Electrical pulse stimulation of primary human skeletal muscle cells

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Abstract

Electrical pulse stimulation (EPS) is an *in vitro* method of inducing contractions in cultured skeletal muscle cells of human and animal origin. Motor neuron activation of muscle fibres can be replaced by applying EPS on differentiated skeletal muscle cells (myotubes) in culture [1,2].

Here we describe two protocols for EPS of human myotubes in 6-well plates: acute, high-frequency (single bipolar pulses of 2 ms, 100 Hz for 200 ms every 5th sec for 5-60 min, 10-30

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V) and chronic, low-frequency (single bipolar pulses of 2 ms, 1 Hz 10-30 V for 48 h) at the end of a 7 days long differentiation.

Key words

Skeletal muscle, myotubes, contractions, electrical pulse stimulations

Running head

Model of *in vitro* contractions

1. Introduction

Skeletal muscle is remarkable in its ability to adapt and remodel in response to contractile activity. Many of the contraction-induced changes in skeletal muscle fibers are well characterized *in vivo*, but molecular mechanisms underlying these adaptations are still not completely understood. Cell culture systems derived from human muscle biopsies have been used in research for decades and represent a valuable alternative to adult skeletal muscle with respects to genetic background, morphological, metabolic and biochemical properties [3]. However, differentiated human skeletal muscle cells (myotubes) are quiescent in cultures, and do not typically contract spontaneously, unless being innervated or electrically stimulated [4,5]. Motor neuron activation can be replaced by electrical pulse stimulation (EPS) in culture [1,2], and this model is widely applied as an *in vitro* model of contractions [6-12].

Both commercially available C-Pace culture pacing systems and home-designed pulse generators have been successfully used on different skeletal muscle cell preparations. Several papers have been published employing C2C12 cell lines, where chronic electrostimulation ("pacing") was used for both *de-novo* sarcomere formation and a hypertrophic response, rather similar to the signaling programs seen in intact muscle upon exercise in skeletal muscle [2,8,13].

Development and application of EPS in human myotubes has mainly been directed towards metabolic and inflammatory responses related to obesity and type 2 diabetes (T2D). By exposing human myotubes to EPS, others and we have been able to induce metabolic adaptations in human myotubes similar to some of the *in vivo* responses to physical exercise. We have shown that acute, high-frequency EPS has similar effects on glucose uptake in human myotubes in culture as *in vivo* muscle contractions, with a 30% increase in glucose uptake, resembling an acute high intensity bout of exercise [14,6]. However, in a clinical

setting, beneficial effects of exercise are more profound after regular physical activity, which may be simulated by exposing human myotubes to chronic, low-frequency EPS. Several research groups using human myotubes from different donors have used this type of EPS, with minor variations. Among the observed effects of chronic, low-frequency EPS of human myotubes are reorganization of the cytoskeleton, *de novo* formation of sarcomeric structures, visible contractions and profound metabolic adaptations reflected in improved oxidative capacity and increased insulin sensitivity [6,9,7]. Importantly, this model of EPS has been an important *in vitro* tool to identify novel contraction-induced myokines [10].

In this chapter, we describe two EPS approaches to stimulate human myotubes: chronic, low-frequency EPS, and acute, high-frequency EPS.

2. Materials

1. Cells: EPS is performed on adherent, differentiated myotubes. We use human myotubes obtained from human satellite cells.
2. Plating medium: Dulbecco` s modified Eagle`s medium (DMEM)-Glutamax™ (5.5 mM glucose) supplemented with 2 % heat-inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 1.25 µg/ml amphotericin B.
3. Proliferation medium: DMEM- Glutamax™ (5.5 mM glucose) supplemented with 2 % heat-inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1.25 µg/ml amphotericin B, 50 mg/ml gentamicin and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
4. Differentiation medium: DMEM- Glutamax™ (5.5 mM glucose) supplemented with 2 % heat-inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1.25 µg/ml amphotericin B, 50 mg/ml gentamicin, 25 mM HEPES and 25 pM insulin.
5. Corning CellBIND six-well plates (Corning Life-Sciences, Schiphol-Rijk, The Netherlands).
6. BD Matrigel™ Basement Membrane Matrix (Bedford, MA, U.S.)
7. Pulse generators and C-Dish electrodes: We use either commercially available IonOptix C-Pace pulse generator (IonOptix, Dublin, Irland), or a self-designed pulse generator. In either case, for chronic EPS, we use commercially available C-Dishes (IonOptix, Dublin, Ireland) arranged to precisely place carbon electrode elements to standard 6-well culturing plates (Figure 1). Self-designed rod carbon electrodes (4 mm diameter), fixed to lid of a culture plate are used for acute, high-frequency stimulation (*see* **Note 1**) (Figure 2).

