Metabolic regulation of collagen gel contraction by porcine aortic valvular interstitial cells
Peter I. Kamel, Xin Qu, Andrew M. Geiszler, Deepak Nagraith, Romain Harmaney, Heinrich Taegtmeyer, K. Jane Grande-Allen

This paper has been published in *Journal of The Royal Society Interface*.

The publisher version can be found at:
http://rsif.royalsocietypublishing.org/content/11/101/20140852.long

It can be cited as:
Metabolic Regulation of Collagen Gel Contraction by Porcine Aortic Valvular Interstitial Cells

Peter I. Kamel, Xin Qu, Andrew M. Geiszler, Deepak Nagrath, Romain Harmancey, Heinrich Taegtmeyer, K. Jane Grande-Allen

1 Summary

1.1 Purpose

Despite a high incidence of calcific aortic valve disease in Metabolic Syndrome, there is little information about the fundamental metabolism of heart valves. Cell metabolism is a first responder to chemical and mechanical stimuli, but it is unknown how such signals employed in valve tissue engineering impact valvular interstitial cell (VIC) biology and valvular disease pathogenesis.

1.2 Methods

Porcine aortic VICs were seeded into 3D collagen gels and analyzed for gel contraction, lactate production, and glucose consumption in response to manipulation of metabolic substrates including glucose, galactose, pyruvate, and glutamine. Cell viability was also assessed in 2D culture.

1.3 Results

Gel contraction was sensitive to metabolic manipulation, particularly in nutrient-depleted medium. Contraction was optimal at an intermediate glucose concentration (2 g/L) with less contraction with excess (4.5 g/L) or reduced glucose (1 g/L). Substitution with galactose delayed contraction and decreased lactate production. In low sugar concentrations, pyruvate depletion reduced contraction. Glutamine depletion reduced cell metabolism and viability.

1.4 Conclusions

Nutrient depletion and manipulation of metabolic substrates impacts the viability, metabolism, and contractile behavior of VICs. Particularly, hyperglycemic conditions can reduce VIC interaction with and remodeling of the extracellular matrix. These results begin to link VIC metabolism and macroscopic behavior such as cell-matrix interaction.

Keywords: Aortic valvular interstitial cell, collagen gel, valve metabolism

2 Introduction

There is increasing recognition of the importance of valvular cell biology in the pathogenesis of disease states such as aortic valve calcification [1]. The current standard of care for valvular diseases, however, relies on mechanical replacement or repair and not on aspects that address cell biology [2].
In particular, there is a paucity of information about the metabolism of heart valves (with the exception of oxygen consumption by valve tissues [3]), which is noteworthy given the reported correlations between Metabolic Syndrome and aortic valve calcification [4]. Furthermore, it has been shown in mouse models that hyperglycemia and mild hyperlipidemia from a high fat, high carbohydrate diet can induce aortic valve disease [5]. As the supplier of energy and building blocks for the cell, metabolism is involved in virtually all cellular processes and would provide a solid foundation upon which to build an understanding of heart valve cell biology to ultimately identify biological treatment options for valvular diseases. VIC interactions with their pericellular environment are also of great interest to the tissue engineering community. Mechanical and chemical stimulation have been widely used in the study and development of tissue engineered heart valves [6][7]. Metabolism however, which is a first responder and fundamental mediator in such processes, has rarely been studied for optimization of valve tissue engineering.

Valvular interstitial cells (VICs) in the aortic valve have been attributed as the cells primarily responsible for the calcification of the valve as a result of VIC interaction with and remodeling of the extracellular matrix (ECM) [8][9]. Metabolic activity underlies such processes. Energy and nutrients available in the system dictate the level and type of biosynthesis in the cell [10], including the synthesis of ECM, the production of integrins that interact with the ECM, and the amount of energy available to maintain cell viability and undergo cytoskeletal remodeling [11]. In vitro, such VIC activity has been demonstrated when VICs are seeded into engineered 3D gels and interact with and remodel the matrix to extrude water, resulting in gel contraction [12].

