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Effects of electrical stimulation in C2C12 muscle constructs

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Abstract

Electrical stimulation affects the deposition of extracellular matrices and cellular differentiation. Type I collagen is one of the most abundant extracellular matrix proteins; however, not much is known about the effects of electrical stimulation on collagen type I deposition in C2C12 cells. Thus, we studied the effects of electrical voltage and stimulation frequency in 3D cultured C2C12 muscle cells in terms of metabolic activity, type I collagen deposition and cell morphology. Electrically excitable C2C12 muscle cells were seeded in collagen scaffolds and stimulated with rectangular signals of voltage (2, 5, 7 V) and frequency (1, 2 Hz), using parallel carbon electrodes spaced 1 cm apart. Metabolic activity was quantified by the glucose: lactate concentration ratio in the medium. Apoptotic activity was assessed by TUNEL staining and changes in collagen deposition were identified by immunohistology. The ultrastructure of the tissue was examined by TEM. Glucose and lactate analysis indicated that all groups had similar metabolic activity. TUNEL stain showed no significant difference in apoptotic damage induced by electrical stimulation compared to the control. Samples stimulated at 2 Hz exhibited reduced collagen deposition compared to the control and 1 Hz stimulated samples. Muscle-protein marker desmin was highly expressed in constructs stimulated with 1 Hz/5 V sample. TEM revealed that the stimulated samples developed highly organized sarcomeres, which coincided with improved contractile properties in the 1 Hz/5 V- and 2 Hz/5 V-stimulated groups. Our data implicate that a specific electrical frequency may modulate type I collagen accumulation and a specific voltage may affect the differentiation of muscle sarcomeres in excitable cells.

Keywords

electrical stimulation; muscle cell C2C12; extracellular matrix; collagen type I; tissue engineering; sarcomere; contractility

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Supplementary material

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1. Introduction

Biophysical (mechanical and electrical) signals modulate cell growth and differentiation by guiding remodelling of the cellular microenvironment and by regulating gene expression (Lutolf and Hubbell, 2005; Boublik *et al.*, 2005). In skeletal muscle, electrical stimulation enhances myosin synthesis, the formation of myofibres and functional properties (Brevet *et al.*, 1976; Pette and Vrbova, 1999; De Deyne, 2000; Xia *et al.*, 2000) and in cardiac myocytes electrical stimulation enhances the functional assembly of contractile proteins (Radisic *et al.*, 2004). In addition, electrical stimulation improves expression of elongation factors and muscle proteins, secretion of vascular endothelial growth factor and insulin response by inducing a functional myogenic signal molecule, insulin growth factor-1, in skeletal muscle cells (Kanno *et al.*, 1999; Aas *et al.*, 2002; Brutsaert *et al.*, 2002). Long-term stimulation of rabbit muscle induces alteration of muscle fibre types and increases the presence of collagen type I and fibrillin (Trumble *et al.*, 2001). Chronic low frequency stimulation induces the formation of slow muscle fibres and glucose uptake in skeletal muscle (Thelen *et al.*, 1997; Pette and Vrbova, 1999; Egginton and Hudlicka, 2000; Hamada *et al.*, 2003).

Murine C2C12 myoblasts maintain their differentiation potential and, upon incubation with differentiation media, the myoblasts fuse with neighbouring cells to form myotubes that are responsible for muscle contraction (Blau *et al.*, 1983). The formation of myofibres in the C2C12 myoblasts is also depending on the cell density and/or the serum concentration in the medium (Yaffe and Saxel, 1997; Dennis *et al.*, 2001). Interestingly, during development, the C2C12 myoblasts express isoforms of cardiac contractile proteins that are incorporated into myofilaments (McMaho *et al.*, 1994) and respond to the co-stimulation of BMP-2 and TGF- β 1 (Katagiri *et al.*, 1994; Pirskanen *et al.*, 2000). In addition, electrical stimulation at 3 V/cm with 2 Hz enhances contractile properties in the C2C12 myotubes (Thelen *et al.*, 1997). The effects of electrical stimulation on muscle development have been studied in several groups, using different muscle cell types and electrical stimulation regimes, but without conclusive results (Dusterhoft and Pette, 1993; Naumann and Pette, 1994; Wehrle *et al.*, 1994; Stern-Straeter *et al.*, 2005; Pedrotty *et al.*, 2005).

