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Research Article

Skeletal muscle perfusion and stem cell delivery in muscle disorders using intra-femoral artery canulation in mice

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ABSTRACT

Muscular dystrophies are among major inherited muscle disorders characterized by progressive muscle damage and fibrosis with no definitive cure. Recently, gene or cell based therapies have been developed to restore the missing gene expression or replace the damaged tissues. In order to test the efficiency of these therapies in mice models of muscular dystrophies, the arterial route of delivery is very advantageous as it provides uniform muscle exposure to the therapeutic agents or cells. Although there are few reports of arterial delivery of the therapeutic agents or cells in mice, there is no in-depth description and evaluation of its efficacy in perfusion of downstream muscles. This study is aimed to develop a practical method for intra-femoral artery perfusion in mice and to evaluate perfusion efficiency using near-infrared-fluorescence (NIRF) imaging as well as histology following stem cell delivery.

Our results provide a practical guide to perform this delicate method in mice. By using a sensitive fluorescent dye, different muscle groups of the hindlimb have been evaluated for proper perfusion. As the final step, we have validated the efficiency of arterial cell delivery into muscles using human iPS-derived myogenic cells in an immunodeficient mouse model for Duchenne muscular dystrophy (NSG-mdx^{4cv}).

1. Introduction

Human skeletal muscle is the largest organ in the body which is composed of post-mitotic multinucleated myofibers. The skeletal muscle has a phenomenal regeneration capacity which allows for fast muscle regeneration and growth following physiological conditions such as training or pathological situations such as injury. In a healthy human being, this tremendous regeneration and growth capability is due to adult muscle stem cells (mainly satellite cells) which start to proliferate and differentiate into mature fibers after any muscle damage [1,2]. However in pathological conditions such as muscle disorders and muscular dystrophies this regeneration ability is eventually disturbed which leads to muscle weakness and fibrosis [3].

Muscular dystrophies (MDs) represent a heterogeneous group of inherited muscle disorders. The main features of these disorders are progressive muscle weakness and fatigability. Many of these disorders are caused by the lack of important structural muscle proteins which lead to myofiber damage and inflammation [3]. Depending on the severity of the damage, muscle inflammation leads to repeated cycles of myofiber necrosis, regeneration and gradual replacement of muscle fibers by fat and fibrotic tissue. One of the most frequent and severe X-linked types of MDs is Duchenne muscular dystrophy (DMD) [4]. It is estimated that 1 in 3500 live born males have DMD mutations. DMD is very progressive and is usually represented by muscle weakness and gait abnormalities at the age of 3–4 years. Unfortunately, the rapid progression of this disease usually leads to lethal respiratory or cardiac failure of the patients in the second or third decade of life. DMD is caused by a defect in the gene encoding for an important membrane associated protein named dystrophin [5–7]. Dystrophin is required for membrane stabilization during muscle contractions. The absence of dystrophin in DMD patients leads to sarcolemmal fragility and eventual necrosis of the myofiber.

In the recent years, two major strategies have been developed for the treatment of MDs. These include replacing the missing gene (gene therapy) or the defective cells by healthy stem cells (cell therapy). In the case of gene therapy, viral delivery of the mini-dystrophin gene [8–10], up-regulation of the other compensating proteins [11–13] or mutation skipping [14–16] has been evolved. Alternative approaches using stem cell therapies have also been developed using myoblasts [17], satellite cells [18,19], mesoangioblasts [20–22] or pluripotent stem cells [23–25]. These cells are able to fuse with the existing myofibers and restore the

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expression of the missing protein. Specifically with the emergence of iPS technology [26,27] and site-specific gene targeting methods [28–32], there are tremendous efforts to develop and evaluate the therapeutic potential of genetically corrected patient-derived pluripotent cells in murine models of MDs such as mdx mice.

In either of these cases, in order to test the therapeutic potential of the gene vectors or stem cells in mice models of DMD, the appropriate route of delivery is crucial. Although many *in vivo* studies have used local intra-muscular (IM) injection of the vectors or cells into the mice muscle, there are major drawbacks using IM route. Low cell survival and diffusion, leakage from injection site, poor engraftment and more importantly, the lack of feasibility in human patients due to generalized muscle involvement of the DMD or other types of MDs are among the major limiting factors for IM delivery [33,34]. Therefore, there has been more attention into systemic ways of delivery such as intra-venous (IV) or intra-arterial (IA) routes. Although the IV route using tail vein injection in mice is a relatively easy and feasible approach, due to sequestration effects of lung capillaries, spleen and other organs, many injected materials and cells will be filtered out by first-pass of circulation through these organs before their arrival at target organs [35,36]. Therefore, the therapeutic agent/ cell availability would be minimal at the target tissue i.e. skeletal muscle.

These short comings have shifted the attention toward IA delivery which provides all advantages of systemic delivery without the drawbacks of IV route. Although few groups including us have tried IA cell delivery in the mice hindlimbs with different success rates, there is no consensus on the methodology and the anatomical site of cell delivery [22,25, 37–41]. Additionally, due to the presence of several small arterial branches and collateral routes in the hindlimb, the location of canula/needle insertion might create huge variations in muscle compartment perfusion outcome [42]. Besides, it is not clear which muscle compartments are properly perfused using this approach. This lack of clarity, makes it difficult to evaluate the therapeutic potential of any agent via IA route.