Pathologic ECM remodeling has been closely linked to human aortic valve disease [13]. This remodeling has been specifically studied in VIC-collagen interaction, which suggests that disruptions in collagen homeostasis can predispose the valve to pathologic disease states including calcific aortic valve disease [14]. One method of investigating VIC-collagen interactions is the collagen gel contraction assay. This assay has been used in a variety of other cell types to monitor responses to cell signaling and subsequent effects on integrin regulation [15]. Compared to 2D culture, suspension of fibroblasts in 3D collagen gels can more closely mimic in vivo behavior in terms of elongation, projections, and collagen synthesis [16][17]. Collagen gels have often been used as scaffolds for VICs in mechanobiologic investigations [18][19] and results have suggested that VICs are less activated in 3D collagen gels than in 2D culture on tissue culture plastic [20]. We hypothesized therefore that collagen gel contraction would be sensitive to metabolic manipulation and serve as a gross indication of a number of cell functions that include cell viability, cytoskeletal remodeling, integrin regulation, and cell-ECM interactions. Consequently, metabolic conditions that negatively affect VIC collagen gel remodeling could be thought to predispose or contribute to aortic valve pathology.

In this study, we monitored contraction of 3D collagen gels seeded with porcine VICs —as well as VIC lactate production and 2D cell viability — in response to the manipulation of metabolic substrates in the culture medium, namely glucose, galactose, pyruvate, glutamine, and Hams F-12 nutrient mixture. Porcine VICs have been frequently used as a model for aortic valve research, as they are readily available, fast-growing, and exhibit similar properties as human valve cells including calcification in long-term culture [21]. The range of metabolic substrates selected offers tools for analyzing macroscopic changes in response to fundamental changes in metabolism through glycolysis and the Krebs citric acid cycle. To our knowledge, this study is the first to investigate metabolism of VICs and macroscopically observe changes in VIC activity in an attempt to characterize a highly understudied phenomenon.

3 Materials and Methods

3.1 Cell Culture Materials

Liquid Dulbecco’s Modified Eagle Medium (DMEM) with glucose (1 g/L) for cell culture and Antibiotic-Antimycotic Solution were purchased from Cellgro (Manassas, VA). Liquid glucose-free DMEM was purchased from Invitrogen and powdered DMEM (−glucose, −pyruvate, −glutamine, −phenol red, −bicarbonate) was purchased from Sigma to prepare the experimental media. HEPES buffer, Hams F-12 Nutrient Mixture, and Bovine Growth Serum (BGS) were purchased from Hyclone (Logan, UT).
3.2 Harvesting and Cell Culture

Aortic valves were dissected from porcine hearts obtained from a local abattoir (Fisher Ham and Meat, Spring, TX) within 6 hours postmortem. In total, cells were obtained from 4 harvests with 1-2 hearts used per harvest. Valves were first soaked in serum-free medium (2% Antibiotic/Antimycotic, 1% HEPES buffer, 1:1 F-12:DMEM) containing 2 mg/mL collagenase type II (Worthington Biochemical Corp, Lakewood, NJ) for 30 minutes in an incubated shaker (37°C, 2.3 Hz). Valves were subsequently wiped with cotton swabs to remove the endothelial cells from the surface, minced, immersed in serum-free medium containing 1 mg/mL collagenase type III and 0.1 mg/mL hyaluronidase (both from Worthington), and returned to the same incubated shaking conditions for 16 hours. Afterwards, the cell suspension was strained through a 70µm cell-strainer (BD Falcon, San Jose, CA), centrifuged at 750×g for 5 min at room temperature and cultured in DMEM:F-12 (1:1, DMEM containing 1 g/L glucose, +pyruvate, +glutamine) with 10% BGS, 2% Antibiotic/Antimycotic, and 1% HEPES buffer at 37°C, 5% CO₂. After this initial cell plating (P0) reached 80-90% confluence, cells were passaged using 0.25% trypsin (Cellgro). All passages after P0 were cultured in the same formulation of medium, with the exception of a 1%, rather than 2%, antibiotic concentration. For all experiments, cells were from passages 2 - 3.

