Metabolic Analysis of Bovine Nucleus Pulposus Cells with Constant and Variable Glucose Supply

by

Rees Alan Rosene

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented May 24, 2022
Commencement June 2022

AN ABSTRACT OF THE THESIS OF

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There is no standardized method for culture of intervertebral disc (IVD) cells when monitoring metabolic processes. We hypothesize that the metabolic rates of cells that are crucial to experiment designs depend on the cell culture method used. The glycolytic pathway and the tricarboxylic acid (TCA) cycle are the pathways through which metabolites are processed into energy for cellular functions in the form of ATP. Imbalances in these pathways are linked to degeneration of the IVD. Key metabolites monitored through this pathway include glucose, lactate, citrate, and non-essential amino acids. Changes in the presence of these metabolites may provide insight into cellular stress, disease, and metabolic demand from different cells. A fluidized bed bioreactor was used to control glucose concentration, allowing metabolic analysis of bovine nucleus pulposus cells. An increase in central metabolic pathways, such as glycolysis and the TCA cycle, was observed in the constant glucose cell culture compared to the standard cyclic feeding method.

Abstract approved:_____

Morgan Giers

Keywords: bNP, Steady-state, bioreactor, metabolomics, metabolites, TCA, glycolysis, IVD

Corresponding e-mail address: rarosene@gmail.com

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APPROVED:

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Morgan Giers, Mentor, representing the School of Chemical, Biological, Environmental Engineering

_____

Christine Kelly, Committee Member, representing the School of Chemical, Biological, Environmental Engineering

_____

Maria Franco, Committee Member, representing the School of Biochemistry and Biophysics

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Rees Alan Rosene, Author

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Introduction:

Back pain is the leading cause of disability worldwide, with significant socioeconomic impacts ranging from decreased productivity to missed workdays [Wu, Hamill]. Intervertebral disc (IVD) degeneration is a primary factor of back pain. IVD degeneration can cause sciatica, herniation, and stenosis [Luoma]. The IVD comprises the annulus fibrosus (AF), cartilaginous endplate (CEP), and the nucleus pulposus (NP) (Figure 1). The AF is a fibrous structure built to connect adjacent vertebral bodies and aid in distributing pressures and forces applied to the IVD [Erwin. The AF is structured as concentric lamellae surrounding the NP. The CEP serves as a point of contact for nutrient transport from blood vessels to the IVD [Moon]. The NP is composed mainly of water and extracellular matrix (ECM) components, such as proteoglycans and collagen II [Urban, Williams, Hwang]. The NP absorbs and redistributes mechanical compressions to the spine [Urban]. Mechanical deformation, genetic factors, phenotype changes, and aging contribute to the degeneration of this area of the IVD [Urban, Williams, Hwang].

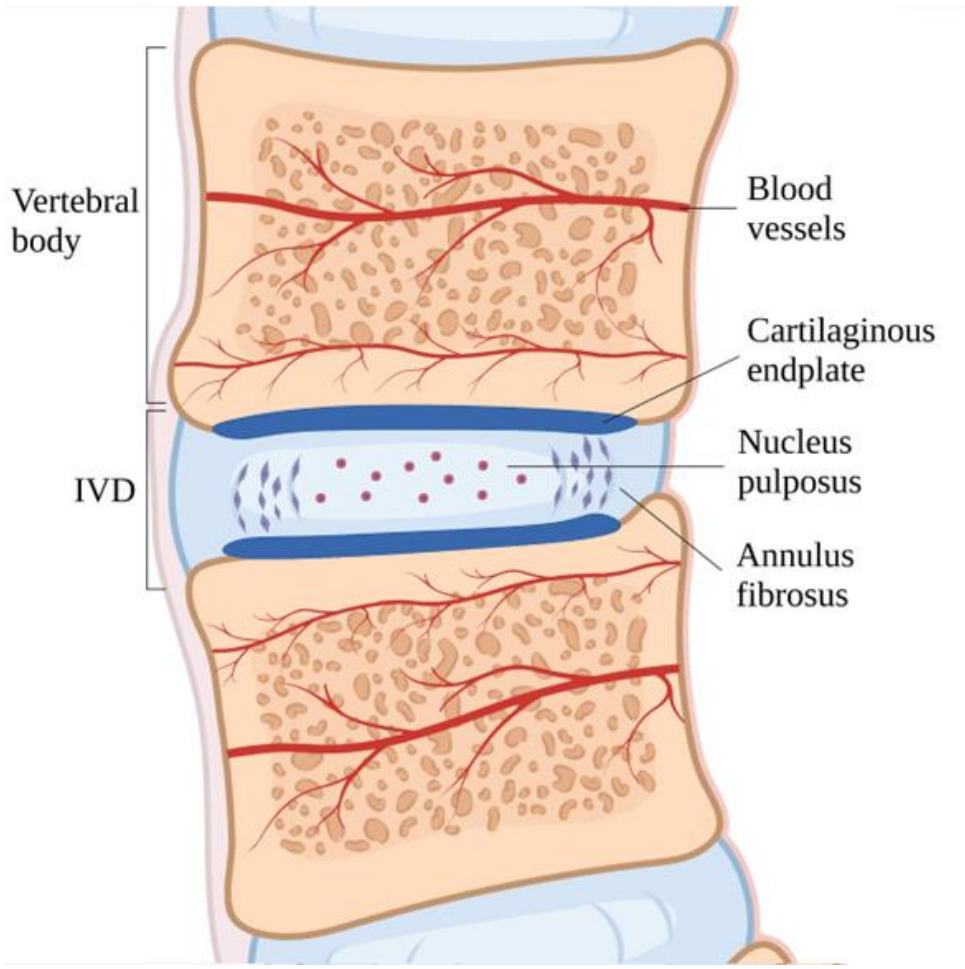

Figure 1: Diagram of the IVD, including adjacent vertebral bodies (Figure credit Rylee Ramsey).

