and dissected from knee biopsies of three patients (age: 60±3 y.o.) undergoing total knee replacement. HMSCs were isolated from human bone marrow aspirates. | DMEM and 10% FBS supplemented with 10 ng/ml FGF2, 100 U penicillin, 1000 U streptomycin, and 2 mM L-glutamine | Hypoxia (oxygen tension 2.5%)                 | Normoxia (oxygen tension 21%) | 35 days      | 1. Gene Expression (Col2a1¹; ACAN¹): ig<cg 2. Gene Expression (RUNC2²; Col10a1¹; ALP¹; Col1a1¹): ig<cg |
| Hung et al. (2011)                | BM-MSC (Human)                    | Three patients (67, 69 and 72 y.o.) undergoing total knee replacement for osteoarthritis | DMEM and 10% FBS supplemented with 4.5 g/L glucose, 1% L-glutamine, 1% penicillin/streptomycin/linzone, and 1% non-essential amino acids | Hypoxia (oxygen tension 1%)                    | Normoxia (oxygen tension 21%) | 28 days      | 1. Size of pallets: ig<cg 2. Mature chondrocyte morphology: ig<cg 3. Gene Expression (Col2a1¹; COMP²; ACAN¹): ig<cg |
| Kalpakci et al. (2014)            | DIAS (Goat)                       | Full-thickness skins from the abdomens of seven adult goats were obtained from a local abattoir | DMEM and 10% FBS supplemented with 4.5 g/L glucose and L-glutamine, 1% penicillin/streptomycin/linzone and 1% non-essential amino acids | Hypoxia (oxygen tension 5%)                    | Normoxia (oxygen tension 20%) | 14 days      | 1. CFU¹: ig<cg 2. Cell growth: ig<cg 3. Total Collagen production¹: ig<cg 4. Collagen type II: ig<cg 5. GAG content: ig<cg 6. Histological evaluation (Collagen type I staining): ig<cg |
| Kanichai et al. (2008)            | BM-MSC (Rat)                      | Three-month old Wistar rats (200–300 g) with the femur and tibia were cut at both epiphyseal and marrow was flushed into a 50 ml tube using 5 ml supplemented DMEM and a 25-gauge needle. | DMEM supplemented with 10% FBS; 100 U/ml penicillin/streptomycin; 2 mM Glutamnax; 1 mM L-glutamine and 1% non-essential amino acids at 37°C in a humidified 5% CO₂ atmosphere | Hypoxia (oxygen tension 2%)                    | Normoxia (oxygen tension 20%) | 21 days      | 1. Chondrogenic Growth Factors (Proteoglycan¹; Col2a1¹): ig<cg 2. Nuclear expression and HIF-1α activity¹: ig<cg 3. Phosphorylation of AKT¹: ig<cg 4. HIF-1α sRNA inhibits proteoglycan deposition |
| Khan et al. (2007)                | IPFP-SC (Human)                   | Three patients (age 67, 69 and 72 y.o.) undergoing total knee replacement for osteoarthritis | DMEM supplemented with 20% (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, with 2 mM L-glutamine at 37°C in a humidified 5% CO₂ atmosphere | Hypoxia (oxygen tension 5%)                    | Normoxia (oxygen tension 20%) | 14 days      | 1. Cell aggregates¹; GAG content¹ and proteoglycan content¹: ig<cg 2. Gene expression (Sox9²; Sox5²; Sox6²; HIF2α²; ACAN¹; VCAN¹; Collagen type II¹; Collagen type III¹; Collagen type X¹): ig<cg |

