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Effects of Low Glucose Concentrations On Oxygen Consumption Rates of Intervertebral Disc Cells

Chun-Yuh C. Huang¹, Tai-Yi Yuan², Alicia R. Jackson², Larry Hazbun², Christopher Fraker³, and Wei Yong Gu^{2,*}

¹College of Dental Medicine, Nova Southeastern University, Fort Lauderdale, FL

²Tissue Biomechanics Laboratory Department of Biomedical Engineering, University of Miami, Coral Gables, FL

³Diabetes Research Institute, University of Miami Miller School of Medicine, University of Miami, Coral Gables, FL

Abstract

Study Design—Investigation of the effects of low glucose concentrations on the oxygen consumption rates of intervertebral disc cells.

Objectives—To determine the oxygen consumption rate of porcine annulus fibrosus (AF) cells at different glucose concentrations and to examine the differences in the oxygen consumption rate between AF and nucleus pulposus (NP) cells at different glucose levels.

Summary of Background Data—Poor nutrient supply has been suggested as a potential mechanism for degeneration of the intervertebral disc (IVD). Distribution of nutrients in the IVD is strongly dependent on transport properties of the tissue and cellular metabolic rates. Previous studies have shown dependence of oxygen consumption rate of IVD cells on oxygen tension, pH levels, and glucose levels outside the physiological range. However, the oxygen consumption rate of AF cells at *in vivo* glucose levels has not been investigated.

Methods—IVD cells were isolated from the outer AF and NP of 4–5 month-old porcine lumbar discs. The changes in oxygen tension were recorded when cells were cultured in sealed metabolism chamber. The oxygen consumption rate of cells was determined by theoretical curve fitting using the Michaelis-Menten equation.

Results—The outer AF cells cultured in high glucose medium (25mM) exhibited the lowest oxygen consumption rate whereas no significant differences in oxygen consumption rates were found among outer AF cells cultured at physiological glucose levels (i.e., 1 mM, 2.5 mM, 5 mM). The oxygen consumption rate of NP cells was significantly greater than that of outer AF cells.

Conclusions—Since the oxygen consumption rates determined in this study are comparable to the findings in the literature, this study has developed a new alternative method for determining oxygen consumption rate. The oxygen consumption rates of IVD cells reported in this study will be valuable for theoretically predicting local oxygen concentrations in IVD, which can provide a better understanding of transport of oxygen in the discs.

*Corresponding author: W.Y. Gu, Ph.D., Department of Biomedical Engineering, College of Engineering, University of Miami, P.O. Box 248294, Coral Gables, FL 33124-0621, USA, Telephone: (305)284-5434, Fax: (305)284-6494, E-mail: E-mail: wgu@miami.edu.

INTRODUCTION

Due to the avascular nature of intervertebral disc (IVD), vital nutrients are delivered to cells by diffusion and convection through the dense complex extracellular matrix. Since the IVD is the largest avascular cartilaginous structure in the human body, poor nutrient supply has been suggested as a potential mechanism for degeneration of the IVD. The IVD is comprised of three major components, with the nucleus pulposus (NP) being centered and surrounded on its periphery by the annulus fibrosus (AF), and superiorly and inferiorly by cartilaginous endplates (CEP) (Figure 1a). Since blood supply is located at CEP and the margin of AF, there are two possible nutrient transport pathways into IVD: the cartilage endplate route and the perianular route [1–9]. *In vivo* animal studies and *in vitro* studies suggest that the endplate route is the main pathway for exchange of solutes between NP (and inner AF) and surrounding blood vessels [1,2,5,10–12]. However, with increasing age and in some disease states, the endplate is reported to exhibit more calcification [13,14] while blood supply in the endplate diminishes with age [15], reducing delivery of nutrients through the cartilage endplate route into the IVD [14,16]. A recent *in vitro* study by Ohshima et al. showed that the diffusion of water for both uptake and washout in the unloaded disc was about 2–3 times larger in the perianular route than in the endplate route [17]. Along the perianular route, the amount of nutrients transported into the NP region strongly depends on the transport properties and cellular metabolic rates of AF tissue. Furthermore, due to the difficulty in investigating the transport of solutes in human subjects *in vivo*, the transport of nutrients in the IVD has been analyzed theoretically [18–20]. In order to obtain realistic prediction of *in vivo* nutrient distribution, metabolic rates of cells at *in vivo* conditions have to be taken into account in the theoretical model. Therefore, the determination of metabolic rates of IVD cells at *in vivo* conditions can provide a better understanding of transport of nutrients in the IVD and useful information for precise theoretical analyses.

