Aus dem Muscle Research Unit, ECRC Experimental and Clinical Research Center
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin und Humboldt-Universität zu Berlin

DISSERTATION

Use of bladder telemetry to evaluate efficacy of muscle stem cells in a rat model of urethral sphincter injury, a preclinical efficacy study

Präklinische Studie zur Wirksamkeit von Muskelstammzellen in einem Rattenmodell für Verletzungen des Harnröhrenschließmuskels

zur Erlangung des akademischen Grades
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List of abbreviations

BaseP	Base Pressure
DSI	Data Sciences International
EEC	Exstrophy-epispadias Complex
FDA	Food and Drug Adminstration
GLP	Good Laboratory Practice
H&E	Hematoxlyin and Eosin
IACUC	Institutional Animal Care and Use Committee
ICI	Intercontraction Interval
LPP	Leak Point Pressure
MuSCs	Muscle Stem Cells
PeakD	Peak Duration
PeakP	Peak Pressure
SMCGM	Skeletal Muscle Cell Growth Medium
SUI	Stress urinary Incontinence

Abstract

Background: Preclinical efficacy studies of cell therapies using representative animal models in an experimental design that closely resembles clinical application are lacking. This study used bladder telemetry to assess efficacy of human muscle stem cells (MuSCs) in a urethral sphincter injury animal model. It was carried out as part of the preclinical regulatory requirement for an upcoming first-in-human trial using MuSCs in children with epispadias.

Methods: Human MuSCs were isolated from a 14-year old male donor who had no neuromuscular disorders. A urethral sphincter injury model using electrocauterization was selected after evaluation of several models using literature review. We implanted telemetry sensors surgically in the bladder of athymic male nude rats. Animals were placed in a standard 12-hour light/dark cycle. After five days, we injected 2 $x10^5$ MuSCs/animal (verum group, n=5) or placebo (cryopreservation medium; control group, n=5). Recording of telemetry variables was done at two time points: pre-injury and post-injection. Six parameters were recorded: Rise, Base pressure (BaseP), Peak pressure (PeakP), Period, Intercontraction interval (ICI) and Peak Duration (PeakD).

Results: Diurnal variation in urodynamics were observed where PeakP, BaseP were higher and ICI and Period shorter during the dark phase. Post-injection, Rise, BaseP and PeakP returned to pre-injury levels in the verum group but remained significantly lower in the control group. (ΔRise; verum = 0.1±0.24, control = -0.69±0.53, p = 0.043; ΔBaseP; verum = 0.2±0.7, control = -1.7±0.5, p = 0.020; ΔPeakP; verum = 0.0±0.7, Control = -2.4±0.7, p = 0.007). Histologic examination revealed injury related changes only in the control group, while the verum group had normal tissue architecture.

Conclusion: MuSCs injection leads to restoration of injured urethral sphincter. Carefully planned preclinical efficacy studies that adhere to regulatory requirements are critical in bridging the translational gap of cell therapies into the clinic.

Zusammenfassung

Hintergrund: Präklinische Studien zur Wirksamkeit von Zelltherapien an repräsentativen Tiermodellen in einem Versuchsaufbau, der der klinischen Anwendung sehr nahekommt, sind kaum zu finden. In dieser Studie wurde die präklinische Wirksamkeit von Muskelstammzellen (MuSCs) in einem Tiermodell mit Verletzung des Blasenschließmuskels unter Verwendung von Blasentelemetrie untersucht. Sie wurde als Teil der behördlichen Anforderungen für eine bevorstehende first-in-human klinische Studie von MuSCs bei Kindern mit Epispadie am Menschen durchgeführt.

Methoden: Humane MuSCs wurden von einem 14-jährigen männlichen Spender ohne neuromuskuläre Erkrankung isoliert. Nach Auswertung mehrerer Modelle anhand einer Literaturstudie entschieden wir uns für ein Modell zur Verletzung des Harnröhrenschließmuskels durch Elektrokauterisation. Wir implantierten Telemetrie-Sensoren chirurgisch an die Blase von männlichen, athymischen Ratten. Die Tiere waren in einem standardmäßigen 12-Stunden-Hell-Dunkel-Zyklus untergebracht. Nach fünf Tagen injizierten wir 2 $x10^5$ MuSCs/Tier (Verumgruppe, n=5) oder Placebo (Kryokonservierungsmedium; Kontrollgruppe, n=5). Die Aufzeichnung der Telemetrie-Variablen erfolgte zu zwei Zeitpunkten: vor der Verletzung und nach der Injektion. Sechs Parameter, nämlich Peak pressure (PeakP), base pressure (BaseP), Rise, Period, Intercontraction interval (ICI) and Peak Duration (PeakD) wurden aufgezeichnet.

Ergebnisse: Es wurden diurnale Schwankungen in der Urodynamik beobachtet, wobei PeakP, BaseP höher und ICI und Period kürzer während der Dunkelphase waren. Nach der Injektion erreichten PeakP, BaseP und Rise in der Verumgruppe wieder die Werte vor der Verletzung, blieben aber in der Kontrollgruppe signifikant niedriger. (ΔRise; Verum = 0.1±0.24, Kontrolle = -0.69±0.53, p = 0.043; ΔBaseP; Verum = 0.2±0.7, Kontrolle = -1.7±0.5, p = 0.020; ΔPeakP; Verum = 0.0±0.7, Kontrolle = -2.4±0.7, p = 0.007). Die histologische Untersuchung zeigte verletzungsbedingte Veränderungen nur in der Kontrollgruppe, während die Verumgruppe eine normale Gewebearchitektur aufwies.

Abschluss: Die Injektion von MuSCs führt zur Wiederherstellung des verletzten Harnröhrenschließmuskels. Sorgfältig geplante präklinische Wirksamkeitsstudien, die den regulatorischen Anforderungen entsprechen, sind entscheidend für die Überwindung der Translationslücke von Zelltherapien in die Klinik.

1 Introduction

1.1. Muscle Diseases

Skeletal muscle is the largest organ and comprises about 40% of the total human body weight.[1] It is responsible for several body functions from regulation of body temperature to mobility and functioning of several internal organs. Muscle wasting defined as loss of muscle mass and strength, is a characteristic of several congenital and acquired disorders and is associated with functional disability and decreased quality of life.[2] Muscular dystrophies represent the most common inherited cause of muscle wasting. They are a debilitating heterogenous group of monogenic disorders with an estimated worldwide prevalence of 3.6 per 100,000 people.[3] They are highly progressive and associated with decreased life span and tremendous disease burden with an estimated annual cost of € 150 million only in Germany.[4] Despite great progress in research in the last three decades, there is currently no cure available for these disorders. With advances in gene therapy, cell therapy is a promising therapy option with several products currently in the pipelines being investigated in preclinical and clinical studies with mixed results.

1.2. Cell therapy in muscular dystrophies

Cell therapy is a rapidly growing field with a wide range of therapeutic applications including muscular dystrophies. However, the path from bench to bedside is challenged by a number of factors, including selection of cell origin, method of in-vivo/in-vitro gene correction, a lack of a representative animal disease model and absence of robust preclinical evidence for safety and efficacy that meets regulatory requirements. Furthermore, clinical use faces hurdles such as effective systemic delivery, adequate immune suppression to enable engraftment and long term survival and a myriad of unpredictable side effects.[5]

Several cell origins including myogenic stem and progenitor cells derived from skeletal muscles, cells from non-skeletal muscle tissues and pluripotent stem cells have been thoroughly investigated.[5] The use of muscle stem cells (MuSCs), also known as satellite cells is, to date, the best possible cell source. Satellite cells are the primary stem cells of skeletal muscles capable of repair and regeneration when activated. Moreover, cells derived from satellite cells can retain their stem cell properties, regenerate muscle tissue, and repopulate the stem cell pool.[6,7] However, isolation and clinical application of these

cells has been challenged by the loss of regeneration potential during ex-vivo cultivation and limited in vivo migration following transplantation.[8] However, Marg et al.,2014.[9] reported an innovative method of cell cultivation that enables the isolation of highly myogenic cells with preserved regenerative potential.

An animal model should recapitulate the disease condition and possess similar gene expression, muscle structure and express the pathologies reported in the disease condition.[10] However, no single animal model that fulfils these criteria exists. Thus, researchers have been using several animal models for specific disease pathologies in muscular dystrophies. To date several animal models such as mouse, pig, rabbit, dog, hamster, and sheep have been used with need for constant improvement.

To date only four drugs have received Food and Drug Administration (FDA) for the treatment of Duchenne muscular dystrophy, while several others are still under development attempting to gather adequate safety and efficacy preclinical data in animals.[11] Given that muscular dystrophies affect several muscles within the body including muscles of the heart and the diaphragm, a systemic delivery of the intended cell therapy is needed. However, this requires administration of a larger dose to demonstrate functional improvement. Larger doses or multiple injections in turn pose a safety concern.[12,13] Thus, muscular disorders where small muscles are locally affected present a great opportunity as a clinical target during the development of such therapies.

1.3. Urinary incontinence in Epispadias as a primary target

For our primary clinical target, we selected a disease with a local skeletal muscle defect. Isolated epispadias represents the mildest form of the exstrophy-epispadias complex (EEC); characterized in males by an abnormal dorsal urethral location, failure of closure of urethral plate and a defect in the urethral sphincter.[14] Although surgical reconstruction successfully ameliorates the genital defect, urinary incontinence remains to be a major problem of patients with epispadias with significant social, psychological, medical, and financial consequences. Incontinence is the result of an anatomic defect characterized by incomplete urethral sphincter muscle due to a developmental anomaly where muscle tissue is replaced by connective tissue (Fig.1).[15] Attempts to preserve continence by major surgical procedures, urinary diversion or repeated catheterization present a major medical challenge as they are associated with low success rates, frequent infections,

prolonged hospital stays and reduced quality of life.[16,17] There is currently no therapy available to repair this urethral defect.