3.3 Glucose Starvation Pre-Treatment

Because prior work with cardiac fibroblasts suggested that culture pre-treatment can increase cell contraction of collagen gels [22], cells were starved from glucose and pyruvate 24 hours prior to gel preparation by replacing the medium with a (−glucose, −pyruvate, +glutamine) DMEM solution containing 10% BGS, 1% Antibiotic/Antimycotic, and 1% HEPES. Although our initial studies showed that depriving VICs of glucose for 24 hours before collagen gel preparation did not appear to affect their collagen gel contraction behavior, the withholding of glucose and pyruvate was performed prior to all experiments to avoid confounding results since those factors were being investigated. Afterwards, cells were trypsinized, seeded into a gel or 2D monolayer culture, and supplemented with experimental media as described in the following section.

3.4 Experimental Media Preparation

Overall, there were five variables in the type of media tested: concentration of sugar, type of sugar, presence of pyruvate, concentration of glutamine, and presence of F-12 (Table 1). All groups were prepared with 10% BGS, 1% Antibiotic-Antimycotic Solution, and 1% HEPES buffer.

Variable Sugar and Pyruvate For the variable sugar and pyruvate studies, base media was prepared using liquid DMEM (−glucose, −pyruvate, +glutamine). D-Glucose (Invitrogen) or D-Galactose (Sigma) was added in various experiments at concentrations of 0 g/L, 0.5 g/L, 1 g/L, 2 g/L, or 4.5 g/L. In order to minimize variability, media formulations were prepared via serial dilutions. Experimental groups containing pyruvate were supplemented with 1.1 g/L pyruvate (Gibco).

Variable Glutamine For the glutamine trials, DMEM was prepared from powder (−glucose, −pyruvate, −glutamine, −phenol red, −bicarbonate) and supplemented with 0 mM, 4 mM, or 8 mM glutamine (Cellgro), as well as 15 mg/mL phenol red (Sigma), and 3.7 g/L sodium bicarbonate (Fisher).

F-12 Presence In the F-12 studies, DMEM without glucose was diluted 1:1 with a solution of F-12 Nutrient Mixture. The F-12 used contained 1.8 g/L glucose and following dilution, the solution was supplemented with glucose to reach a final concentration of 1 g/L, 2 g/L, or 4.5 g/L.

Each group was tested with at least three replicates (n = 3 to n = 6) and experiments were repeated at least twice from different VIC harvests.
3.5 2D Culture

In order to assess their viability, cells were seeded into 24-well plates at approximately 20,000 cells/cm² with 500 µL of the above experimental media. After 24, 48, and 96 hours, triplicates of these wells were washed with PBS and incubated for 30 minutes with 2 µg/mL Calcein AM (Invitrogen, OR) in PBS. Plates were subsequently measured for fluorescence intensity in a SpectraMax M2 Plate Reader (Molecular Devices, Sunnyvale, CA) with excitation at 485 nm and emission at 538 nm.

3.6 3D Collagen Gel Preparation

Collagen gels were prepared using 1 part 10× DMEM, 8 parts collagen solution, and 1 part 1× DMEM culture medium (containing VICs). 10× DMEM was prepared from powder (−glucose, −pyruvate, −glutamine, −phenol red, −bicarbonate) at 83 g/L in ultra-pure water and supplemented with 150 mg/L phenol red, 10 mM pyruvate, 37 g/L sodium bicarbonate, and 40 mM L-glutamine. Collagen solution was prepared from a stock of rat tail collagen type I (BD Biosciences, Bedford MA) diluted to 2 mg/mL using 0.02 M acetic acid, giving a collagen concentration of 1.6 mg/mL in the final gel. On ice, 10× DMEM was mixed with collagen solution and neutralized by adding 1M NaOH dropwise until the solution turned a peach color (approximately physiologic pH). Afterwards, pelleted cells were resuspended to produce a final concentration of 1 × 10⁶ cells/mL in 1× DMEM without sugar and thoroughly mixed into the solution containing the 10× DMEM and collagen. A 500 µL volume of this solution was immediately placed in each well of a 24-well plate.