The IVD is the largest avascular structure in the human body and depends on the diffusion of nutrients from the cartilaginous endplate (CEP) [Urban, Hwang]. One prominent issue with the health of NP cells is that they can be as distant as 6-8 mm from the CEP [Johnstone]. Transport via diffusion is inefficient in providing essential nutrients for cell function, such as glucose, and removing unwanted metabolic waste products compared to vascularized transport of nutrients. Therefore, the NP is vulnerable to degeneration, as it lacks easy access to nutrients required for remodeling and repairing damaged cells and tissues [Urban, Roughly, Johnstone]. As NP cells degenerate, they tend to cluster together [Roughly]. The clustering of cells results in changes in

the ECM expression of proteoglycans, centering primarily around the degenerated cell clusters while assembling little in the area between the groups [Johnstone]. The uneven distribution of proteoglycans results in a non-uniform negative charge distribution across the NP, reducing the ability of the IVD to retain water and distribute loads placed upon it [Johnstone]. As degeneration continues in the NP, nutrient transport limitations become an even more significant threat to the ability of the IVD to repair itself [Roughly]. Nutrient deprivation can cause cellular senescence, apoptosis, and degenerative disc disease in the IVD [Hammil, Bibby].

Several animal and human studies have shown promising potential for stem cell injections to restore disc health and alleviate back pain compared to current therapies such as conservative therapy, discectomy, disc replacement, and spinal fusion [Williams]. However, the potency of cell therapy is reduced in degenerated IVDs due to the limited supply of nutrients [Dou]. Additionally, biologics are commonly researched as a regeneration strategy but are best examined in a setting that models the IVD microenvironment [Evans, Machado, Vasiliadis]. One way of incorporating this principle into this research is to implement a feeding pattern for cells that mimics the *in vivo* supply of nutrients and removes waste products. IVD cell cultures, whether in monolayer, 3D, or whole organ, are typically fed every few days, introducing frequent changes to nutrient concentrations. Because cells sense their chemical environment, changes to nutrient levels induce stress on the cells, requiring them to adjust their phenotypic expression to cope with the new environment [Hamill, Salvatierra, Wu].

Bioreactors are a standard cell culture method in the bioprocessing industry, but they are not commonly used in IVD studies because of their large scale. Bioreactors are ideal for studying cellular metabolism and their response to biologics because they can provide an environment that controls variables such as oxygen content and media feed rate. Because bioreactors are designed

for holding variables that affect metabolic behaviors as constants, they are suitable for lab-based scale-down of experiments. Cell behavior is assumed to remain constant across orders of magnitude larger bioreactors if media nutrient concentrations remain constant.

Uniform cell culture methods are crucial for evaluating variables when modeling the IVD. Assumptions for future *in vivo* experiments involving IVD metabolic parameters such as basal metabolic rate, lactate production, and cellular phenotypic expression are derived from literature values of *in vitro* studies [Rolfe]. It is in the interest of IVD research to base these variables on values that most closely represent how cells would behave in their native environment [Rolfe, Wu].

The lag phase of cellular growth describes a state of little to no division of cells. A tremendous amount of energy is required to assemble proteins to cope with the stresses placed on cells adapting to a new chemical environment. The assembly of proteins increases energy demands to the extent that cell division is slowed to compensate [Salvatierra]. Additionally, energy demands are of particular concern for the efficacy of NP cell cultures because, *in vivo*, the IVD operates at hypoxic concentrations, promoting hypoxia-inducible factor 1-alpha (H1F-α) expression, forcing the cells to source their energy from simple glycolysis rather than oxidative phosphorylation [Weidemann]. Lactate is the main metabolic byproduct of simple glycolysis, and its secretion reduces the pH of the ECM. Low pH is a significant inducer of cellular stress and IVD degeneration [Hamill].

Currently, cell culture methods do not account for removing inflammatory factors, such as lactate, which lowers the pH of the cell culture [Kushioka]. A uniform cell culture method to replicate the *in vivo* conditions of adding new nutrients while maintaining the ability to remove waste products has not been established [Kushioka, Piprode]. This work seeks to develop a method

of achieving a steady-state glucose bioreactor. A steady-state bioreactor serves by holding nutrient supply constant and removing waste products. Cellular stress increases overall metabolic demand, as chemoreceptors will provoke increased protein production to adapt to their changing chemical environment [Hamill, Salvatierra].