Only few studies have reported the rates of oxygen consumption by IVD cells [5,21,22]. Holm et al. showed that the oxygen consumption rates of the tissues obtained from the NP and inner AF regions of canine lumbar disc were similar and strongly depended on the oxygen concentration at low concentration levels [5]. Ishihara and Urban also showed a similar oxygen tension dependence on the oxygen consumption rates of the NP and outer AF tissues from the bovine caudal discs [21]. More recently, the study of Bibby et al. demonstrated that the oxygen consumption rate of bovine NP cells was regulated by the levels of oxygen and pH in culture medium, but insensitive to glucose level [22]. However, oxygen consumption rate of AF cells at *in vivo* level of glucose has not been investigated. Since the previous theoretical analysis showed a steep gradient of glucose concentration within the AF region [19], the objective of this study is to determine the oxygen consumption rate of the porcine AF cells in the culture medium of different glucose concentrations and examine differences in oxygen consumption rate between AF and NP cells at different glucose levels.

MATERIALS AND METHODS

Cells were isolated from 4–5 month-old porcine lumbar discs which were obtained from a local abattoir. Discs were exposed by making a transverse cut on either superior or inferior margin of the discs (Figure 1 b) and then NP and outer AF tissues were carefully harvested from the discs without removing endplate. The tissue harvest was completed within 2 hours of slaughter. The AF tissues were finely chopped before cell isolation. Cells were released from AF and NP tissues by enzyme digestion in the high glucose (25mM) Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp.) and 1% antibiotic-antimycotic (Invitrogen Corp.) for 24 hours. The enzyme solution contained 1 mg/ml collagenase (Worthington Biochemical Corp., Lakewood, NJ) and 0.6 mg/ml protease (Sigma Chemical, St. Louis, MO) for AF tissues and 0.5 mg/ml collagenase