The aim of this study was to investigate the effect of electrical field stimulation of varying voltage and frequency on three-dimensional (3D) skeletal muscle constructs. Our data suggest that collagen deposition may be modulated by electrical stimulation frequency and muscle differentiation may be modulated by stimulation voltage.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES), phosphate-buffered saline (PBS, calcium- and magnesium-free), 1× trypsin-EDTA solution in PBS, Hank's balanced salt solution and 1× penicillin-streptomycin were all from Invitrogen (Grand Island, NY, USA); Matrigel® was from Becton-Dickinson (Bedford, MA, USA); 10% buffered formalin was from Sigma Diagnostic (St. Louis, MO, USA). The orbital shaker (type BTB) was from Bellco Inc. (Vineland, NJ, USA).

2.2. Cells

Mouse muscle cell line C2C12 was obtained from ATCC (Manassas, VA, USA). The cells were grown in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin (Pen-Strep) for 3 days. At 85% confluence, the cells were harvested and seeded into 3D collagen scaffolds for further studies.

2.3. Electrical circuit

A custom-built electronic circuit was designed to deliver six independent output channels at 1 or 2 Hz and 0–10 V. Two independent 555 integrated circuits were tuned with 100 k potentiometers to deliver a 1 Hz and 2 Hz signal, as verified by oscilloscope. These outputs were fed to a 123 pulse generator, which output a 5 V, 2 ms pulse at each respective input frequency. These two pulse outputs were tied to an LPC66 X2 op amp buffer, generating a 10 V pulse. Six mechanical switches tied either 1 Hz or 2 Hz pulse to six separate 50 K potentiometers (Clarostat 392JB), allowing the 10 V pulse input to be scaled down to the desired output voltage. Finally, each of these signals was fed through an AD8570 output buffer, providing six independent channels available for stimulation.

2.4. Scaffold and cell seeding

The collagen scaffold was from Davol Inc. (Cranston, RI, USA), which is a commercially available Ultrafoam[®] haemostat cut into a $6 \times 8 \times 1.5$ mm dimension. Each collagen scaffold was hydrated in muscle culture medium (DMEM supplemented with 10% serum, 1% HEPES, 1% Pen/Strep) for 2 h at 37 °C in a 5% CO₂ humidified incubator to increase cell binding on the scaffold. The soaked scaffolds were inoculated with the C2C12 cells at a density of 1.35×10^8 cells/cm³, as described previously (Park *et al.*, 2005). Briefly, the cells were collected by centrifugation (1200 r.p.m., 5 min) and resuspended in 5 µl of Matrigel/1 million cells. The pre-wetted scaffolds were briefly dried and the cell–gel suspension was applied evenly to each scaffold. Gelation of the cell–gel construct was achieved within 20 min in the 37 °C incubator and the inoculated scaffolds were transferred into a six-well plate containing the cell culture medium. Six constructs per group were tested.

2.5. Electrical stimulation

After 3 days of pre-culture, the cell–gel constructs were transferred to stimulation dishes and 10 ml of the muscle culture media was added. A platinum wire from the stimulation dish was connected to an electrical circuit and continuous stimulation was initiated for an additional 5 days. For control, a cell–gel construct in a stimulation dish was cultured without stimulation for 8 days. The culture medium was replenished 2 days after the stimulation.

2.6. Metabolic assay

Glucose and lactate concentrations in the culture medium were measured using glucose and L-lactate analyser Model 2300 STAT Plus (Yellow Springs Instruments, Yellow Springs, OH, USA). For total DNA and protein measurement, the constructs were resuspended in an extraction buffer (1 N ammonium hydroxide, 0.2% Triton X-100) and homogenized using a steel ball bead beater (Biospec Products, Bartlesville, OK, USA) and the extraction solution was used for the following assays. The DNA content was measured fluorometrically with Hoechst dye 28 and the protein content was measured colorimetrically using the DC protein assay system (Bio-Rad, Hercules, CA, USA). The lactate dehydrogenase (LDH) activity from the culture medium was measured using LDH-L reagent (Bayer, Tarrytown, NY, USA). Six constructs per group were tested.