When analyzing the overall metabolite content of a sample or the basal metabolic rate, it is crucial to have a known number of cells. Alginate Cell culture provides a promising method for studying metabolic behavior. It provides a method for cell culture that allows extracellular matrix formation and restricts cell proliferation [Yu]. These properties allow for observation of metabolic processes in an environment that more closely mimics the 3D structure of the IVD while holding the overall cell count as a constant [Yu].

Here we show a method for a fluidized 3D alginate bioreactor that can maintain a steady-state glucose concentration over an extended culture period of 10 days. Bovine nucleus pulposus cells were expanded, encapsulated in 3D alginate culture, then analyzed for cell viability and metabolite concentrations through nuclear magnetic resonance (NMR). The bioreactor specifications were modeled from a mass balance on glucose entering, exiting, and consumed by the cells in the bioreactor. This cell culture method also removes waste products from the system, more closely modeling the body's ability to remove waste products than cyclically fed cell culture.

Methods/Results:

Alginate Cell Culture

This protocol was adapted and modified from Le Maitre (2005). Bovine nucleus pulposus (bNP) cells were cultured in 175 cm^2 t-flasks using low-glucose, phosphate-free Dulbecco's modified eagle media (DMEM) (Sigma-Aldrich, St. Louis, USA). Once cells were approximately

80% confluent, cells were isolated using a 0.1% trypsin solution (Sigma-Aldrich, St. Louis, USA) and centrifuged at an RCF of 300 for 10 minutes. Cell concentrations were determined using a Countess II FL (Life Technologies, Carlsbad, USA). Direct samples from trypsinized cell solution were added in a 1:1 ratio with trypan blue, and Countess II estimated cell concentration in the solution. Because the total volume of trypsinized cells was known, the culture's total number could be calculated from the cell concentration. A solution of 300,000 cells/mL was mixed in 2% alginate (Sigma-Aldrich, St. Louis, USA) in 150 mM NaCl (Sigma-Aldrich, St. Louis, USA) solution and added to a container containing $CaCl_2$ (Sigma-Aldrich, St. Louis, USA) using a 16-gauge syringe. The entire method for forming the alginate beads is outlined in Table 1. The beads were allowed 15 minutes to develop a gel matrix in the 102mM $CaCl_2$ solution. After hardening, the alginate beads were washed twice in fresh 150 mM NaCl [Le Maitre].

Bioreactor

The bioreactor was assembled for steady-state operation using a two-pump system. One pump circulated media in the bioreactor, while a second pump was used to maintain a constant feed of fresh media to hold the glucose concentration steady inside the bioreactor (Figure 4). The 50 mL conical tube bioreactor was constructed with a 50 mL conical tube mixing vessel, and the culturing bioreactor was kept in a sterile incubator at 37 ºC. The mixing vessel had a sterile 0.2µm air filter attached to ensure that gas exchange with the incubator's interior would be provided to the cells. One peristaltic pump was used to ensure that the media inside of the system would be well mixed. This pump was operated faster than the feed pump, 5 mL/min compared to 0.3 mL/min.

Cyclic feeding was achieved using the same setup as the steady-state glucose group but without the feed, pump attached to the mixing vessel. Media was removed and replaced with new media through the sampling port every three days.

A mass balance on glucose was performed for the system, measuring the overall glucose consumption rate of the bNP cells based on cell count and literature metabolic rates. The cell count is constant because cells cannot proliferate in a sodium alginate matrix, so the overall cellular metabolic rate is constant. The remaining variable in the mass balance was the flow rate of DMEM in and out of the system. The mass balance was used to solve the flow rate of new media into the bioreactor to achieve a steady-state for a given number of bNP cells.

$$0 = F(Cg, in - Cg, out) - BMR \cdot n$$

Equation 1. Mass balance of glucose in the bioreactor system. F_{in} was equal to $F_{out,}$ simplifying the entering and exiting terms of the mass balance to have a single F representing the flow rate. $C_{g,in}$ is the concentration of glucose into the system, and $C_{g,out}$ is the concentration of glucose leaving the system. BMR is the literature basal metabolic rate of bNP cells, and n is the number of cells in the bioreactor. Modeling nutrient consumption is simplified by the knowledge that cells remain at the outer edges of the alginate beads, as the nutrients do not have to travel through the gel matrix. The concentration of nutrients reaching the cells can be assumed to be the same as their concentrations in the media. The mass balance was performed to determine F, the flow rate of new media into the mixing vessel, as a function of the number of cells in the alginate culture.