3. Methods

All work involving cells and C-Dish electrodes is carried out in a cell culture safety hood using aseptic working technique. Cells are cultured in a humidified 5% CO_2 atmosphere at 37°C.

3.1 Establishment of differentiated human skeletal muscle cells (myotubes)

3.1.1 A bank of myoblasts established from human muscle biopsy samples, obtained with informed consent and according to regulations of national ethic committees, as previously described [15] is stored in liquid nitrogen.

3.1.2 Human myoblasts are taken out from liquid nitrogen, thawed and seeded at a density of 100 000 cells/well in a Corning CellBIND (*see* **Note 2**) 6-well plate, using 1-2 ml/well (*see* **Note 3**) of plating medium. After 24 h, plating medium is replaced by equal volume of proliferation medium, which is then changed every 2-3 days. At approximately 80% confluence (usually after 1-2 weeks), medium is changed to differentiation medium, which induces differentiation of myoblasts into multinucleated myotubes. Differentiation medium is changed every 2-3 days, and cells are differentiated for 7 days.

3.1.3 Myotubes should be attached to the surface of the culturing plate and fully differentiated (at least 4 days of differentiation) prior to EPS. Inspect the cells frequently under microscope, both during culturing, before the onset of, and during EPS (*see* **Note 4**).

3.2 Electrical pulse stimulation (EPS)

1. Chronic, low-frequency EPS should be applied on day 5 of the differentiation period. Onset of acute, high-frequency EPS is on day 7 of the differentiation.
2. For chronic, low-frequency EPS, use C-Dishes from Ionoptix. For acute, high-frequency EPS, use rod carbon electrodes.

3. Sterilize the C-Dishes and rod carbon electrodes immediately before use by spraying with 70% ethanol, and allow them to air dry completely.
4. Fresh, warm (37°C) differentiation medium should be added to the cells before EPS is started.
5. Fit the C-Dish on top of the culturing plate so that C-Dish electrodes descend into the media. The cell culture plate lid will fit the groove on top of the C-Dish. Rod carbon electrodes are already fixed to the lid of a culturing plate.
6. Transfer the assemblies of culturing plates, C-Dishes/rod carbon electrode lids into the incubator.
7. Connect the assemblies to the pulse generator with the appropriate cables, run the cables through the door opening of incubator, and apply the stimulation protocol as anticipated. For chronic, low-frequency stimulation, we apply single, bipolar pulses of 2 ms, 30 V and 1 Hz continuously for the last 24 or 48 h of a 7 days differentiation period (*see* **Note 5**). For acute, high-frequency stimulation, we generally apply single bipolar pulses of 2 ms, 100 Hz for 200 ms every 5th sec for 5-60 min, 10-30 V, on day 7 of the differentiation.
8. Change culturing medium once per 24 h during chronic, low-frequency EPS. This is carried out by carefully removing the C-Dish from the culturing plate. Fresh medium is then added to the cells. During medium change, C-Dish electrodes are again sterilized by spraying with 70% ethanol, allowed to dry in the safety hood, before being placed back on the top of cell culturing plates.
9. After ended stimulation, cells are harvested and C-Dishes and rod carbon electrode lids are cleaned and sterilized as described in the following section.

3.3 Cleaning and sterilization of C-Dishes and rod carbon electrode lids

Electrodes must be thoroughly cleaned and sterilized after each use, following next procedure (*see* **Note 6**):

3.3.1 Cleaning

1. After ended application, soak the entire C-Dish or rod carbon electrode lid in distilled water for a couple of days with daily changing the water. The water should be changed until the distilled water stops turning pink. Additionally, measurement of the water's pH may indicate whether ions or other chemicals still leach from the electrodes.
2. Use a soft toothbrush to scrub the surface of the electrodes under rinsing distilled water to avoid accumulation of absorbed salts (*see* **Note 7**).
3. After the cleaning procedure, electrodes must to be sterilized.