2.7. Histological analysis

For histological evaluation, the constructs were fixed in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, embedded in paraffin, bisected in cross-section through the centre and sectioned to 5 µm thickness. The sections were stained with haematoxylin and eosin (H&E) for general evaluation. Three constructs per group were used for histological analysis.

2.7.1. Apoptosis assay—Tissue constructs cultured under the various electrical stimulations might induce cell necrosis and apoptosis. The tissue apoptosis was examined using an apoptosis kit (R&D, Minneapolis, MN, USA), following the manufacturer's instructions. Deparaffinized sections were permeabilized in protease K for 30 min and then labelled with a labelling reaction mixture for 1 h in a 37 °C humidified chamber. After washing, streptavidin–horseradish peroxidase (HRP) solution was added to the slide, which was incubated for 10 min at room temperature, and then TACS blue labelling solution was applied to the slide and incubated for 10 min. After counterstaining with fast red, the sections were mounted with cytoaseal mounting medium (Tissue-Tek®, MA, USA).

2.7.2. Immunostaining—To assess the morphology and the distribution of myotubes, the sections were stained with monoclonal anti-collagen type I (Neomarker, Canada) and monoclonal anti-desmin (Chemicon International Inc., CA, USA). For immunohistochemical staining, the sections were deparaffinized and the antigen was retrieved by heat treatment for 20 min at 95 °C in a decloaking chamber (Biocare Medical, CA, USA). Subsequently, the sections were blocked with 10% horse serum for 30 min at room temperature (RT) and then incubated for 1 h at 37 °C with collagen type I (1: 150) and desmin (1: 150) antibodies diluted in PBS containing 0.5% Tween 20 and 1.5% horse serum. For anti-collagen type I staining, the sections were further incubated for 30 min at RT with secondary antibodies (anti-mouse IgG, 1: 200; Standard Elite ABC kit, Vector Laboratories), and then with an avidin–biotin complex agent for 30 min (RT) and 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) for 15 min at RT. For anti-desmin, the sections were further incubated with secondary antibodies (FITC-conjugated-anti-rabbit IgG, Texas red-conjugated-anti-mouse IgG, 1: 200; Vector Laboratories) for 30 min at RT. A humidified chamber was used for all incubation steps. Construct architecture and cell distribution were assessed from stained tissue sections using a fluorescent microscope (Axioplan, Zeiss, Germany) and Open Lab software.

2.8. Contractile activity

The contractile function of the engineered constructs was evaluated by monitoring the occurrence frequency of contractile activity upon electrical stimulation. Each construct was placed between two carbon electrodes connected to a cardiac stimulator (Radisic *et al.*, 2004; Nikon Kohden, Japan), in a 60 mm Petri dish filled with 10 ml culture medium. The temperature of the Petri dish was maintained at 37 °C using a heating tape fixed to the bottom of the Petri dish and connected to a temperature controller. The entire set-up was placed on an optical microscope and contractile responses to electrical stimuli (rectangular pulses, 2 ms duration) were monitored using $\times 10$ magnification. The signal amplitude was increased in 0.1 V increments to 10 V and the stimulation frequency was increased up to 4 Hz. We looked for the presence of random and spontaneous contractions (an indicator of immature tissue) vs. synchronous contractions in response to electrical pacing (an indicator of more mature tissue, consisting of electromechanically coupled cells). Two parameters were measured to evaluate the contractile behaviour in response to electrical stimulation: excitation threshold (ET, the minimum voltage of electrical stimulation required to elicit sustained synchronous contractions of tissue constructs at 1 Hz) and maximum capture rate (MCR, the maximum frequency of sustained synchronous contractions that could be achieved at a stimulation voltage corresponding to 1.5 ET). Six constructs per group were used for the measurements.

2.9. Transmission electron microscopy (TEM)

The tissue was fixed in 2.5% glutaraldehyde and 3% paraformaldehyde with 5% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4. The tissue was then post-fixed in 1% OsO₄ in veronal acetate buffer. The tissue was stained *en bloc* overnight with 0.5% uranyl acetate in veronal acetate buffer, pH 6.0, then dehydrated and embedded in Spurr's resin. Sections were cut on a microtome (Reichert Ultracut E) with a diamond knife (Diatome) at a thickness setting of 50

nm, and stained with 2% uranyl acetate followed by 0.1% lead citrate. Samples were examined using an EM410 TEM instrument (Philips, Eindhoven, The Netherlands) at 80 kV. One construct per group was used for the TEM analysis.