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Table 1: (Continued)

| Author            | Type of MSC (Donor) | Donor characteristics | Cells preparation | Intervention to the main group | Control(s) | Duration | Scores/Results |
|-------------------|---------------------|-----------------------|-------------------|--------------------------------|-------------|----------|----------------|
| Khan et al. (2010) | BM-MSC (Human)      | The bone marrow was extracted following fully informed consent of three 18–40 y.o. patients | MSC media supplemented with 5 ng/ml rh-FGF-2 at a density of 166,000 cells per cm² in a T25 cell culture flask. Nonadherent cells were removed after 24 h by washing twice in Dulbecco’s phosphate buffered saline (DPBS) and changing the medium | Hypoxia (oxygen tension 5%) | Normoxia (oxygen tension 20%) | 14 days  | 1. Cell aggregates*: GAG accumulation*: Proteoglycan content*: Ig<cg  
2. Gene Expression (Sox9*: Collagen type II*: VEGFA*: BMP4*: ACAN*: Collagen type XI*): Ig<cg  
3. Collagen 1*: and relaxed moduli*: Ig<cg  
4. Tensile strength; compressive property; and relaxed moduli*: Ig<cg |
| Kishimoto et al. (2006) | ATDC5 Cells derived from murine embryonic carcinoma (Murine) | RKIEN cell Bank (Tsukuba, Japan) | 1:1 mixture of DMEM and Ham’s F-12 (Invitrogen) supplemented with 5% FBS and antibiotics (Invitrogen: penicillin 50 U/ml, streptomycin 50 mg/ml; Expansion medium) at 37°C in a humidified 5% CO₂ atmosphere | Hypoxia (oxygen tension 1%) | Normoxia (oxygen tension 20%) | 7 days | 1. Gene expression (sox9*: HIF-1α*: Ig<cg  
2. Cell expansion: Ig<cg  
3. Increment Chondrogenic matrix (BMP4*: ITS+: ACAN*: Collagen type I*: Ig<cg  
4. Tensile strength; compressive property; and relaxed moduli*: Ig<cg |
| Koay et al. (2008) | ESC (Human) | The National Institutes of Health (NIH)-approved H9 line (WiCell, Madison, WI, USA) was cultured according to standard protocols using a defined medium (www.wicell.org) and a gamma-irradiated CF-1 mouse embryonic fibroblast (MEF) feeder layer on T75 culture plates (Nunc, Rochester, NY, USA). Frozen ESCs at passage 33 (p33) were thawed according to standard protocol and sub-cultured. | HG-DMEM, 10⁻⁵ M dexamethasone, ITS+ Premix (6.25-ng/ml insulin, 6.25-mg/ml streptomycin, 1.25-mg/ml bovine serum albumin, and 5.35-mg/ml linoleic acid), 40-µg/ml L-proline, 50-mg/ml ascorbic acid, 100-mg/ml sodium pyruvate, and 1% FBS at 5% CO₂ | Hypoxia (oxygen tension 2%) | Normoxia (oxygen tension 20%) | 21, 49 days | 1. Gene expression (Sox9*: Collagen type I*: ACAN*: Collagen type XI*): Ig<cg  
2. Hypoxic culture suppressed chondrogenesis-induced apoptosis  
3. Hypoxic condition inhibited activation of caspase-8 and caspase-3 during chondrogenesis  
4. Tensile strength; compressive property; and relaxed moduli*: Ig<cg |
| Lee et al. (2013) | BM-MSC (Human) | Three male Asians with age ranged from the third to fifth decade, who received a spine surgery for spinal disorders | a-MEM supplemented with 16.6% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine at 37°C; 5% CO₂ | Hypoxia (oxygen tension 1%) | Normoxia (oxygen tension 21%) | 7, 14 days | 1. Gene expression (Sox9*: Collagen type I*: ACAN*: Collagen type XI*): Ig<cg  
2. Hypoxic culture suppressed chondrogenesis  
3. Hypoxic condition inhibited activation of caspase-8 and caspase-3 during chondrogenesis  
4. Tensile strength; compressive property; and relaxed moduli*: Ig<cg |
| Lee et al. (2015) | cAMSC (Dog) | 4-month-old beagle dogs (n = 5) | DMEM supplemented with 1% penicillin streptomycin and 10% FBS and maintained in a humidified incubator at 5% CO₂ and 37°C | Hypoxia (oxygen tension 5%) | Normoxia (oxygen tension 21%) | 21 days | 1. Survival Gene Expression (HIF-1α*: VEGFA*: Ig<cg  
2. Chondrogenic gene expression (Collagen type I*: Sox9*: Collagen type XI*): Ig<cg  
3. Collagen 1*: and relaxed moduli*: Ig<cg  
4. Tensile strength; compressive property; and relaxed moduli*: Ig<cg |
| Mahyudin et al. (2018) | BM-MSC (Rabbit) | Bone marrow of healthy male New Zealand rabbit strain | α-MEM with 1-glutamine, without ribonucleoside or deoxyribonucleoside; FBS; 1 Glutamine, 200 ml (Nitrogen); Penicillin G (10,000 units/ml) and streptomycin sulfate (10,000 µg/ml) in 0.85% NaCl solution; Ficol-Paque; Phosphate buffered saline (PBS), without Ca or Mg²⁺; pH 7.4; Trypsin (0.25%) EDTA 4 NA (0.38 g/dL) | Hypoxia (oxygen tension 1%) | Normoxia (oxygen tension 21%) | 35 days | 1. Gene Expression (Collagen type II*: Sox9*: Ig<cg  
2. Chondrogenic gene expression (Collagen type I*: ACAN*: Collagen type XI*): Ig<cg  
3. Collagen 1*: and relaxed moduli*: Ig<cg  
4. Tensile strength; compressive property; and relaxed moduli*: Ig<cg |