3.7 Gel Maintenance and Quantification of Lactate and Glucose

Gels were not manually detached from well sides, but were left to detach and contract spontaneously. Media was changed every three days in gels. This change frequency was determined based on initial observations of changes in the media color (from red to yellow) with accumulation of acidic metabolites. Media was collected and stored at −80°C at each change. Lactate and glucose concentrations were analyzed in the media using a YSI 2300 STAT Plus Glucose & Lactate Analyzer (YSI Life Sciences, Yellow Springs, Ohio).

The individual wells were periodically imaged with a 3MP digital camera over the course of the contraction period. Contraction was quantified digitally using ImageJ by measuring the area of the gel and normalizing it to the area of the base of the well. Following full contraction, gels were blotted dry, weighed, and frozen for future analysis (not included in this report).

3.8 Statistical Analysis

Data are reported as mean with standard error of the mean (SEM). Statistical analysis to detect differences in gel contraction was performed via two-factor ANOVA of normalized gel size over each of the time points during the contraction period. Thus, a significant difference in contraction would be detected if either the contraction rate was slower between groups or the onset of contraction was delayed between groups. A single factor ANOVA between groups was performed for comparisons at a single time point, such as for cell viability or lactate concentration at a point in time. Tukey Tests were used for post-hoc analysis to identify significant differences between groups. Statistical analysis was performed in Microsoft Excel and Minitab 16.

4 Results

4.1 Variable Glucose, F-12 Present

When F-12 was present in the culture medium, cells seeded in 2D wells exhibited similar viability regardless of the concentration of glucose present even up to 96 hours after cell seeding (Figure 1a).

In 3D culture, VICs began contracting collagen gels within 24 hours of gel construction. Control gels not containing cells did not contract over the course of the experiment. Gels containing 2 g/L glucose media exhibited slightly greater contraction at the various time points than those with 1 g/L glucose.
or 4.5 g/L glucose (Figure 1b). Differences were small, but significant \((p = 0.007, \text{ two-factor ANOVA, 26-47 hours})\).

Following contraction, lactate concentration in the media was significantly reduced in 1 g/L glucose media compared to groups with 2 and 4.5 g/L glucose \((p < 0.001)\), but a significant difference was not observed in lactate concentration between the 2 g/L and 4.5 g/L glucose groups \((p = 0.07)\) (Figure 1d). Glucose was depleted in the initial 1 g/L glucose media \((0.007 \pm 0.002 \text{ g/L, SEM})\) and in the 2 g/L media \((0.15 \pm 0.01 \text{ g/L, SEM})\), with some remaining in the 4.5 g/L media \((1.38 \pm 0.04 \text{ g/L, SEM})\), \((p < 0.001, \text{ single-factor ANOVA})\).

### 4.2 Variable Sugar, F-12 Absent

When F-12 and pyruvate were removed, a reduction in 2D cell viability was observed in groups containing less glucose. Differences were not significant at 24 \((p = 0.83)\) or 48 hours \((p = 0.17)\), but became significant after 96 hours \((p < 0.001, \text{ single-factor ANOVA})\) (Figure 2a). The cell viability at 96 hours was not reduced by using 1 g/L galactose instead of 1 g/L glucose (data not shown).

In collagen gels lacking F-12, VICs typically did not begin contracting gels until 2-5 days after gel construction. Groups with 0 g/L and 0.5 g/L glucose did not contract at all during the experiment (data not shown). Similar to the experiments containing F-12, there was faster contraction for the groups containing 2 g/L glucose than for the other two groups but the difference was more pronounced \((p = 0.016 \text{ overall, two-factor ANOVA, 77-120 hours})\) (Figure 2b).

Substitution of glucose with galactose generally slowed gel contraction, and this was especially pronounced at higher concentrations of sugar \((p < 0.001 \text{ for 4.5 g/L glucose vs. galactose, two-factor ANOVA, 47-71 hours})\) (Figure 2c). Following contraction, significantly more lactate was found in the culture media in groups containing greater concentrations of glucose \((p < 0.001, \text{ single-factor ANOVA})\). With galactose, however, there was no significant difference in lactate produced at any concentration of galactose \((p = 0.49)\) (Figure 2d).