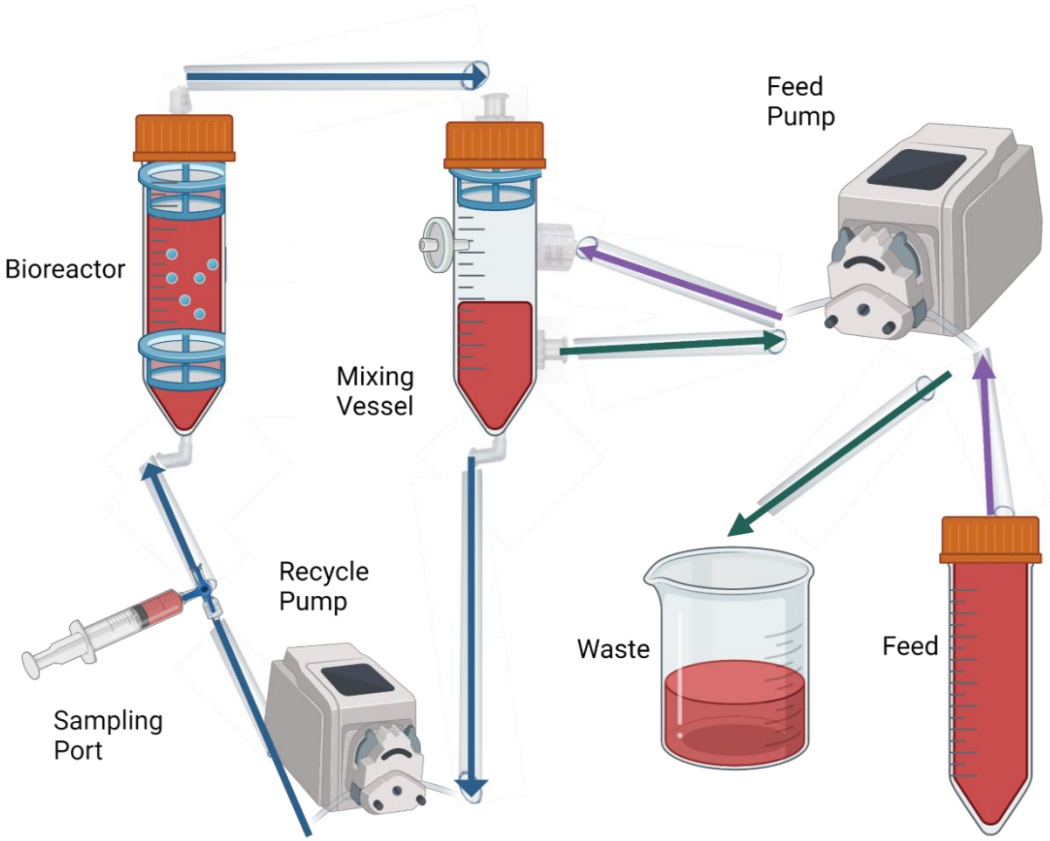

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Figure 4. Bioreactor Pump System as assembled. Blue arrows indicate the tubing and flow direction of the recycle pump, ensuring the media is well mixed. The feed pump had multiple channels for flow, so multiple sets of tubes could be set to the same flow rate from the pump. The purple arrows indicate channel 1, the feed into the mixing vessel, while the green arrows indicate channel 2 for flow out of the mixing vessel.

The bioreactor and mixing vessel were both constructed from 50mL conical tubes. Sterile stopcocks were used to allow for sterile sampling of the bioreactor media. The sampling point was placed between the mixing vessel and the bioreactor's bottom entry to sample the well-mixed media glucose concentrations. A 5 mL syringe was used to pull three 1mL samples from the

bioreactor every three days. A Reli On Prime handheld glucose monitor (Novo Nordisk, Bagsværd, Denmark) was used to measure the glucose concentrations of each sample against a standard curve of 0, 50, 110, 270, and 470 mg/dL glucose (Fisher Scientific, Waltham, USA) measured on the same day as each sample.

Tubing through the peristaltic pump was MasterFlex 2.4 mm ID autoclavable silicone tubing (Cole-Parmer, Vernon Hills, IL). In Figure 4, this tubing is represented by the peristaltic pump images. After connectors, this diameter dropped to 1.6 mm ID PTE autoclavable tubing, as depicted in Figure 4 by the blue, green, and purple arrows.

The two pump setups provided options for each bioreactor; the first configuration could run with only the mixing pump to circulate the media, as depicted by the blue arrows in Figure 4, as a control setting for data collection. The second configuration could also be run with the feed pump, as depicted by the purple and green arrows in Figure 4, for the experimental group to achieve steady-state glucose.

Live Dead Staining

A Live Dead Cell Viability Assay Kit for 3D and 2D cell culture (CBA415 Sigma-Aldrich, St. Louis, USA) was used to determine the viability of bNP cells inside alginate beads (Figure 2). Cells migrated to the outside edges of the alginate beads, indicating that the cells would be readily able to uptake nutrients and would not have to compete with a gradient of nutrients across the bead, simplifying the cell culture analysis (Figure 3).