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Table 1: (Continued)

| Author            | Type of MSC (Donor)          | Donor characteristics                          | Cells preparation                                                                 | Intervention to the main group | Control(s) | Duration | Scores/Results                                                                 |
|-------------------|-----------------------------|------------------------------------------------|-----------------------------------------------------------------------------------|-------------------------------|------------|----------|--------------------------------------------------------------------------------|
| Markway et al.    | BM-MSC (Human)              | Ten ml bone marrow was taken from iliac crest of healthy donors | LG-DMEM supplemented with 10% FBS and 50 μg/ml gentamicin and placed in tissue culture flasks | Hypoxia (oxygen tension 2%)   | Normoxia (oxygen tension 21%) | 14 days   | 1. GAG: Ig<cg  
2. Detection of Collagen I and Collagen II in hypoxic cultivation  
3. Gene expression (Sox9; ACAN; Collagen I; Collagen II; Collagen X; RUNX2/CBFA1); Ig<cg |  
4. CFU: Ig=cg  
5. ALP: Ig<cg  
6. Matrix deposition and higher level of cellularity occurred peripherally in hypoxic cultivation  
7. Gene expression (Sox9; Collagen I; Collagen III Ratio; Collagen X); Ig<cg |
| Merceron et al.   | AMSC (Human)                | Three different patients undergoing abdominal plastic surgery who had provided prior informed consent | DMEM containing 1% penicillin-streptomycin, 1% L-glutamine, and 10% FCS (control medium) | Hypoxia (oxygen tension <5%)  | Normoxia (oxygen tension 20%) | 28 days   | 1. Gene expression (Collagen II; CollagenIII*; Collagen X): Ig<cg  
2. DNA content: Ig<cg  
3. Hydroxyproline content: Ig<cg  
4. GAG synthesis: Ig<cg  
5. ALP: Ig<cg  
6. Mitochondria number and morphology: Ig=cg |
| Meretoja et al.   | Articular Chondrocyte and Bovine-MSC (Bovine) | Harvested from 7 to 10 day old calves and marrow isolated from tibia and femoral bone of the bovine | DMEM, 10% FBS, 1% non-essential amino acids, 50 mg/mL ascorbic acid, 10 mM L-proline, 20 mM HEPES, PSF | Hypoxia (oxygen tension 5%)   | Normoxia (oxygen tension 20%) | 14 days   | 1. GAG production occurred in D7, D14 and D28  
2. Gene expression (ACAN; Collagen X): Ig<cg |
| Munir et al.      | Human AMSC (Human)          | Four human donors adipose tissue was harvested using a tumescent technique with pump-assisted aspiration. Subcutaneous fat were collected from one female and three male donors, with fat from the abdomen and the hips as the primary source | α –MEM supplemented with 10% FCS in a standard humidified atmosphere containing 5% CO₂ at 37 °C | Hypoxia (oxygen tension 5%)   | Normoxia (oxygen tension 21%) | 28 days   | 1. CFU: Ig<cg  
2. mitochondria number and morphology: Ig=cg  
3. GAG: Ig<cg  
4. attachment of SDSC: Ig<cg |
| Ohara et al.      | SDSC (Human)                | Human synovium was harvested during total knee arthroplasty from 33 donors diagnosed with knee osteoarthritis and some synovium were used for several experiments and the average age was 76±6 years | 10 mL α-MEM containing 10% FBS, 100 unit/mL penicillin and 100 mg/mL streptomycin | Hypoxia (oxygen tension 5%)   | Normoxia (oxygen tension 21%) | 14 days   | 1. CFU: Ig<cg  
2. mitochondria number and morphology: Ig=cg  
3. GAG: Ig<cg  
4. attachment of SDSC: Ig<cg |
| Porton et al.     | Human AMSC (Human) Rabbit AMSC (Rabbit) | Human patients (HumanMSC) undergoing liposuction and who had given written consent AMSC harvested from the inguinal region of the Rabbit | Serum-free DMEM supplemented with 1% penicillin/streptomycin, 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL sodium selenite, 50 nM sodium L-ascorbate, 10 M dexamethasone and 10 ng/mL TGF-β1 with the cultivation in hypoxia 5% and normoxia 21% | Hypoxia (oxygen tension 5%)   | Normoxia (oxygen tension 21%) | 21 days   | rAMSC: (Co2Za1*; ACAN*); Ig<cg  