### 4.3 Variable Pyruvate

The absence of pyruvate reduced gel contraction at the various time points compared to groups with pyruvate present, particularly at lower concentrations of sugar (Figure 3a, 3b). Differences were statistically significant in 1 g/L glucose \((p = 0.026)\) and in 2 g/L glucose \((p = 0.035)\) but not in 4.5 g/L glucose \((p = 0.226, \text{ two-factor ANOVA, 74-120 hours})\). Likewise, lactate production was slightly but significantly reduced when pyruvate was absent and media contained 1 g/L galactose \((p < 0.001)\) or 1 g/L glucose \((p = 0.034, \text{ Tukey Test})\) (Figure 3c).

### 4.4 Variable Glutamine

When glutamine was removed from the media, a significant reduction in cell viability was observed as early as 24 hours later \((p = 0.004 \text{ with 1 g/L glucose, } p = 0.015 \text{ with 2 g/L glucose, single factor ANOVA})\) (Figure 4a). Differences in 2D cell viability were not significant between 4 mM and 8 mM glutamine at 1 g/L and 2 g/L glucose. Lactate production was consistent with this trend and a significantly lower concentration of lactate was observed when glutamine was removed (Figure 4c). Likewise, glucose consumption was significantly reduced at 0 mM glutamine \((p < 0.001, \text{ single factor ANOVA})\) (Figure 4d). However, no significant differences in gel contraction were consistently observed, though there was a tendency for groups with low glucose (1 or 2 g/L) to exhibit reduced contraction with 8 mM glutamine \((p = 0.071, \text{ two-factor ANOVA, 4 mM glutamine vs. 8 mM glutamine, 1 g/L glucose})\) (Figure 4b).

## 5 Discussion

In this study, we analyzed gel contraction, lactate production, and glucose consumption by VICs in 3D collagen gels under different concentrations of sugar, nutrients, pyruvate, and glutamine. We also examined viability of VICs seeded in 2D culture under these conditions. Overall, gel contraction was
Discussion

sensitive to media formulation, particularly when fewer metabolic substrates were present, and greater cell viability did not necessarily correlate with an increase in gel contraction. To our knowledge, no previous studies have analyzed such an effect of metabolic changes on the macroscopic properties of VICs in vitro.

5.1 Glucose

Despite trends in viability, glucose was observed to have an optimal concentration for VIC contraction, with glucose excess or deprivation resulting in reduced contraction. In the experiments with F-12, cell viability was not significantly reduced in groups with lower glucose concentrations, indicative of the metabolic robustness of healthy cells, which can metabolize the variety of nutrients and cofactors present in F-12 through other metabolic pathways [23]. Absent these nutrients, cells exhibited reduced viability in groups with less glucose, yet 3D collagen gel contraction was greatest with an intermediate level of glucose around 2 g/L. A low concentration of glucose likely reduced the energy available for processes such as actin filament remodeling, biosynthesis of integrins and ECM components, and assembly of integrin-based focal adhesions linking the ECM to the cytoskeleton, which may have caused the reduction in gel contraction [10][11]. The detrimental effect of excess glucose may have been caused by increased production of metabolic by-products that adversely affected cell activity. This includes the generation of lactic acid or toxic reactive oxygen species that are products of active respiration [24]. Prior research in other cell types have implicated the activation of other processes, including hexosamine biosynthesis involving glutamine:fructose-6-phosphate amidotransferase, as contributors to the adverse effects of excess glucose [25][26]. Furthermore, metabolism — as measured by lactic acid production — plateaued when increasing the concentration of glucose in the media. Specifically, increasing glucose concentration from 1 to 2 g/L nearly doubled lactate production, but there was no significant difference in final lactate concentration between 2 and 4.5 g/L glucose when F-12 was present in the media (Figure 1d). This finding supports our proposed interpretation of an optimal range for glucose concentration for VIC metabolism and gel contraction.

The collagen results comparing 1 g/L to 2 g/L glucose in VICs are comparable to those observed with cardiac fibroblasts, in which increased gel contraction was observed with an increase in glucose concentration from 1 g/L to 4.5 g/L [22]. Results were attributed to an observed increase in the expression of α1 and β1 integrins, which account for increased cell-ECM interaction and adhesion. In cardiac fibroblasts, increased glucose concentration also increased collagen production, which has been proposed to promote a profibrotic response in diabetes [27][28][29], and may also be relevant to valvular dysfunction in diabetics.