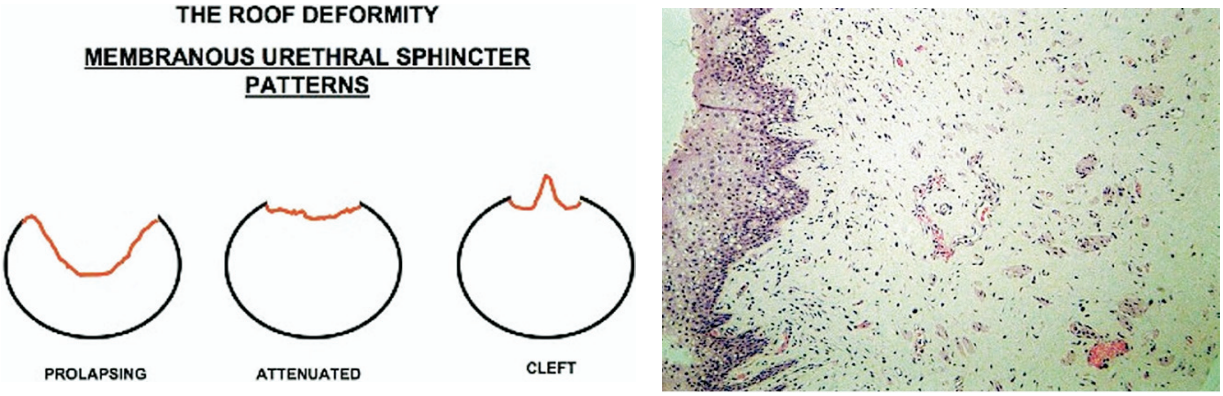

Figure 1: Urethral defect in Epispadias

A. Schematic drawing of roof deformity in the urethral sphincter region in epispadias. B. Histological staining of excised roof deformity depicting normal epithelium (left) and sparse smooth muscle with connective tissue (right). (Taken from Canon et al. 2008[15])

Despite significant advances in the last two decades, clinical application of MuSCs in urinary incontinence is still a long way off. Among others, inability to produce an adequate number of cells with regenerative and preserved myogenic capacity, studies with inconclusive results, lack of adequate cell characterization to enable reproducibility, lack of animal models and preclinical experiments that closely resemble clinical use and the heterogeneity of voiding dysfunctions are attributing factors.[18] This study was aimed at demonstrating preclinical efficacy of well-characterized MuSCs in a representative animal model using an experimental design that closely resembled clinical application.

Establishment of cell-based therapies for urinary incontinence necessitates functional assessment of efficacy in preclinical studies. Urodynamic measurements using conventional cystometry are frequently used to evaluate lower urinary tract function. This often includes a one-time, terminal measurement in awake or sedated animals where bladder pressure and voided volume are assessed while the bladder is continuously filled to stimulate the micturition reflex.[19] A frequently used method is leak point pressure (LPP) measurement, which requires transection of the spinal cord and animal sacrifice.[20,21] This method has several drawbacks: the use of anesthesia alters bladder function,[22,23] measurement in conscious animals necessitates restraint, which influences the micturition cycle,[24,25] and it is a one-time terminal measurement with no possibility of longitudinal assessments. Bladder telemetry allows for continuous recording of

urodynamic parameters in freely moving, unrestrained and awake animals with no need for artificial bladder filling[26]. It provides a more physiologic and accurate measurement than conventional cystometry and enables multiple measurements at different time points in the same animal, making it an invaluable tool in efficacy assessments.[27]

1.4. Objectives

This study was conducted as part of a regulatory requirement for an upcoming first-in-human clinical trial in children with epispadias (Eudra-CT Nr. 2021-002004-13). It is a preclinical efficacy assessment of MuSCs in urethral sphincter injury using a carefully selected animal model that closely resembles the defect in epispadias and bladder telemetry to assess outcome within each animal across time in a near-GLP (Good laboratory practice) experimental setup.

2 Methods

2.1. Cells

MuSCs were prepared using preparation methods as described in Marg et al.,2014.[9]. We obtained muscle tissue from a biopsy of a vastus lateralis muscle of a 14-year-old boy without any muscular diseases (ethical approval EA1/203/08, Charité) to approximate the upcoming trial where cells would be isolated from children. The isolation of muscle stem cells was carried out by the in-house technical assistants. Following biopsy, the specimen was transferred into a sterile tube containing transport medium with 30 mM HEPES, 130 mM NaCl, 3 mM KCl, 10 mM D-glucose, and 3.2 µM Phenol red (pH 7.6). Excess fat and connective tissue were removed, and each specimen manually dissected using forceps under stereomicroscope (Leica Microsystems) to obtain human muscle fiber fragments (HMFF). Single HMFFs with a length of 2-3mm were carefully examined and further dissected to remove any remaining connective tissue. HMFFs were then placed in a hypothermic treatment (4-6°) for 7 days. Afterwards each fragment was cultivated in Skeletal Muscle Cell Growth Medium (SMCGM, Provitro) supplemented with 10% FCS, glutamax, and gentamicin and cultured in a humidified atmosphere containing 5% CO2 at 37°C. Outgrowing cells were then propagated and characterized using Desmin and Trypan blue. Desmin staining demonstrates myogenic potential and excludes contaminating fibroblasts.[7] Cells that consisted of > 95% myogenic cells and were >95% viable were selected, pooled and placed in a cryopreservation medium at a concentration of 10 x 10^6 cells/ml. (Fig. 2)

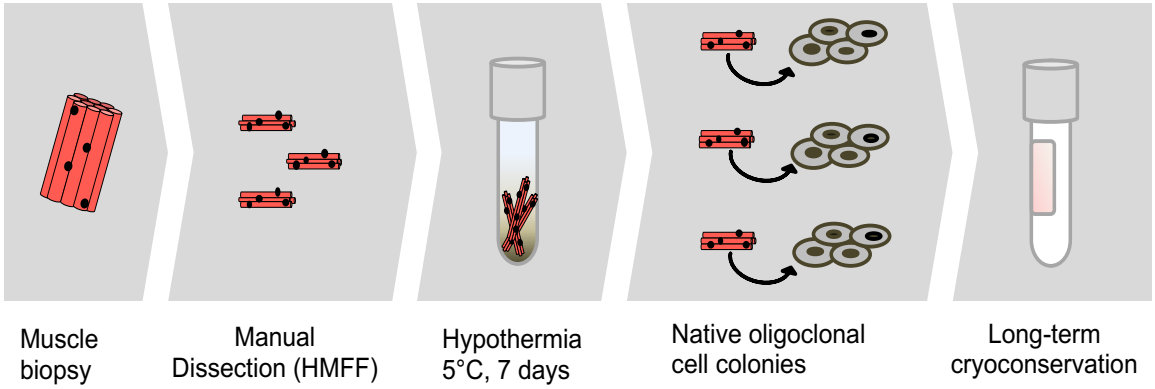

Figure 2: Cell isolation and cultivation

HMFFs are prepared using manual dissection followed by hypothermia treatment. Cell colonies grow out of HMFFs within 3 weeks of cultivation and can be preserved long-term. (Modified from Marg et.al 2019[7])

HMFF – Human muscle fiber fragments

2.2. Selection of animal model

There is currently no animal model for epispadias. Previous attempts to develop a suitable model failed due to high costs and a low survival rate.[28] The use of an incontinence model with urethral sphincter damage is a close approximation to the urethral defect seen in epispadias. However, this injury model must fulfil the following criteria:

- Closely mirror the disease pathology in humans. In addition to anatomic differences among species, the fact that multiple factors play a role in urinary incontinence is a major limitation to fulfillment of this criterion.
- Effect must be measurable and reproducible. Urethral dysfunction must be confirmed with urodynamic measures and effect reproduced every time injury is sustained by the animal model.
- Must result in a permanent urethral sphincter damage. Effects induced must be persistent and not able to be repaired through normal host muscle regeneration.
- Must be large enough to minimize technical difficulties in injury and urodynamic measurements.

Following a thorough review of the literature and a comparison of various injury methods (see Table 1), we determined that electrocauterization in a rat urethra was the best approximate small animal model for the sphincter defect in isolated epispadias. Yiou et al., 2003[29] initially established this model and showed that electrocauterization resulted in irreversible destruction of both sphincter myofibers and nerve endings resulting in a dysfunctional sphincter and long-lasting decrease in urethral resistance. In addition, Chermansky et al., 2004[30] used a similar model in which tissues lateral to the urethra were electrocauterized without affecting bladder function, resulting in decreased LPP maintained up to 16 weeks post injury.

Table 1: Comparison of urethral injury models

Publication	Injury method	Advantages	Disadvantages
Yiou et al., 2002[29]	Notexin (myotoxic agent)	- Reproducible results	- No persistent damage - Activation of satellite cells was seen at about 3 weeks after trauma

Praud C. et al., 2007[31]	Freezing of the proximal urethral wall	- Technically easier	- Non-reproducible - No persistent damage
Lee et al., 2003[32]	Denervation	- Reproducible damage	- Slow tissue injury - Resultd in only nerve damage
Eberli et al., 2009[33]	Surgical resection of urethra segment (Canine model)	- Persistent	- Not reproducible in small animal models
Yiou et al., 2003[29] Chermansky et al., 2004	Electrocoagulation	- Reproducible damage - Persistent damage - Combined tissue and nerve damage	- Technically difficult in small animals

For the current study, we used ten athymic rats (Crl:NIH-Foxn1^{rnu}, 250-300 g; Charles River Laboratories, Inc., Raleigh, NC or Kingston, NY). Considering the higher incidence of epispadias in males (M:F ratio of 13:1)[34] and significant anatomic differences, only male animals were selected. All animal experiments were performed at Charles River's AAALAC-accredited animal facility (Mattawan, MI) after approval by the Institutional Animal Care and Use Committee (IACUC) under the study number 3268-001 d. The animals were subjected to a 12-hour light/dark cycle and water and food provided ad libitum. Urethral sphincter was injured using electrocauterization as described previously[30] with modifications. Under anesthesia, animals were placed in supine position and bladder exposed via a midline laparotomy. We placed a urinary catheter in the urethra to enable better visualization and cauterized tissues 1cm caudal to the bladder on each side using a high-temperature fine tip cautery (Bovie Medical, Antioch, USA). Duration of electrocauterization was increased from 30 seconds to 60 seconds to ensure adequate tissue damage.