and 0.3 mg/ml protease for NP tissues. After the resulting digestions were filtered by a sterile 70 μm cell strainer (BD Biosciences, Bedford, MA), cells were recovered by centrifugation and then resuspended in high-glucose DMEM containing 10% FBS and 1% antibiotic-antimycotic. Finally, cells were encapsulated in 2% agarose disks (8mm in diameter, 1 mm thick) at a density of 2.4×10^7 cells/ml. Three-dimensional agarose culture has been shown to maintain the phenotype of IVD cells [23–25]. After an initial 24-hour culture in the high-glucose DMEM containing 10% FBS and 1% antibiotic-antimycotic at 37°C , the agarose disks were separated into four groups which were incubated in the DMEM with glucose concentrations of 1, 2.5, 5, and 25 mM for another 24 hours before the oxygen consumption rate measurement. The low glucose levels were chosen based on the calculation in the finite element study of human IVD [19]. This study also included the standard culture medium (25 mM glucose) in which the previous *in vitro* measurements of AF oxygen consumption were performed [5,21]. The 24-hr incubation allowed cellular glycogen levels to adapt to low concentration of glucose [22]. In the experiment, the agarose disk (1.2×10^6 cells) was cut into small pieces (about 1 mm cubes) to minimize the concentration gradient of glucose and oxygen within the agarose gel and then placed into a metabolism chamber containing a culture medium with the same concentration as in the 24-hr incubation and pH 7.4 (Figure 2a). The culture medium used in the metabolism chamber had been pre-heated to 37°C and equilibrated with room air to establish the initial oxygen concentration. The metabolism chamber was equipped with an optic oxygen sensor system (Ocean Optics Inc., Dunedin, FL) and maintained at 37°C using a circulator (Fisher Scientific International Inc., Hampton, NH). After the chamber was sealed, the oxygen concentration measurement was recoded during the experiment. The concentration of dissolved oxygen in the culture medium at 37°C and atmospheric pressure was 0.206 mM (or 6.59 mg/L). After adding cells and sealing, the reading of oxygen concentration varied nonlinearly with time initially (probably due to cellular adaptation to new culture environment); then steadily decreased with time (initial linear region of the curves in Figure 2b). In most cases, the initial point for the steady decrease in concentration started at around 0.16 mM (Figure 2b). The recording of oxygen concentration below 0.16 mM was used for theoretical curve-fitting (Figure 2b). At the end of the experiments, the glucose concentration of the culture medium was measured using a quantitative enzymatic assay (Sigma Chemical) and pH of the culture medium was examined using a pH meter (Beckman Coulter, Inc, Fullerton, CA). In this study, the decrease in glucose concentration was found to be minimal. The pH of the culture medium was also measured after the experiment and the change was negligible (less than 2.7%). Furthermore, cells were released from agarose samples by the 3-hr digestion in 2 ml PBS containing 200 $\mu\text{g/ml}$ agarase (Sigma Chemical) at 37°C . Total number of cells was determined and cell viability was examined by trypan blue exclusion. The results were excluded when the viability of the cells was less than 80%.

The Michaelis-Menten equation was used to describe the relationship between the oxygen consumption rate and oxygen concentration. The governing equation of the system is given as

$$\frac{dC}{dt} = -R_o = -\frac{V_{\max}C}{K_m + C}\rho \quad (1)$$

where R_o is the oxygen consumption rate (unit: $\mu\text{M/hr}$), t is the time (unit: hr), C is the concentration of oxygen (unit: μM), $V_{\max}$ is the maximum consumption rate (unit: nmol/million cells/hr), K_m is Michaelis-Menten constant (unit: μM), and ρ is the cell density in the chamber (unit: million cells/ml). The analytical solution of Eq.(1) is derived as

$$t = \frac{K_m}{V_{\max}\rho} \ln \frac{C_0}{C} + \frac{C_0 - C}{V_{\max}\rho} + t_0 \quad (2)$$

where C_0 is the initial concentration of oxygen and t_0 is the time at the beginning of the experiment. V_{max} and K_m were determined by curve fitting the experimental data with Eq.(2) using MATLAB (MathWorks, Natick, MA). After V_{max} and K_m were obtained from curve fitting, the oxygen consumption rates of AF and NP cells were calculated at 5 % oxygen tension (i.e., in vivo condition) using Eq. (1). Two-way ANOVA analysis of variance was performed to examine the effect of glucose concentration on V_{max} and K_m and differences on both parameters and oxygen consumption rate at 5% oxygen tension between AF and NP cells using the program SPSS (SPSS Inc., Chicago, IL).

RESULTS

There were very good agreements between the experimental data and theoretical curve fitting for AF ($r^2=0.994\pm0.005$) and NP ($r^2=0.997\pm0.003$) cells (Figure 2). V_{max} and K_m determined for outer AF and NP cells from theoretically curve fitting are shown in Table 1. Significant effects of glucose level were found on V_{max} of outer AF cells ($p=0.045$) with the cells cultured in the highest glucose medium (25mM) exhibiting a smaller V_{max} than those cultured in the lowest glucose medium (1mM) (Figure 3). No significant differences were found in V_{max} among the experimental groups of NP cells (Figure 3). There were no significant differences in K_m among the experimental groups of either outer AF cells or NP cells (Figure 3). K_m of outer AF cells was significantly greater than that of NP cells ($p<0.001$) whereas NP cells exhibited significantly greater V_{max} than outer AF cells ($p=0.01$) (Figure 4). At the levels of 5% oxygen tension, the oxygen consumption rate of NP cells was significantly higher than that of outer AF cells ($p<0.001$) (Figure 5) wherein the outer AF cells cultured in the highest glucose medium (25mM) glucose exhibited a lower oxygen consumption rate than those cultured in the lowest glucose medium (1 mM) ($p=0.035$) (Figure 5).

