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The effects of oxygen level and glucose concentration on the metabolism of porcine TMJ disc cells

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SUMMARY

Objective: To determine the combined effect of oxygen level and glucose concentration on cell viability, ATP production, and matrix synthesis of temporomandibular joint (TMJ) disc cells.
Design: TMJ disc cells were isolated from pigs aged 6–8 months and cultured in a monolayer. Cell cultures were preconditioned for 48 h with 0, 1.5, 5, or 25 mM glucose DMEM under 1%, 5%, 10%, or 21% O_2 level, respectively. The cell viability was measured using the WST-1 assay. ATP production was determined using the Luciferin–Luciferase assay. Collagen and proteoglycan synthesis were determined by measuring the incorporation of [2, 3-^{3}H] proline and [^{35}S] sulfate into the cells, respectively.
Results: TMJ disc cell viability significantly decreased ($P < 0.0001$) without glucose. With glucose present, decreased oxygen levels significantly increased viability ($P < 0.0001$), while a decrease in glucose concentration significantly decreased viability ($P < 0.0001$). With glucose present, decreasing oxygen levels significantly reduced ATP production ($P < 0.0001$) and matrix synthesis ($P < 0.0001$). A decreased glucose concentration significantly decreased collagen synthesis ($P < 0.0001$). The interaction between glucose and oxygen was significant in regards to cell viability ($P < 0.0001$), ATP production ($P = 0.00015$), and collagen ($P = 0.0002$) and proteoglycan synthesis ($P < 0.0001$).
Conclusions: Although both glucose and oxygen are important, glucose is the limiting nutrient for TMJ disc cell survival. At low oxygen levels, the production of ATP, collagen, and proteoglycan are severely inhibited. These results suggest that steeper nutrient gradients may exist in the TMJ disc and it may be vulnerable to pathological events that impede nutrient supply.

Introduction

The temporomandibular joint (TMJ) is a load-bearing joint, consisting of the condyle of the mandibular bone and the fossa eminence of the temporal bone, separated by a fibrocartilaginous disc. Temporomandibular joint disorders (TMJD) affect approximately 35 million people in the United States with tremendous morbidity and financial cost, yet its etiology remains poorly understood[1]. In approximately 30% of TMJD patients, mechanical dysfunction of the TMJ disc, especially displacement due to tissue degeneration, is a common event[2]. The mean age of onset of degenerative changes in the TMJ is between 18 and 44 years[3], which for unknown reasons is a decade earlier than in post-cranial joints[4]. In contrast to other joints, attempts to surgically reconstruct the TMJ is often unsuccessful and may result in severe disabilities[5]. Thus, research to understand the pathophysiology of TMJ disc degeneration for earlier diagnosis and management are essential.

The mechanical function of the TMJ disc is determined by the composition and structure of its extracellular matrix (ECM). The TMJ disc has a distinctive ECM composition when compared to hyaline cartilage and other fibrocartilaginous tissues [e.g., the intervertebral disc (IVD)]. The TMJ disc is comprised primarily of water with a significant amount of collagen type I and a small amount of proteoglycan[6,7]. The normal human TMJ disc is a large avascular structure[8], so the nutrients required by the disc cells for maintaining a healthy matrix are supplied by synovial fluid at the margins of the disc as well as through nearby blood vessels at the connection to the posterior bilaminar zone[9]. The balance between the rate of nutrient transport through the matrix and the rate of consumption by disc cells establishes a concentration gradient

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across the TMJ disc. In articular cartilage, these gradients of essential nutrients can profoundly affect chondrocyte viability, energy metabolism, matrix synthesis, and the response to inflammatory factors[10–13]. Studies have shown that oxygen and glucose play critical roles in the metabolism of chondrocytes and are essential for both adenosine triphosphate (ATP) production and matrix synthesis[14]. In the IVD, cellular energy metabolism is dominated by anaerobic glycolysis, thus glucose levels play a significant role in ATP production and matrix protein synthesis[15,16]. A disrupted nutrient supply has long been implicated in the development of IVD disc degeneration, including cartilage end-plate calcification and a further decrease in oxygen and glucose levels. In TMJ disc cells, recent studies have shown that hypoxia with inflammation modulates the gene expression of tenascin-C and matrix metalloproteinases[17,18]. However, unlike the chondrocytes and IVD cells, the effect of essential nutrients (e.g., oxygen and glucose) on the energy metabolism and matrix synthesis of TMJ disc cells is still largely unknown.

Our recent studies have shown that solute diffusivities in the TMJ disc are much lower than the values in articular cartilage and the IVD[19–21], and compressive mechanical strain can further impede solute diffusion in the TMJ disc. Moreover, our cell metabolic studies have shown that the TMJ disc has a higher cell density and higher oxygen consumption rates compared to articular cartilage and the IVD[22]. Therefore, it is likely that a steeper nutrient gradient may exist in TMJ discs and thus, it is more vulnerable to pathological events which impede nutrient supply, including sustained joint loading due to jaw clenching and bruxism. To understand the biological consequence of a limited nutrient supply, it is necessary to examine the impact of nutrient levels on TMJ disc cells.

