FAST AND NON-VIRAL METHOD TO DELIVER PLASMID DNA TO HUMAN MUSCLE-TENDON GRAFT USED IN ANTERIOR CRUCIATE LIGAMENT RECONSTRUCTION

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Abstract

Muscle tissues attached to the proximal ends of hamstring tendon grafts are routinely discarded during anterior cruciate ligament (ACL) reconstruction. Preserving the muscle on the ACL graft was shown to improve the surgery outcomes, possibly due to the osteogenic differentiation of muscle satellite cells, accelerating graft integration into the underlying bone. The enhancement of muscle cell osteogenic differentiation was previously demonstrated via adenoviral transfer of bone morphogenetic protein-2 (BMP-2). We evaluated gene electrotransfer as a fast, non-viral and clinically relevant alternative for the delivery of plasmid DNA to muscle-tendon ACL grafts. Human muscle-tendon tissues remaining were collected from 13 patients undergoing ACL reconstruction procedures, and standardized tissue samples were injected twice with 20 µg plasmid DNA or not treated. A combination of high voltage (600 V, 100 µs) and low voltage (80 V, 100 ms) electric pulses or medium voltage (MV; 200 V, 20 ms) pulses were first tested using plasmid DNA encoding the green fluorescent protein. The selection of MV protocol was confirmed with a luciferase plasmid and subsequently used to test a therapeutic BMP-2 plasmid. Upon detailed evaluation of individual tissue sample properties (*i.e.*, donors, thickness and volume) and their BMP-2 release, further optimization of tissue selection and preparation for gene electrotransfer was defined. This study indicates the feasibility of gene electrotransfer as a method to deliver plasmid DNA easily and rapidly to muscle tissue preserved on ACL grafts.

Keywords: Gene therapy, plasmid DNA, gene electrotransfer, anterior cruciate ligament.

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List of Abbreviations

ACL	anterior cruciate ligament
BMP-2	bone morphogenetic protein-2
BMPs	bone morphogenetic proteins
HV-LV	high voltage and low voltage electric pulses
MV	medium voltage electric pulses
GFP	green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
RLU	relative light unit

Introduction

Anterior cruciate ligament (ACL) reconstruction is a commonly used orthopaedic surgical technique for the treatment of ACL tears, aiming to replace damaged ligaments with graft tissue. The efficacy of the ACL reconstruction depends on graft choice, integration of the graft into bone and post-operative rehabilitation. ACL grafts are easy and safe to harvest, exhibit favourable mechanical properties and result in positive clinical outcomes. Although graft selection remains a topic of debate (Migliorini *et al.*, 2023; Thaunat, Fayard and Sonnery-Cottet, 2019), hamstring tendons have been in the past decades more commonly used ACL graft type than patellar tendon (Thaunat, Fayard and Sonnery-Cottet, 2019). Thereat, their biomechanical but also the biological properties of the graft must be considered. More efforts have recently been put into improving critical aspects of ACL reconstruction, including graft remodelling, maturation and integration into bone (Hexter *et al.*, 2018). Repair strategies mainly target enhancing the osteogenic properties of ACL grafts by delivery or recruitment of bone morphogenetic proteins (BMPs), known to promote bone formation (Bez *et al.*, 2018; Han *et al.*, 2019). Towards this objective, most studies focused on the grafted tendon tissue, while the regenerative potential of muscle tissue routinely separated from the end of the tendons has largely been neglected. However, skeletal muscle tissue is known to contain satellite cells that can differentiate into osteocytes (Asakura, Komaki and Rudnicki, 2001; Musgrave *et al.*, 2001; Yin, Price and Rudnicki, 2013). Osteogenic progenitor cells were also demonstrated to reside in *gracilis* and *semitendinosus* muscles harvested from the ends of human ACL hamstring tendon grafts (Levy *et al.*, 2001). The adult stem cells obtained from these human muscle tissues showed higher expression of early bone differentiation markers than cells derived from tendons, suggesting their capacity to enhance ossification and healing (Cuti *et al.*, 2017). The same type of cells was previously successfully transduced with an adenoviral construct harbouring bone morphogenetic protein-2 (BMP-2) (Rod *et al.*, 2019), resulting in increased expression of osteogenic markers. Furthermore, implanting ACL grafts with muscle remnants in combination with recombinant BMP-2 protein delivery promoted bone formation on the implant site in rabbits (Germann *et al.*, 2018). Likewise, keeping the muscle tissue on the ACL graft improved intra-articular healing and remodelling of the graft in another ACL repair study in rabbits (Sun *et al.*, 2013) and the functional outcomes in a recent clinical study (Funchal *et al.*, 2021).

Although viral approaches have commonly been tested and used in clinics (Bezeljak, 2022; Chen *et al.*, 2016; Kawakami *et al.*, 2017; Zhao, Anselmo and Mitragotri, 2022) and the adenoviral transduction protocol was successfully shortened to 30 min for the delivery of BMP-2 into the muscle of ACL graft (Rod *et al.*, 2019), they come along with concerns of being unsafe for patients due to the inflammatory immunological response, toxicity and exogenous insertion into the genome (Chuah, Collen and VandenDriessche, 2003). As an alternative approach, non-viral gene delivery of plasmids exhibits lower risks, due to the lack of viral protein components and unlikely insertions into the host genome (Hacobian and Hercher, 2018).