2. GAG content: (Co2Za1*; ACAN*; LUM, COMP*; HIF-1α*); Ig<cg |
| Ranera et al.     | BM-MSC (Horse)              | Bone marrow aspirates were obtained from a total of five castrated male horses | The cells were rinsed twice with PBS (Gibco), counted, and plated at 2 × 106 nucleated cells/cm² in 6-well plates (Becton Dickinson) in growth medium consisting of low glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich) supplemented with 10% Foetal Bovine Serum, 1% Glutamine (Sigma) and 1% Streptomycin/Penicillin. | Hypoxia (oxygen tension 5%)   | Normoxia (oxygen tension 21%) | 21 days   | 1. sGAG content*: Ig<cg  
2. Gene expression (Co2Za1*; ACAN*; LUM, COMP*; HIF-1α*); Ig<cg |  
3. sGAG: Ig=cg  
4. matrix deposition and higher level of cellularity occurred peripherally in hypoxic cultivation  
5. Bone matrix deposition and higher level of cellularity occurred peripherally in hypoxic cultivation  
6. Gene expression (Sox9; Collagen I; Collagen III Ratio; Collagen X); Ig<cg |
| Author                  | Type of MSC (Donor) | Donor characteristics                                                                 | Cells preparation                                                                 | Intervention to the main group                          | Control(s)          | Duration | Scores/Results                  |
|------------------------|---------------------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------|--------------------|----------|---------------------------------|
| Silva et al. (2020)    | BM-MSC and SDSC     | Bone marrow aspirates (healthy male 36 years) were obtained from Instituto Português de Oncologia Francisco Gentil, Lisboa Portugal and an additional sample of fresh unprocessed bone marrow sample (Male 24 years) was purchased from Lonza (Basel, Switzerland) Synovium aspirates from donors undertaking routine arthroscopic surgery with no history of joint disease (healthy male 22 years and healthy male 28 years) were obtained from Centro Hospitalar de Lisboa Ocidental, E.P.E, Hospital São Francisco Xavier, Lisboa, Portugal | DMEM supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin, Pen-strep, Gibco) and cryopreserved in liquid nitrogen tanks until usage | Hypoxia (oxygen tension 5%) | Normoxia (oxygen tension 21%) | 21 days | 1. Gene expression (Sox9*, ACAN*) BM-MSC: ig>cg SDSC: ig>cg |
| Tian et al. (2013)     | BM-MSC (Human)      | Iliac crests of 9 healthy volunteers (three males and five females)                   | LG-DMEM supplemented with 10% FBS, 1% penicillin Streptomycin and 2 mmol/L L-glutamine at 37 °C with 5% CO₂ | Hypoxia (oxygen tension 5%) | Normoxia (oxygen tension 21%) | 21 days | 1. Gene expression (ACAN*, Collagen type II*): ig>cg 2. Diameter*: ig>cg 3. GAG*: ig>cg |
| Wan Safwani et al. (2017) | AMSC (Human)         | Adipose tissues were harvested from 6 healthy female donors aged 25e35 years who were undergoing Caesarean section with prior informed written consent | DMEM/F12 with 10% FBS, 200 μM indomethacin, 0.5 μM isobutyl-1-methyl xanthine, 1 μM dexamethasone and 10 μM insulin | Hypoxia (oxygen tension 2%) | Normoxia (oxygen tension 21%) | 21 days | 1. Cell number*: ig>cg 2. Gene expression (Col2*, Sox9*, ACAN*): ig>cg |
| Xu et al. (2007)       | AMSC (Mice)          | The inguinal fat pads from 3-week-old FVB mice were carefully dissected and washed sequentially in a Betadine and phosphate buffered saline (PBS) solution | DMEM, 1% FBS, 1% penicillin/ streptomycin, 37.5 mg/mL ascorbate-2-phosphate, ITS premix, and 10 ng/mL TGF-β1 | Hypoxia (oxygen tension 2%) | Normoxia (oxygen tension 21%) | 11 days | 1. Proliferation rate*: ig>cg 2. sGAG (proteoglycan accumulation)*: ig>cg 3. Gene expression (Collagen type II*): ig>cg (ACAN; Sox9): ig>cg |

