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Influence of oxygen levels on chondrogenesis of porcine mesenchymal stem cells cultured in polycaprolactone scaffolds

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Abstract

Chondrogenesis of Mesenchymal stem cells (MSCs) is known to be regulated by a number of environmental factors, including local oxygen levels. The hypothesis of this study is that the response of MSCs to hypoxia is dependant on the physical and chemical characteristics of the substrate used. The objective of this study was to explore how different modifications to polycaprolactone (PCL) scaffolds influenced the response of MSCs to hypoxia. PCL, PCL-hyaluronic acid (HA) and PCL-Bioglass® (BG) scaffolds were seeded with MSCs derived from bone marrow and cultured for 35 days under normoxic or low oxygen conditions , and the resulting biochemical properties of the MSC laden construct were assessed. Low oxygen tension has a positive effect over cell proliferation and macromolecules biosynthesis. Furthermore, hypoxia enhanced the distribution of collagen and GAGs deposition through the scaffold. On the other hand MSCs displayed certain material dependent responses to hypoxia. Low oxygen tension had a positive effect on cell proliferation in BG and HA scaffolds, but only a positive effect on GAGs synthesis in PCL and HA scaffolds. In conclusion, hypoxia increased cell viability and expression of chondrogenic markers but the cell response was modulated by the type of scaffold used.

Keywords

Hypoxia, chondrogenesis, mesenchymal stem cells, hyaluronic acid, Bioglass.

Introduction

Tissue engineering of functional articular cartilage is still a major challenge in orthopedics, due to the absence of long lasting quality substitutes in clinical practice[1,2]. Autologous chondrocyte implantation, and its variants, are considered to be the gold standard treatment option, but its utility is generally limited to small defects in healthy young patients. This may

be due, at least in part, to the difficulty in obtaining large numbers of chondrocytes and developing tissues with properties similar to native cartilage[3]. MSCs constitute a promising cell source for cartilage engineering, as they can be easily isolated from the patients (bone marrow aspirate, lipoaspirate, etc.), expanded *in vitro* and show multilineage potential[4]. Nevertheless, in vitro tissue engineered cartilage generated using MSCs is generally inferior to that obtained from chondrocytes [5], and there are serious concerns about the phenotypic stability of chondrogenically primed MSCs[5]. It has been pointed out that chondrogenically induced MSCs usually fail to produce a stable adult hyaline cartilage chondrocyte, turning instead into hypertrophic chondrocytes, which ultimately leads to calcification and cell death with corresponding degeneration of the implant[6,7,8]. This has led to increased interest in developing strategies to avoid this terminal differentiation. To this end the bio-physiology of chondrogenesis is being deciphered, definition of differential markers of adult hyaline vs transient chondrocytes is being drawn and practical methods for obtaining functional chondrocytes and cartilage are being tested. Among them, it is worth mentioning the use of viral vectors aimed to stabilize the phenotype[9], supplementation with cytokines[10] (e.g. PTHrP, blockers of Smad1/5/8, MMP13 inhibitors), coculture with chondrocytes[11], the application of biomechanical stimulation [12,13] or the use of low oxygen tension during expansion or differentiation[14,15]. Articular cartilage is an avascular tissue, with oxygen levels ranging from less than 1% to 7% through the depth of the tissue[16]. Hypoxia has been seen to regulate chondrogenic induction of MSCs, through the activation of hypoxia inducible factors (HIFs)[17], mainly HIF1-a which accumulates inside the nucleus in hypoxic conditions and modulates DNA binding affinity of various gene promoters[18]. Moreover, hypoxia may hold some clues in avoiding terminal differentiation as well as to improving the quality of the

formed tissue. As many cell processes and pathways are regulated by cell adhesion and hypoxia has been associated to an altered integrin expression[19], we formulated the hypothesis that cell response to hypoxia would be modulated by the scaffolding material used. Most studies published use pellet cell culture[14] or cells embedded in hydrogels[20] in order to study the effect of hypoxia on chondrogenesis, but to date there are few studies focusing on the crosstalk between hypoxia and material microenvironment[21] in the modulation of chondrogenesis.