Plasmid vectors are also not limited by the coding sequence and their production is safe and easy. Furthermore, the poor transfection efficiency of non-viral vectors (Hacobian and Hercher, 2018; Ramamoorth and Narvekar, 2015) can be compensated by codon optimization and intron insertion in a native gene sequence. For example, transfecting the cells with a plasmid harbouring a modified BMP-2 promoted the expression and osteogenic differentiation compared to conventional BMP-2 plasmid (Hacobian *et al.*, 2016). Delivery of this modified BMP-2 in rat calvarial defects has been shown to accelerate the formation of bone tissue (Raftery *et al.*, 2018). Transfection approaches used for the BMP-2 plasmid delivery in the above-described studies were lipofection and nanoparticles, respectively. Although these are non-viral approaches, they do not hold promise from a clinical perspective as they require long incubation periods that would not be possible during ACL reconstruction. In contrast, gene electrotransfer of plasmid DNA could be a more suitable method, as it allows an on-site tissue treatment alongside graft harvesting and it is easy, fast, low costs and does not require additional therapeutic components (Sokolowska and Blachnio-Zabielska, 2019). Gene electrotransfer is based on electroporation, a physical method where the application of electric pulses transiently increases the permeability of the cell membrane, enabling the entry of molecules such as drugs or genetic material into cells. It is suitable for both *in vitro* and *in vivo* applications (Cemazar *et al.*, 2006; Groselj *et al.*, 2022; Markelc *et al.*, 2015; Potocnik *et al.*, 2022) and has been used in clinical practice to treat tumours and metastasis (Gehl *et al.*, 2018). The efficacy of gene electrotransfer varies between different tissues, thus the optimal electric pulses must be determined for each tissue type. Specifically, the amplitude and duration of electric pulses, number of pulses, pulse repetition frequency and electrode geometry must be considered depending on the experimental setup (Andre *et al.*, 2008; Forjanic *et al.*, 2019; Luft and Ketteler, 2015; Peri *et al.*, 2020; Potocnik *et al.*, 2022). In previous studies, the efficacy of gene electrotransfer to murine *musculus tibialis cranialis* was extensively researched to finally show that the combination of high (600 V/cm) and low (80 V/cm) voltage electric pulses results in a larger

transfected muscle area than low or medium (100 and 200 V/cm) voltage electric pulses alone (Andre *et al.*, 2008; Tevz *et al.*, 2008). Furthermore, a combination of 1 high voltage and 4 low voltage pulses was more efficient than the combination of 1 high and 1 or 8 low voltage electric pulses (Tevz *et al.*, 2008). However, parameters for gene electrotransfer to human muscle tissue preserved on the ACL tendon grafts have not yet been established.

The objective of our study was to evaluate the gene electrotransfer as an alternative to the viral approach for the delivery of plasmid DNA to human muscle tissue preserved on the ACL tendon graft. We conducted *ex vivo* experiments on human muscle-tendon grafts freshly harvested from 13 patients undergoing ACL reconstruction procedures. Using green fluorescent protein (GFP) and luciferase reporter plasmids, we found that human muscle tissue retained on the grafts can be efficiently transfected by two times injection of 20 µg of plasmid DNA and application of electric pulses with a parallel orientation of electrodes and 8 medium voltage pulses (200 V/cm, 20 ms, 1 Hz). Based on the BMP-2 detection in the culture medium after gene electrotransfer, tissue selection parameters were determined to further improve the efficacy of the method.

Materials and Methods

Harvesting and preparation of human muscle-tendon tissue

The experiments were conducted on *gracilis* and *semitendinosus* muscle-tendon tissues harvested from patients undergoing arthroscopic ACL surgery at the University Hospital Sveti Duh in Zagreb, Croatia. Tissues were harvested without fascia from the ends of the ACL grafts that are usually removed during the surgery and were as such considered biological waste. Tissue collection was conducted with the approval of the University Hospital Sveti Duh ethics committee (approval number 01-1095/2). Tissues from a total of 13 patients were used in the study, including 9 males and 1 female (mean age 28 ± 9 years) and 3 patients where data on age and gender were not available. The viable tissues were harvested and placed in 20 mL of high glucose Dulbecco's Modified Eagle medium (Thermo Fischer Scientific, Waltham, MA, USA) supplemented

with 10 % foetal bovine serum (FBS; Thermo Fischer Scientific) and 1 % penicillin and streptomycin (Pen/Strep; Thermo Fischer Scientific) and transported on ice to the laboratory. The harvested tissues were cut to a total of 34 samples of approximately 10 × 10 × 4 mm in size (Fig. 1**A**). The amount of harvested tissue varied between the patients; therefore, a different number of tissue samples were used from individual patients in experiments with different plasmids (Table 1).

Table 1. Overview of the gene electrotransfer studies performed on human muscle-tendon ACL grafts. The use of plasmids, sample distribution and settings for gene electrotransfer protocol are shown. L, length; W, width; T, thickness; HV-LV, high voltage and low voltage electric pulses; MV, medium voltage electric pulses; NA, not analysed.

Plasmid	Plasmid injection	No of patients	No of muscle-tendon tissue samples	Average tissue size (L mm × W mm × T mm)	Electric pulses
pMaxGFP	2 × 20 μg	4	8 (6 transfected)	12.6 × 9.7 × 3.6	HV-LV MV
pLuc	2 × 20 μg	3	4 (2 transfected)	NA	MV
pBMP-2-advanced	2 × 20 μg	7	23 (13 transfected)	11.1 × 9.6 × 4	MV

Gene electrotransfer delivery of plasmid DNA
An overview of the gene electrotransfer protocols used for muscle-tendon tissue is shown in Table 1. Selection of the optimal gene electrotransfer parameters was determined with pMaxGFP plasmid encoding the green fluorescent protein (GFP; Amaxa™, Lonza, Bern, Switzerland) and confirmed with luciferase plasmid (pLuc) reporter plasmid encoding Metridia luciferase. The gene electrotransfer delivery of a therapeutic plasmid was performed using the BMP-2-advanced plasmid for enhanced expression of the BMP-2 protein. The pLuc and pBMP-2-advanced plasmids were made in-house by cloning the *MetLuc* and *BMP-2* genes, respectively, into the pBMP-2-advanced backbone under the control of the elongation factor 1 alpha promoter (Hacobian *et al.*, 2016). The cloning was performed via restriction enzyme digest and verified by plasmid sequencing.