(Contd...)
Table 1: (Continued)

| Author                  | Type of MSC (Donor) | Donor characteristics | Cells preparation                                                                 | Intervention to the main group | Control(s) | Duration | Scores/Results |
|-------------------------|--------------------|-----------------------|-----------------------------------------------------------------------------------|-------------------------------|-------------|----------|----------------|
| Yodmuang et al. (2015) | ESC (Human)        |                       | DMEM-F12 supplemented with 10% KnockOut™ serum replacement (KSR), 1 mM L-glutamine, 0.1 mM MEM amino acids, 0.1 mM 2-mercaptoethanol and 4 ng/ml bFGF | Hypoxia (oxygen tension 5%)   | Normoxia (oxygen tension 21%) | 7, 14, 21 days | 1. Gene Expression (Sox9*): ig>cg 2. Transient hypoxia: (Col2a1*: DNA content*; GAG*): ig<cg (Col1a1*: Col10a1*): ig<cg |

1. Regarding MSCs
a. BM-MSC: Bone Marrow Mesenchymal Stem Cells
b. SDSC: Synovium-derived Mesenchymal Stem Cells
c. AMSC: Adipose Tissue Mesenchymal Stem Cells
d. UC-MSC: Umbilical Cord Blood-Mesenchymal Stem Cells
e. HAC: Human Articular Chondrocyte
f. hMSC: Human Mesenchymal Stem Cells
g. SM-MSC: Synovial Membrane- Mesenchymal Stem Cells
h. hAC: Human Articular Chondrocyte
i. IPFP-SC: Infrapatellar Fat Pad-Stem Cells
j. ESC: Embryonic Stem Cells
k. iAMSC: Canine Adipose Derived-Mesenchymal Stem Cells

