Plasmid Electrotransfer into Mouse *Soleus* Muscle

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Introduction. Plasmid transfer into the skeletal muscle cells *in vivo* is an important method in assessment of gene function. Unfortunately, gene delivery into the skeletal muscle *in vivo* is particularly hard and even the most elaborated methodologies fail to accomplish this goal [1]. One of the main approaches to achieve the delivery of a specific gene into the specified tissue cells is related with muscle electroporation. In this approach plasmid coding for functional gene or short interfering RNA (siRNA) is injected into target tissue and then the tissue is treated with electric field pulses [1 - 3]. In most of the cases plasmid has been electrotransfered into the mouse *tibialis anterior* (TA) muscle [2, 3]. Unfortunately, assessment of whole muscle function in TA is highly complicated, because of relatively large muscle mass, which may make muscle more susceptible to hypoxic central core development. In addition, TA has no tendon at proximal side and therefore cannot be attached to dynamometer with being left intact. In contrast, soleus muscle in standard laboratory adult mouse has a small mass (~9 mg) and tendons at both ends therefore rendering it well suited and thus widely used for assessment of contractile function in vitro [4]. In addition, TA muscle has limitations as a model because it is dominated by type 2B fibres [5] not present in human muscles. Mouse *soleus*, on the other hand, contains a proportion of type I and type II fibres [6, 7] that is comparable to human locomotive muscle [8]. To the best of our knowledge, there are only few studies that used plasmid electrotransfer into the mouse *soleus* and therefore there is lack of evidence of transfection efficiency [9]. The standard and arguably most efficient electroporation protocol to transfer plasmid into the muscle uses eight 175 - 200 V/cm strength electrical pulses 20 ms of pulse duration delivered at 1 Hz frequency [10]. The aim of this study was to evaluate the efficiency of standard electroporation-mediated plasmid delivery into the mouse *soleus* muscle.

Materials and methods. In this experiment we used ~12-week-old BALB/c male mice (n = 5). Animals were kept in 12/12-h light/dark cycle, 20 - 22 °C temperature and 55 ± 10% humidity in standard mouse cages (267 \ 207 \ 140 mm), 1 - 3 mice per cage. Animals received tap water and were fed with standard chow diet *ad libitum*. All experiments were approved by the Lithuanian Republic Alimentary and Veterinary Public Office (Nr. 0223).

Hind limbs were depilated 24 hours before the procedure. Before transfection, animals were anesthetized by intraperitoneal injection with anaesthetics: ketamine (120 mg/kg; Richter Pharma AG, Wels, Austria) and xylazine (14 mg/kg; Eurovet Animal Health B.V., Bladel, Netherlands). When animals showed no reaction to a foot pinch, incision in leg skin and fascia over the muscle was made and *gastrocnemius* muscle was retracted to expose *soleus* muscle. Thereafter, 20 μg of plasmid DNA coding green fluorescent protein (Snap25-GFP, a generous gift of Stefano Schiaffino) in 10 μl of 0.9% NaCl (final concentration 2 μg/μL) was injected using Hamilton 100 μl volume syringe with 34G needle. Skin incision was sewed up using polyamide threads (4–0 Ethilon; Ethicon, Norderstedt, Germany). Then conductive gel (Comed, Strasbourg, France) was applied over the leg skin to ensure good contact between electrodes and the target tissue during of the muscle electroporation. On the experimental leg, two stainless steel electrodes were placed over the skin on both sides (laterally and medially) of *gastrocnemius* (*soleus* anatomical position is deeper underneath lateral *gastrocnemius*). Then custom made electroporator generated eight square-wave pulses, with a length of 20 ms and a voltage of 200 V/cm at 1 Hz frequency. Contralateral leg was injected with the plasmid however did not receive electroporation and served as control.