Muscle-tendon tissue samples were prepared as described above to allow two-site injection of the plasmid and prevent plasmid leakage from the muscle (Fig. 1**A**). Using a 29 G insulin syringe (Chirana, Stara Tura, Slovakia), 2 × 20 μg of plasmid DNA prepared in 20 μL of saline were injected into the muscle (Fig. 1**B**) on two opposite sides of the tissue sample. The injection was limited to muscle tissue (*i.e.*, between 2 and 5 mm) and targeted only the superficial and central layers of the tissue, avoiding needle contact with the tendon. Immediately after plasmid injection, the tissue was placed between the stainless-steel plate electrodes of a 6 mm distance to ensure maximum contact between the electrodes and the tissue, supported by the presence of medium remains surrounding the tissue (Fig. 1**C**). The electric pulses were applied to the tissue with a Cliniporator® electroporator (IGEA, Carpi, Italy) using two different, previously established electric pulse protocols: (1) a combination of high voltage (HV) and low voltage (LV) pulses: 1 pulse (600 V/cm, 100 μs), 1 s pause, 4 pulses (80 V/cm, 100 ms, 1 Hz) and (2) medium voltage (MV) pulses: 8 pulses (200 V/cm, 20 ms, 1 Hz) (Andre *et al.*, 2008; Forjanic *et al.*, 2019; Pasquet *et al.*, 2018). Transfected tissues and non-treated controls were placed in 6 well plates in 2 mL of phenol-free advanced RPMI 1640 cell culture medium (Gibco, Waltham, MA, USA) supplemented with 1× GlutaMAX (Gibco, Life Technologies, Carlsbad, CA, USA), 5 % FBS (Gibco) and 1× Pen/Strep (Sigma-Aldrich, St. Louis, MO, USA). The well plates with the tissues were placed in a humidified incubator at 37 °C and 5 % CO_2 and

the cell culture medium was changed every 4 days (days 4, 8, 12 and 16). For the analysis of cell culture media using luciferase assay or enzyme-linked immunosorbent assay (ELISA), 500 μL of media was harvested and stored at − 80 °C on days 1, 3, 7 and 21 after gene electrotransfer.

Fig. 1. Optimized protocol for gene electrotransfer of plasmid DNA into human muscle-tendon ACL grafts. The images show (**A**) a typical specimen consisting of the tendon on the bottom side and dense muscle tissue on the top of average 10 mm width and length and 4 mm thickness, (**B**) injection of 2 × 20 μg of plasmid DNA into the superficial and central layers of muscle tissue and (**C**) medium voltage pulses (200 V/cm, 20 ms, 1 Hz) applied to the tissue positioned between two electrodes of 6 mm distance.

Evaluation of GFP signal

Muscle-tendon tissue samples transfected with pMaxGFP and non-treated tissue samples were imaged 1, 3 and 7 days after gene electrotransfer using a SteREO Lumar.V12 fluorescence stereomicroscope (Zeiss, Jena, Germany) equipped with an MRc.5 digital camera (Zeiss). To obtain 16-bit grayscale images of emitted fluorescence light, the appropriate filters (excitation: 470/40 nm; emission: 525/50 nm) were used. The GFP transfected area of the tissue was quantified using Imaris software version 9.7.2 (Bitplane, Belfast, United Kingdom).

Luciferase assay

Luciferase activity was quantified in a non-diluted cell culture medium harvested 1, 3, 7, 14 and 21 days after gene electrotransfer. Luciferase activity was quantified using the Gaussia Luciferase Assay kit (#319, NanoLight Technology, Pinetop, AZ, USA), according to the manufacturer's instructions. The luminescence signal was measured in two technical replicates using a Polarstar Omega (BMG Labtech, Ortenberg, Germany) and the data was normalized to blank control.

ELISA

The release of BMP-2 from transfected tissue samples was assessed in tissue culture media

collected on days 1, 3 and 7 after gene electrotransfer using an enzyme-linked immunosorbent assay (ELISA) kit against human BMP-2 (#ab119581, Abcam, Cambridge, UK), according to the manufacturer's instructions. Briefly, non-diluted culture media were incubated in 96 well-plates pre-coated with antibodies. The end-product developed in enzymatic peroxidase reaction was measured in two technical replicates at 450 nm using a microplate reader (Promega, Madison, WI, USA) and calculated according to the standard curve.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Upon verification of the normality by Shapiro-Wilk tests, the data underwent unpaired parametric *t*-test analysis. The correlation coefficient was calculated using Pearson correlation analysis and a two-tailed *p* value. The differences were considered statistically significant when $p < 0.05$.

