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Effect of perfluorotributylamine-enriched alginate on nucleus pulposus cell: Implications for intervertebral disc regeneration

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ABSTRACT

Various scaffolds have been attempted for intervertebral disc regeneration, but their effectiveness was limited by loss of nutrients within the scaffolds. It has been suggested that the disc is not severely hypoxic and limited availability of oxygen results in disc degeneration. Therefore, a certain oxygen level might be beneficial for disc regeneration, which has not been given enough attention in previous studies. Here, we used perfluorotributylamine (PFTBA) for the first time as an oxygen regulator in alginate scaffold for disc regeneration *in vitro* and *in vivo*. We found that the characteristics of alginate were not affected by PFTBA and the oxygen level of the scaffold was regulated. Then, human nucleus pulposus (NP) cells were cultured in the PFTBA-enriched alginates. It was found that PFTBA could promote NP cell survival and proliferation. In addition, 2.5% PFTBA was capable of regulating extracellular matrix (ECM) to a disc-like tissue graft with little effect on the expression of NP cell markers. Finally, 2.5% PFTBA-enriched alginate was found to restore the disc height and the ECM in a mouse disc degeneration model, indicating its beneficial effect on alleviating disc degeneration. These findings highlight the promising application of PFTBA in further intervertebral disc regeneration.

1. Introduction

Intervertebral disc degeneration (IDD) is a worldwide health issue with comprehensive socio-economic consequence. It is generally associated with low back pain, which affects almost 80% of the population during their life [1]. However, current treatment strategies are still in an early stage with regard to disc regeneration. The intervertebral disc sits between the vertebrae and is responsible for the support, durability and flexibility of the spine [2]. As the largest avascular tissue, the disc is a structurally complex organ that consists of three sub-parts, the central nucleus pulposus (NP), the outer anulus fibrosus (AF) and the cartilage endplates connecting adjacent vertebras [3]. The central NP is a gel-like region that composed of loosely organized extracellular matrix (ECM) such as collagen II and proteoglycan molecules (including aggrecan, versican, decorin, biglycan, fibromodulin, lumican, chondroadherin, etc), whereas the surrounding AF contains higher type I collagen component organized into circumferential lamellae.

The etiology of IDD has been ascribed to various factors, *ie* abnormal mechanical stress, increased cell death, gene polymorphisms, nutrient decline and aberrant inflammatory cytokines [4]. Amongst those factors, one of the primary causes of IDD is thought to be associated with a failure of nutrient transport from the peripheral blood vessels to the disc cells [5]. In IDD, the abnormal oxygen concentration across the disc has been observed in many studies [6]. Moreover, the calcification of the endplates, which act as an impermeable barrier to oxygen transport, has been considered to be a factor leading to IDD [7]. Studies have shown that NP cells require nutrients such as oxygen to synthesize matrix

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molecules. In addition, different oxygen concentrations have been shown to affect the ECM ingredient ratio [8]. Therefore, oxygen concentration plays an important role in maintaining the disc function. However, the role of oxygen in disc metabolism remains a point of controversy. On one hand, a number of studies support the idea that IDD might be associated with a fall in nutrient such as oxygen supply [9–11], and therapies with hyperbaric oxygenation is capable of hampering IDD and increasing ECM production [12,13]. On the other hand, it has also been reported that low oxygen concentration microenvironment plays an essential role in maintaining disc physiological function [14]. Therefore, a certain oxygen tension may exist for optimal regeneration of disc structures. Thus far, little information has been available for the role of oxygen tension in disc tissue engineering.

In the regeneration of the disc, various scaffolds have been attempted *in vitro* and *in vivo* for NP regeneration [15–17]. However, the loss of nutrients within the scaffold limits their effectiveness in promoting disc regeneration [18]. Therefore, it is necessary to provide regulated oxygen in scaffolds for disc engineering to maintain NP cell viability and ECM homeostasis. Perfluorotributylamine (PFTBA), which belongs to the family of perfluorocarbons (PFCs), has a high solubility of oxygen with advantages in its commercial availability, chemical and biological inertness, and ability to be easily sterilized [19,20]. In addition, linear relationship has been found between oxygen partial pressure and oxygen in PFTBA emulsions [21]. In our previous study, we have shown that PFTBA is capable of promoting Schwann cell function and nerve regeneration in cellular nerve grafts [22–24]. All these properties make PFTBA an attractive option to regulate oxygen tension for disc reconstruction. Given that specific oxygen concentration is crucially involved in disc function, as well as in disorders characterized by abnormal apoptosis and ECM composition, we assumed that regulated oxygen might play a role in the tissue engineering of the NP. Accordingly, the current study was designed to investigate the role of PFTBA as an oxygen regulator in alginate scaffold in disc regeneration.