5.2 Galactose

Substitution of glucose with galactose reduced the metabolic activity of VICs as suggested by decreased metabolite production and was consequently observed to delay contraction. Galactose is metabolized by the cell through the Leloir pathway, which first requires a conversion to glucose (Figure 5) [30]. This conversion process requires approximately the same amount of energy as that produced by glycolysis. It was as expected then that galactose substitution reduced the rate of gel contraction. More interesting was the observation that nearly a fivefold increase in galactose concentration did not significantly alter lactate production (Figure 2d). Lactate is produced by the cell through the reduction of pyruvate, particularly when metabolism exceeds oxygen availability. The lack of significant difference in lactate production with an increased concentration of galactose — in contrast to the increased lactate production with increased glucose — may be an indication that galactose conversion is rate-limiting, perhaps with UDP-galactose-4-epimerase, which is speculated as being the rate-limiting enzyme in galactose metabolism [31]. This may be a factor in reducing toxicity of a high sugar concentration.

5.3 Pyruvate

Pyruvate absence was observed to significantly reduce gel contraction and lactate production, but only when glucose concentrations were low. Pyruvate, an end product of glycolysis, is typically metabolized through the Krebs cycle to supply energy via oxidative phosphorylation. Pyruvate absence
was significant when less energy was available to the cell through sugar metabolism, as was the case with 1 g/L glucose media, or when additional energy was required to metabolize the sugar, as with 1 g/L galactose. At higher concentrations of glucose, glucose metabolism was sufficient to compensate for the lack of pyruvate.

5.4 Glutamine

Absence of glutamine in the culture media significantly decreased 2D cell viability and metabolic activity in collagen gels. Glutamine, in addition to a large set of other cellular functions, enters the Krebs cycle in the form of α-ketoglutarate [32][33]. Glutamine participates in an anaplerotic pathway to feed the cycle by participating as a direct intermediate that can be recycled. When glutamine was absent in the experimental media, the supply of Krebs cycle intermediates was limited, reducing the cells ability to undergo cellular respiration and consequently decreasing metabolism (Figure 4c) and cell viability (Figure 4a). Since Krebs cycle intermediates can be recycled and are maintained at a relatively stable level, it follows that doubling the glutamine concentration did not have a significant effect on cell viability or metabolism.

5.5 Clinical Implications

These results have implications in clinical research related to diabetes. It has been shown that persistently elevated serum glucose levels in diabetes are directly linked to the development of the microvascular complications of retinopathy, nephropathy, and neuropathy [34]. If it is the case that persistent hyperglycemia is additionally a direct cause of valvulopathy through pathologic collagen remodeling, the pathogenesis of the disease may advocate for strict glycemic control. Intensive insulin regulation could prove to potentially reduce the development of valvular complications that have been associated with metabolic syndrome [4]. Perhaps even dietary modifications in terms of the type of sugar consumed (glucose v. galactose) may be beneficial in a diabetic diet.

Furthermore, there is active research in optimizing tissue engineered heart valves that can be used in place of mechanical valves or cadaveric transplants [35]. While studies have investigated factors such ideal oxygen conditions [3] and conditioning [36] in engineering the valves, few studies have investigated ideal metabolic conditions, which would be essential to optimize bioreactor conditions.