2. Characteristics
a. y.o.: years old
b. TKA: Total Knee Arthroplasty
c. Related to preparation procedures:
   a. MEM: Minimum Essential Medium
   b. PBS: Phosphate Buffered Saline
   c. FBS: Fetal Bovine Serum
   d. DMEM: Dulbecco's modified eagle medium
   e. LG-DMEM: Low-Glucose Dulbecco's modified eagle medium
   f. HG-DMEM: High-Glucose Dulbecco's modified eagle medium
   g. IM-DM: Iscove's modified Dulbecco's medium
   h. FFP: Fresh Frozen Plasma
   i. FCS: Fetal Calf Serum
   j. HEPES: hydroxyethyl piperazineethanesulfonic acid
   k. PSF: Penicillin/streptomycin/amphotericin
   l. ITS: Internal Transcribed Space+

4. Score
a. ig: intervention group
b. cg: control group
c. CFU: Colony-Forming Unit
d. GAG: Glycosaminoglycan
e. DNA: Deoxyribonucleic acid
f. AGC: Aggrecan

2. Data extraction and synthesis
Two authors (R.S. and S.R.) independently performed all the assessments. A thorough discussion was done to resolve discrepancies within authors.

Data extraction and synthesis
Two authors (R.S. and S.R.) recorded data from all included studies independently to extract the following data: Study design, type of cell donor, control and intervention given, duration of experiment, and result evaluation. Discussion was done to resolve any disagreements between the two authors.

Study outcomes are shown in Table 1. Meta-analysis could not be performed due to the heterogeneity of the data (i.e., source of MSCs, different hypoxic oxygen level, follow-up duration, and outcome measurement).
Table 2: In vivo study result

| Author | Type of MSC (Donor) | Donor characteristics | Type of controlled laboratory experiments | Intervention to the main group | Control(s) | Duration | Scores/Results |
|--------|--------------------|----------------------|------------------------------------------|-------------------------------|------------|----------|----------------|
| Duvai et al. (2012) | BM-MSC (Human) | Iliac crest of adult donors (ages 54-75 y.o. with median 68 y.o.) | In vivo (5-week-old rats, mice, and rabbits) | Group 2 (received alginates with human chondrocytes cultured in hypoxia 5%) | Group 1 (received alginates containing stem cells cultured in normoxia 21%) | 21 days | 1. Macroscopic evaluation (hypoxia looked like hyaline cartilage): ig>cg 2. Histological examination (cartilage-like matrix): ig>cg 3. Immunohistological analysis (Co21): ig>cg 4. Cell expressing HIF-1α induced chondrocyte-like cell in normal expression: ig>cg |
| Porton et al. (2013) | Human ASC (Rabbit) | Adult female New Zealand White rabbits weighing 3 to 3.5 kg and 1-month-old female Swiss nude mice | In vivo | Group 1b: Implantation of hASC in nude mice subsinds | Group 1a: Implantation of autologous rabbit nasal chondrocytes (RNCs) | 48 days | hASC (rabbit): GAG and Co21 were detected in histologic precondition hASC (mouse): formation of cell aggregates occurred and immune-reactive for type II collagen |

Results

Study selection

A PRISMA flow diagram (Figure 1) summarizes the study selection process. A total of 438 studies were identified. After screening of the titles and abstracts, 52 articles were considered eligible for further evaluation. After full-text assessment, 34 in vitro studies and two in vivo studies were included in this systematic review.

Study characteristics

This review is presented in Tables 1 and 2 to specifically explain about the type of MSC used, cell preparation, control and intervention groups, duration of observation, and study results.