2.3. Telemetry

We used the Data Sciences International (DSI) HD-S10 bladder telemetry transducer. Prior to implantation, we verified the zero-offset of the pressure channels prior to implantation with accuracy of ± 3 mmHg. A purse string suture was used to secure transducers in the bladder, and the transmitter was placed subcutaneously or in the abdominal cavity. We recorded a total of six parameters: three pressure parameters

namely Rise, Base pressure (BaseP) and Peak pressure (PeakP) and three temporal parameters namely Peak duration (PeakD), Period and Inter-contraction interval (ICI). PeakP was defined as the pressure at the peak of contraction, while BaseP was the pressure at the beginning of a contraction and RiseP was the difference between PeakP and BaseP. Period is the time between one contraction and the next, whereas PeakD is the time from beginning to the end of a single contraction. ICI describes the time between the end of one peak contraction and the start of the next. (See Fig. 3 Below)

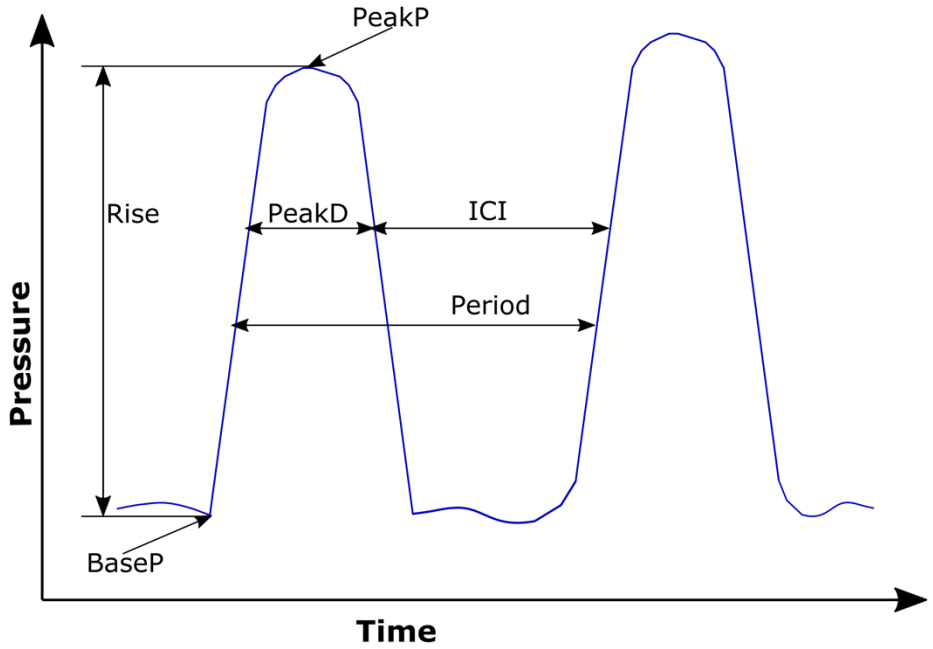

Figure 3: Telemetry parameters graphic representation

PeakP – Peak pressure, BaseP – Base pressure, PeakD – Peak duration, ICI – Inter-contraction interval (taken from Bekele et.al 2022[35])

2.4. Experimental design

Bladder telemetry transducers were transplanted in the bladder of each animal and the animals were allowed to recover for 7 days. We injured the urethral sphincter using cauterization on day 10. On day 15, we divided the animals into two groups; the verum group (n=5), where MuSCs in cryopreservation medium were injected, and the control group (n=5), where only placebo (cryopreservation medium) was injected. Urodynamic parameters were measured every five minutes for a total of 24 hours at two-time points; day 7 (pre-injury) and day 37(post-injection). Animals were then sacrificed, and tissues were examined histologically. (Fig. 4)

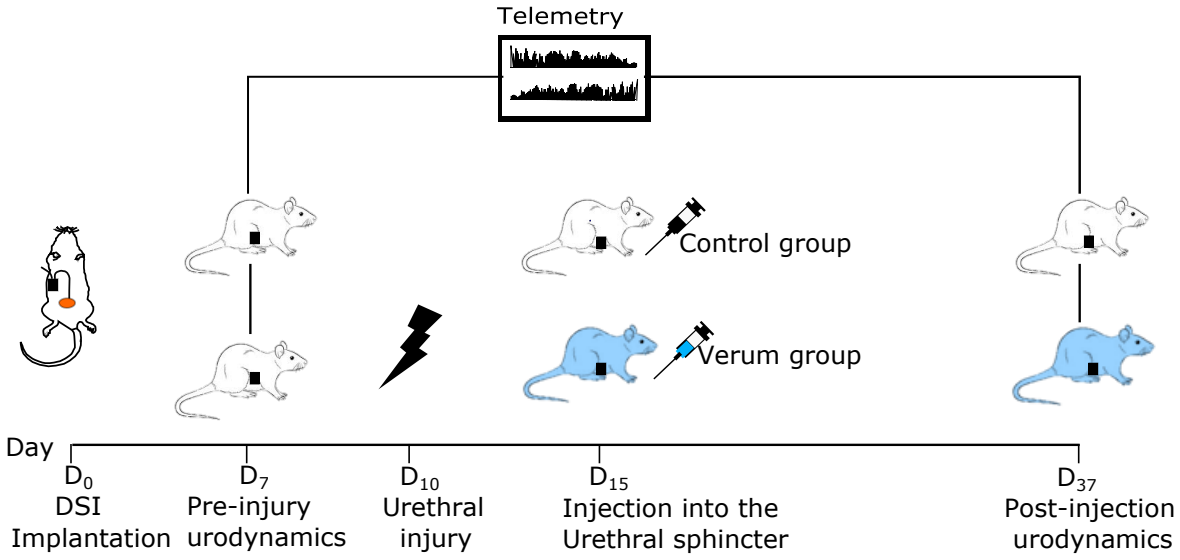

Figure 4: Experimental design

Bladder telemetry transducer (DSI) surgically implanted on day 0 (D_0). First urodynamic measurement (pre-injury, D_7) after a recovery period. Urethral sphincter injury on day 10 (D_{10}) followed by injection of MuSCs/placebo on day 15 (D_{15}) and the second urodynamic measurement (post-injection) on day 37 (D_{37}).

2.5. Injection

We performed a midline lower abdominal incision and identified the urethral sphincter. We injected four periurethral injections with 5 µl per site (two injections on each side) into the sphincter. We injected a total of 2 x10^5 MuSCs/animal in cryopreservation medium in the verum group and an equal volume of placebo (cryopreservation medium) in the control group.

2.6. Histologic examination

Animals were sacrificed on day 38 and bladder and urethra removed in-toto. We trimmed the urinary sphincter administration site to include the entire distal portion of the bladder, prostatic sphincter region and adjacent distal urethra. After identifying the lumen microscopically, we obtained sections and stained with hematoxylin and eosin (H&E).

2.7. Statistical analysis

Sample size calculation was based on effect seen in Chermansky et al.,2004[36]. Given the small sample size in the study, variability was estimated with a conservative standard

deviation of 3. Following simulations with 1000 replications, a sample size of 4 animals per group was deemed sufficient and one animal per group to account for potential drop-outs.

Urodynamic parameter recordings were adjusted for extreme values using winsorization at the 1st and 99th percentiles. A summary of each parameter is presented as median ± SD. Difference between the two points of measurement was used as a primary outcome variable to eliminate inter-individual variability. We used t-test or Wilcoxon test, as appropriate, and *p* values less than 0.05 were considered statistically significant. All statistical analysis was performed using R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

2.8. Fulfillment of regulatory requirements

In light of the upcoming clinical trial and the regulatory requirements, national regulatory authorities were involved from the early stages of this study. Several points, including MuSCs characterization and validation of in-vitro evidence, choice of animal facility, choice of animal model, experimental design, and final results, were thoroughly discussed and deemed appropriate by experts. Particular attention was given to the following points:

- Use of a MuSCs with similar quality and quantity criteria as intended to be used in the clinical trial
- Conduct of experiments in near-GLP (Good laboratory practice) conditions
- Pre-study sample size calculation and justification
- Selection of animal model that recapitulates the pathology in epispadias
- Experimental design adhering to the current and intended clinical practice

3. Results

3.1. Preliminary study

Chermansky et al., 2004[30] demonstrated that electrocoagulation of the urethra resulted in lower LPP starting at 2 weeks and sustained upto 16 weeks post-injury. However, whether electrocoagulation results in similar alterations in urodynamic measurements measured by telemetry has not been shown so far. Before performing the preclinical efficacy study, we conducted a preliminary study where we assessed whether injury induced by electrocoagulation resulted in persistent reproducible damage that can be measured by telemetry. For this study, bladder telemetry was implanted a total of five athymic rats followed by a urethral injury by electrocoagulation. Urodynamic parameters were assessed at three time-points: pre-injury, post-injury and post-dye. Additionally, rats were injected with a placebo solution (2 x 10 µl blue dye) in the urethral injury site to demonstrate adequate localization of injection. (Fig. 5)

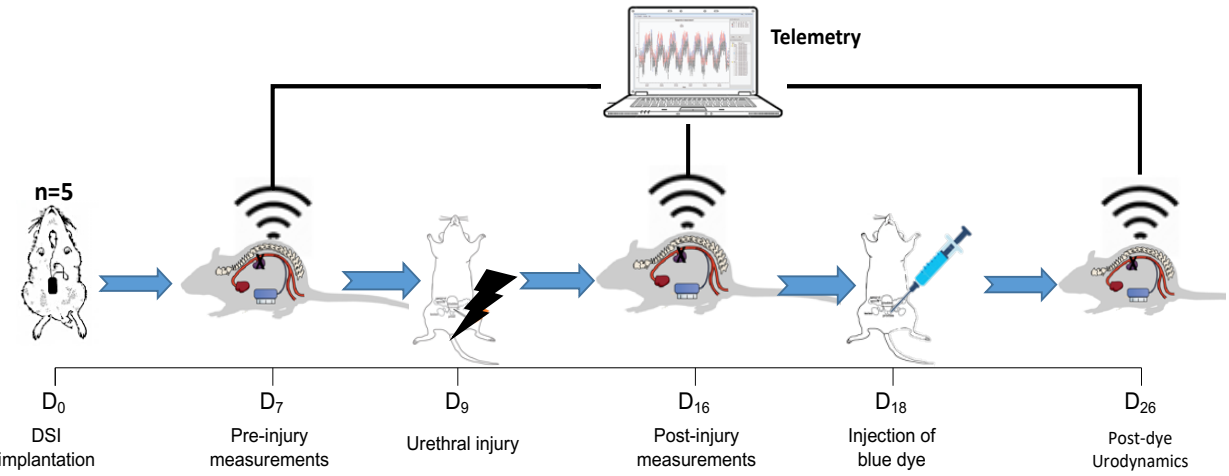

Figure 5: Design of preliminary study

Bladder telemetry transducer (DSI) surgically implanted on day 0 (D_0). First urodynamic measurement (pre-injury, D_7) after a recovery period of 7 days. Urethral sphincter injury on day 9 (D_9) followed by second urodynamic measurement (post-injection) pm day 16 (D_{16}). Afterwards blue dye was injected on day 18 (D_{18}) followed by the third urodynamic measurement (post-dye) on day 26 (D_{26}).

We observed a significant reduction in PeakP between pre-injury (20.2 ± 2.7 mmHg versus 15.9 ± 1.9 mmHg, **p = 0.031**) and post-injury measurements indicating a measurable change in urodynamic parameters. Furthermore, PeakP remained significantly lower during the post-dye injection measurement (20.2 ± 2.7 mmHg versus 17.1 ± 2.9 mmHg, **p = 0.04**). (Fig. 6) This preliminary study established that

electrocoagulation resulted in change in urodynamic measurement that can be assessed by telemetry.