Results

Establishing the optimal gene electrotransfer parameters for human muscle-tendon tissues

Following *pMaxGFP* gene electrotransfer with both HV-LV and MV protocols, a GFP fluorescence signal was detected in the superficial

layers of muscle tissues already on day 1 after gene electrotransfer, whereas there was no GFP signal visible in the control group (Fig. 2**A**). The GFP expression could still be detected in transfected tissues 3 and 7 days after gene electrotransfer. The GFP-positive signal area in the transfected muscle tissues was quantified to determine which of the two tested gene electrotransfer protocols was more efficient. MV electric pulse protocol resulted in a larger area of transfected tissue compared to the *HV-LV* gene

electrotransfer protocol on days 3 and 7 after gene electrotransfer (Fig. 2**B**). Further analysis showed that the transfection was more efficient in thicker tissues, namely ≥ 3.7 mm and significantly in tissues of higher volume for day 1, even though two out of three samples treated with MV pulses underwent plasmid leakage (Fig. 2**C,D**). Taken together, our results suggested that the gene electrotransfer performed on a thick muscle-tendon tissue using MV pulses resulted in the most effective transfection.
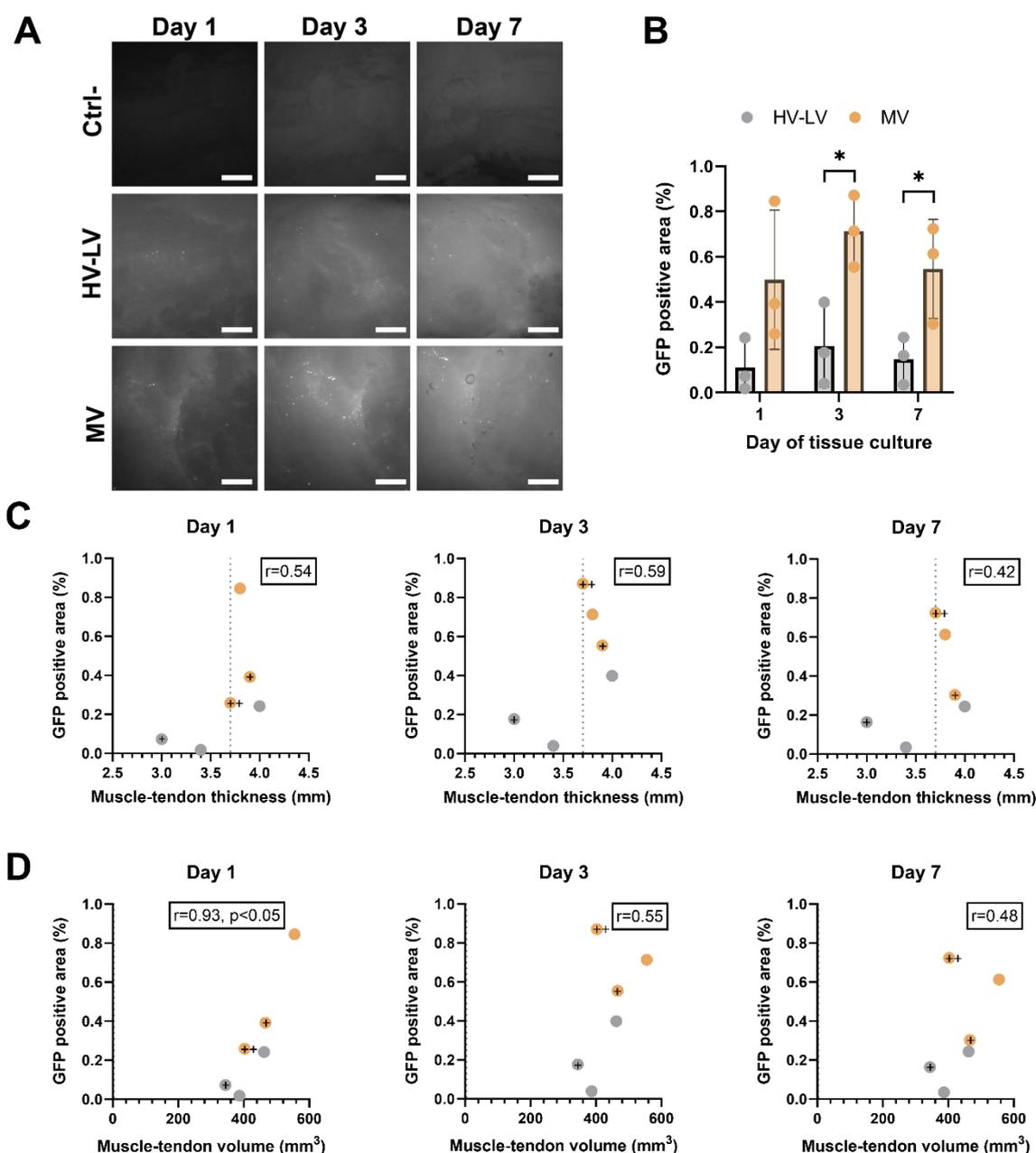
Fig. 2. The efficiency of pMaxGFP gene electrotransfer in muscle-tendon tissue using a combination of high and low voltage (HV-LV) or medium voltage (MV) electric pulse protocol. Representative

sample images (**A**) and the percentage of GFP positive area quantified in the superficial layer of muscle tissues from 3 biological replicates per group shown as a mean value ± standard deviation (**B**), and individually for each time point with regards to tissue thickness (**C**) and tissue volume (**D**). The dashed line indicates a thickness of 3.7 mm at which the efficacy appeared higher. One and two crosses assigned to GFP positive dots indicate low and medium leakage, respectively, observed during plasmid injection in the sample. Statistical significance was calculated using a parametric *t*-test (* *p* < 0.05). R stands for correlation coefficient. Scale bar: 1 mm.

To further verify the efficacy of gene electrotransfer of plasmid DNA using the MV electric pulse protocol, muscle-tendon tissue was transfected with a plasmid encoding luciferase gene. The signal from the excreted luciferase was detected in the culture medium of both transfected tissues throughout the whole 21-day culture period with the peak of luciferase activity detected on day 7 after gene electrotransfer (Fig. 3). The signal reduced to low levels on day 21 of tissue culture and was in the range of luciferase activity in non-transfected tissues, which remained very low at every assayed time point.