Hydrogels are similar to cartilage and favour the *in vitro* maintenance of the chondrocytic phenotype but *in vivo* hydrogels may not have appropriate biomechanical properties as chondral implants and can limit colonization by host cells. Alternatively, hydrophobic materials such as polylactic acid or polycaprolactone (PCL) have mechanical properties matching those of articular cartilage [22], even when are used to manufacture highly porous scaffolds[23] with an interconnected pore structure, and they favor cell colonization and integration into host tissue [24-26]. We have previously developed biodegradable polyester based scaffolds with interconnected spherical pores, which have shown interesting properties and promoted osteochondral repair *in vivo* [24,25]. With a view towards designing scaffolds that would enhance the formation of MSC-based cartilage, we modified previously developed PCL scaffolds using hyaluronic acid or Bioglass. We used hyaluronic acid coating because the literature describes that hyaluronic acid have a positive effect over chondrogenesis, probably due to the interaction trough CD44 that is thought to be a positive regulator of chondrogenesis[27-30] and combines the described advantages of both materials with positive results [23-24]. On the other hand the use of Bioglass in polymeric scaffolds is not common in cartilage tissue engineering [39, A, B], but composites evaluated showed an improvement of the cell seeding efficiency, cell migration and chondrogenic phenotype compared to bare

polymer scaffold. Bioglass not only increase the hydrophilicity of sample improving the cell migration [39,40] it contains silicon too, which has been linked to healthy collagen and polysaccharides metabolism on connective tissues[31] and could improve the ECM secretion. Scaffolds were then seeded with porcine mesenchymal stem cells and cultivated in normoxic (20% O_2) or hypoxic/low oxygen (5% O_2) conditions up to 35 days to evaluate the our hypothesis.

Materials and Methods

Scaffold fabrication: Scaffolds used in the present work were obtained using a combined process of freeze extraction (a modification of freeze drying proposed by Wang and co-workers[32]) and particle leaching as described in previous works[23,26] using polycaprolactone (MW 43000-50000 Da, Polysciences) dissolved in dioxane at 20% w/w and low molecular weight polyethylmethacrylate beads (Lucite International) were used as porogen. Samples were dry and cut at 5 mm of diameter and 3 mm of height. Composite samples were obtained as in previous works[33] mixing PCL at 16% and BG at 4% of weight referred to total dissolution weight.

Hyaluronic acid scaffold coating: Polycaprolactone-hyaluronic acid (HA) samples were obtained following a protocol previously described[B]. Briefly hyaluronic acid (Sigma-Aldrich) at 2% (w/v) in 0.2M NaOH was crosslinked using divinyl sulfone (Sigma-Aldrich) at 2:1 molar ratio inside PCL scaffolds at 37ºC for 48 hours.

Cell harvesting and subculturing: Porcine mesenchymal stem cells from bone marrow were obtained as described in previous works[34] using a modified protocol for human MSC isolation [35]. The cells were seeded at $4X10^5$ cells/cm in a T75cm2 culture flask with DMEM GlutaMAX (Gibco), enriched with 10% of FBS (Gibco), 2% penicillin/streptomycin (Gibco)

and 125µg/ml amphotericin B (Sigma). Then from passage 2 to 3 the cells were cultured in T175 cm2 culture flasks at $2.8X10^5$ in presence of culture media supplemented with 5ng/mL recombinant human fibroblast growth factor-2 (Prospec Bio).

Cell culture: Scaffolds were sterilized in ethanol 70% for 72 hours at 4ºC and conditioned overnight in DMEM supplemented with 10% FBS, finally scaffolds were sigthly dried and deposited in 3% agarose moulds with a hole with same dimensions than scaffold and seeded following the figure X description. PCL and BG scaffolds were seeded pipetting 20µl/sample of cell suspension (containing $5x10^5$ cells) onto top of the scaffold to allow penetrate the scaffold. HA scaffolds have the pores partially closed by hyaluronic acid and cells were seeded injecting 20µl/sample (containing $5x10^5$ cells) with a Hamilton syringe of cell suspension inside each scaffold. After seed all the samples were incubated at 37ºC during 15 minutes to allow cell adhesion. After this period, was added 1.5ml of chemically defined chondrogenic medium (CDM) [8] and incubated 2 hours more to avoid cell leakage to de culture medium. Finally 1 ml of fully supplemented culture medium was added to the samples covering them and were cultured at 37ºC in hypoxic conditions (5% CO_2 - 5% O_2) or in normoxic conditions (5% CO_2 - 16% O_2). Culture medium was changed two times per week and samples were collected at 1, 17 and 35 days.