2.10. Statistical analysis

Statistical analysis was done using multivariate ANOVA with the Tukey HSD post hoc programme (Statistica, version 7).

3. Results

We investigated the effects of electrical stimulation on C2C12-based muscle constructs seeded in 3D collagen sponge scaffolds. Electrical field stimulation was provided by a custom-designed electrical circuit. The electrical circuit was designed to provide three different voltages (2, 5 or 7 V) at two different frequencies (1 or 2 Hz). The circuit was connected to a stimulation dish in which carbon rods and platinum wires were placed to generate the electrical field.

3.1. Cellularity and metabolic activity of the engineered tissue

Overall cell density of the engineered tissue was assessed by measuring total DNA and protein content (Table 1). It was found that there was no significant difference in total DNA and protein content in the stimulated and control groups, indicating similar cell concentrations in these groups (Table 1). The metabolic rates of lactate produced/glucose consumed and LDH activity in the stimulated group were similar to the controls (Table 2). These data indicate that electrical stimulation in the stimulation regime used here did not change metabolic rate of the C2C12 cell constructs. Although cell concentrations in both stimulated and control groups were similar, it is conceivable that electrical stimulation may induce cell damage and death. Therefore, the cell death of constructs was assessed by apoptosis staining (Figure 1). All of the stimulated tissues showed similar apoptotic responses throughout the scaffold and only a small apoptotic area was detected near the outer layer of the construct. However, the overall apoptotic area identified by the stain was comparable to control groups (Figure 1), indicating no significant differences in the rate of cell apoptosis in the stimulated constructs.

3.2. Histomorphology

The cells were more uniformly distributed in the stimulated groups (1 Hz/5 V, 1 Hz/7 V, 2 Hz/5 V, 2 Hz/7 V) compared to the controls (see Supplementary Figure 1, available in Wiley InterScience at: <http://www.interscience.wiley.com/jpages/1932-6254/suppmat/>). The presence of muscle cells in the stimulated groups was visualized by immunohistology of the intermediate filament protein desmin. Electrical field stimulation at 5 V with 1 Hz enhanced the presence of desmin protein in muscle cells. The control cells maintained a rounded cell morphology, which is an indication of undifferentiated phenotype (Figure 2).

Accumulation of the collagen matrix in the 3D C2C12 muscle constructs was assessed by Masson's trichrome stain, which can distinguish newly deposited collagen matrix (blue) from muscle cells (red). In the group stimulated at 2 Hz, cells were distinctively elongated and developed uniform cell layers containing muscle cells (Figure 3). In particular, cells stimulated at 5 V with 2 Hz for 5 days formed highly developed myotubes and showed less collagen deposition in the tissue (arrow indicates myotube); cells stimulated with 1 Hz accumulated more collagen matrix (appearing as blue stain) than 2 Hz-stimulated cells (Figure 3). Immunohistology using collagen type I antibodies confirmed high levels of collagen type I in controls and in the 1 Hz-stimulated groups (Figure 4). Only the outer layer of the 2 Hz-stimulated groups was stained positively with collagen type I (Figure 4).

TEM analysis showed well-developed ultrastructural features in 8 day-stimulated groups. Myofibrils in cells grown without stimulation did not form functional contractile sarcomeres (Figure 5). Instead, they contained disarrayed thin myofibrils distributed in the cytosol. However, cells in the stimulated constructs (5 V stimulation) contained well-developed sarcomeres, which correlated with the construct contractilities measured for these groups (Figure 5). The mitochondria were found in between sarcomere bundles (Figure 5).

Contractile properties were assessed by measuring ET and MCR in both control and stimulated groups (Figure 6). Stimulated constructs were capable of contracting synchronously under electrical field stimulation. In contrast, control constructs could not respond to pacing by synchronous contractions, thus the measurement of MCR and ET in this group was not possible. Within the stimulated culture groups, there was no significant difference in the MCR. However, the intensity of the field applied during culture significantly affected the ET of the constructs, resulting in the best performance for tissues cultivated at 5 V/cm with either 1 or 2 Hz (Figure 6).

4. Discussion

By varying frequency and voltage of electrical signals, we were able to affect the electrical excitability and contractile properties of 3D C2C12 constructs and modulate accumulation of the collagen type I matrix within 8 days of culture.