DISCUSSIONS

Vital nutrients are delivered to the cells in the center of the avascular IVD from the vasculature at the periphery of the tissue by diffusion and convection through two main pathways: the cartilage endplate route and the perianular route. Since delivery of nutrients through the cartilage endplate route often reduces due to various factors [13–16], the perianular route may become a major pathway for nutrient transport into the IVD [17]. Determining the metabolic rate of AF cells can provide a better understanding in nutrient transport along the perianular route into the IVD tissue. To the authors' knowledge, this study is the first to measure the oxygen consumption rate of outer AF cells under different glucose concentrations including physiological glucose levels. Generally, cells exhibit a higher rate of glycolysis in high glucose culture. Inhibition of cellular respiration by high rate of glycolysis, described as the Crabtree effect, have been demonstrated on chondrocytes [26,27]. This study found a similar effect on the outer AF cells which exhibited a significantly lower oxygen consumption rate in the highest glucose (25 mM) culture. However, there were no significant differences in oxygen consumption rate among the outer AF cells cultured at physiological levels of glucose such as 1 mM, 2.5 mM, 5 mM glucose. Insensitivity of oxygen consumption rate of porcine NP cells to glucose level found in this study also confirms the study of Bibby et al., who found the oxygen consumption rate of bovine NP cells was not regulated by glucose levels. Furthermore, the oxygen consumption rates of outer AF and NP cells were dependent on the oxygen concentration. This is consistent with the previous studies in the literature [5,21,22].

The NP and AF tissues derive from different embryonic origins with the NP tissue being synthesized by notochordal cells and the AF tissue by mesenchymal cells [28]. The differences in cell morphology, phenotypic expression, and matrix production between the NP and AF cells have been recognized in the literature [29–35]. In this study, the finding that NP cells exhibited significantly higher V_{max} than the outer AF cells (Figure 4) indicates that the

consumption rate of NP cells is higher at the high level of oxygen tension. Significant difference in K_m between the outer AF and NP cells further increases difference in oxygen consumption rates between the NP and outer AF cells under physiological condition (e.g., at the levels of 5% oxygen and 5mM glucose, Figure 5). Since the NP cells obtained from pigs of young age in this study were primarily notochordal, differences in oxygen consumption rates may be due to different cell types. The previous study reported that the oxygen consumption of NP tissue was similar to that of outer AF tissue for bovine caudal disc [21]. Since the cell density in the AF tissue tends to be higher than that in the NP tissue [2,34] (e.g., 4000 cells/mm³ and 9000 cells/mm³ in human NP and AF regions, respectively [2]), the previous finding may also indicate that the oxygen consumption rate of NP cells may be higher than that of outer AF cells. Furthermore, since the outer AF cells exhibited lower oxygen consumption rate under *in vivo* conditions, it would allow more nutrients transported into the center of the IVD than those predicted in the previous theoretical studies [19,20] which assumed the same oxygen consumption rate for NP and AF cells.

Based on the average values of V_{max} and K_m determined in this study, the relationship between the oxygen consumption rate and oxygen concentration for porcine NP and outer AF cells can be described by the Michaelis-Menten equation (i.e., Eq. (1)) (Figure 6). The oxygen consumption rate of NP cells of porcine lumbar disc determined in this study is similar to that of NP cells of canine lumbar disc reported in the previous study [1] (Figure 6). The pH-dependent relationship between the oxygen consumption rate and oxygen concentration for NP cells of bovine caudal disc has also been determined and described by a modified Michaelis-Menten equation [22] and the relationship at pH 7.4 is shown in Figure 6 for comparison. Although the oxygen consumption rate of NP cells of bovine caudal disc is lower than that of NP cells of porcine and canine lumbar discs (Figure 6), they are of the same order of magnitude. This difference may be due to different cell types since the majority of NP cells in young porcine and canine IVD are notochordal cells while bovine NP cells are chondrocyte-like.

