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Membrane culture and reduced oxygen tension enhances cartilage matrix formation from equine cord blood mesenchymal stromal cells *in vitro*

C. Co †, M.K. Vickaryous †, T.G. Koch †‡*

† *Department of Biomedical Sciences, University of Guelph, Guelph, ON N1G 2W1, Canada*
‡ *Department of Clinical Medicine, Orthopaedic Research Laboratory, Aarhus University, Aarhus, Denmark*

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SUMMARY

Objective: Ongoing research is aimed at increasing cartilage tissue yield and quality from multipotent mesenchymal stromal cells (MSC) for the purpose of treating cartilage damage in horses. Low oxygen culture has been shown to enhance chondrogenesis, and novel membrane culture has been proposed to increase tissue yield and homogeneity. The objective of this study was to evaluate and compare the effect of reduced oxygen and membrane culture during *in vitro* chondrogenesis of equine cord blood (CB) MSC.
Methods: CB–MSC (n = 5 foals) were expanded at 21% oxygen prior to 3-week differentiation in membrane or pellet culture at 5% and 21% oxygen. Assessment included histological examination (H&E, toluidine Blue, immunohistochemistry (IHC) for collagen type I and II), protein quantification by hydroxyproline assay and dimethylmethylene assay, and mRNA analysis for collagen IA1, collagen IIA1, collagen XA1, HIF1α and Sox9.
Results: Among treatment groups, 5% membrane culture produced neocartilage most closely resembling hyaline cartilage. Membrane culture resulted in increased wet mass, homogenous matrix morphology and an increase in total collagen content, while 5% oxygen culture resulted in higher GAG and type II collagen content. No significant differences were observed for mRNA analysis.
Conclusion: Membrane culture at 5% oxygen produces a comparatively larger amount of higher quality neocartilage. Matrix homogeneity is attributed to a uniform diffusion gradient and reduced surface tension. Membrane culture holds promise for scale-up for therapeutic purposes, for cellular preconditioning prior to cytotherapeutic applications, and for modeling system for gas-dependent chondrogenic differentiation studies.

Introduction

Articular hyaline cartilage in performance horses routinely experience high intensity, repetitive loading. Spontaneous orthopaedic injuries resulting from these activities represent a major cause of loss of performance days and wastage[1]. The intrinsic repair capacity of cartilage is low and current palliative and surgical intervention measures have not been able to demonstrate long-term relief. Tissue replacement strategies using autologous sources are limited by the difficulty of acquiring suitable graft material, and the potential for donor site morbidity[2]. Because of these limitations, cellular replacement strategies, such as tissue-engineered implants or the injection of cells for tissue repopulation have become a topic of interest.

Autologous chondrocytes isolated from hyaline cartilage have been investigated as a source of replacement cells for focal cartilage defect repair[3]. Improved healing was observed, however the limited availability and loss of phenotype upon *in vitro* expansion remains a problem. Hyaline cartilage characteristically contains a low number of chondrocytes, and isolation protocols are only able to recover approximately 22% of total available chondrocytes[4]. *In vitro* expansion of the isolated chondrocytes are therefore required to obtain a therapeutic cell number This expansion step is often associated with a phenotypic alteration towards a fibroblast-like morphology and reduction of characteristic type II collagen and Aggrecan gene expression[5]. Efforts to maintain or redifferentiate chondrocytes to their native phenotype represents an ongoing field of investigation[6].

* Address correspondence and reprint requests to: T.G. Koch, Ontario Veterinary College, University of Guelph, 50 Stone Road, Guelph, ON N1G 2W1, Canada.
E-mail addresses: cco@uoguelph.ca (C. Co), mvickary@uoguelph.ca (M.K. Vickaryous), tkoch@uoguelph.ca (T.G. Koch).

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Multipotent mesenchymal stromal cells (MSC) hold great promise as an alternative, or complementary, strategy for repopulating chondrocytes during cartilage defect repair. MSC can be isolated from a variety of sources, most commonly bone marrow (BM), adipose tissue (AT), and peripheral or cord blood (CB)[7,8]. Defining characteristics of MSC are their ability for self-renewal and trilineage differentiation into osteocytes, chondrocytes and adipocytes[9]. The main advantage of MSC is their ability to maintain proliferative and differentiation potential during *in vitro* expansion[10,11]. CB–MSC are isolated from blood collected non-invasively from the umbilical vessels at the time of birth. CB–MSC are cryotolerant and can be cryobanked for later use. Establishment of CB–MSC cultures ahead of time provides time for cell function screening and/or manipulation for optimal efficacy prior to clinical use. Most other MSC sources are associated with some degree of invasive collection method such as surgical AT harvesting or BM aspiration[11]. In addition, CB–MSC have demonstrated greater chondrogenic potential than other MSC sources, such as BM and AT, in both humans and horses[11–14].

Although MSC are readily acquired in large numbers and capable of undergoing chondrogenic differentiation, the quality of the generated neocartilage is inferior in comparison to normal hyaline cartilage[7,13]. Majority of *in vitro* generated neocartilage is of hybrid fibro-hyaline phenotype and express hypertrophic markers such as type X collagen[15,16]. To address this issue, various culture parameters have been investigated. Two of the most important factors are oxygen tension and culture method.