Continuous electrical stimulation can alter cellular metabolism positively or negatively, with a possibility of inducing the apoptotic response and reduction of cell viability in a frequency- and voltage-dependent manner (Thelen *et al.*, 1997). We tested the apoptotic activity in the stimulated samples. The apoptosis stain revealed that the tested electrical stimulation regimen did not increase apoptosis (Figure 1), a result consistent with those of Pedrotty *et al.* (2005). The presence of apoptotic cells in these constructs is consistent with oxygen's diffusional limitations observed under static culture (Radisic *et al.*, 2005). Therefore, C2C12 muscle constructs stimulated by a certain voltage (2–7 V) and frequency (1–2 Hz) for 8 days were used for analysis of collagen deposition and functional analysis.

Although the metabolic data did not show any dramatic changes, it is worth mentioning that in the stimulated group at 2 Hz, the LDH activity was slightly lower than in the 1 Hz-stimulated and control groups (Table 2). Electrical stimulation not only changes cellular architecture and gene expression but also influences mass transport of nutrients and gases in tissues, by inducing contractions (Connor *et al.*, 2001). Our data suggested that 2 Hz stimulation possibly increases oxygen delivery to the tissue indirectly, through the construct contractions of the tissue.

Remodelling of the cellular environment by physical stimulation has been extensively explored, and induction of cellular development by biomimetic physical stimulation is closely related to transcriptional signal transduction cascades (McDevitt *et al.*, 2001). We showed that 2 Hz stimulation led to reduction of collagen type I matrix deposition (Figures 2, 3) compared to the control and 1 Hz-stimulated groups. In rabbit, long-term stimulation with 10–20 Hz at 2 V for 6–12 weeks increased collagen type I and reduced collagen type III deposition (Trumble *et al.*, 2001). This discrepancy might be due to the different frequency and voltage which were applied to the experiment. The 2 Hz stimulation might have induced high expression of matrix metalloproteinase 1 (MMP1), which degrades collagen type I, resulting in reduced amounts of collagen type I. Another possibility is that the 2 Hz stimulation downregulated collagen type I expression. These possibilities are yet to be identified. Ultimately, it is desired for the muscle constructs to exhibit high cellularity, synchronous contractility and electromechanical coupling, rather than the increased deposition of ECM proteins that may be compared to the formation of scar tissue. We demonstrated here that electrical field stimulation at a specific

voltage (5 V) and frequency (2 Hz) improves excitability while reducing the presence of collagen type I.

When the C2C12 cells are confluent, the cells fuse, become multinucleated and develop functional myotubes, which contract spontaneously (Blau *et al.*, 1983; Kislinger *et al.*, 2005). Development of functional myotubes in 2D culture of C2C12 takes 2 weeks in differentiation medium, but our 3D culture system promoted myotube formation and cell differentiation within 8 days, identified by desmin staining and TEM. Desmin is an early myogenic marker and an abundant intermediate filament protein that plays a role in stabilizing sarcomeres in skeletal muscle and in slow twitch muscle (Balogh *et al.*, 2003, 2005; Capetanaki *et al.*, 1997). Elevated presence of desmin was found in 1 Hz-stimulated groups but to significantly lesser extent in control and 2 Hz-stimulated groups (Figures 2, 6). This might be indication of enhanced maturation of muscle cells.

Early development of the myotube could be due to the stimulation of electrical signal, our 3D culture system, or a conjunction of electrical stimulation and 3D system, which triggered cell fusion and myotube formation and enhanced signal transduction for muscle development. It was demonstrated previously that avian skeletal muscle embedded in 3D collagen gel also differentiated into highly contractile myotubes (Vandenburgh *et al.*, 1988). 3D cell culture promotes the formation of cellular junctions supported by integrin and Flk receptors (Milkiewicz *et al.*, 2003; Reinecke *et al.*, 2000). However, the molecular mechanism underlying the observed phenomena in 3D muscle tissue constructs in the presence of electrical field stimulation needs to be further investigated.