BMP-2 release in tissue culture medium

The analysis of culture media indicated no significant difference in BMP-2 release between gene electrotransfer of BMP-2 plasmid and non-treated when all tissue samples were pulled together (Fig. 4**A**). The concentration of released BMP-2 varied from not detectable to 300 pg/mL between the two groups, tissue donors and individual tissue samples. Plotting of samples that gave positive signals for at least one of the time points on days 1 and 3 indicated an ascending trend in the control group for 4 out of 6 samples and descending in the treated group for 6 out of 7 samples (Fig. 4**B**).

Individual tissue sample properties and plasmid delivery were further analysed with regard to subsequent BMP-2 release (Table 2 and Fig. 5). The results indicated that using thinner tissues and those of small volume impeded the injection and delivery of plasmid to the tissue, thereby reducing the chances of a successful gene electrotransfer (Table 2: P1, P3, P5). Particularly tissue samples ≤ 3.8 mm commonly give no or weak BMP-2 signal. If the injection could not be properly performed, plasmid leakage was found to occur even in thicker tissues and of higher volume, similarly reducing the chances for efficient gene electrotransfer (Table 2: P7). Tissues ≥ 3.8 mm were more likely to release BMP-2 both with or without gene electrotransfer. Altogether, tissue thickness and consequentially volume seemed to be major factors for a successful plasmid injection and gene electrotransfer of the tissue, although other factors may have played a role since some tissues ≥ 4 mm did not express BMP-2 (Table 2: P6). This effect was further confirmed statistically when the correlation of BMP-2 expression and tissue thickness and volume (*i.e.,* r = 0.74, *p* < 0.005 and r = 0.56, *p* < 0.05, respectively) was significant at day 1 for the treated group.

Discussion

Our study aimed to explore an alternative to viral methods, a rapid and clinically relevant approach for plasmid DNA delivery in human muscle-tendon ACL grafts. We provided a proof-of-concept that muscle residues on the tendon of a human ACL graft can be transfected using the gene electrotransfer approach, thus holding promise for future application in ACL surgical reconstructions. For reproducible delivery of therapeutic growth factors such as BMP-2, the method requires further optimization and validation as discussed hereafter, particularly regarding tissue selection.

Gene electrotransfer as a method for biological enhancement of surgery outcome is of particular interest for use in a commonly performed orthopaedic procedure of ACL reconstruction (Sanders *et al.*, 2016). Plasmid injection and gene electrotransfer on human muscle-tendon tissue collected from patients undergoing ACL surgery required only a few minutes of labour in *ex vivo* conditions and could thus be performed as a part of ACL graft harvesting and preparation prior to implantation. Compared to other regenerative approaches such as the use of mesenchymal stem/stromal cells from bone marrow or adipose tissue (Hevesi *et al.*,
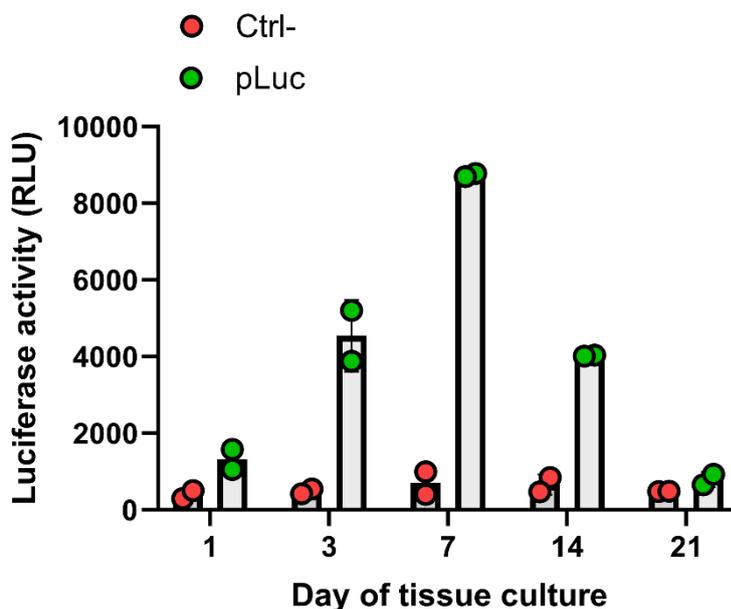
Fig. 3. Luciferase signal measured in the medium culture of non-transfected (Ctrl-) human muscle-tendon tissues and after gene electrotransfer of luciferase plasmid (pLuc) using medium voltage (MV) electric pulse protocol. Analysis was performed on in total of 4 tissue pieces from 3 patients (2 transfected tissues originating from the same patient), 2 being assigned to each group. RLU, relative light unit.
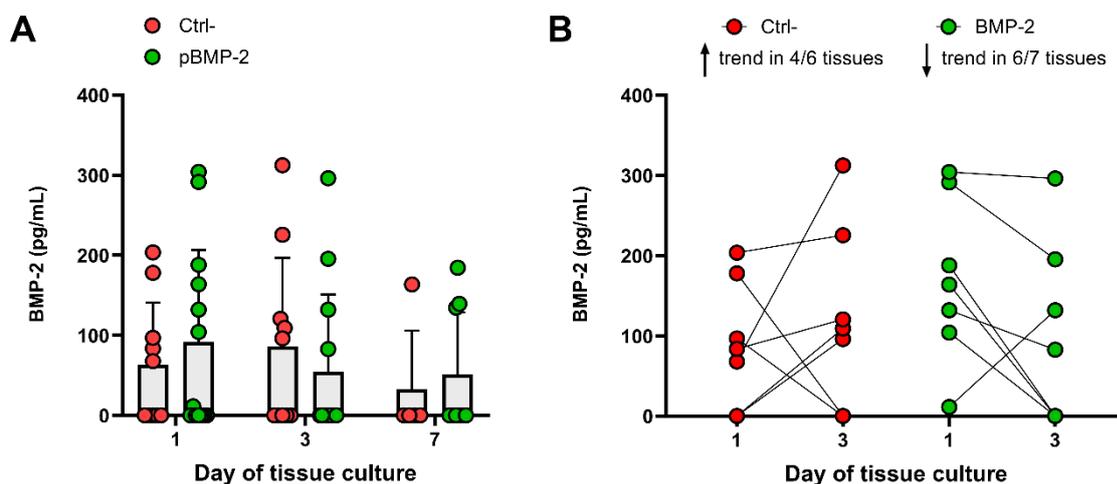
Fig. 4. Release of BMP-2 in culture media after gene electrotransfer of pBMP-2-advanced plasmid and no treatment (Ctrl-) in 23 muscle-tendon tissues harvested from 7 patients. BMP-2 release for all samples at different time points (**A**) and the ascending or descending trend of release from day 1 to day 3 for individual samples in which signal could be detected for at least one of the time points (**B**). Data are shown as a mean ± standard deviation (**A**). Lines connect the release from the same sample at 2 time points (**B**).