3.3.2 Sterilization

1. Autoclave should not be used for this purpose (*see* **Note 8**). Instead, sterilization is performed using either of the following procedures:
   a) Disinfection with 70% ethanol, followed by placing the C-Dish or rod carbone electrode lid under UV germicidal lamp in the cell culture hood for 15 minutes,
   b) Heating the C-Dish or rod carbon electrode lid in a drying oven at 100°C for 3 h.

2. Cleaned and sterilized C-dishes and rod carbon electrode lids are placed on top of sterile 6-well culturing plates, with the culture plate lid fitted on top, rapped in aluminum foil and stored at room temperature (*see* **Note 9**).

4. Notes

1) C-Dishes from Ionoptix are, according to producer, specifically designed to enable chronic electrostimulation ("pacing") of cultured cells. We have tested C-

Dishes in combination with acute, high-frequency EPS as described in current protocol, which resulted in overheating, detachment from culturing surface and cell death. Thus, as implied by the producer, C-Dishes can not be used for this type of EPS in our cell culture system. Rod carbon electrodes we use for the purpose of acute, high-frequency EPS, on the other hand, have smaller diameter, and can be used for high-frequency stimulation without damaging the myotubes.

2) The cells should be attached to the surface of cell culturing plate. We generally use Corning CellBIND plates for EPS. However, in our hands, human myotubes originating from different donors may respond differently to EPS, and proper coating should be used to avoid cell detachment. For cells that detach under EPS when cultured on Corning CellBIND plates, we have successfully performed 48 h of EPS by coating the plates with BD Matrigel™ Basement Membrane Matrix. For that purpose, BD Matrigel™ Basement Membrane Matrix solution is thawed in the fridge for 2 hours, and diluted 1:50 in DMEM. Cell culture plates are coated for 2 h at room temperature, and BD Matrigel™ Basement Membrane Matrix is removed by aspiration.
3) Electrodes must be immersed in liquid during EPS, and volume of medium is adjusted accordingly. Volume of 2 ml is sufficient when Corning CellBIND six-well plates and the compatible 6-well C-Dishes are used. For acute, high-frequency EPS, performed using carbon rod electrodes, cells are cultured in 1 ml/well medium. Thus, the volume required will vary depending of the length of electrodes and types of plates. Always check that the electrodes are immersed in media, and adjust volume if necessary.
4) Visualizing contractions in primary human myotubes during EPS may be challenging. Not all cells contract at all times. Avoid vigorous movements when

taking the cells out of the incubator under stimulation, as both movements and temperature variations may affect contractions. For optimal visualization of cells during EPS, a microscope within temperature-controlled chamber may be used. Take time when looking for contractions under microscope.

5) Preferably, voltage should not be higher than 30 V for the described protocol designed around a 6-well assembly. Higher voltage increases the risk of over-heating. For chronic, low-frequency EPS, we have been using voltage from 8 to 30 V, and for acute high-frequency stimulation we have used 10-30 V.

6) Carbon electrodes absorb salts, electrolysis by-products and proteins from the media. Toxic chemicals may leach from the electrodes into the media and could induce unspecific effects or even kill the cells. Although it is time-consuming, performing the entire cleaning and sterilization procedure is an essential part of a successful EPS experiment.

7) Toothbrush will often be black once the rubbing of electrodes is finished, but carbon is a solid material, and this should not be a matter of concern.

8) While Ionoptix strongly discourages using autoclave for sterilization of C-Dishes, it is specified in their manual that if the dishes must be completely sterilized, autoclaving cycles should be limited to 20 min at 121°C. Temperature should not exceed 145°C, as it may damage the C-Dishes. Following the described cleaning and sterilization procedure in our protocol, however, we have not experienced contamination, and we do not recommend using autoclave for sterilization.

9) Due to substantial cleaning routines and the fact that carbon is a porous material, it is highly recommended to have multiple C-Dishes available and rod carbon electrodes available. This allows the researcher to switch between the electrodes

in different experiments, while performing proper maintenance (cleaning and sterilization) after each use.