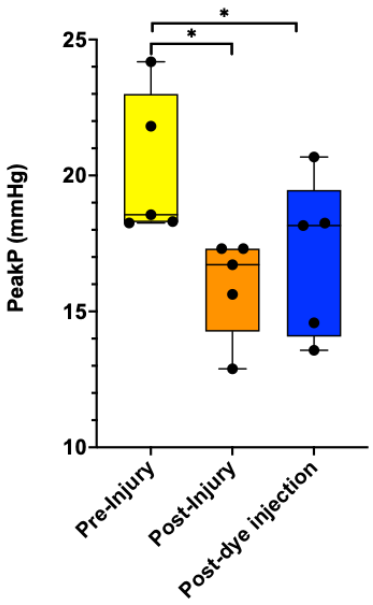

Figure 6: Change in urodynamic parameters in a preliminary study

A Box plot representing the change in PeakP between three time points: pre-injury, post-injury and post-dye injection. PeakP- Peak pressure

3.2. Diurnal variation

Rats were placed in a 12-hour light/dark cycle and we recorded telemetry parameters for a duration of 24 hours. Pre-injury measurements were used to evaluate circadian variations in bladder function. We observed significantly higher BaseP, PeakP and shorter ICI and period during the dark phase. (For details see Table 2 below)

Table 2: Telemetry parameters recorded pre-injury during light and dark phase (Taken from Bekele et al.,2022)

Parameter	Light phase	Dark phase	*p**
PeakP (mmHg)	11 ± 10.4	12 ± 2.9	**0.001**
BaseP (mmHg)	7.5 ± 1.8	7.8 ± 1.9	**0.001**
Rise (mmHg)	3.9 ± 1.8	4.0 ± 2.7	0.215
Period (sec)	12.5 ± 19.2	10.9 ± 11.1	**0.001**
PeakD (sec)	3.6 ± 0.8	3.6 ± 0.7	0.424
ICI (sec)	8.4 ± 19.6	7.2 ± 11.4	**0.002**

*Wilcoxon test, All values indicate Median ± SD, PeakP – Peak pressure, BaseP – Base pressure, PeakD – Peak duration, ICI – Intercontraction interval

3.3. Changes in urodynamic parameters

Due to technical errors, we excluded three animals (n=2 control group and n=1 verum group) from further analysis of post-injection changes. Urodynamic recordings were abnormally high indicating misplaced sensors in the freely moving rats. Pre-injury and post-injection pressure tracings of selected animals in each group are shown in Fig. 7.

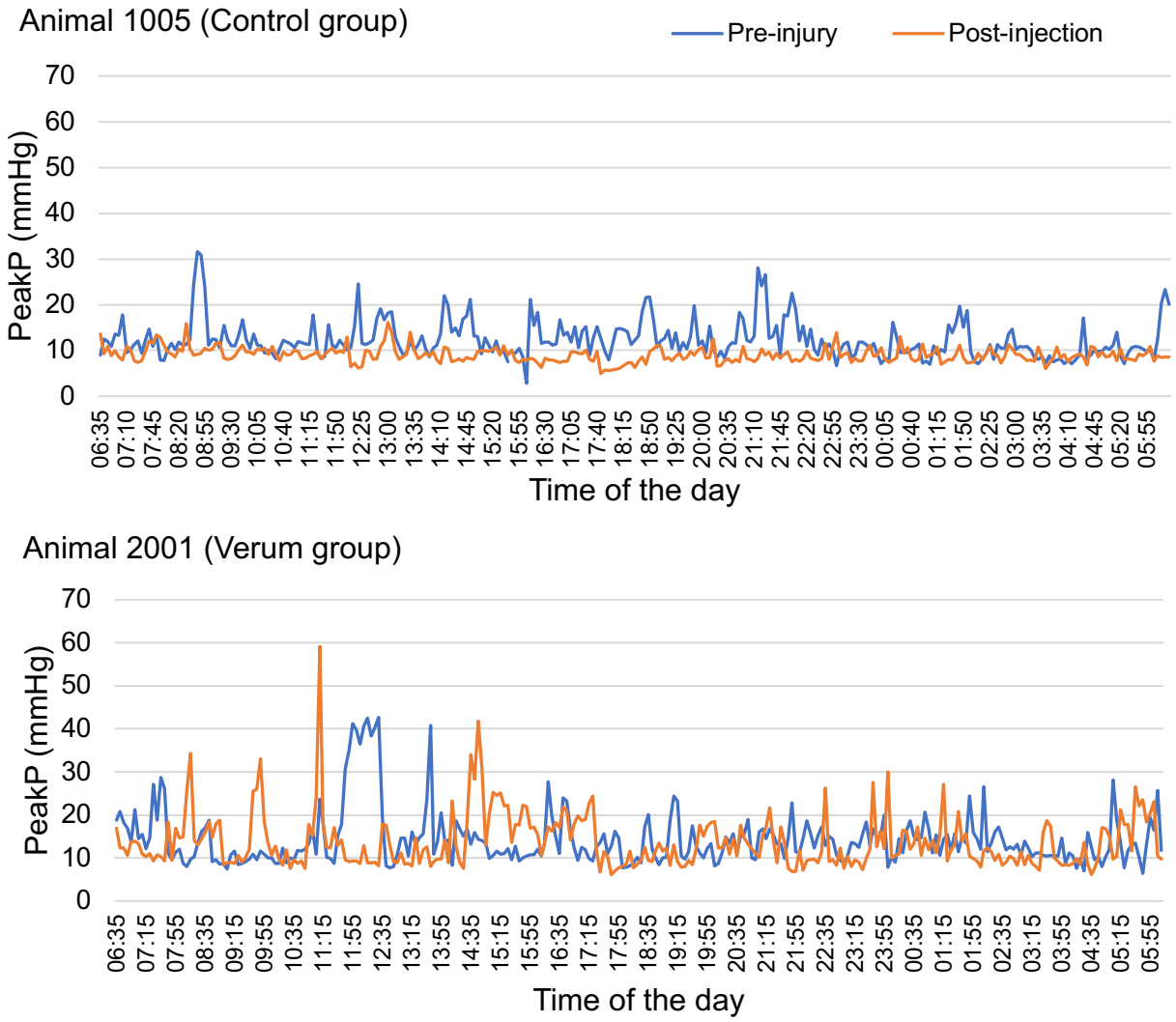

Figure 7: Representative pre-injury and post-injection bladder telemetry pressure tracings

Depicted measurement are across 24 hours of measurement with corresponding time of day in x-axis. PeakP – Peak pressure

During both the pre-injury and post-injection time points, we found no statistically significant differences between the two groups. However, comparing the changes in

urodynamic parameters between the two time points, we saw a significantly higher decrease in all pressure parameters in the control group (ΔRise; verum = 0.1±0.24, control = -0.69±0.53, **p = 0.043**; ΔBaseP; verum = 0.2±0.7, control = -1.7±0.5, **p = 0.020**; ΔPeakP; verum = 0.0±0.7, Control = -2.4±0.7, **p = 0.007**). (See Fig. 8) None of the temporal parameters showed a significant change. In the verum group none of the urodynamic parameters showed a significant change between pre-injury and post-injection measurements.

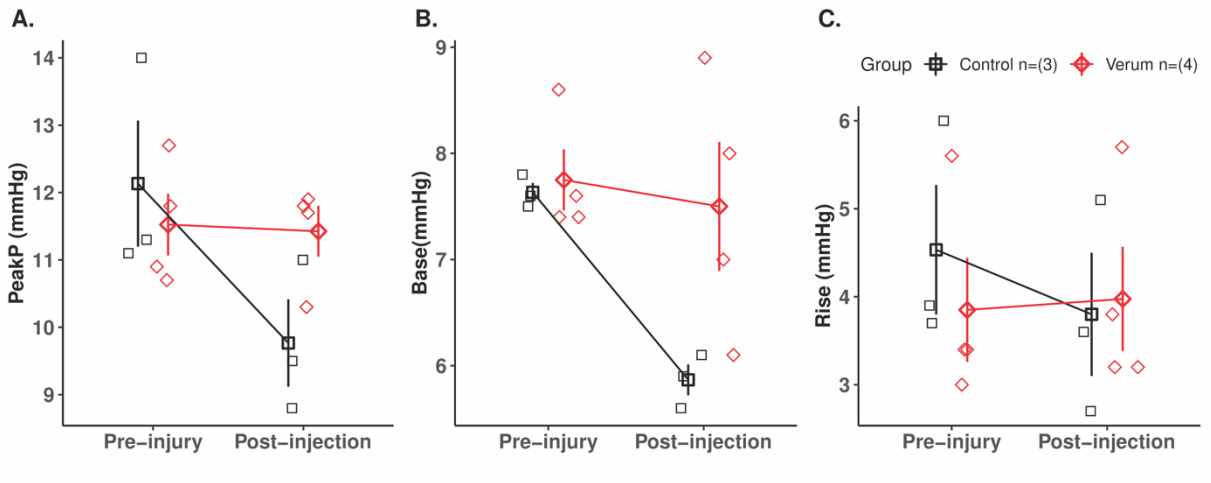

Figure 8: Change in urodynamic parameters between two time points

Values represented are median ± SD for each animal. Rise- defined as the difference between PeakP and BaseP, PeakP- Peak pressure, BaseP – Base pressure(Taken from Bekele et al.,2022[35])

3.4. Histologic changes

Animals were sacrificed on day 38 and the bladder and urethra removed intact and histologically examined. Only the control group showed injury-related tissue changes, while the verum group had normal sphincter tissue architecture. (Fig. 9)

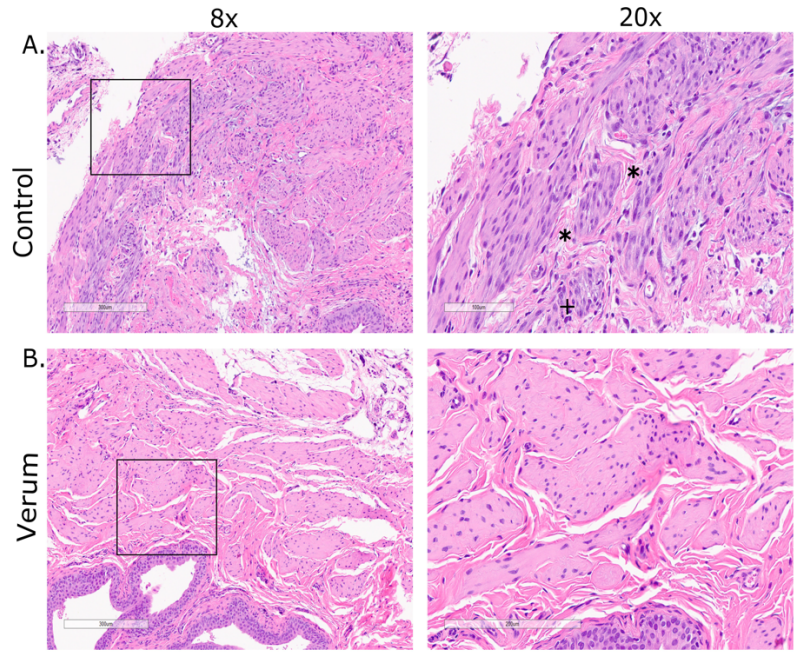

Figure 9: Post-injection histologic findings in the urethral sphincter of rats

A. Hematoxylin & Eosin staining depicts tissue changes related to injury such as scarring and degenerative changes in the control s group (*) B. Hematoxylin & Eosin staining of the urethral sphincter in verum group showing normal urethral sphincter tissue architecture (Taken from Bekele et al.,2022[35])

4. Discussion

This study assessed the efficacy of human MuSCs in repair of urethral injury in a rat animal model. An injury model was chosen based on well documented evidence and close resemblance to defect in the target disease; epispadias. We used bladder telemetry to assess changes within the same animal before and after injection. Moreover, it provided a less invasive, more accurate and physiologic measurement of urodynamic parameters in freely moving animals. We show how pressure parameters returned to pre-injury levels in animals injected with MuSCs whereas animals in the control group had persistent decrease. Our histology findings, which showed that tissue alterations attributable to injury were exclusively evident in the control group, further supported these results. All these findings suggest a functional restoration of the damaged urethral sphincter following injection of MuSCs.