Table 2. Overview of BMP-2 release in culture media after gene electrotransfer of pBMP-2-advanced plasmid and no treatment (Ctrl-) in 23 muscle-tendon tissues harvested from 7 patients (P) with respect to donor, biological replicates, tissue thickness (bold) and plasmid leakage. One and two crosses indicate low and medium leakage, respectively, observed during plasmid injection in the muscle sample. The coloured values indicate positive BMP-2 signals after 1 and 3 days of tissue culture in control (red) or transfected (green) tissues ≥ 3.8 mm. NA, not analysed; ND, not detected.

Patient	Sample	Size (L mm x W mm x T mm)	Plasmid leakage	Group	BMP-2 (pg/mL)		
					Day 1	Day 3	Day 7
P1		14.1 x 11.3 x **4.5**		Ctrl-	ND	ND	ND
		11.2 x 9.7 x **3**	+	pBMP-2	11.2	131.9	184.4
		11.2 x 10 x **3.4**		pBMP-2	ND	ND	139.3
		10.6 x 11.9 x **3.4**		pBMP-2	ND	ND	ND
P2		13.4 x 10.6 x **3.6**		Ctrl-	ND	ND	ND
		12.1 x 9.9 x **4.5**	+	pBMP-2	187.9	0.0	134.5
P3		11.6 x 10.4 x **2.6**		Ctrl-	ND	ND	ND
		12.6 x 8.3 x **4.6**		pBMP-2	131.9	82.7	ND
		11.4 x 10.7 x **2.9**	+	pBMP-2	ND	ND	ND
P4		12.8 x 7 x **3.8**		Ctrl-	83.6	120.6	163.6
		9.7 x 8 x **3.8**		pBMP-2	163.8	ND	ND
		10 x 6.4 x **4.2**		pBMP-2	104.1	ND	ND
P5	NA	9 x 10.3 x **4.6**		Ctrl-	203.6	225.5	NA
	NA	9.6 x 10.7 x **4.8**		Ctrl-	67.8	312.3	NA
	NA	8.3 x 12 x **4.8**		Ctrl-	178.1	ND	NA
	NA	10.9 x 9.6 x **3.8**		pBMP-2	ND	ND	NA
	NA	10 x 10.1 x **4.4**		pBMP-2	304.2	296.1	NA
	NA	9.5 x 13 x **4.8**		pBMP-2	291.4	195.4	NA
P6		11 x 8.6 x **3.8**		Ctrl-	ND	96.0	NA
		12.9 x 8.5 x **4**		Ctrl-	96.8	ND	NA
		10.2 x 8.5 x **4**		pBMP-2	ND	ND	NA
P7		10 x 7.4 x **5.6**		Ctrl-	ND	109.2	ND
		12.6 x 7.4 x **4**	++	pBMP-2	ND	ND	ND
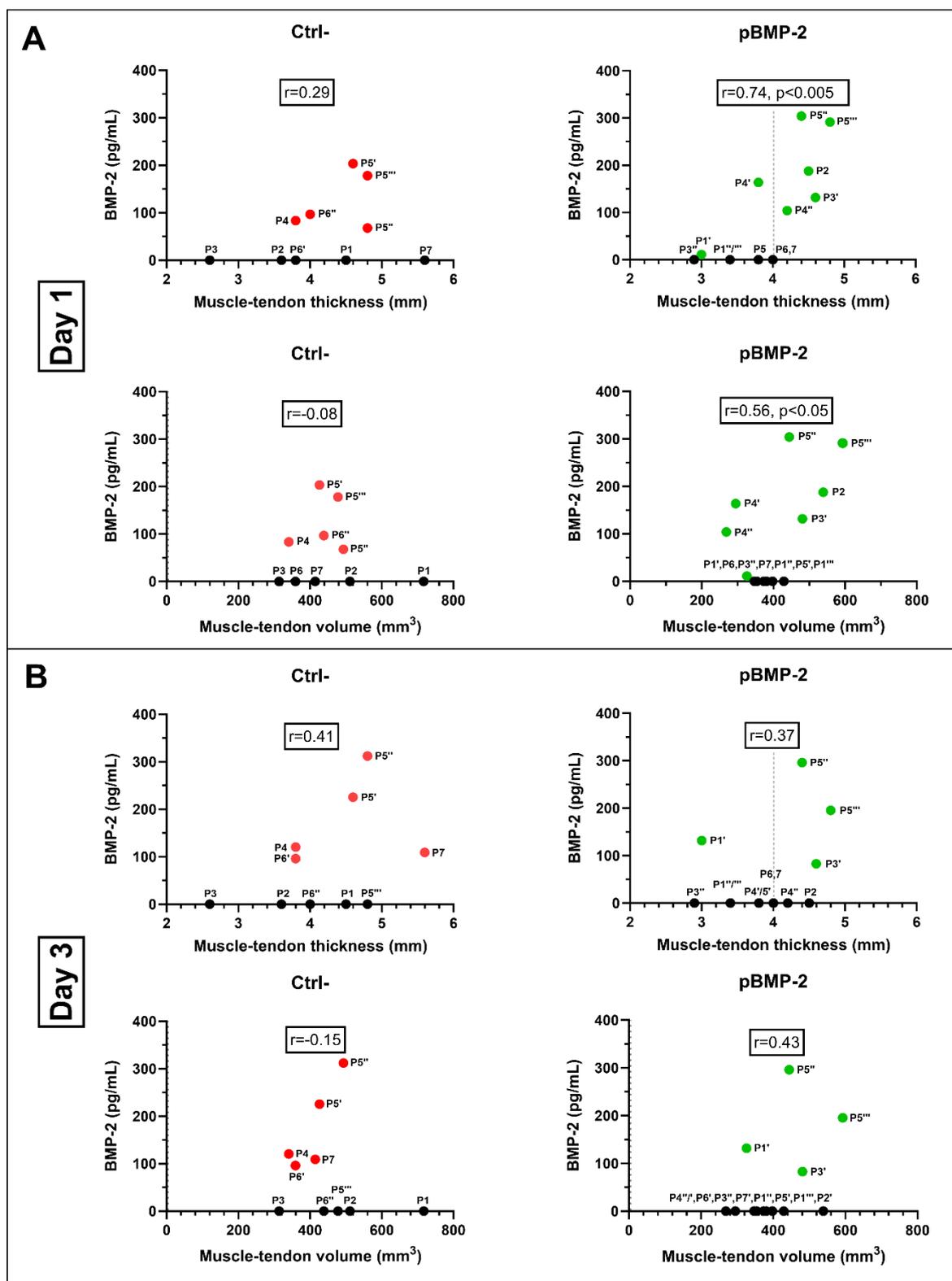
Fig. 5. Correlation between muscle-tendon tissue thickness and volume and BMP-2 release in non-treated tissues and tissues transfected with pBMP-2-advanced after 1 day (A) and 3 days (B) of sample culture. Samples for which the BMP-2 release was below the detection range of the used method are equalized to 0. The dashed line indicates a thickness of 4 mm, which appeared important for the efficacy of gene electrotransfer with the injection of 2 × 20 μL plasmid volume. The patient (P)