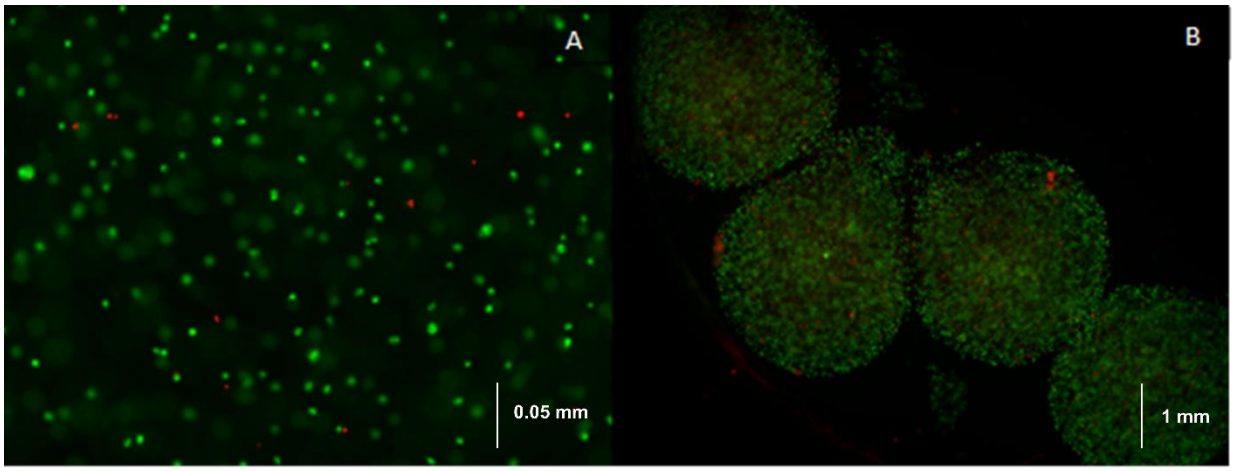

Figure 2. Cell Viability assay on bNP cell cultures in alginate beads. A) 10x objective on the top layer of a bNP alginate bead. B) 2x objective on the middle layer of bNP alginate beads. Cells in focus are present in that layer, while blurred cells belong to a different layer.

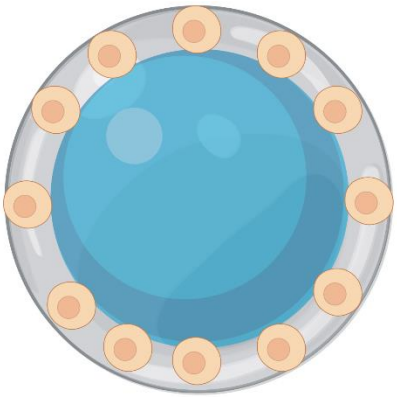

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Figure 3. Schematic of alginate bead with encapsulated cells. The alginate gel forms a rigid outer matrix while the inside remains softer. This phenomenon encourages the cells to settle on the outer edge of the bead.

Alginate Bead Dissolution

Before analysis, alginate beads were collected and washed twice in NaCl for 10 minutes. bNP cells were extracted from the alginate beads by gentle pipetting in a pH 7.4 dissolving buffer containing 55 mM sodium citrate (Sigma-Aldrich, St. Louis, USA), 30 mM EDTA (Sigma-Aldrich, St. Louis, USA), and 150 mM NaCl in distilled water. The cell-containing solution was incubated at 37°C with agitation for 10 minutes. The cells were then pelleted in a centrifuge at an RCF of 300 for 10 minutes. The cell pellet was reconstituted in 5 mL 1X PBS and used to perform cell viability and metabolomics assays.

NMR Metabolomics

Metabolites from cells fed in constant glucose versus those cultured in fluctuating glucose were compared using NMR metabolomics. Cells were rescued from the alginate culture using the dissolving buffer, then sent to NMR analysis. Metabolite concentrations were normalized to cell count in the 3D culture, then normalized to be a scalar multiple compared to the baseline of the current cyclical feeding that is standard for research.

Table 1. Common issues for cell culture, bioreactor, and pump system process with troubleshooting guidance and solutions for each case.

Issue	Solution
Contamination of system	Use sterile equipment, reduce airflow in the cell culture room by closing the door, and spray and wipe surfaces with ethanol before and after use

Frequent leaking from tubing	Use MasterFlex tubing on peristaltic pump heads. Frequently inspect the tubing for leaks or signs of wear.
Cells becoming confluent too quickly	Use a larger T-flask size; 75 cm^2 flasks for a bNP culture would usually take three days to become confluent. A 175 cm^2 T-flask would usually take a week to become confluent.
Not enough cells are removed during trypsinization	Allow longer time for trypsinization. Ten minutes at 37 ºC should be sufficient for most of the cells in the culture.
Cells resuspending after centrifugation	To avoid cells resuspending while moving the conical tube from the centrifuge to the culture hood, centrifuge for a more extended period at an RCF of 300. Ten minutes should be sufficient in most cases. Walk from the centrifuge to the culture hood slowly, holding the tube with both hands to limit shaking.
Beads not forming properly	Beads should be created using a syringe, one drop at a time. The syringe should be held close to the surface of the $CaCl_2$ solution to make uniform spherical beads. Slowly agitate the solution if beads remain at the top of the surface.

Preliminary Results

Cell viability was found to be 90%, indicating that the 3D alginate culture method is viable for analyzing the metabolic behavior of healthy cells. Additionally, imaging from the cell viability assays provided critical information on the behavior of cells in an alginate culture. Cells tended to migrate to the outer edge of the alginate bead, simplifying our cellular glucose consumption analysis because cells all experienced nutrient concentrations assumed to be the same as the solution they were cultured.