The objective of this study was to examine the combined effect of oxygen level and glucose concentration on TMJ disc cell viability, energy metabolism, and matrix protein synthesis. Specifically, the cell viability, ATP production, and radioactive proline and sulfate incorporation (i.e., collagen and proteoglycan synthesis) were measured in porcine TMJ disc cells under defined oxygen levels and glucose concentrations.

Materials and methods

Cell isolation and culture

A total of nine porcine heads (American Yorkshire, male, aged 6–8 months) were collected from a local abattoir within 2 h of slaughter (i.e., three porcine heads on three independent experimental days). Both left and right TMJs were removed *en bloc* with the capsule intact from each porcine head. The six TMJ discs were pooled together and harvested under sterile conditions and then digested overnight at 37°C with 0.1% (w/v) collagenase II (Worthington Biochemical Corp., Lakewood, NJ) in standard 25 mM glucose DMEM (HyClone) containing 10% fetal bovine serum (FBS) (Invitrogen). Digestions were strained through a 70 μm filter, washed with PBS, and re-suspended in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco Brl) and 25 μg/mL ascorbic acid. Isolated TMJ disc cells were plated at 1×10^4 cells/cm^2 at 21% O_2 and 5% CO_2 at 37°C in 25 mM glucose DMEM. The media was changed every 2 days, and upon reaching confluence typically within 2 weeks, first-passage (P1) cells were detached with trypsin-EDTA (Invitrogen). Cells were re-plated at a 1:2 ratio and cultured in a monolayer to second passage (P2) for use in experiments. After cell viability was quantified by trypan blue exclusion (0.4% in buffered saline solution), the P2 cells were seeded at 1×10^4 cells into 96 wells. At 90% confluence, the culture medium was replaced by DMEM plus 10% FBS at four different glucose concentrations. These mediums were prepared by the supplementation of glucose-free DMEM with 0, 1.5, 5, or 25 mM glucose and the osmolality was measured within the range of 290–310 mosmol (Vapro Vapor Pressure Osmometer, Elitech Group). The FBS (Invitrogen) was filtered by the manufacturer until glucose levels were <5 mg/dL, which equates to approximately 0.27 mM glucose, therefore, the presence of glucose due to the presence of FBS in the testing medium is minimal. A 25 mM glucose concentration is normally adopted for *in vitro* cell culture, and the typical glucose concentration in plasma is 5 mM. Although the exact glucose environment has not been determined, it can be expected that the glucose concentration in TMJ disc tissue can range from 0 to 5 mM. Studies on IVDs have shown that the oxygen level and glucose concentration can be as low as almost 0 inside the tissue[15,16]. For each glucose concentration, cells were further cultured under various oxygen levels (1%, 5%, 10%, and 21% O_2) for 48 h in a triple gas incubator in which N_2 was used to reduce O_2 levels[18].

WST-1 assay for examining metabolically active cell viability

Cell viability of the preconditioned experimental groups was measured after 48 h using the WST-1 kit (Roche Molecular Biochemicals, Mannheim, Germany). Water-soluble tetrazolium salt, 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1, 3-benzen disulfonate (WST-1), has been demonstrated to be a simple and rapid measurement of cell viability with extremely low cytotoxicity. A ten percent working solution was made by mixing one part volume of the cell viability reagent WST-1 with nine parts volume of media. Quantification of the formazan dye produced by metabolically active cells was done via a scanning multi-well spectrophotometer (420–480 nm)[23]. Absorbance values collected from cells cultured at 25 mM glucose and 21% oxygen level were considered the control measurement due to the initial *in vitro* expansion culture conditions. All other absorbance values from other cell culture conditions were normalized to the control. A standard curve was performed to show the relationship between different numbers of seeded porcine TMJ disc cells and absorbance.

ATP measurement

Levels of intra- and extra-cellular ATP of the preconditioned experimental groups after 48 h were determined using the Luciferin–Luciferase kit (PerkinElmer, Wellesley, MA). At the end of an oxygenated or hypoxic incubation period, 100 μL of the cell suspension was mixed with 50 μL of a mammalian cell lysis solution (0.1M alkaline solution to inactivate endogenous ATPases and to stabilize the released ATP) in a 96-well microplate and mixed for 5 min. The mixture was then combined with 50 μL of the substrate (Luciferase/Luciferin) solution and mixed for an additional 5 min. The plate was allowed to dark-adapt for 10 min in the luminometer before luminescence counting was initiated. The total per viable cell based ATP production was then calculated by normalizing to the WST-1 absorbance values[24]. The total ATP production per viable cell was then normalized to the control (25 mM glucose and 21% oxygen level).

[2, 3-^{3}H] Proline incorporation assay

Cellular synthesis of collagen was determined by measuring the incorporation of radioactivity (derived from [2, 3-^{3}H] proline) into collagen. The TMJ disc cells were exposed to 20 μCi/mL [2, 3-^{3}H] proline in 2 mL of medium for the final 24 h of the total 48 h incubation. The cell layer was washed three times with PBS and homogenized with a polyton in 0.2% Triton-X 100 and 50 mM Tris/HCl. The cell homogenate was digested with 0.02% collagenase