2. Materials and methods

2.1. Formulation of alginate scaffolds with PFTBA

The PFTBA emulsion was prepared as the previous protocol [20,22]. In brief, the PFTBA emulsion (~98% purity, Sigma) was filtered through a 0.2 μm nylon filter. The sodium alginate solution was diluted with sterile saline to reach a 1.5 mg/ml concentration. Then, the PFTBA (~98% purity, Sigma) emulsion was added and sonicated for obtaining alginate supplemented with 2.5 wv.%, 5 wv.% and 10 wv.% PFTBA. The mixture was sterilized by filtering through a 0.8 membrane filter (Millipore, Billerica, MA). To reach coagulation, 102 mmol/L $CaCl_2$ bath was fixed above the level of the solution for 20 min. Then, the calcium chloride was decanted. For one of the control groups, the alginate without PFTBA was also prepared.

2.2. Swelling and degradation

The degree of swelling was measured as previously reported [25]. Briefly, 100 μl scaffolds were formulated and the dry weight (Wd) was obtained following freeze-drying for 24 h. Thereafter, the scaffolds were incubated in 500 μl PBS at 37 °C. After 24 h, the PBS was removed and the gels re-weighed (Ws). The degree of swelling was calculated as (Ws-Wd)/Wd by using triplicate samples for each group. The degradation of scaffolds was determined over a period of 12 days using triplicate samples for each group. Scaffolds were synthesized and incubated in serum-free culture DMEM/F12 medium at 37 °C. The buffer was changed every day. At specific time points, excess buffer was detached and then the scaffolds were weighed. The degree of degradation was calculated as a % of the original mass.

2.3. Oxygen release behavior

The oxygen release behavior was measured as previously described [22]. Briefly, The alginates with or without PFTBA were placed in the 6-well plate. Then, 2.88 ml serum-free culture DMEM/F12 medium was added to each well. After that, the alginates were incubated at 37 °C in a humidified environment with 0.5% O_2/5% CO_2 (Whitley DG250, England). The level of oxygen from each medium of alginate was measured by a blood-gas analyzer (Bayer, Germany) at each time point after incubation under hypoxic conditions.

2.4. Scanning electron microscopy

Morphological observation of the alginate scaffolds with or without PFTBA was performed by scanning electron microscopy (SEM; JSM-4800; Hitachi) on each group. Briefly, scaffolds were formulated and dried under partial vacuum. Then scaffolds of each group were sputter-coated with gold and analyzed at an accelerating voltage of 5–10 kV.

2.5. NP cells isolation and cultures

The study was approved by the Institutional Ethics Review Board of Xijing Hospital with written informed consents obtained from each patient. Human disc tissues were obtained from patients with idiopathic scoliosis (n = 20, age 19.0 ± 0.7years, range 15–24years). Samples were obtained within 2 h after surgery. NP tissue was identified and separated by a stereotaxic microscope. Specimens were minced and digested for 40 min in 0.2% pronase (Gibco-BRL, Carlsbad, CA) under gentle agitation. After being washed with PBS, specimens were incubated in 0.025% type II collagenase (Gibco-BRL) at 37 °C under gentle agitation. The remaining tissue debris was removed through a 45 μm pore-size nylon mesh after 4 h incubation. Cells were then washed and seeded in culture flasks cultured with DMEM/F12-based culture medium (containing 10% FBS, 1% P/S) in 5% CO_2 and 20% oxygen incubator at 37 °C. For the *in vivo* study, NP cells were obtained from C4–C5 tail discs of C57 mice aged 10–12 weeks in the same method.

2.6. Fabrication of 2D or 3D cultures

Passage 2 NP cells were digested with a 0.05% trypsin solution. Cells were washed and resuspended in DMEM. For 2D cultures, cells were seeded on a glass slide for adhesion with a density of 5×10^4. The glass slides seeded with NP cells were then placed on the upper surface of Transwell chambers (NUCN, Denmark), which were immersed in DMEM/F12 medium containing alginate with or without PFTBA. For 3D cultures, the cells were resuspended with sterile alginate to reach a density of 1×10^5/ml. Droplets of cell suspension were released into 6-well plates and incubated in a 102 mmol/L $CaCl_2$ solution for 20 min to polymerize. Thereafter, the final alginate mixture was washed twice and incubated in DMEM/F12 (Fig. 3). The 2D and 3D cultures were then placed in a humidified hypoxic incubator (0.5% O_2, 5% CO_2, 37 °C), respectively. The cultures were also placed in a normoxic incubator (21% O_2, 5% CO_2, 37 °C) as control groups.