Ishihara and Urban found that the oxygen consumption of the outer AF tissue of bovine caudal disc remained constant at high level of oxygen tension but strongly depended on the level of oxygen tension when oxygen tension was lower than 5% [21]. This finding is similar to the relationship between the oxygen consumption rate and oxygen concentration for the porcine AF cells determined in this study (Figure 6). Although the oxygen consumption of outer AF tissue of bovine caudal disc can not be compared to the data of this study due to the lack of information about its cell density, the oxygen consumption rate of porcine AF cells at high oxygen tension (i.e., V_{max}) in this study is comparable to that of cells in other avascular tissue such as articular chondrocytes which was reported in the range of 1.7–17 nmol/million cells/hr in the recent studies [26,37–39]. Furthermore, the oxygen consumption rate of the inner AF cells of canine lumbar disc was found to be similar to that for NP cells which is similar to that of porcine NP cells but higher than that of porcine AF cells (Figure 6). This indicates that the oxygen consumption rate of inner and outer AF cells may be different.

The approach used to determine oxygen consumption rate of IVD cells was different from that in the literature. The most common way is to determine “averaged” oxygen consumption rate of cells for the oxygen tension of interest by taking the ratio of the change in oxygen concentration over a short time period. Since oxygen consumption rate changes rapidly when the oxygen tension was around K_m , this common method may not provide an accurate measurement of oxygen consumption rate at oxygen tensions near K_m . In our study, all of the experimental data (at least 20,000 data points) from the oxygen concentration measurement were used on theoretical curve fitting with the analytical solution of the Michaelis-Menten equation. This large amount of data points provided a high degree of reliability on theoretical curve fitting. Since there were very good agreements between the experimental data and theoretical curve fitting in this study, V_{max} and K_m are believed to have been accurately

determined. Variation in V_{max} and K_m shown in Figure 4 may be due to the fact that cells were obtained from different pigs. Furthermore, since the results of oxygen consumption rate measurements in this study are comparable to the findings in the literature, the new method used in this study can be an alternative for determining oxygen consumption rate.

Osmolarity (ranging from 316–600 mOsm) was shown to have little effects on the oxygen consumption rate of bovine articular chondrocytes [26]. In our study, since osmolarity of medium with different glucose concentrations was found in a small range (314–335 mOsm), it was assumed to have minimal effects on the measurement of oxygen consumption rate. Furthermore, a previous study has shown that the oxygen consumption rate of the bovine NP cells was regulated by pH [22]. Although this study did not investigate the effect of pH on the oxygen consumption rate of outer AF cells, it could be expected that the regulation of oxygen consumption rate of outer AF cells by pH is similar to that of NP cells reported in the literature [22]. Based on the modified Michaelis-Menten equation used to describe the pH-dependent oxygen consumption rate of bovine NP cells in the study of Bibby et al. [22], V_{max} and K_m reduce to zero at pH 4.95 and pH 4.59, respectively. A similar equation can be proposed to describe possible pH dependence for oxygen consumption rate of porcine NP and AF cells based on the average values of V_{max} and K_m as:

$$R_o = \frac{V'_{max}(pH - 4.95)C}{K'_m(pH - 4.59) + C} \quad (3)$$

where $V'_{max}(pH-4.95)$ is the pH dependent maximum consumption rate ($V'_{max}=5.27$ nmol/million cells/hr for NP cells and 3.64 nmol/million cells/hr for outer AF cells), $K'_m(pH-4.59)$ is the pH dependent Michaelis-Menten constant ($K'_m=3.4$ μ M for NP cells and 12.3 μ M for outer AF cells), and the unit of oxygen concentration C is the same as K'_m . It can be utilized in the theoretical analysis on oxygen transport in the IVD tissue.