5.6 Study Limitations

Several factors need to be considered when extending these results to a physiologic context. In healthy valves in vivo, aortic VICs express a quiescent fibroblast-like phenotype. When isolated and cultured, VICs have been shown to spontaneously differentiate into a myofibroblastic contractile phenotype, often characterized by the expression of α-smooth muscle actin (α-SMA). This phenotype is considered to be activated and is associated with pathologic changes in vivo [37][38]. 3D free-floating collagen gels have been shown to be superior in maintaining the quiescent VIC phenotype over 2D culture and stressed 3D models under standard media preparations similar to baseline experiments in this study [20][18]. However, prior studies have suggested that various types of stress — be it metabolic or otherwise — may precipitate pathologic VIC activation. For example, hypercholesterolemia, both in vivo and in vitro can modulate phenotypic behavior of VICs by inducing endoplasmic reticulum stress [39]. Perhaps the starved or hyperglycemic states may induce similar phenotypic changes that may have mediated the results that were observed in this study. Furthermore, the activation of VICs would likely result in an increase in metabolic activity and demand. Future study would analyze whether metabolic changes such as nutrient deprivation or excess glucose availability can induce the activated state in VICs and if such activation alters VIC metabolism.

Furthermore, while glucose concentrations of 1 to 4.5 g/L are common practice for in vitro culture, physiological blood sugar typically does not reach a concentration as high as 4.5 g/L in normoglycemic patients, with fasting levels maintained below 1 g/L and any random sugar above 2 g/L classified as diabetes [40]. The cyclic change in the concentration of glucose in diabetic conditions in vivo as well commonly comorbid dyslipidemia may also have an important effect on metabolism in addition to the increased magnitude of the serum glucose concentration. As with any in vitro study, there
Discussion

are a variety of other pathways and factors that can affect metabolism, but were not considered in this study, including but not limited to the aortic valve microenvironment [41], lymphocytic and macrophage infiltration of the valve [42], and the variety of infectious, congenital, and systemic factors that have been shown to contribute to valvulopathy. However, our results suggest that metabolism may play a role in disease progression and may lead to clinical studies investigating the link between diabetes and aortic valve disease. Future study may also utilize fibrin — as opposed to collagen — gels, which offer less suppression of ECM production and may better mimic in vivo conditions [43]. Our preliminary tests with fibrin gels have shown similar trends to those observed in collagen gels.

5.7 Conclusion

These results begin to link VIC metabolism with macroscopic behavior such as cell-matrix interaction and contraction. They demonstrate that an alteration in metabolic substrate availability can affect valvular phenotypic properties such as contractility and establish the collagen gel contraction assay as a novel and viable option in investigating metabolism through tissue engineering approaches. The data provide an initial understanding of the metabolic behavior of heart valve cells and the potential clinical relevance. Inhibition of gel contraction by higher concentrations of glucose is particularly relevant to physiological conditions like diabetes, where elevated blood-sugar levels are observed. This line of investigation opens the door for future experimentation including analysis of heart valve calcification in response to diabetic metabolic conditions as well the incorporation of biologically relevant factors such as mechanical stimulation or disease states, to ultimately improve our understanding of the link between metabolism and valvular diseases.

Acknowledgments

Special thanks go to Dr. Daniel Harrington for his critical evaluation of the research, Shannon Burke for assistance in experimentation, and Dr. Melissa McHale for her review of the manuscript. This research was funded by the Rice University Century Scholars Program, a Hamill Innovation Award from the Rice University Institute for Biosciences and Bioengineering, and NIH R03EB011576.

Data Accessibility

Data from this study can be accessed at http://hdl.handle.net/1911/72186.
Figures

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameters Tested</th>
<th>Function in Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM Sugar Concentration</td>
<td>0 g/L, 0.5 g/L, 1 g/L, 2 g/L, 4.5 g/L</td>
<td>Input for glycolysis and subsequent metabolic pathways</td>
</tr>
<tr>
<td>DMEM Sugar Type</td>
<td>Glucose, Galactose</td>
<td>Input for glycolysis with variable conversion costs</td>
</tr>
<tr>
<td>Nutrient Mixture</td>
<td>F-12 present in 1:1 dilution, F-12 absent</td>
<td>Provides a variety of amino acids, vitamins, and metabolic cofactors for the cell; contains glucose</td>
</tr>
<tr>
<td>DMEM Pyruvate Concentration</td>
<td>0 g/L, 1.1 g/L</td>
<td>Input for the Krebs cycle and other metabolic pathways</td>
</tr>
<tr>
<td>DMEM Glutamine Concentration</td>
<td>0 mM, 4 mM, 8 mM</td>
<td>Converted to α-ketoglutarate, feeding into the Krebs cycle</td>
</tr>
</tbody>
</table>