Previous studies have reported a circadian variation in bladder capacity and micturition pattern in rodents.[37–40] Our results are consistent with these findings where rats displayed shorter ICI and Period during dark (active) phase, and less frequent contractions during light (inactive) phase. However, our study demonstrates a difference in bladder pressure that was not previously reported. We observed increased BaseP, and PeakP during dark (active) phase, and lower BaseP, PeakP during light (inactive) phase. In fact, Herrera et al., 2010[41] reported no difference in bladder pressure despite differences in bladder capacity and micturition frequency. This could be explained by the differences in experimental design. Herrera and colleagues used saline infusion into the bladder which eliminates urine production and oral uptake. Circadian variation in urodynamics is highly dependent on daily urine production, properties of the bladder, and neuro-hormonal control through the kidney.[42] Furthermore, urodynamic parameters were only recorded for 30-90 minutes, and comparisons were made between two distinct groups (light versus dark group). In our study, urodynamic parameters were measured for 24 hours with 12-hour light/dark phases under physiologic conditions with natural filling, and comparisons were made within the same animal.

This study used bladder pressure buildup as an indicator of urethral sphincter function and continence based on established preclinical evidence and current clinical practice. It is well documented that volume/pressure relationship significantly affects bladder compliance, and a well-functioning sphincter is imperative for rise in bladder pressure.[43] Moreover, decreased bladder pressure and urinary incontinence are highly correlated. In

fact, in children with epispadias, LPP measurement serves as an objective determinant of continence and a deciding factor for further surgical interventions.[44] Following MuSCs injection, we observed a return of Rise, BaseP and PeakP to pre-injury levels in the verum group, indicating a restoration of the injured urethral sphincter. A similar effect was not seen in the control group, which received an equal volume of cryopreservation medium. Our histologic examinations further showed restoration of the injured sphincter tissue in the verum group, evidenced by the observed normal tissue architecture. In the placebo group, injury-related changes such as scarring were still present. Our findings are further corroborated by previous studies that reported similar urodynamic and histologic findings.[30,32,36,45–47]

Our findings represent a great potential solution to a long-standing medical problem in children with epispadias, whereby functional restoration of the urethral sphincter can be achieved by injection of autologous cells in a minimally invasive manner. Current therapy options for incontinence in epispadias patients include: the most widely practiced surgical repair known as bladder neck reconstruction (BNR) and the less frequent injection of bulking agents. BNR is a major surgical operation with long hours of general anesthesia, significant perioperative risks, and lengthy recovery time. In this procedure, surrounding tissues are anatomically approximated to achieve some form of functional sphincter that enables continence. However, despite significant progress in the evolution of this surgical method, urinary continence has been a challenging goal to achieve so far; with reported continence rates varying from 37 to 88% and inconsistent among studies.[48–55] In fact, only 25% of EEC are expected to void normally per urethra without the use of catheterization or urinary diversion.[55] Injection of bulking agents has low success rates, very short-term results, and is associated with risks such as immune reaction and migration to other organs. In fact, it has been proven ineffective as a standalone therapy.[56] Our study demonstrates the use of a biocompatible autologous therapy in a low-risk surgical procedure and low risk of immune reactions resulting in a functional urethral sphincter. This in turn can potentially allow children with epispadias to achieve controlled voiding through the urethra without the need of further augmentation such as catheterization or urinary diversion.

The implications of this study extend beyond patients with epispadias and address the unmet medical need in all patients with urinary incontinence due to defects in the urethral sphincter. Urinary incontinence affects around 20% of people throughout their lifetime[57] and has shown an increase in the past few years to about 60% of adult women being

affected only in the United States.[58] The most common type of incontinence is Stress urinary incontinence (SUI) accounting for about 88% of all incontinent patients.[59] SUI is often a result of intrinsic urethral sphincter deficiency (anatomic and physiologic) and urethral hypermobility.[60] A recent review identified a total of 17 clinical trials conducted and openly published until the year 2020 using cell therapy in patients with stress incontinence.[61] Despite the heterogeneity of these studies in terms of cell source, cell cultivation techniques, cell number, injection techniques and patient population, all showed acceptable functional results in both male and female patients with minimal complications. Thus, use of MuSCs injection in combination of adjuvant therapies such as pelvic training in patients with urinary incontinence other than epispadias is foreseeable. Major challenges observed were the need for optimization of cell isolation and cultivation and adherence to regulatory requirements. Our study demonstrates the successful isolation of pure and highly myogenic MuSCs from a small muscle biopsy tissue contending the challenges of satellite cell cultivation. These cultivation techniques have shown reproducible in-vivo and in-vitro results and are scalable.[7,9]

Cell therapy belongs to the newly emerging category of therapy known collectively as advanced therapy medicinal products (ATMPs). ATMPs are highly innovative therapies that have gained focus in the last decade addressing several high unmet medical needs. These therapies are costly, highly individualized therapies that face several hurdles including higher quality and safety concerns, significant risks inherent to cell and gene therapies, ethical concerns, higher regulatory requirements, and unpredictable market success. Up to date, only 19 products have been approved for the market in the EU since 2008.[62] There are ongoing efforts to establish flexible and expedited processes and product-specific decision making from regulatory bodies to enable more products access to the market.[63,64] However, according to 2019 reports, ATMPs have a success rate of around 59%, which is lower than that of biopharmaceuticals (76%) and has withdrawal rates after approval of about 36%.[65,66] Adherence to regulatory requirements is not only crucial for approval and marketing authorization but also for success afterwards. Given that the 73.2% of ATMP products are sponsored by non-commercial organizations where 37% are investigator-initiated products, extensive preparation is needed from the investigator's end.[67] For our study, we actively involved national regulatory bodies in determining the relevant parameters of the preclinical efficacy study. We used a well-characterized cell product with documented reproducible results. Our cell product fulfilled the same criteria as would have been used in the upcoming clinical trial and all

experiments were conducted in near-GLP conditions. We are hopeful that this can help eliminate some of the hurdles during approval and clinical application.

Our study is the first study to use bladder telemetry for preclinical efficacy assessment in rats. Almost all previous studies used conventional cystometry, which requires restraint, anesthesia, saline infusion, and sacrifice. A longitudinal assessment within the same animal was never performed, and the interpretation of results was inconclusive due to interindividual differences. The use of bladder telemetry allowed measurement in physiologic conditions and longitudinal assessments within the same animal minimizing confounding factors and further validating the findings of previous studies. Furthermore, we were able to use a smaller number of animals while providing a more robust data collection from each animal.

We have attempted to bring our experimental design as close to clinical practice as possible by using a representative animal model, a functional urodynamic measurement tool and clinically relevant outcome parameters. Current clinical practice in epispadias patients involves the measurement of LPP around the age of 3 when continence training can adequately be undertaken. However, there have been several attempts in introducing the use of either catheter-based sensors or wireless implantable sensor in the bladder to allow determination of urodynamic parameters continuously.[68]

Marg et al., 2014[9] also showed that isolated MuSCs can successfully be genetically modified. In-situ genetic repair of mutations is now made feasible as a consequence of the development of gene editing technologies like the CRISPR-Cas system and more accurate editing tools like base and prime editors. This provides new opportunities to address the unmet medical need of patients with muscular dystrophy. Recent studies published by our group have shown the successful isolation of MuSCs from patients and successful *in-vitro* repair of genetic mutations in SGCA and LGMD genes thereby providing a cure to conditions such as Limb girdle muscular dystrophies (LGMDs).[69,70] Furthermore, the repaired MuSCs were able to regenerate muscle and repopulate the stem cell pool when transplanted into mice models. Muscular dystrophies are monogenetic disorders, where mutations in a single gene result in progressive and debilitating muscle degeneration and wasting. Thus, transplantation of genetically repaired MuSCs can provide functional improvement, delay progression of disease and perhaps even provide a cure in this patient population.

There were some limitations to our study. The small sample size and lack of replacements for excluded animals are worth mentioning. Our study did not record voiding behavior or bladder capacity. This could provide more insight into the change in the voiding pattern of the animals. Finally, the long-standing question of whether regeneration is solely caused by injected MuSCs or attributed to inherent inflammatory changes remains open to speculation. Furthermore, our study did not assess the long-term results of MuSCs injection. Only two timepoints of measurements were done. Additional measurements further in time could help determine whether the observed functional changes are persistent over time. However, the lack of non-invasive cell tracking of long-term survival and functionality has long been a focus of interest in cell therapy, with no promising results to date.

5. Conclusions

Preclinical studies with relevant animal models, design mirroring clinical practice and functional physiologic assessments are critical in yielding data needed to bridge the translational gap to the clinic. Moreover, we encourage pre-study counselling and adherence to national and international regulatory requirements from early design to analysis to bring innovative therapies to clinical practice at a faster pace. Despite its limitations, our study combined careful planning and review of existing evidence to demonstrate efficacy of our cell product. In light of our findings, translation into clinical practice is foreseeable.