In summary, this study showed that the outer AF cells cultured in the high glucose medium (25 mM glucose) exhibited a lower oxygen consumption rate whereas there were no differences in oxygen consumption rate among the outer AF cells cultured at physiological levels of glucose (i.e., 1 mM, 2.5 mM, 5 mM). The oxygen consumption rate of NP cells was found to be significantly greater than that of outer AF cells. Since the oxygen consumption rates determined in this study are comparable to the findings in the literature, this study has developed a new alternative method for determining oxygen consumption rate. The oxygen consumption rates of IVD cells reported in this study will be valuable data for theoretically predicting the local concentration of oxygen in the intervertebral disc, which can provide a better understanding on transport of oxygen in the intervertebral discs.

- A new, alternative method for determining oxygen consumption rates has been developed.
- Oxygen consumption rates of outer AF cells are affected by high glucose concentrations.
- Oxygen consumption rates are independent of glucose concentration at physiological glucose levels.
- NP cells have significantly higher oxygen consumption rates than outer AF cells.
- Results are useful for theoretical prediction of oxygen concentration in IVD and in understanding transport of oxygen in IVD.

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Figure 1.
(a) Anatomy of the intervertebral disc. (b) Different regions of porcine intervertebral disc.

Figure 2.

(a) Schematic of experimental setup. (b) Typical experimental data of oxygen concentration measurement for NP and outer AF cells along with theoretical curve fittings. Cells were cultured in the metabolism chamber containing DMEM medium and 1 mM glucose (pH=7.4).

Figure 3. Comparison of (a) V_{max} and (b) K_m among the outer AF or NP cells cultured in the media with different glucose concentrations (n=5 for each group).

Figure 4. Comparison of the average of (a) V_{max} and (b) K_m between the outer AF or NP cells (n=20 for each group).

Figure 5.

Comparison of oxygen consumption rate at 5% oxygen tension (a) between the outer AF and NP cells (n=20 for each group) and (b) among the outer AF or NP cells cultured in the media with different glucose concentrations (n=5 for each group). The oxygen consumption rate was calculated using Eq. 1 based on V_{max} and K_m which were determined from theoretical curve fitting.

Figure 6.

Comparison of oxygen consumption rate within the range of oxygen tension from 0 to 20% between this study and those in the literature. The consumption rate of bovine NP cells at pH 7.4 was calculated based on the modified Michaelis-Menten equation proposed in the study of Bibby et al [22]. The oxygen consumption rate of canine NP cells was converted from the oxygen consumption of canine NP tissue based on its cell density reported in the study of Holm et al. [5].

Table 1 V_{max} and K_m of NP and outer AF cells

Glucose Concentration	Outer AF		NP	
	V_{max} (nmol/million cells/ hr)	K_m (μ M)	V_{max} (nmol/million cells/ hr)	K_m (μ M)
1 mM (n=5)	12.0 $\pm$ 2.4	34.1 $\pm$ 17.5	13.4 $\pm$ 7.3	5.5 $\pm$ 3.1
2.5 mM (n=5)	9.1 $\pm$ 4.2	31.0 $\pm$ 18.4	11.6 $\pm$ 3.9	11.5 $\pm$ 4.2
5 mM (n=5)	8.6 $\pm$ 3.3	37.7 $\pm$ 25.1	15.2 $\pm$ 4.8	14.5 $\pm$ 13.3
25 mM (n=5)	6.0 $\pm$ 1.6	35.7 $\pm$ 14.9	11.5 $\pm$ 6.3	6.8 $\pm$ 3.3
Average (n=20)	8.9 $\pm$ 3.6	34.6 $\pm$ 17.9	12.9 $\pm$ 5.5	9.6 $\pm$ 7.7