number is accompanied by an upper dash that refers to the respective tissue pieces listed in Table 2 if more than one was harvested from the same patient. R stands for correlation coefficient.

2019), gene electrotransfer requires neither additional surgery for tissue harvesting for cell isolation nor tissue transportation to/from the laboratory and cell processing for isolation and expansion. Furthermore, the delivery of plasmid DNA in the absence of viral vectors and transfection reagents, such as via the gene electrotransfer, is anticipated to be safer and reduce immunological response while resulting in efficient transfection of endogenous cells (Hardee *et al.,* 2017).

Using the gene electrotransfer on human muscle-tendon ACL graft, we have found the MV electric pulse protocol resulted in a larger area of transfected tissue than the HV-LV electric pulse protocol. This is in contrast to previous reports that suggested the high and low voltage pulse settings for gene electrotransfer of plasmid DNA to murine muscle tissues (Andre *et al.,* 2008; Tevz *et al.,* 2008; Vicat *et al.,* 2000). Our results thus imply that the HV-LV combination of pulses might not be optimal for all types of skeletal muscles and origins. Indeed, some studies have shown that lowering voltage, but increasing plasmid injection loading time or the number of pulses can result in an efficient transfection of skeletal muscles (Yamamoto *et al.,* 2012).

Using the MV electric pulse protocol, we detected the GFP signals 7 days in the superficial layers of muscle after gene electrotransfer, which is consistent with studies on murine skeletal muscle where the signal was observed after 7 (Florio *et al.,* 2022) and 10 days (Spugnini *et al.,* 2020) post-gene electrotransfer. However, the signal was not tested at the end of the 21-day tissue culture. To verify the efficacy, it would be of interest to further quantify the signal at the established time point of 7 days with respect to the cell number using, for example, common nucleus stains both in superficial and deeper layers of the tissue. We further showed that the luciferase signal peaked at 7 days after gene electrotransfer and was still detectable after 14 days, which should be long enough to induce the effect of therapeutic genes in the muscle (Kawai *et al.,* 2005). However, some reports indicated that gene electrotransfer of luciferase plasmid to

murine skeletal muscle may result in a long-term expression of the protein, even up to 6 months, if suitable pulses are chosen for gene delivery (Vicat *et al.,* 2000). In contrast to reporter genes, the expression and stability of BMP-2 released from treated samples seemed to persist in the medium for a short time, as the protein levels reduced in most samples already 3 days after gene electrotransfer. Recent research suggests that transiently expressed BMP-2 remains cell-associated by binding to type I receptors where it takes part in autocrine signalling (Atasoy-Zeybek *et al.,* 2023). This could explain the signal reduction in 6 out of 7 treated samples on day 3 compared to day 1 in our study. Considering that such BMP-2 was shown to remain available for signalling, we would expect osteogenic effects to take place after longer incubation of treated samples. Previous reports showed that the release of BMP-2 after gene electrotransfer to mesenchymal stem cells peaked after 21 days (Ferreira *et al.,* 2012). Furthermore, the period of only 10 and 20 days post gene electrotransfer of BMP-2 plasmid in murine skeletal muscle was sufficient to induce endochondral ossification (Kawai *et al.,* 2005) or bone formation (Yamamoto *et al.,* 2012), respectively. However, our histological analysis of samples cultured in standard conditions for 3 weeks revealed no difference in tissue mineralization between the treated and control samples (data not shown). We thus suggest conducting future studies on *ex vivo* tissues in a culture environment to better recapitulate the native tissue environment after surgery (*e.g.,* within a cylindrical bone explant) or in an animal model where the effect could be studied at the site of graft attachment to bone.