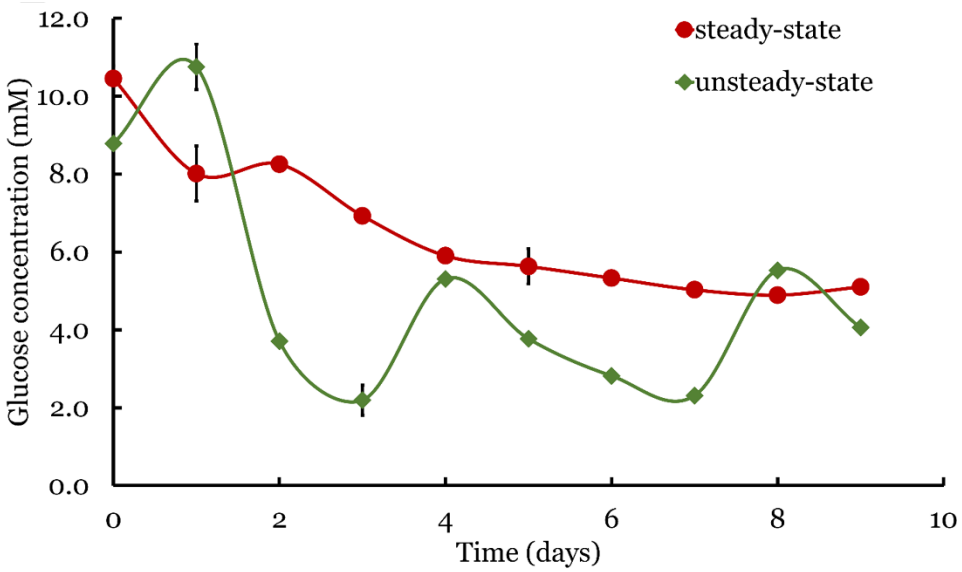

Figure 5: Bioreactor glucose concentrations over ten-day culture time. Steady-state glucose feed and unsteady-state cyclic glucose feeding are compared.

Preliminary results showed that a steady state of glucose could be achieved through our bioreactor after about four days. A slight lag period to reach steady-state was expected as the cells' glucose consumption, and the feed rate of fresh media equilibrated to a steady-state glucose concentration, in this case, about 5.5 mM.

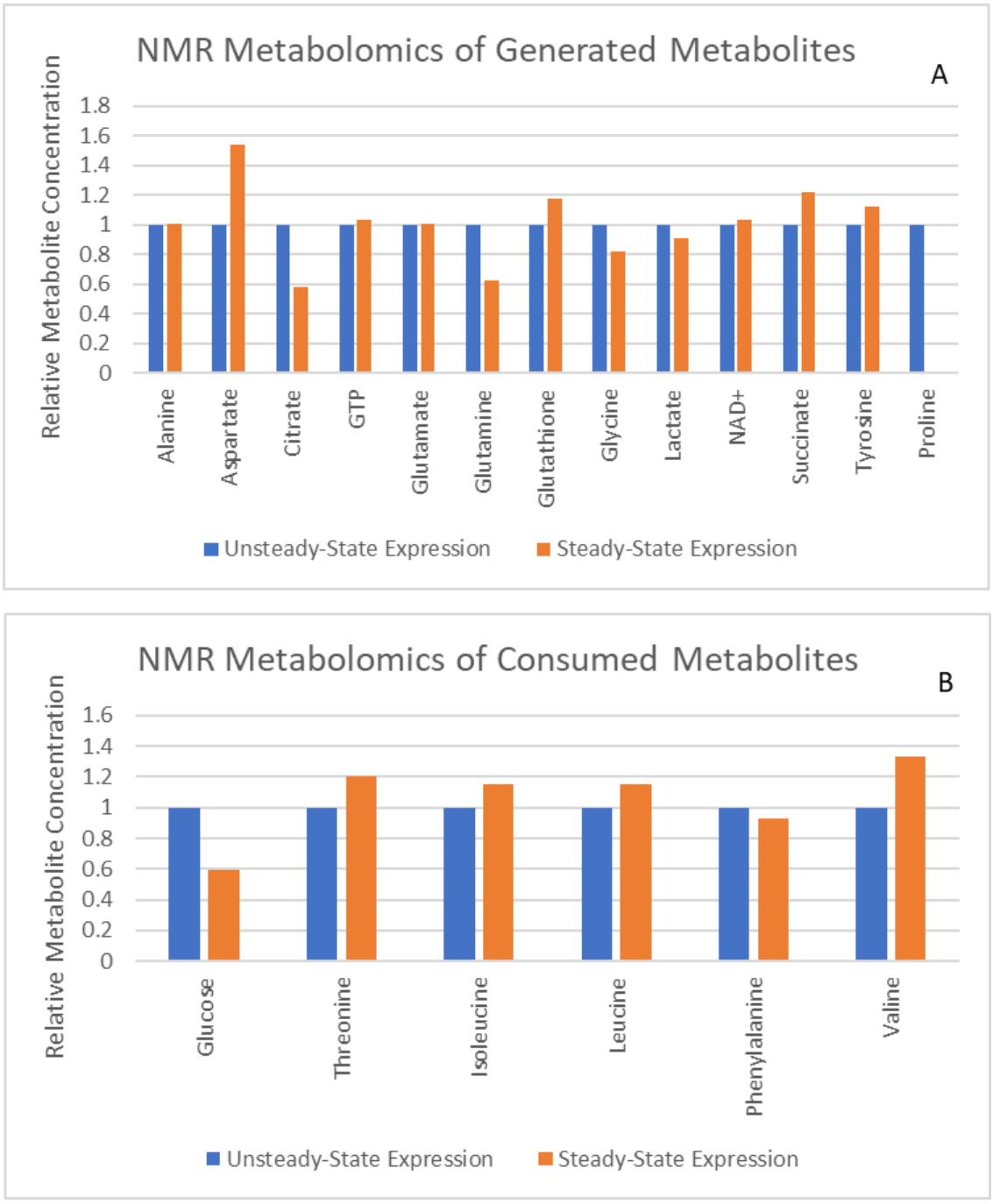

Figure 6. NMR metabolomics of Glycolysis and TCA cycle metabolites from bNP cells when cultured in changing glucose or constant glucose levels. A) depicts the relative concentrations of metabolites generated through glycolysis or the TCA cycle B) depicts relative concentrations of