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Statutory Declaration

“I, Biniam Melese Bekele, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Use of bladder telemetry to evaluate efficacy of muscle stem cells in a rat model of urethral sphincter injury; a preclinical efficacy study”/” Präklinische Studie zur Wirksamkeit von Muskelstammzellen in einem Rattenmodell für Verletzungen des Harnröhrenschließmuskels”, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

Biniam Melese Bekele contributed the following to the below listed publication:

Publication 1: Bekele BM, Schöwel-Wolf V, Kieshauer J, et al. Human primary muscle stem cells regenerate injured urethral sphincter in athymic rats. Anim Model Exp Med. 2022; 00:1-8. doi:10.1002/AME2.12280
Contribution:

- Conceptualization: formulation of study endpoints, study planning and design, literature review
- Methodology: selection of animal model, interim analysis and modification of chosen model
- Analysis and interpretation of data: Data clean-up and review, statistical analysis, interpretation, and presentation of results. Statistical analysis was initially done by Biniam Bekele and confirmed with an independent statistician as part of the regulatory requirement.
- Manuscript: preparation and creation of published work, all images and tables, Coordination with co-authors with review and editing of manuscript, Editing of manuscript according to journal guidelines, submission, response to reviewers and final submission for publication.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

Excerpt from Journal Summary List

The journal “Animal Models and Experimental Medicine” is currently not listed in the Journal summary list provided by the Charité. A detailed description of the journal and application for acceptance will be submitted by the supervisor.

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ORIGINAL ARTICLE

Human primary muscle stem cells regenerate injured urethral sphincter in athymic rats

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Abstract

Background: The aim of the study was to demonstrate the efficacy of human muscle stem cells (MuSCs) isolated using innovative technology in restoring internal urinary sphincter function in a preclinical animal model.

Methods: Colonies of pure human MuSCs were obtained from muscle biopsy specimens. Athymic rats were subjected to internal urethral sphincter damage by electrocauterization. Five days after injury, 2×10^5 muscle stem cells or medium as control were injected into the area of sphincter damage ($n = 5$ in each group). Peak bladder pressure and rise in pressure were chosen as outcome measures. To repeatedly obtain the necessary pressure values, telemetry sensors had been implanted into the rat bladders 10 days prior to injury.

Results: There was a highly significant improvement in the ability to build up peak pressure as well as a pressure rise in animals that had received muscle stem cells as compared to control ($p = 0.007$) 3 weeks after the cells had been injected. Only minimal histologic evidence of scarring was observed in treated rats.

Conclusion: Primary human muscle stem cells obtained using innovative technology functionally restore internal urethral sphincter function after injury. Translation into use in clinical settings is foreseeable.

KEYWORDS
human muscle stem cells, sphincter injury, telemetry, urinary incontinence, urodynamics

1 | INTRODUCTION

Muscle regenerates through activation and proliferation of satellite cells, the primary skeletal muscle stem cells (MuSCs). Moreover, cell populations derived from satellite cells retain their stem cell capacity and promote regeneration.[1] Transplantation of muscle stem cells is a potential treatment option for a variety of diseases. However, clinical application to date has been hampered by (i) lack of a validated definition of the cell product to be administered, (ii) lack of well defined medical conditions to be treated, and (iii) limitations in producing sufficiently large number of cells with high regenerative myogenic capacity. We have introduced a new method to successfully isolate highly myogenic satellite cells.[3,4] In this study, we aimed to demonstrate the efficacy of such isolated cells in a preclinical model of internal urethral sphincter injury.

Urinary incontinence is a very common disorder, with an estimated prevalence of 53% in women and 11% in men.[2] In recent decades, transplantation of MuSCs raised expectations of an effective therapy but results are still inconclusive.[7–11] The diversity of underlying pathology and the consequent difficulty in finding adequate models pose significant challenges for preclinical studies.[3] Furthermore, conventional cystometry measurements, necessary to characterize changes in lower urinary tract function, often entail a single, terminal measurement in sedated or awake animals through artificial bladder filling to stimulate the micturition reflex.[4] A commonly used approach is the leak point pressure (LPP) measurement,[5,6] which is defined as the pressure at which leakage occurs. However, this measurement creates a drastic diversion from normal physiology as transection of the spinal cord is required to acquire the desired measurement.[7,8] Bladder telemetry, however, enables continuous recording of more accurate and physiologic urodynamic parameters in awake, freely moving unrestrained animals without the need for artificial bladder filling.[9,10] In addition, it allows repeated measurements across different time points within the same animal,[10–14] making it ideal to assess the efficacy of a therapy.

We selected urinary incontinence in isolated epispadias as the first-in-human indication to demonstrate functional efficacy of MuSCs prepared by our innovative methods[1] (Eudra-CT Nr. 2021-002004-13). In epispadias, there is a defined anatomical congenital defect in the internal urethral sphincter muscle that cannot be functionally restored surgically. The defect leads to lifelong incontinence unless major and debiliating operations like bladder neck reconstruction are performed. There is no animal model of epispadias. The chosen protocol reported here comes as close as possible to the anatomical defect of epispadias and has been presented as an efficacy study to regulatory bodies.

2 | METHODS

2.1 | Preparation of cells

MuSCs were prepared as described in Marg et al., 2014.[1] A muscle specimen was acquired through biopsy from a 14-year-old boy without neuromuscular disorders (ethical approval EA1/203/08, Charité) after written informed consent was obtained. Using manual dissection, muscle fragments were isolated and placed in hypothermic treatment (4–6°C) for 7 days. Muscle fragments were cultivated in skeletal muscle cell growth medium (SMCGM, Provitro) in a humidified atmosphere containing 5% CO_2 at 37°C. The outgrowing muscle stem cells were propagated, characterized and frozen. Selected >95% desmin positive colonies were thawed and concentrated as 10×10^6 cells/ml in a cryopreservation medium.

2.2 | Animals

Ten nude male rats (Crl:NIH-Foxn1^{rnu}, 250–300 g; Charles River Laboratories, Inc.) were used. Animals were kept in a regular 12-h light/dark cycle with food and water provided ad libitum. All animal experiments were conducted at Charles River's AAALAC-accredited animal facility and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Procedures were conducted under veterinary supervision with appropriate anesthesia and analgesia protocols.

2.3 | Experimental design

A pressure transducer was surgically implanted into the bladder of each animal. After a recovery period of 7 days, baseline urodynamic measurement (pre-injury) was done. On day 10, the urethral sphincter was injured in all animals by electrocauterization. Five days later, in the verum group ($n = 5$) MuSCs and in the placebo group ($n = 5$) placebo (cryopreservation medium) was injected. Three weeks after injection, a second urodynamic measurement (post-injection) was done, and all animals were sacrificed. Figure 1 shows a timeline of the experimental design.

2.4 | Telemetry

The transducer used was the Data Sciences International (DSI) HD-S10, which allows pressure, activity and temperature measurement in small animals. Zero-offset of the pressure channels was verified prior to implantation (accuracy ± 3 mmHg). Each rat was anesthetized with isoflurane (0.5%–3%) and placed in the supine position with the lower legs abducted. We performed a midline laparotomy and secured the pressure transducer in the bladder using a purse string suture and additional stay sutures as needed. The transmitter was secured in the abdominal cavity or subcutaneously.

2.5 | Urethral injury

The urethral sphincter injury model was used as previously described.[15] In anesthetized animals, bladder and urethra were exposed through a midline laparotomy. A urinary catheter was placed

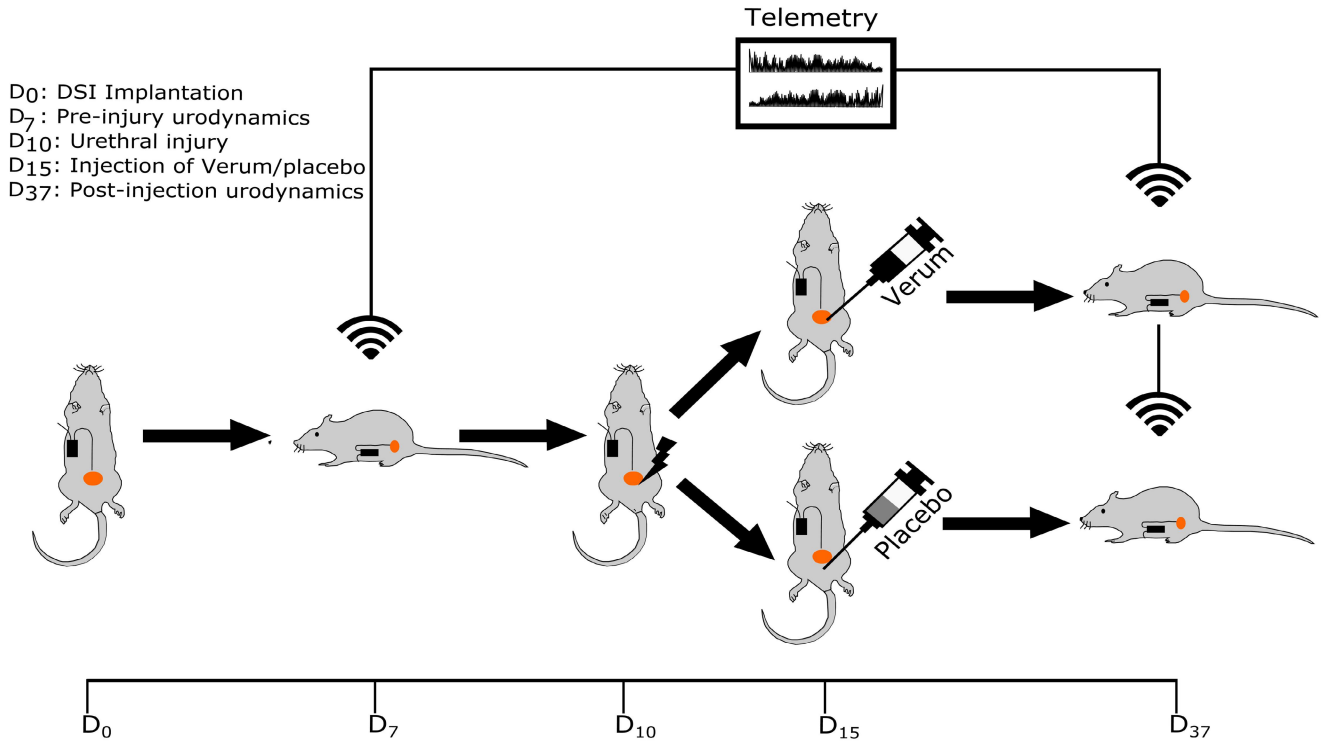

FIGURE 1 Experimental design. DSI was surgically implanted (D_0) followed by the first urodynamic measurement (pre-injury, D_7). On day 10 (D_{10}) the urethral sphincter was injured using electrocauterization followed on day 15 (D_{15}) by the injection of either MuSCs or placebo into the urethral sphincter. The second urodynamic measurement (post-injection) was done on day 37 (D_{37})

in the urethra for better visualization. Tissues approximately 1 cm caudal to the bladder (caudal to the prostate) and extending to the edge of the pelvis were cauterized using a fine tip high-temperature cautery (Bovie Medical). Both sides were cauterized for 60 s.