We could also detect the BMP-2 signal in several untreated tissues after 1 day of culture, increasing after 3 days in 4 out of 6 tissues. Muscle tissue is known to express endogenous BMP-2 during regeneration (Borok, Mademtzoglou and Relaix, 2020). We thus hypothesise that due to muscle damage during tissue harvesting and preparation, the osteogenic progenitor cells contained within the tissue could have induced BMP-2 production and release. Such endogenous

BMB-2 might initiate signalling and mineralization response, as we observed to occur in non-treated tissues (data not shown).

The gene electrotransfer of BMP-2 plasmid resulted in a high variation of detected protein concentrations in the culture medium between the treatment groups, individual patients and tissue samples from individual patients. *In vivo* studies on canine muscle to deliver a therapeutic gene (*i.e.,* interleukin 12) using gene electrotransfer also detected a high variation of the protein released in the serum, which was not in correlation with the amount of plasmid delivered to the muscle (Pavlin *et al.,* 2008), thus partially confirming our results of therapeutic plasmid delivery for application on *ex vivo* human muscles. The heterogeneity of material concerning cell shape, density and biochemistry was previously shown to affect the cell response to gene electrotransfer (Canatella *et al.,* 2004; Luft and Ketteler, 2015). Our results support these observations, whereby the muscle morphology and thickness with regards to a different position on the ACL graft are likely to play a more important role than a biological potency related to age or sex, as all tissues in this study were harvested from relatively young (*i.e.,* on average 28 years old) and predominately male patients. We found that a more efficient gene electrotransfer of therapeutic plasmids injected in a volume of 2 × 20 μL occurred in tissues of minimally 4 mm thickness and a total higher tissue volume (Fig. 5). Considering that we often observed leakage, it would be worth testing in another *ex vivo* study if the same effect appears after adjustment of plasmid volume to tissue volume and particularly its thickness. In contrast to *in vivo* studies on mice where primarily the intact muscles were used to electrotransfer the plasmid DNA, the gene electroporation was in our study limited to a relatively small area of a cut muscle tissue (*i.e.,* 10 mm). However, in clinical settings in which gene electrotransfer would be performed on the intact ACL graft, it would be possible to optimize the site of the plasmid injection depending on the tissue availability. We suggest that upon evaluation of the graft, the site of a single injection on each side of the graft gets restricted to abundant and compact muscle preserved at the ends of the graft, which would also be the site of implantation and where

integration into bone is expected to take place. Studies on an intact tissue would further allow the establishment of injection protocols concerning tissue volume to prevent leakage, track the efficacy with respect to the injection site (*e.g.,* with high-resolution microscopy techniques) and muscle morphology and compare it to the non-treated tissue surrounding the injection site. Additionally, *in vivo* studies on whole length ACL graft are also recommended to establish the long-term effects of BMP-2, as it would be crucial to limit them to the ends of the graft while retaining tendon's functional role as replacement for ligament and restoration of knee mobility.

To further increase the efficiency of *BMP-2* gene electrotransfer, other protocol improvements could be considered. Shortly delaying the delivery of electric pulses after the injection of plasmid DNA to allow it to diffuse to the cells in the interior of the tissue could further enhance the efficacy (Canatella *et al.,* 2004), as it was shown to occur after a 5 to 15-minute delay of the delivery of electric pulses in tumors (Mesojednik *et al.,* 2007). Prolonging the post-injection loading time may also increase the gene electrotransfer efficacy (Yamamoto *et al.,* 2012). Additionally, a sterile electroconductive gel could be used to increase the contact surface between the electrodes and the muscle tissue, thus optimizing the distribution of electric fields.

Conclusions

We have demonstrated gene electrotransfer as a rapid alternative to viral approaches for the transfection of muscle-tendon grafts used for ACL reconstructions. We found that MV electric pulse protocol resulted in a successful transfection of GFP and luciferase reporter plasmids, with a signal duration of up to 7 and 14 days, respectively. Delivery of a potentially therapeutic BMP-2 plasmid proved to be more variable between individual tissue samples, likely due to the BMP-2 attachment to cell receptors after transient expression. Nevertheless, the data indicated that tissue samples thicker than 4 mm stand a higher chance of being successfully transfected using our protocol. The improvements in plasmid delivery about site and volume of plasmid injection require further *ex vivo* or animal testing and

optimization for a therapeutic factor delivery and eventually osteodifferentiation. This method is of particular interest for use in the clinical setting (< 5 minutes) as it has the potential to improve the critical points of gene therapy in ACL reconstruction—clinical performance in the sense of time and post-operative limitations of the graft integration into the bone and ligament repair.

Author contributions

AŠ: Conceptualization, Validation, Formal analysis, Writing- original draft, Visualization, Project administration, Funding acquisition. BM: Methodology, Validation, Formal analysis, Writing – Review & Editing, Visualization. IM: Review & Editing, Investigation. AF: Review & Editing, Investigation. AH: Review & Editing, Methodology, Resources. TJ: Review & Editing, Methodology, Validation, Formal analysis, Visualization. MC: Conceptualization, Resources, Writing – Review & Editing. HR: Review & Editing, Conceptualization, Resources, Funding acquisition. DMP: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. AI: Review & Editing, Conceptualization, Resources, Funding acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Tissue collection was conducted with the approval of the University Hospital Sveti Duh ethics committee (approval number 01-1095/2).

Conflict of interest

The authors declare no conflict of interest.

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