metabolites solely consumed by glycolysis or protein synthesis. Concentrations are expressed as normalized to the changing glucose group, showing scalar multiple values of the fluctuating glucose group in comparison.

NMR metabolomics showed increased relative concentrations of succinate, glutamate, aspartate, and most amino acids in the steady-state glucose group. Glucose, lactate, citrate, asparagine, glutamine, and glycine showed decreased relative concentrations in the steady-state glucose group.

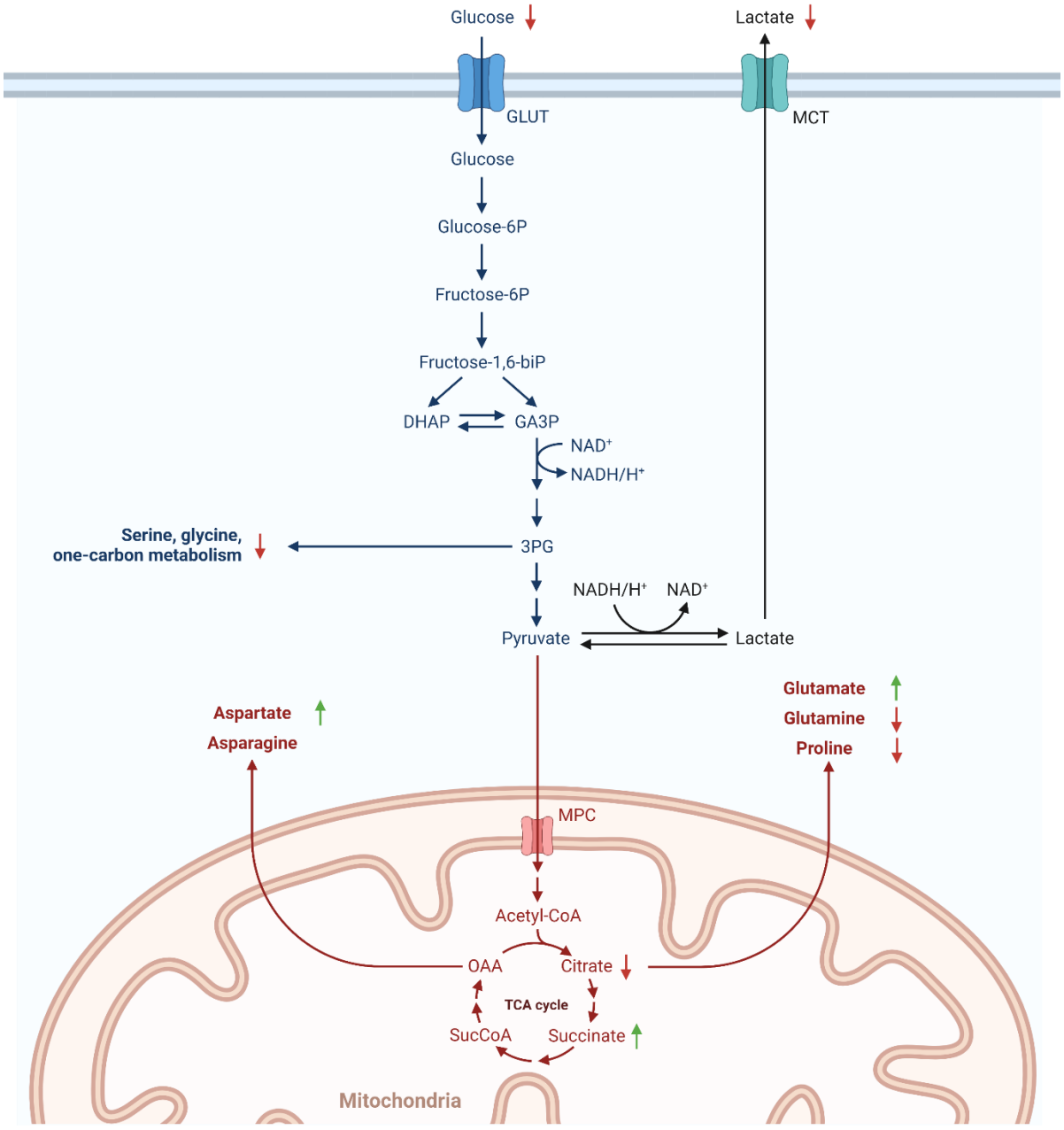

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Figure 7. Diagram of glycolysis and TCA cycle including amino acids present in NMR metabolomic analysis. Green arrows indicate the upregulation of the metabolite in the steady-state

glucose group compared to the cyclically fed group. Red arrows indicate the downregulation of the metabolite in the steady-state group compared to the cyclically fed group.