2.6 | Injection

Five days after electrocauterization, a midline lower abdominal incision was made and the internal urethral sphincter identified. Four periurethral injections (5 μl per site) were directed into the sphincter with two injections on each side. The verum group received an injection of MuSCs (2×10^5 cells/animal) suspended in a cryopreservation medium while the control group received an equal volume of cryopreservation medium only.

2.7 | Histologic evaluation

On day 38 animals were sacrificed, the urethra and urinary bladder were removed in toto, embedded in paraffin, sectioned, mounted on glass slides and stained with hematoxylin and eosin (H&E). For the urinary sphincter administration site, the prostate was removed in such a way as to leave the tissue immediately surrounding the urethra intact. The urinary sphincter administration site was trimmed to include the whole intact distal portion of the urinary bladder, urinary sphincter region (prostatic urethra), and adjacent distal urethra. The dorsal surface was marked with tissue dye to indicate orientation for embedding. Once the lumen of the urethra was identified microscopically, sections were obtained and stained with H&E.

2.8 | Statistical analysis

All urodynamic parameters are presented as median values ± SD and *p* values less than 0.05 were considered as statistically significant. The raw data from the 24-h recordings was adjusted for extreme values, using winsorization at the 1st and 99th percentiles. To account for inter-individual variability, the difference between pre-injury and post-injection values was calculated for each animal and analyzed as the primary outcome variable. We used Student's *t* test or the Wilcoxon test, as appropriate, to compare values between groups. All statistical analysis was performed using R Statistical Software (version 2.14.0; R Foundation for Statistical Computing).

3 | RESULTS

Three pressure parameters, namely peak pressure (PeakP), base pressure (BaseP) and rise, and three temporal parameters, namely period, peak duration (PeakD) and inter-contraction interval (ICI), were recorded using the bladder telemetry. These parameters were defined as follows: PeakP is the pressure at the peak of a contraction, while BaseP is the pressure at the beginning of a contraction,

and the difference between the two is defined as Rise. Period refers to the time between beginning of one contraction and the next while PeakD is the time from start to end of one contraction and ICI represents the time between the end of one peak and the beginning of the next (Figure 2).

3.1 | Diurnal variation

Telemetry parameters were continuously reported for a duration 24 h with measurements taken every 5 min. Typical 24-h monitoring traces using bladder telemet are depicted in Figure 3.

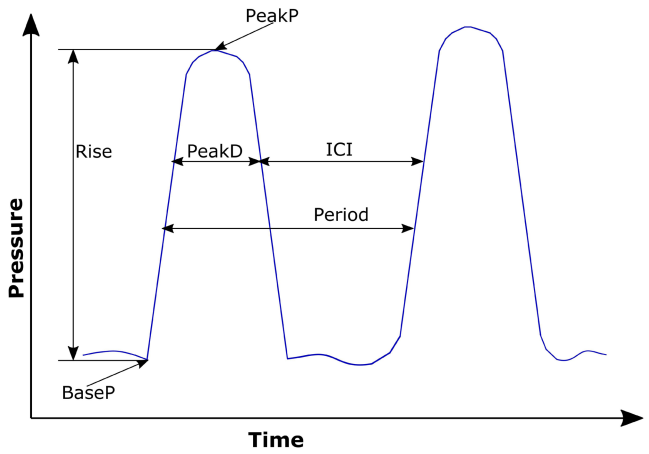

FIGURE 2 Graphic description of telemetry parameters. BaseP, base pressure; ICI, inter-contraction interval; PeakD, peak duration; PeakP, peak pressure

Rats were entrained to a regular 12-h light/dark cycle. We compared telemetry parameters between light and dark phases for all animals using the pre-injury recordings. Significantly higher PeakP, BaseP and shorter period and ICI values were seen during the dark phase while no difference was seen in Rise and PeakD. (See Table 1 below).

3.2 | Urodynamic parameters

Three animals (n = 1 verum group and n = 2 control group) were excluded from analysis of changes post-injection due to technical errors. Post-injection values in these animals were abnormally high due to misplaced sensor chords.

Representative bladder pressure tracings post-injection are depicted in Figure 4. There were no statistically significant differences between verum and control groups at both pre-injury and post-injection time points. Comparing changes in urodynamic parameters between the two time points, a significantly higher decrease in all three pressure parameters was seen in the control group (Delta PeakP: control = −2.4 ± 0.7, verum = 0.0 ± 0.7, p = 0.007; Delta BaseP: control = −1.7 ± 0.5, verum = 0.2 ± 0.7, p = 0.020; Delta rise: control = −0.69 ± 0.53, verum = 0.1 ± 0.24, p = 0.043). The changes in period, PeakD and ICI were not statistically significant between the two groups (Delta period: control = 9470 ± 9947, verum = 1690 ± 8045, p = 0.703; Delta PeakD: control = 1666 ± 3519, verum = 1490 ± 3003, p = 0.394, Delta ICI; control = 1870 ± 2215, verum = −578 ± 1138, p = 0.385) (See Figure 5). There was no statistically significant difference in all values between pre-injury and post-injection in the verum group.

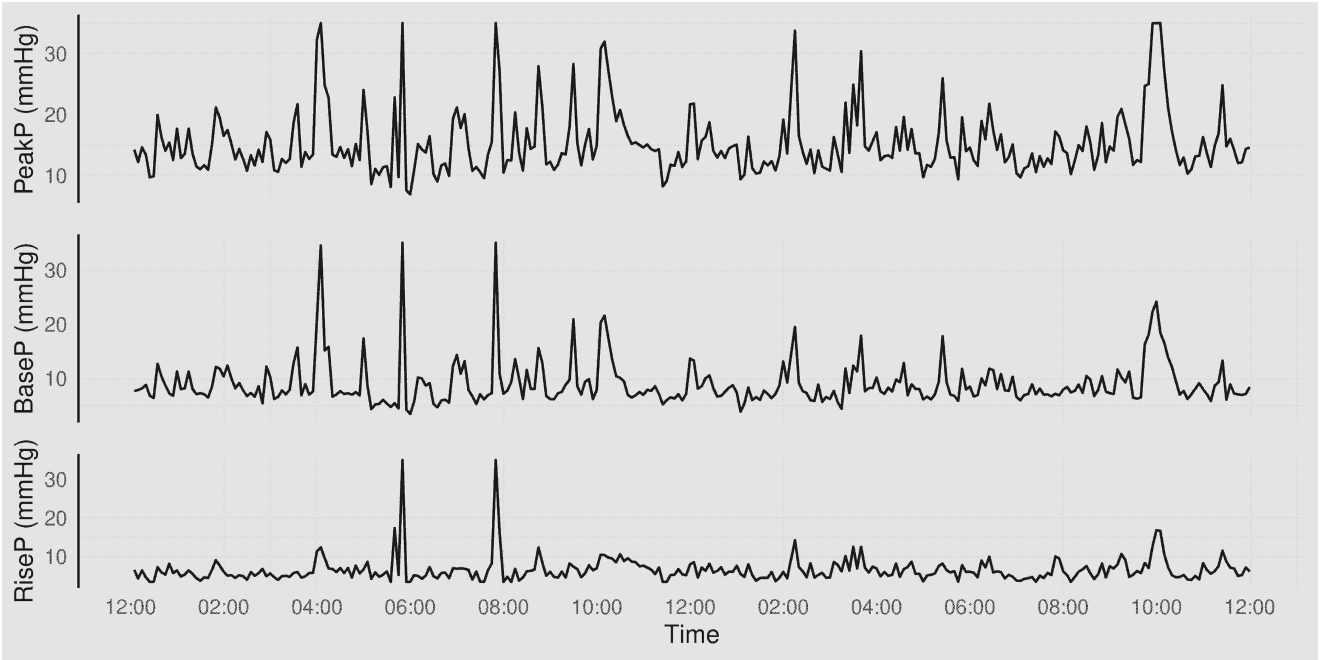

FIGURE 3 A full 24-h monitoring trace of conscious rats using bladder telemetry. These traces were recorded pre-injury. Animals were kept in a 12-h light/dark cycle. The first 12 h represent the light hours

3.3 | Histologic findings

In the complete removed bladder-urethra unit, injury-related tissue changes were seen only in the placebo group. The verum group showed normal sphincter tissue architecture with no signs of injury related changes. (Figure 6).

TABLE 1 Comparison of telemetry parameters pre-injury between light and dark phase

Parameter	Light phase	Dark phase	*p**
PeakP (mmHg)	11 ± 10.4	12 ± 2.9	**0.001**
BaseP (mmHg)	7.5 ± 1.8	7.8 ± 1.9	**0.001**
Rise (mmHg)	3.9 ± 1.8	4.0 ± 2.7	0.215
Period (sec)	12.5 ± 19.2	10.9 ± 11.1	**0.001**
PeakD (sec)	3.6 ± 0.8	3.6 ± 0.7	0.424
ICI (sec)	8.4 ± 19.6	7.2 ± 11.4	**0.002**

Note: All values indicate median ± SD. Significant results are highlighted in bold.

Abbreviations: BaseP, base pressure; ICI, intercontraction interval; PeakD, peak duration; PeakP, peak pressure.

*Wilcoxon test.

4 | DISCUSSION

In this study, we evaluated the efficacy of human MuSCs in a urethral sphincter injury rat model. By using bladder telemetry, longitudinal and minimally invasive assessments of urodynamic parameters under physiologic conditions was possible. More importantly, it enabled the assessment of intraindividual changes within animals before and after the injection. Furthermore, the return of pressure parameters to pre-injury levels suggested a functional restoration of the damaged sphincter after injection of MuSCs.