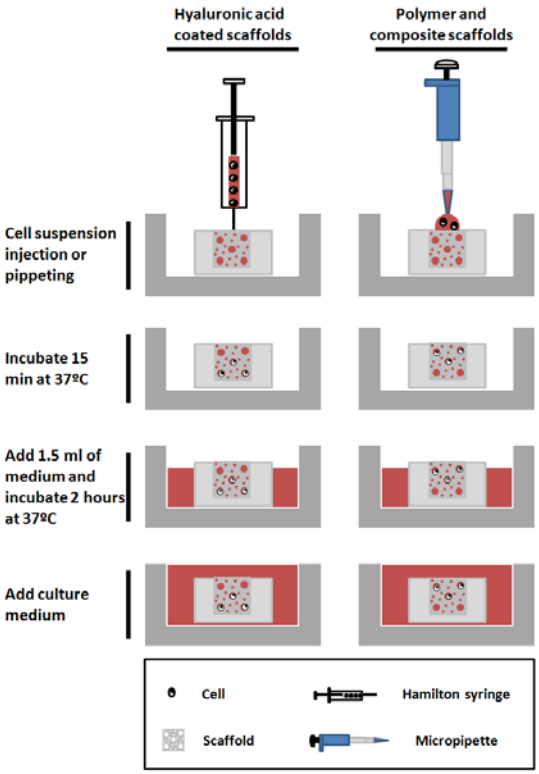

Figure X: **Mesenchymal stem cells seeding in polymeric scaffolds using agarose molds.**

Biochemical analysis: DNA, collagen and glycosaminoglycans content was analyzed at at 1, 17 and 35 days. Four samples for each type of scaffold and culture condition were collected and stored at -80°C until they were analysed. The biomolecules of the extracellular matrix (ECM) were solubilized through enzymatic digestion with papain enzyme at 125µg/ml (Sigma-Aldrich) during 18 h at 60°C.

Collagen content was determined by the hydroxyproline assay using the protocol described by Kafienah[36]. Signal was measured by reading the absorbance at 570nm in a microplate reader (SynergyTM HT from BioTek). Hydroxyproline content was determined from the calibration curve obtained from hydroxyproline standard concentrations and the collagen content was calculated using a value of hydroxyproline-to-collagen ratio of 1:7.69[37]. Sulphated glycosaminoglycans content was determined using Blyscan assay kit . GAG signal was

measured by reading the absorbance at 656nm in a microplate reader and GAG content was calcultated from a standard curve realized using chondroitin sulphate standard provided in the kit. DNA content present in the samples was measured using DNA Quantitation Kit (Sigma) as described previously[38]. Signal was read at 460 nm in a black P96 multiplate with a microplate reader. Quantities of DNA were determined from a calibration curve realized using DNA standards.

Histological analysis: Samples were removed at day 17 and 35, immersed in 4% paraformaldehyde (PFA) at 4ºC for 24 hours and stored at 4ºC in DPBS until analysis were performed. Samples were included in polyester wax (Electron Microscopy Sciences) and were cut longitudinally using the microtome Leica RM2025 in 10 μm thick sections.

Sections were stained for glycosaminoglycans and collagen. GAGs stain was performed with Alcian Blue at 1%. Cell's nuclei counterstain was performed with nuclear fast red at 0.1%. On the other hand total collagen was stained using Picro-Sirius Red at 0.1% and cells nucleus were counterstained with Harris hematoxylin.

Inmunohistochemical collagen staining was carried out following a modified Dako kit staining protocol (EnVision®+dual Link System-HRP, DakoCytomation). Samples were incubated with pepsin (5mg/ml in 5mM HCl) 45 minutes at 37ºC as antigen retrieval. Anti-collagen I antibody (1:100) (Abcam) or anti-collagen II antibody (1:100) (ChemiconInc) were chosen as primary antibody and were incubated 1 hour. Finally samples was dehydrated and mounted with Entellan mouting media (Electron Microscopy Sciences).

Statistical analysis: Statistical analysis was selected after samples homogeneity analysis with Levene's test. Student t-test or one factor ANOVA was chosen when Levene's test was

positive; on the other hand if it was negative a Kruskal-Wallis test was used; differences were considered significant for $p<0.05$.

Results

The evolution of DNA content in the constructs is shown in **Figure 1**. Initial cell number at day 1 showed that seeding efficiency in BG (79.63 ± 12.26) and PCL (78.27% ± 8.02) scaffolds was similar, whereas seeding efficiency in HA constructs (48.75% ± 11.71) was lower. In all constructs cells proliferated up to day 17, showing that materials support mid-term survival of MSCs. Proliferation was slower in hypoxic conditions (HY) than in normoxia (NO) up to day 17, except for HA samples, where proliferation was comparable in both conditions. From day 17 to day 35, DNA content remained nearly constant for all samples in hypoxic conditions and for PCL NO. However, there was a significant decrease in DNA content in BG NO and HA NO samples over the same time period.