Discussion:

Cell culture methods were adapted to improve the consistency of the number of cells and the quality of the alginate beads. Increasing the total surface area of the 2D culture improved cell yields so that the desired cell concentrations were obtained. Adding the alginate solution dropwise near the surface did not gain enough velocity to break the surface tension of the 102 mM $CaCl_2$ solution. This created uniform spherical beads, as the alginate bead formed a sphere on the surface of the $CaCl_2$ solution to maximize surface area and minimize surface energy. The sphere then entered the solution, as the pressure on the surface increased when a sphere was formed, creating uniform spherical beads of ~ three mM diameter.

The assembly of the bioreactor went through many adjustments. A few critical adjustments that were made throughout the design process included: adding sterile parafilm to any holes in the 50 mL conical tubes that fittings were added to; using MasterFlex tubing on the peristaltic pumps to reduce degradation and leaks in the tubing; Adding cell strainers to the assembly to prevent loose alginate from clogging the flow of the system. These adjustments helped to allow the system to run smoothly for the entire ten-day cycle required for the culture time.

The preliminary NMR metabolomics data show that a change in metabolic behavior of cells occurs when glucose is held constant in the bioreactor. The overall effects of state-state glucose culture on cellular metabolism can be mapped using cellular stress and metabolic consumption rates as our guiding principles for analysis in conjunction with Figure 6.

The cells expressed an upregulation of metabolic activity in glycolysis and TCA cycle metabolism under steady-state glucose conditions (Figure 7). However, the metabolite concentrations were not as expected for the direct metabolites involved in these pathways. Simple glycolysis was downregulated, as shown by a decrease in lactate content. Succinate, a component of the TCA cycle and critical intermediate in oxidative phosphorylation, was upregulated (Figure 7).

A reduction in glucose present in the cells indicates that the cells have metabolized glucose into useful components, such as amino acids or intermediate metabolites like pyruvate and succinate. Additionally, a reduction in lactate content indicates that the cells reduce their use of the simple glycolytic pathway [Iacobazzi]. The presence of lactate causes a drop in pH that creates additional stress on the cells in culture [Yu, Urban]. Because lactate is reduced in cells fed with constant glucose, the cells may be experiencing less stress, thereby more closely mimicking the behavior of healthy functioning cells in culture [Yu, Salvatierra].

Of note in these preliminary results is the increase in glutamate, aspartate, leucine, and isoleucine. Aspartate and glutamate are non-essential and thus can be produced through mammalian cellular metabolism. However, leucine and isoleucine are essential amino acids. The trend over essential and non-essential amino acids indicates that fewer proteins are being constructed by the cells, resulting in a lower overall metabolic demand for protein formation. Glutamate and aspartate are formed at higher rates when cellular stress is reduced due to a lack of metabolic demand for protein formation [Hertz, Alkan]. Increased lactate in cyclic feeding indicates increased cellular stress overall, likely explaining the increase in the assembly of these amino acids through TCA cycle intermediates.

Succinate, an intermediate of the TCA cycle, was found at increased concentrations in the cells analyzed. Succinate is involved in many metabolic pathways, so its accumulation is of interest for further study of alternative pathways that could change their regulations in constant glucose culture [Giorgi-Coll].

A limitation of this study is that the cell culture analysis under hypoxic conditions may be another step that helps retain the NP phenotype throughout the culture time of the cells [Jaworski]. The lengthy culture period is the most challenging part of troubleshooting this process, and NP cells have been shown to change their metabolic phenotype over time when in normoxic conditions.

While the bioreactor was created with 3D alginate cell culture, the system could be used in whole tissue cultures or other scaled-up bioreactor processes. The bioreactor system assembled could benefit from more environmental controls, such as a hypoxic culturing environment and monitoring pH throughout the culture. These additions would help present a complete view of how cells behave *in vivo*.

A limitation of this work is controlling for the heightened oxygen levels present in the bioreactor compared to *in vivo*. This study was performed under atmospheric normoxic conditions but provided a baseline for constructing a bioreactor that can analyze the metabolomic data of cells in extended culture. Cells will favor using the TCA cycle for ATP production with more available oxygen, unlike the expected hypoxia-driven glycolysis metabolism of NP cells *in vivo*. However, when considering NMR metabolomics, a reduction in citrate shows that the cells' need for energy supplied by the TCA cycle was reduced.

Conclusion:

A fluidized bed 3D alginate cell culture bioreactor was created. The efficacy of the novel technology was confirmed through pilot studies on metabolite contents of cells cultured using cyclic and steady-state feeding modes of the bioreactor. This study shows the development of a small-scale bioreactor that can be adapted to different sizes, gas compositions, temperatures, and culture contents. The versatility of this bioreactor culture method could help to standardize cell culture for research of the IVD. Standardization of cell culture is crucial because we base our assumptions of metabolic consumption and production rates on a common practice in researching the IVD.

This cell culture method shows promise in use to characterize the metabolic activity of IVD cells. Future research would require further NMR analysis of a greater sample size, preferably n = 3 or more, to produce statistically significant results. Additionally, research into the pathways in which succinate is aggregating to slow down would be of further interest to view the whole picture of the metabolic analysis of this work.

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