References

1. Thelen MH, Simonides WS, van Hardeveld C (1997) Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca2+-ATPase gene. The Biochemical journal 321 (Pt 3):845-848

2. Fujita H, Nedachi T, Kanzaki M (2007) Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes. Exp Cell Res 313 (9):1853-1865. doi:S0014-4827(07)00106-1 [pii] 10.1016/j.yexcr.2007.03.002

3. Aas V, Bakke SS, Feng YZ, Kase ET, Jensen J, Bajpeyi S, Thoresen GH, Rustan AC (2013) Are cultured human myotubes far from home? Cell and tissue research. doi:10.1007/s00441-013-1655-1

4. Nikolic N, Gorgens SW, Thoresen GH, Aas V, Eckel J, Eckardt K (2017) Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise - possibilities and limitations. Acta physiologica 220 (3):310-331. doi:10.1111/apha.12830

5. Askanas V, Kwan H, Alvarez RB, Engel WK, Kobayashi T, Martinuzzi A, Hawkins EF (1987) De novo neuromuscular junction formation on human muscle fibres cultured in monolayer and innervated by foetal rat spinal cord: ultrastructural and ultrastructural--cytochemical studies. J Neurocytol 16 (4):523-537

6. Nikolic N, Bakke SS, Kase ET, Rudberg I, Flo Halle I, Rustan AC, Thoresen GH, Aas V (2012) Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. PLoS One 7 (3):e33203. doi:10.1371/journal.pone.0033203

7. Feng YZ, Nikolic N, Bakke SS, Kase ET, Guderud K, Hjelmesaeth J, Aas V, Rustan AC, Thoresen GH (2015) Myotubes from lean and severely obese subjects with and without type 2 diabetes respond differently to an in vitro model of exercise. Am J Physiol Cell Physiol 308 (7):C548-556. doi:10.1152/ajpcell.00314.2014

8. Nedachi T, Fujita H, Kanzaki M (2008) Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. Am J Physiol Endocrinol Metab 295 (5):E1191-1204. doi:90280.2008 [pii] 10.1152/ajpendo.90280.2008

9. Lambernd S, Taube A, Schober A, Platzbecker B, Gorgens SW, Schlich R, Jeruschke K, Weiss J, Eckardt K, Eckel J (2012) Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. Diabetologia 55 (4):1128-1139. doi:10.1007/s00125-012-2454-z

10. Raschke S, Eckardt K, Bjorklund Holven K, Jensen J, Eckel J (2013) Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. PLoS One 8 (4):e62008. doi:10.1371/journal.pone.0062008

11. Danilov K, Sidorenko S, Milovanova K, Klimanova E, Kapilevich LV, Orlov SN (2017) Electrical pulse stimulation decreases electrochemical Na+ and K+ gradients in C2C12 myotubes. Biochem Biophys Res Commun 493 (2):875-878. doi:10.1016/j.bbrc.2017.09.133

12. Tarum J, Folkesson M, Atherton PJ, Kadi F (2017) Electrical pulse stimulation: an in vitro exercise model for the induction of human skeletal muscle cell hypertrophy. A proof-of-concept study. Exp Physiol. doi:10.1113/EP086581

13. Nedachi T, Hatakeyama H, Kono T, Sato M, Kanzaki M (2009) Characterization of contraction-inducible CXC chemokines and their roles in C2C12 myocytes. American journal of physiology Endocrinology and metabolism 297 (4):E866-878. doi:10.1152/ajpendo.00104.2009

14. Aas V, Torbla S, Andersen MH, Jensen J, Rustan AC (2002) Electrical stimulation improves insulin responses in a human skeletal muscle cell model of hyperglycemia. Ann N Y Acad Sci 967:506-515

15. Henry RR, Abrams L, Nikoulina S, Ciaraldi TP (1995) Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects. Comparison using human skeletal muscle cell cultures. Diabetes 44 (8):936-946

Figure captions

Figure 1. C-Dish with carbon electrodes used for chronic, low-frequency EPS.

Figure captions

Figure 2. Rod carbon electrodes fixed to a culture plate lid, used for acute, high-frequency EPS.