MSC chondrogenic differentiation was studied by assessing the production of cartilage specific ECM components, specifically sGAG and collagen. GAGs content normalized to DNA content is shown in **Figure 2A**. GAG secretion increased with time for all samples. Hypoxia led to significant enhancement for most conditions (PCL HY at all times, HA HY at 35 days and BG HY at 17 days). GAG secretion per cell was comparable for hypoxic cultures in all materials. In normoxic conditions, total GAGs synthesis remained low (increase seen in BG NO sample is due to lower DNA levels at 35 days). Results for collagen secretion are shown in **Figure 2B**. Collagen secretion per cell also increased over time, being superior at day 35 for all samples. At day 17, both HA (NO and HY) and BG (NO and HY) scaffolds showed inferior collagen secretion compared to PCL scaffolds in both normoxic and hypoxic conditions, whereas at day 35 only HA (NO and HY) showed inferior collagen level. Hypoxia did not have a significant

effect on collagen synthesis. The only two conditions where there was a significant difference was for PCL HY and BG HY at day 17, where normoxic samples showed increased collagen content with respect to the hypoxic ones.

A qualitative observation of ECM secretion at 35 days is provided in **Figure 3** and **4**. Accumulation of glycosaminoglycans over culture time is shown in **Figure 3A**. In normoxic conditions, staining of mucopolysaccharides (shown in blue in **Figure 3A**) is limited, with a smooth blue staining filling the pores in PCLn and BGn scaffolds, whereas in HA NO constructs the secretion was limited to isolated cell clusters. In hypoxic conditions, in all materials there were significant amounts of matrix filling the scaffolds pores and in some zones of intense blue color, cells were embedded in lacunae within a GAG-rich matrix. Collagen deposition shown in red in **Figure 3B**; consistent with the findings of the biochemical analysis, collagen deposition is higher in PCL and BG samples, while in HA samples less intense staining is observed. Stained parts show either a rough and fibrous texture (such as in BG (NO and HY) samples or PCLn sample) or a non textured pink background such as in PCLh and HAh samples.

Secretion of cartilage specific collagen (type II) was assessed using immunochemical stain against collagen type I (**Figure 4A**) and collagen type II (**Figure 4B**). In all samples both types of collagen are present; hypoxic culture led to a reduction in collagen type I specific staining, whereas collagen type II staining was slightly more pronounced. In HAn sample there was negligible staining for collagen type I or collagen type II, but in HAh sample there was a pronounced staining for collagen type II.

Discussion

The hypothesis of this study was that hypoxia would improve viability and chondrogenesis in

in vitro within different scaffolds in a substrate dependent manner. We selected a bioactive glass (BG) and one of the principal cartilage polysaccharides (HA) with that purpose. BG incorporation could improve protein adsorption on the pore surfaces and cell invasion.[39,40] and silicon released by BG is an essential element in bone and cartilage development[31]. On the other hand the HA is a component of cartilage that show specific cues recognised trough CD44 membrane protein.[27,28]. On the other hand, normoxic culture of chondroinduced MSCs during up to 35 days in synthetic scaffolds resulted in a decrease in DNA content in both HA NO and BG NO groups with respect to 17 days, whereas a slight decrease was seen in PCL NO group. DNA increment under hypoxia conditions is widely described in the literature[41,42] probably through apoptosis inhibition of chondrocytes and chondroinduced MSCs via both the HIF-1α and the PI3K/Akt pathway[14]. This could explain why hypoxia was necessary in order to maintain constant DNA content over a large culture period when cultivating MSCs along the chondrogenic pathway in our materials. Supplementing with TGFβ3, in our model, was not sufficient to maintain cell viability in high oxygen conditions over a large period. Hypoxia increased GAGs/cell synthesis in PCL HY and HA HY materials, whereas no significant difference was seen in BG HY materials. Consistent with findings from the literature, total collagen content per cell in normoxic culture in PCL NO and BG HY washigher at 17 days than in their hypoxic counterparts, whereas in all other conditions no significant differences between normoxia and hypoxia were found.