Therefore, in this research work, we have developed a step by step practical guide to describe the methodology for proper perfusion of mice hindlimb using intra-femoral artery canulation. Furthermore, by using a water soluble dye and a sensitive near-infrared-fluorescence (NIRF) imaging, we have compared two nearby potential anatomical sites for canulation in mouse femoral artery (proximal or distal to superficial caudal epigastric artery – SCEA) to evaluate their efficiencies for muscle perfusion. By comparing all muscle groups in the thigh and shin region, we have demonstrated IA method′s efficiency for muscle perfusion. Our data has demonstrated that small deviation from proper canulation site is enough to create a huge difference in hindlimb muscle perfusion.

Furthermore, we have also evaluated this method′s efficiency for cell perfusion into hindlimb muscle vasculature. For this, we have used human iPS-derived myogenic progenitors as a potential cell therapy in an immunodeficient mouse model for DMD (NSG-mdx^{4cv} mouse). Presented data demonstrate IA ability for cell delivery into skeletal muscles. We believe this study provides a practical guide for proper and efficient perfusion of hindlimb muscles in the mice which can benefit researchers involved in the field of gene or cell therapy for skeletal muscle regeneration.

2. Materials and methods

2.1. Mice

Two to four month old NSG-mdx^{4cv} mice [43] were used for these experiments. Mice were housed at the barrier facility at the Brown Institute of Molecular Medicine at the University of Texas at Houston. Mice were group-housed in ventilated cages, given acidified water and irradiated rodent diet (Purina, St. Louis, MO) ad libitum, and maintained on a 12:12-h light: dark cycle. All experimental studies were carried out in accordance and approved by the Institutional Animal Care and Use Committee guidelines of The University of Texas Health Science Center at Houston.

2.2. Intra-arterial canulation

Canulation of the femoral artery was performed under a dissection microscope (VWR, Radnor, PA). A 1 cm incision was made at the inguinal region on the right hindlimb parallel to the femoral vascular bundle. The superficial fascia layers were carefully dissected to visualize the femoral artery and vein. After visualization of the branches of the femoral artery, canulation of the femoral artery was performed proximal or distal to the SCEA. The inguinal fat tissue was carefully dissected from the neurovascular bundle using blunt dissecting forceps and hemostasis was achieved with brief pressure or cautery if needed. After careful isolation of femoral nerve from the bundle, the femoral artery was separated from the vein using fine dissecting forceps. A 6-0 silk suture (Ethicon, Blue Ash, OH) was used proximal to each canulation site for the temporary ligation of the artery during procedure. After making a partial thickness incision in the artery wall using micro iris scissors (Fine Science, Foster City, CA), the 32-gauge intrathecal catheter (Harvard Apparatus, Holliston, MA) was guided into the artery. After proper insertion of the guide wire and subsequently, the overlaid plastic catheter, the wire was drawn back gradually as the catheter was advanced forward. At this stage, the distal suture was placed over the canulated artery to secure the catheter inside the artery. Then the guide wire was completely removed and a 1 ml insulin syringe containing the perfusate or cell was attached to the catheter.

2.3. Hindlimb perfusion

For muscle perfusion visualization, a 0.2% trypan blue dye (Life technologies, NY, USA) was injected using a perfusion pump (Kent Scientific Corporation, Torrington, CT) at a rate of 50 μl/min. For near-infrared fluorescent imaging, indocyanine green (ICG-Patheon Italia, Milano, Italy) was reconstituted to a concentration of 0.5 mg/ml and infused. For cell perfusion we have used human iPS derived myogenic cells (5×10^5 cells/400 μl PBS) with the same flow rate. Cell preparation was done as previously described [24,44].

2.4. Imaging

Perfusion of the hindlimb was evaluated using customized near-infrared-fluorescence imaging (NIRF) as described before [45]. Imaging was performed by excitation from a laser diode operating at 785 nm (500 mW, Intense Ltd., North Bruswick, NJ). Emission light was collected after it passed through two interference filters (830.0-2.0, Image Quality, Andover Corp., Salem, NH). For the excitation and emission system, a convex lens and diffuser were used to create a uniform excitation field and filtered light was focused onto an electron-multiplying charge-coupled device (EMCCD) camera (Photon Max 512, Princeton Instruments, Trenton, NJ) with 200 ms integration time for static imaging, using a Nikon camera lens (AF Nikkor 28 mm f/2.8 D, Nikon Inc., Melville, NY). To acquire white-light images for registration purposes, a low-power lamp illuminated the animal. Image acquisition was accomplished by V++ software (Digital Optics, Aukland, New Zealand).

2.5. Immunohistochemistry

Dissected muscles were washed in PBS and flash frozen in Tissue-Tek OCT compound (Sakura, Japan). 8 μm Cryosections were then rehydrated and blocked with 0.3% Triton for 30 min followed by blocking buffer (3% BSA) for 1 h. The slides were incubated with primary