7 days after the procedure animals were sacrificed by cervical dislocation. *Soleus* muscles where dissected and frozen in isopentane precooled in liquid nitrogen. Cryostat (Leica CM1520, Leica Bio systems, Nussloch, Germany) was used to make 10 μm cross sections in the middle of the *soleus* muscle at –21 °C. Cross sectional images were taken using green fluorescent microscopy. Transfected muscle fibres were counted using ImageJ software (NIH – version 1.43, Bethesda, Maryland, USA). All results are presented as mean ± SD.

Results. Total *soleus* muscle fibre count averaged 751.4 ± 54.0. Muscle fibers were considered transfected, if they were brighter than control muscle fibers. We transfected 160.4 ± 77.1 fibres which was 21.6 ± 10.4% of transfection efficiency. No transfection was observed in control muscle. Also, we did not observe any signs of muscle damage (necrotic fibers) after electroporation. Fig. 1 demonstrates an example of cross sections of control (A) and experimental (B) *soleus* muscles.

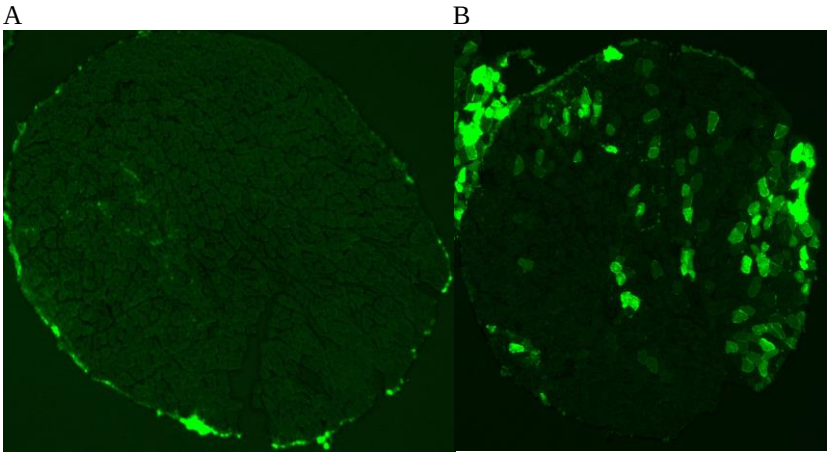

Fig. 1. Example of control (A) (Snap25-GFP injection without electroporation) and experimental (B) (Snap25-GFP injection plus electroporation using eight 20 ms square-wave pulses of 200 V/cm at 1 Hz) *soleus* muscles. Experimental muscle shown in the figure contains about 18% of transfected fibres.

Discussion and conclusions. Using standard TA muscle electroporation protocol for *soleus* muscle, we achieved ~ 22% of transfection efficiency. Nevertheless, to establish the functional effect of the gene of interest, higher transfection efficiency may be required. Higher voltage or increased length and/or number of the pulses may provide such benefits, but it also increases probability of damaging the target muscle and inducing skin burns. It was also demonstrated that additional injection of hyaluronidase TA transfection efficiency [10] suggesting that hyaluronidase may also increase transfection efficiency of the *soleus* muscle. In addition, another approach to increase transfection efficiency is to treat *soleus* muscle with low voltage pulses (50 V/cm) using spatula electrodes that are placed directly over the exposed muscle. This methodology has been reported to be extremely efficient (~80% transfected fibres) for the TA muscle in CD-1 line mice [3].

In summary, the standard protocol used for electrotransfection of TA muscle resulted in about 22% of transfected fibres of *soleus* muscle. In order to investigate functional effects of genes of interest in *soleus* muscle higher percentages of transfected fibres are needed and therefor further optimisation of methodology is required.

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Plasmid electrotransfer into mouse *soleus* muscle may help to assess gene effect on muscle function. The aim if this study was to evaluate transfection efficiency of superficial *tibialis anterior* muscle plasmid electrophoretic transfer protocol for deeper *soleus* muscle. Plasmid with green fluorescent protein was injected into *soleus* muscle and thereafter eight square-wave 20 ms and 200 V/cm electrical pulses at 1 Hz were used to induce the transfection. Transfection efficiency was ~22 % thus methodology adjustments are needed when higher level of transfection is required.