Most of the studies utilized stem cells from human (22 studies). Six studies used bovine, murine, and horse stem cells, with two studies for each stem cell type. Human and rabbit, rabbit, mice, rat, goat, and dog were used in the past six studies.

The most common MSC type was from bone marrow (14 studies), followed by AMSC (7 studies), ESC (two studies), and SDSC (two studies). With one study each, the others used BM-MSC and SDSC, UCB-MSC, SM-MSC and BM-MSC, murine MSC, bovine MSC, hAC/hMSC, DIAS, IPFP-SC, and Chondroprogenitor derived cells.

Hypoxia as intervention group varied from 1% to 5% oxygen level compared to normoxia with 21% oxygen level. Treatment duration varied from 7 to 49 days. Most of studies evaluated chondrogenic gene and protein expression including Col1a1, Col1a2, ACAN, Sox9, COMP, and RUNX2.

In vitro study outcomes

In vitro study outcomes are summarized in Table 1. According to most studies, there were higher chondrogenic gene and protein expression including ACAN, Col1a1, Col1a2, Col2a1, and Sox9 [5], [9], [10], [11], [14], [15], [16], [17], [18], [19], [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31], [32], [33], [34], [35], [36], [37], [38], [39], [40], and higher extracellular matrix including Glycosaminoglycan (GAG) [5], [9], [10], [18], [19], [24], [25], [29], [31], [33], [38], [41], [42], higher expression of HIF-1α and HIF-2α [5], [17], [18], [22], [29], [36], [39], GAG production [5], [9], [10], [18], [19], [24], [25], [29], [31], [33], [41] and presence of Transforming Growth Factor-β (TGF-β) [5], [9].

The presence of TGF-β1 and Bone Morphogenetic Protein (BMP)-2 had no effect on chondrogenesis activity [40]. There was no difference between hypoxic and normoxic group in cell surface
markers including CD13, CD29, CD44, CD73, CD90, CD105, and CD151 [5]. IL-1β was higher in hypoxic group [37]. Expression of miR-124 [14] and hydroxyproline content [26] was lower in hypoxic group than in normoxic group.

Evaluation of culture size diameter [10], [31], [37], cell number [32], proliferation rate [33], histological evaluation (collagen type 1 staining) [41], and phosphorylation of Akt [17] was higher in hypoxic group. However, study by Hung et al. stated that the size of pallets and mature chondrocyte morphology was lower in hypoxic group [16]. Kalpakci et al. also stated that cell growth was lower in hypoxic group than normoxic group [41]. Mitochondria number and morphology [43], cell expansion [20], and ALP [26] had no difference in between two groups.

Study by Koay et al. was evaluated for construct diameter and thickness of the cultured cell. The hypoxic group was more inferior to normoxic group. However, the tensile strength, compressive property, and relaxed moduli were found higher than normoxic group [34]. There was inconsistent result in CFU [5], [9], [10], [41], [43]. Adesida et al. and Bae et al. stated that CFU was higher in hypoxic group compared to normoxic group [5], [9]. Ohara et al. stated that there was no CFU difference between normoxic group and hypoxic group [43]. However, Bornes et al. and Kalpakci et al. concluded that there was lower CFU amount in normoxic group compared to hypoxic group [10], [41].

**In vivo study outcomes**

In vivo study outcomes are summarized in Table 2. There were only two studies that evaluate in vivo study. Study by Duval et al. revealed that there were superior results in macroscopic evaluation, histological examination, immunohistological analysis, and chondrocyte-like cell induction after HIF-1α expression in hypoxic group compared to normoxic group [36]. Study by Portron et al. concluded that there were GAG and Col2a1 detection in hypoxic precondition, and formation of cell aggregates for collagen type 2 [28].