Tab. 1: Variables tested in this paper and their relevant functions within the cell.
Fig. 1: (a) 2D cell viability at 96 hours of VICs supplemented with media containing 1 part DMEM and 1 part F-12 at variable levels of glucose. Differences in cell viability were not significant even after 96 hours ($p = 0.054$). (b) Contraction of VICs in collagen gels with the same formulations of media. Contraction with 2 g/L glucose was slightly, but significantly faster than that with 1 or 4.5 g/L glucose (two-factor ANOVA, 26-47 hours). (c) Stereotypical example of gels in wells with variable glucose media, illustrating fastest contraction with 2 g/L glucose. (d) Lactate concentration in media following contraction. Media with 1 g/L glucose contained significantly less lactate than did media with 2 or 4.5 g/L glucose ($p < 0.001$). There was no significant difference in lactate concentration observed between 2 and 4.5 g/L glucose ($p = 0.07$). Data represent mean ± SEM.
Fig. 2: (a) Calcein viability assay of VICs after 96 hours in 2D cell culture with media composed of DMEM with variable glucose and no F-12. Differences between groups were significant with reduced viability in groups with less glucose ($p < 0.001$, single-factor ANOVA). (b) Contraction of VICs in collagen gels in the same formulations of media. Contraction with 2 g/L glucose was significantly faster than that with 1 or 4.5 g/L glucose. (c) Gel contraction comparing 4.5 g/L glucose and 4.5 g/L galactose DMEM media. Contraction with galactose was significantly delayed ($p < 0.001$, two-factor ANOVA, 47-71 hours). (d) Lactate concentration in media following contraction in variable sugar DMEM. Differences were significant between all concentrations of glucose ($p < 0.001$) but not between concentrations of galactose ($p = 0.49$, single-factor ANOVA). The dotted line indicates the baseline level of lactate in the culture medium as measured from media supplied to collagen gels without any cells. Data represent mean ± SEM.
Fig. 3: Contraction of collagen gels with and without pyruvate in DMEM media containing (a) 1 g/L glucose and (b) 2 g/L glucose. Differences in contraction between groups with and without pyruvate were significant at 1 g/L glucose ($p = 0.026$) and 2 g/L glucose ($p = 0.035$), but not at 4.5 g/L glucose ($p = 0.226$) (two-factor ANOVA, 74-120 hours). (c) Lactate concentration following contraction in gels of groups with variable sugar and pyruvate. Lactate production was slightly, but significantly reduced when pyruvate was absent in 1 g/L glucose and 1 g/L galactose media. The dotted line indicates the baseline level of lactate in the culture medium as measured from media supplied to collagen gels without any cells. Data represent mean ± SEM.
Fig. 4: (a) Calcein cell viability assay of VICs after 24 hours in 2D cell culture with 0 mM, 4 mM, and 8 mM glutamine in 1 g/L or 2 g/L glucose DMEM. Groups with 0 mM glutamine had significantly reduced 2D viability compared to either of the groups with 4 mM or 8 mM glutamine. (b) Contraction of VICs in collagen gels with 1 g/L glucose and variable glutamine. No significant differences were observed between groups. (c) Lactate concentration following contraction of collagen gels with variable glutamine in 4.5 g/L glucose. Groups with 0 mM glutamine produced significantly less lactate in collagen gels than either the groups with 4 mM or 8 mM glutamine. (d) Concentration of glucose remaining in media after contraction with variable glutamine and an initial 4.5 g/L glucose. Significantly more glucose remained in groups lacking glutamine. Data represent mean ± SEM. * indicates $p < 0.001$, compared to groups with glutamine.
Fig. 5: A condensed illustration of the main metabolic pathways in this study. Galactose is converted to glucose via the Leloir pathway, which requires ATP. Glucose is broken down to pyruvate with a net yield of ATP. Pyruvate proceeds into the Krebs cycle, producing ATP and reduced electron carriers for oxidative phosphorylation. Glutamine feeds into the Krebs cycle as α-ketoglutarate.
References


Title for page headings: VIC Metabolism