Electrical stimulation clearly improved myofibre formation and the functional properties of the muscle cells (Xia *et al.*, 2000). Our data also demonstrated that electrical stimulation promoted the development of the contractile apparatus in the C2C12 cells (Figure 5). Without electrical stimulation, the control constructs had formed poorly developed myofibrils, with sarcomeres scattered in the cytoplasm (Figure 5), resulting in non-functional muscle fibres without contraction (Figure 6). In contrast, in stimulated groups the muscle fibres were functional (Figure 6) and ultrastructural analysis identified well-developed sarcomeres arranged into myofibrils in the cytoplasm (Figure 5). These data clearly suggested that electrical pacing not only acted as a signalling cue for protein expression but also promoted the assembly of appropriate ultrastructural features. The exact mechanism of protein induction by electrical pacing and the translation mechanism of electrical signal into cellular signals need to be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Apoptosis analysis. Fixed sections were stained for apoptotic nuclei. Arrows indicate apoptotic cells in the tissue construct. In control, cells were cultured without electrical stimulation. Scale bar = 400 μ m

Figure 2.

Immunohistochemistry of desmin, which was highly expressed in the 1 Hz/5 V stimulated group. Arrows indicate desmin-positive stain. In control, cells were cultured without electrical stimulation. Red indicates desmin-positive cells and blue indicates nuclei. Scale bar = 100 μm

Figure 3.

Collagen stain by Masson's trichrome; blue represents collagen and pink represents muscle cells. Samples stimulated with 2 Hz showed less collagen than the control and 1 Hz-stimulated groups. Arrow indicates myotube. In controls, cells were cultured without electrical stimulation. Scale bar = 100 μ m

Figure 4.

Immunohistochemistry of collagen type I. Arrow indicates collagen scaffold and dotted arrow indicates collagen type I localization. Inserts show high magnifications. In control, cells were cultured without electrical stimulation. Scale bar = 100 μ m

Figure 5.

Ultrastructure of engineered muscle tissues. Muscle tissues were stimulated at either 2 or 5 V with 1 or 2 Hz for 5 days. Control was not stimulated. Arrows indicate sarcomeres. M, mitochondria

Figure 6.

Contractile properties of 3D engineered muscle tissue. (a) ET measurement; lower ET represents improved electrical excitability, which suggests better functional properties of the tissue. (b) MCR measurement. Higher MCR refers to the formation of functional contractile units in the tissue. Six samples from each group were measured. *Statistically significant difference

Table 1

Assessment of total DNA and proteins in the engineered tissues. There was no significant difference in cellularity of each group. Six samples from the each group were used for the measurement

	DNA ($\mu\text{g}/\text{construct}$)	Proteins ($\text{mg}/\text{construct}$)
Control	5.5 ± 0.14	0.35 ± 0.003
1Hz/2V	4.9 ± 0.08	0.28 ± 0.002
1Hz/5V	5.0 ± 0.08	0.33 ± 0.006
1Hz/7V	4.6 ± 0.49	0.31 ± 0.006
2Hz/2V	4.7 ± 0.38	0.32 ± 0.008
2Hz/5V	5.3 ± 0.39	0.33 ± 0.005
2Hz/7V	4.7 ± 0.4	0.32 ± 0.006

Table 2

Metabolic assessment of the engineered tissues. All the measurement was performed within 3 days after media collection. There was no significant difference in metabolic rate of each group. Six samples from the each group were used for the measurement

	Lactate produced/ Glucose consumed (mol/mol)	Lactate dehydrogenase (10^{-3} U/hr/construct)	Glucose consumption (μ mol/hr/construct)
Control	1.94 $\pm$ 0.56	4.78 $\pm$ 1.74	0.63 $\pm$ 0.22
1Hz/2V	1.97 $\pm$ 0.55	4.6 $\pm$ 1.72	0.60 $\pm$ 0.23
1Hz/5V	1.87 $\pm$ 0.53	5.23 $\pm$ 0.62	0.65 $\pm$ 0.22
1Hz/7V	1.81 $\pm$ 0.48	5.14 $\pm$ 1.53	0.70 $\pm$ 0.29
2Hz/2V	1.78 $\pm$ 0.35	5.08 $\pm$ 1.89	0.68 $\pm$ 0.33
2Hz/5V	1.83 $\pm$ 0.46	4.76 $\pm$ 1.65	0.71 $\pm$ 0.32
2Hz/7V	1.84 $\pm$ 0.53	4.15 $\pm$ 1.71	0.69 $\pm$ 0.29