Literature findings show that the cells under hypoxia regulate the secretory profile from a non-specific one to a more hyaline cartilage like. Increased GAGs synthesis as a result of hypoxic culture was observed in many [15,43] but not all published works [21,44] in the literature. Increased collagen secretion by cells is not a common finding (in some cases even inferior

values are found in hypoxic conditions [45]) although more specific staining for collagen type II and less staining for collagen type I is generally described [15**]**. It has been reported that the content of collagen in hyaline cartilage is 15% and that of proteoglycans 9% percent of total cartilage wet weight[46], thus the total collagen/proteoglycans ratio is 1.67. In the present study we found that PCL NO and HA NO showed aberrant total collagen/GAGs ratios of 6 while this ratio was 3 in BG NO. On the other hand in the same sample compositions (bare PCL, Bioglass® composite and PCL coated with hyaluronic acid) under hypoxia a ratio around 1.5 was determined. Our results suggest that ECM evaluation in base to levels of biochemical components levels is not conclusive, whereas total collagen/GAGs ratio provides direct measurement of tissue quality. In our work hypoxia showed a powerful positive effect over cell differentiation more important than cell substrate. On the other hand, both collagen type I and collagen type II deposition was observed, although collagen type I deposition was limited and collagen type II increased under hypoxia; culture of the cells on a rigid substrate that appears as two-dimensional for cells due to the pore size may be related to the presence of collagen type I, which is very limited in hydrogel cultures [15]. This is concordant with the observation of marked fibrous texture observed in picrosirius stain in normoxic cultures. Interestingly, a great qualitative difference was observed under hypoxia, where a diffuse staining was observed whereas cells appeared more prone to cluster formation. Around these clusters that seem to mimic the condensation stage, glycosaminoglycans deposition was increased. Hyaline cartilage ECM is mainly formed of glycosaminoglycans, which provide compression resistance through osmotic retention of water in their negatively charged chains, and fibrillar collagen type II, which provides tensile resistance. Promotion of GAGs secretion through hypoxia improves the balance between collagen and glycosaminoglycans content in the formed tissue, leading to a

cartilage-like appearance in all three materials. At the contrary, lack of glycosaminoglycans in the secreted ECM (which in our model is not mimicked by the presence of an hydrogel phase, as when chondrocytes are cultured in alginate or agarose gels) leads to the formation of highly fibrous extracellular matrix that likely promotes excess cytoskeletal tension, which may lead to further phenotypic mismatch and production of collagen type I instead of collagen type II. Moreover, traditional models (hydrogel or pellet) enhance the retention of ECM[15]; in the case of pellet or aggregates, the dimension of the construct is determinant in restricting the diffusion of secreted macromolecules into the culture medium [47].

In conclusion, despite of these pitfalls of our system with respect to *in vitro* culture (as they can be seen as positive for *in vivo* applications where the constraints are different), significant amounts of ECM were observed in all materials except HA NO. Hypoxia proved a more decisive influence than the material modification with respect to chondrogenesis. Modification with Bioglass® seemed to lead to some increase in glycosaminoglycans production and collagen type II staining in normoxic conditions, (although total collagen per cell was significantly lower than PCL control at 17 days in both hypoxic and normoxic conditions) but had no significant effect in hypoxic conditions.

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Figure 1. Total DNA of porcine mesenchymal stem cells after seeding at day 1, 17 and 35 in scaffolds under normoxia (NO) and hypoxia (HY). Results are averaged from n=4 experiments. Error bars represent standard deviation. Significance ($p<0.05$) compared to similar group for normalized scaffolds is signalled as: (P) PCL, (B) BG and (H) HA samples for each group at the same time and culture condition and (#) statically cultured sample from the same type at the same time.

Figure 2. Glycosaminoglycans (GAGs) (A) and Collagen (B). Total levels (A1 and B1), normalized to total DNA (A2 and B2) at day 17 and 35 under normoxia (NO) and hypoxia (HY) and GAGs/DNA (A3) and Collagen/DNA (B3) values normalized to normoxia. Results are averaged from n=4 experiments. Error bars represent standard deviation. Significance ($p<0.05$) compared to similar group for normalized scaffolds is signalled as: (P) PCL, (B) BG and (H) HA samples for each group at the same time and culture condition and (#) statically cultured sample from the same type at the same time.

Figure 3. Alcian Blue staining for glycosaminoglycans(A) and Picro-Sirius Red staining for collagen(B) at day 17 and 35 under normoxia (NO) and hypoxia (HY). Glycosaminoglycans appear in blue and cells in pink; Collagen appears in red and cells in purple; Textured zones

correspond to the microporous structure of the scaffolding material (pointed with arrows). (magnification/scale bar = X1.25/1mm and X10/100 μm).

Figure 4. Microscopic views of anti- collagen type I(A) and anti- collagen type II(B) immunohistochemical staining of scaffolds cultured with mesenchymal stem cells at day 35 under normoxia (NO) and hypoxia (HY). Scaffold appears gray and collagen is brown. (magnification/Scale bar = X10/100μm)