**Hypoxic condition (oxygen concentration)**

Thirty-four in vitro studies used various oxygen level, 18 studies (52.9%) used 5% oxygen, eight studies (23.5%) used 2% oxygen, five studies (14.7%) used 1% oxygen, two studies (5.9%) used 3% oxygen, and one study (2.9%) used 2.5% oxygen (Table 1).

Two in vivo studies all used 5% oxygen concentration (Table 2).

**Discussion**

There were many studies that evaluated the chondrogenic genes and proteins. Collagen type II contributes more than 80% of normal articular cartilage extracellular matrix. It provides tensile strength and cartilaginous scaffold [40]. ACAN is a proteoglycan in articular cartilage that constitutes 80–90% of all articular cartilage proteoglycan [40]. It serves as fluid regulator in the cartilage matrix and has elastic and compressive strength in articular cartilage [5], [40]. Transcriptional factor Sox9 was found to be an essential factor for chondrogenesis gene including Col2a1 [5]. Collagen type 10 has more osteogenic than chondrogenic differentiation process and also has hypertrophic property marker for chondrocyte [5], [9], [15]. Some studies stated the downregulation and upregulation of this gene. RUNX2 is one of osteogenic mRNA [15]. Expression of Bone Morphogenetic Protein-2 (BMP-2) alone or BMP-2 with TGF-β can increase chondrogenesis process [9], [40]. Adesida et al. wrote that there was improvement of TGF-β Receptor II (TGF-β RII) significantly and TGF-β Receptor I in hypoxic culture condition [5].

Hypoxia state seems to play an important role in chondrocytes proliferation, differentiation, and matrix production [17], [18]. There was no specific consensus for the best hypoxic condition in chondrocyte scaffold culture. In this review, the hypoxic state varied from 1% to 5% oxygen level. Most of in vitro studies showed that hypoxic condition can induce production of ACAN, Collagen type II, and Sox9. There was still inconsistent result in improvement of BMP-2, TGF-β, TGF-β RII, TGF-β R, and reduction of Collagen type 10, RUNX2. Most studies that evaluated GAG extracellular matrix showed that there were more production of GAG in hypoxic state of stem cell culture compared to normoxic group [5], [9], [10], [18], [19], [24], [25], [29], [31], [33], [41].

Cell proliferation rate, cell number, cell diameter, and CFU were still unable to be determined due to inconsistent results. Some studies revealed superior result and the others showed inferior result compared to normoxic group. Hypoxic state can induce cartilage formation and chondrocyte proliferation. Normoxic oxygen level state may be stressful to MSC and may induce an oxidative stress response [33].

Based on in vivo studies, there was supportive result in two studies. Portron et al. stated that there was an increment in production of GAG and collagen type II [28]. Study conducted by Duval et al. concluded that there were increments in macroscopic evaluation, histological examination, immunological analysis, and HIF-1α-induced chondrocyte like cell [36]. Low oxygen tension culture before implantation can enhance chondrogenesis in stem cells. These cells produce chondrocyte markers (type II collagen, ACAN) and
chondrogenesis marker (Sox9) [36]. Hypoxic oxygen level in vitro can influence the regenerative potential of cartilage after in vivo implantation [28]. Chondrogenic stimuli affect stem cells chondrogenesis and cartilage maturation tissue [11], [44].

There were several limitations to this study. The included studies were mostly done with in vitro study with only two studies that reviewed in vivo study. The included studies have several hypoxic oxygen level state, difference in stem cells used, different duration of study evaluation, and different end-point evaluation. Therefore, it was hard to perform a quantitative analysis. Further research is needed to evaluate the exact hypoxic oxygen level that produces the best chondrogenic properties in stem cell culture. More in vivo study is required to achieve better result from further studies.

Conclusion

Application of hypoxic oxygen level in stem cell culture is a promising method to trigger the chondrogenic lineage differentiation and proliferation. However, more pre-clinical studies are needed to further evaluate the exact hypoxic oxygen level to produce the most supportive environment for stem cell culture.

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