Compared with atmospheric oxygen levels of ~21%, the oxygen concentration of *in vivo* cartilage is low, ranging between 1% and 10% depending on thickness and proximity to subchondral bone or synovial fluid[17]. Reduced oxygen conditions may therefore be considered 'normoxic' for cartilage cell cultures, whereas those at an atmospheric concentration of 21% represent a state of 'hyperoxia'. The effect of low oxygen culture on chondrogenesis of MSC has been studied in human and animal models, using articular chondrocytes, BM–MSC and AT–MSC[18–20]. Low oxygen culture was shown to enhance the phenotype of neocartilage generated *in vitro*. In addition, hypoxia inducible factors (HIF), which have been characterized as major response molecules to low oxygen, have been correlated with chondrogenic markers such as Sox9 and downstream Collagen IIA1and Aggrecan mRNA expression[21]. Detailed review is given by Schipani[22].

Another important parameter of *in vitro* chondrogenesis is the mode of culture. Comparative chondrogenic potency studies are generally conducted using pellet or micromass differentiation systems. Although pellet differentiation systems permit multiple cell lines to be simultaneously screened for chondrogenic potency using (for example, 96-well culture plates), the resulting 3D cell aggregates are limited in size due to diffusion limitations: as pellet volume increases, oxygen and nutrient diffusion diminishes. Pellets often display areas of necrosis as well as cortical deposition of type I collagen upon histological evaluation[15]. Consequently, there are physical limitations to the overall number of chondrogenic cells that can be generated from each pellet. With a starting MSC seeding density of 2.5×10^5–5.0×10^5 cells, the pellet differentiation system is impractical for large scale MSC cartilage replacement strategies[23]. A more recent approach for promoting MSC differentiation *in vitro* is a membrane culture method[24]. Membrane culturing is a scaffold-free method, wherein high numbers of MSC are seeded on a porous, inert, collagen coated polytetrafluoroethylene (PTFE) membranes. In contrast to pellets, the porosity of the membranes allow for more efficient diffusion between culture medium and developing tissues, possibly contributing to tissue homogeneity. Lee and colleagues reported the formation of a homogenous neocartilage, with abundance of type II collagen and glycosaminoglycans (GAG) using membrane culture methods for chondrogenic differentiation of ovine BM–MSC[24]. Comparable techniques using transwell inserts or agarose gel substrates (termed self-assembly cultures) were associated with similar findings[25,26].

In this study, we hypothesized that mode of culture and oxygen level significantly affects the quality of *in vitro* chondrogenesis of equine CB–MSC. To test this, we compared the effect of two different oxygen concentrations during *in vitro* chondrogenesis of CB–MSC in two different culture systems, pellets and membranes. We determined that both oxygen concentration and culture method influence chondrogenesis of CB–MSC, with low oxygen membrane culturing yielding the most optimal outcome.

Materials and methods

CB–MSC isolation and expansion

The nuclear cells (NC) were isolated from five equine CB samples using PrepaCyte®-WBC (CytoMedical Design Group LLC; St. Paul, MN, USA) according to manufacturer's instructions. As previously described[7], the NC fraction was resuspended in isolation media consisting of low-glucose Dulbecco's modified Eagle medium (LG-DMEM: Lonza; Walkersville, MD, USA), 30% Fetal Bovine Serum (FBS) (Invitrogen; Burlington, ON, Canada), 100 IU/mL Penicillin (Invitrogen; Burlington, ON, Canada), 0.1 mg/mL streptomycin (Invitrogen; Burlington, ON, Canada), 2 mM L-glutamine (Sigma–Aldrich; Oakville, ON, Canada) and 0.1 mM dexamethasone (Sigma–Aldrich; Oakville, ON, Canada). The cell fraction was then plated on polystyrene tissue culture flasks at a density of 1×10^6 cells/cm^2 (Sigma–Aldrich; Oakville, ON, Canada). Colonies were trypsinized (0.04% trypsin-EDTA) at 70–80% confluency and further culture expanded in expansion medium (EM) consisting of LG-DMEM, 30% FBS, 100 IU/mL Penicillin, 0.1 mg/mL streptomycin and 2 mM L-glutamine at 21% oxygen. At passage 2 the cells were cryopreserved in EM with 10% dimethylsulfoxide (DMSO) (Sigma–Aldrich; Oakville, ON, Canada) by overnight control-rate freezing canisters at −80°C (Nalgene® Mr. Frosty; Fischer Scientific; Markham, ON, Canada) before transfer to long-term storage in liquid nitrogen.

Cryopreserved CB–MSC were thawed and plated at a cell density of 5,000 cells/cm^{2}in EM at 21% oxygen. EM was completely changed every 2–3 days. Cells were harvested at 60–80% confluency by enzymatic digestion with trypsin-EDTA and counted with automated cell counter (Nucleocounter® NC100, Mandel, Guelph, ON, Canada) prior to chondrogenic differentiation.

Differentiation

Chondrogenic differentiation was carried out in both pellet culture and membrane culture at 21% (referred to as normoxic

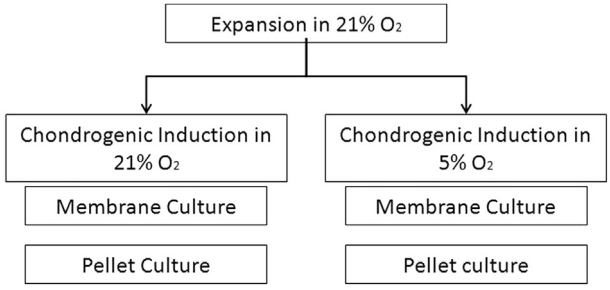

Fig. 1. Study design. CB–MSC from five horses were expanded at 21% oxygen and then differentiated in 21% and 5% oxygen in membrane and pellet culture. The passage number at time of differentiation was 5 or 6. Pooled samples (four pellets, three membranes) were used per each biological replicate for biochemical protein assays and gene expression quantification.