Our results demonstrate, as expected, a diurnal variation in bladder function. The dark phase, which corresponds to the rats' active phase, was characterized by increased PeakP and BaseP, and frequent contractions, as evidenced by shorter Period and ICI. Rats demonstrated lower PeakP, BaseP, and fewer frequent contractions during the light (inactive) phase. This is consistent with prior studies that showed a circadian difference in bladder capacity and micturition frequency in rodents with no surgery[16,17] and chronically catheterized animals.[18,19] We do, however, report a difference in diurnal bladder pressure that has not previously been reported. In fact, Herrera et al,[20] observed no difference in the average bladder pressure between light and dark phases. This could be attributed to differences in experimental design. In this study, urodynamic parameters were measured in response to artificial bladder filling

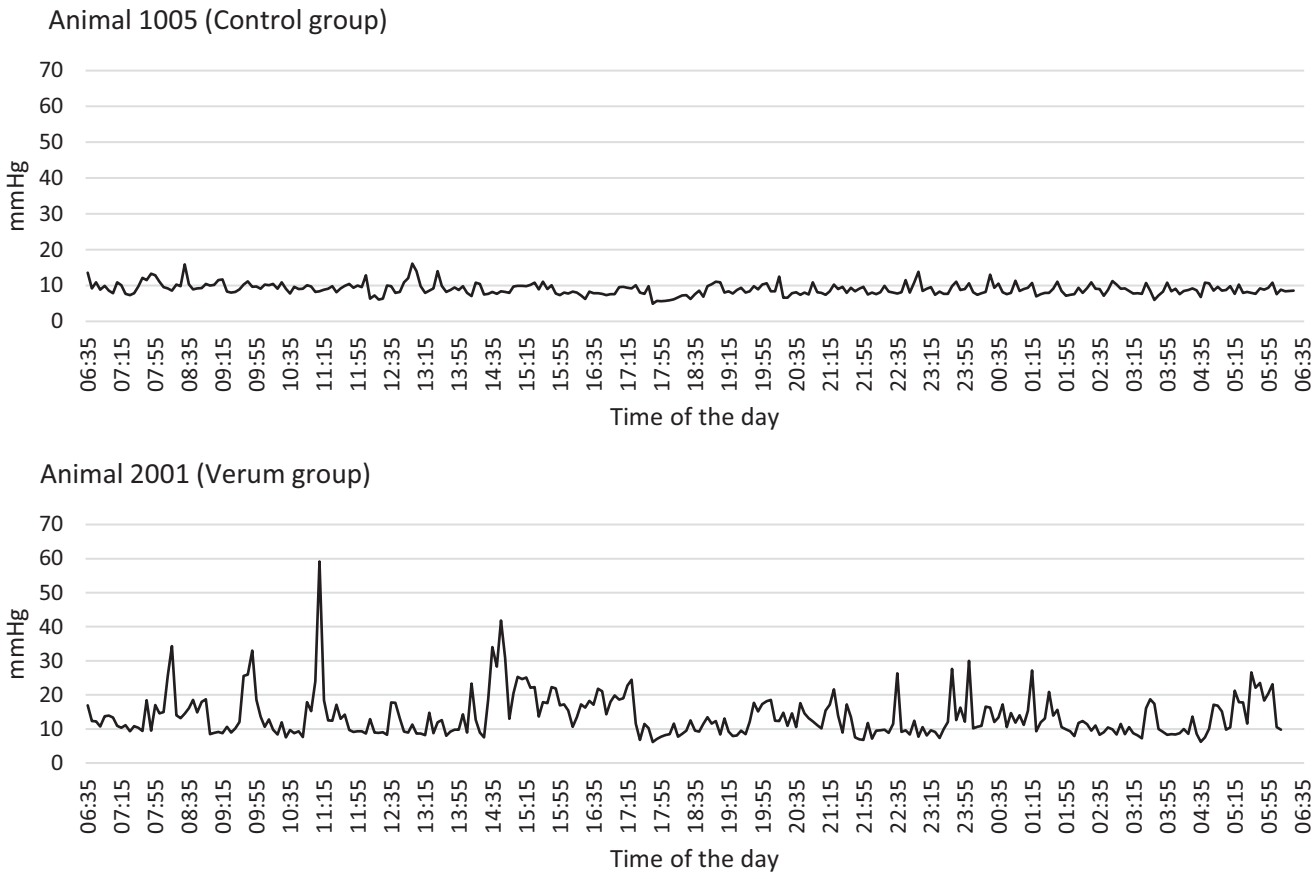

FIGURE 4 Representative post-injection bladder pressure tracings during 24 h of measurement. Pressure is measured in mmHg and corresponding time of the day is indicated in the x-axis

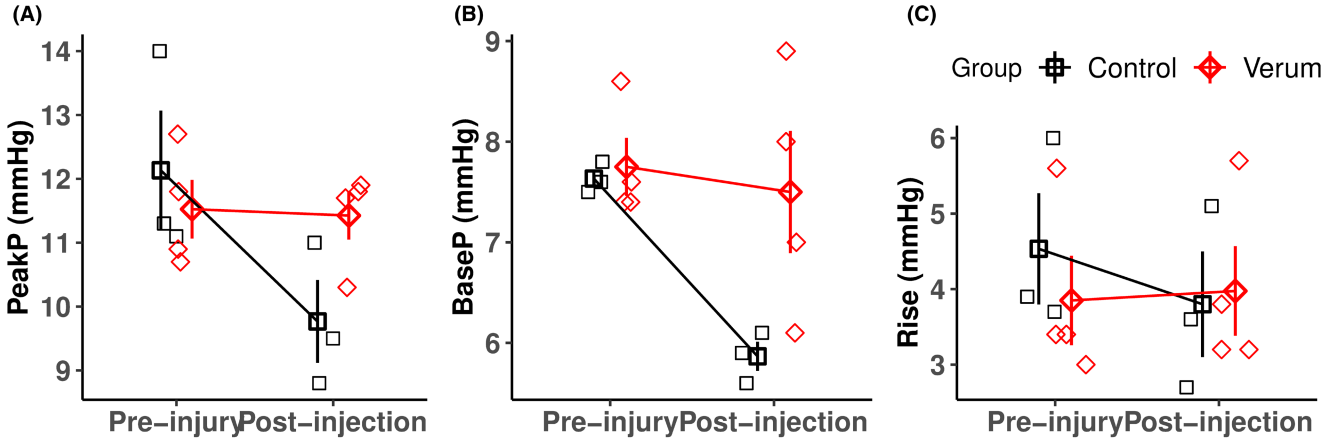

FIGURE 5 Change in urodynamic parameters between pre-injury and post-injection. Each point represents the 24 h median value of each animal. Control $n = 3$, verum $n = 4$. BaseP, base pressure; PeakP, peak pressure; Rise, difference between PeakP and BaseP

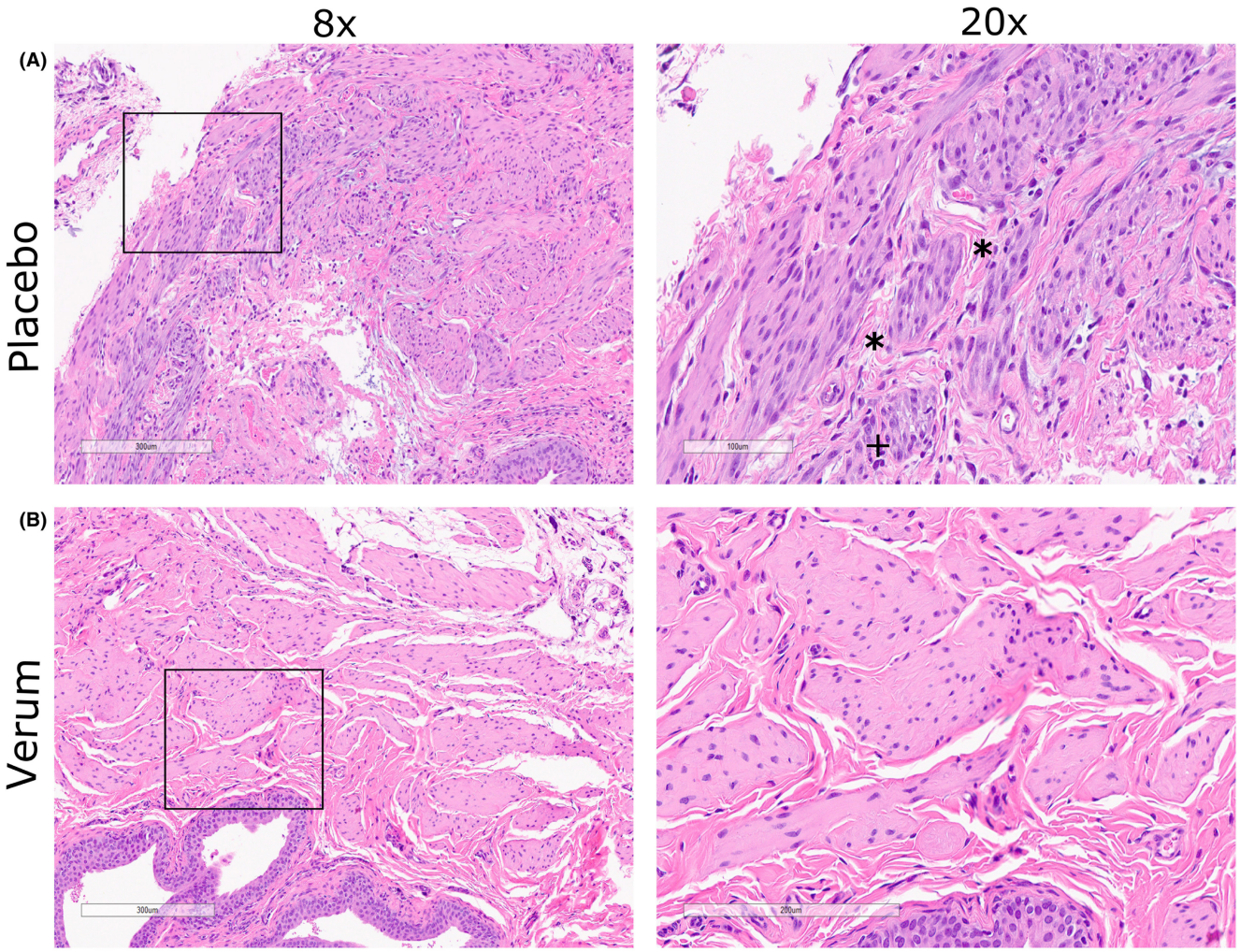

FIGURE 6 Histologic findings in urethral sphincter post-injection. (A), Hematoxylin & Eosin staining showing injury related tissue changes in the placebo group (*, scarring; +, degenerative changes) (B), Hematoxylin & Eosin staining showing normal urethral sphincter tissue architecture in the verum group

with saline, which does not represent the physiologic urine production cycle. Cystometrograms were measured only for a period of 30–90 min and pressure differences were compared between two different groups (light versus dark group). Thus, results are significantly impacted by inter-individual differences. In our study, comparisons were made within the same animal using continuous

recording over a 12-h light/dark period and urine production was under physiologic control with natural filling.

The presence of a well-functioning urethral sphincter is crucial for bladder pressure buildup. The relationship between incontinence and decreased bladder pressure is well documented.[21] In our study, injection of MuSCs resulted in return of PeakP, BaseP and Rise to pre-injury levels in the verum group. This suggests restoration of the injured urethral sphincter. In the control group, no similar effect was observed, indicating that the improvement in sphincter function was most likely associated with MuSCs. Other studies showing similar results in animal models of urethral sphincter injury further support these findings.[5,15,22] However, in all these studies, functional restoration was assessed using conventional cystometry, specifically LPP measurement. This necessitates anesthesia, restraint, and infusing saline into the bladder. In addition, it is a terminal procedure with spinal cord transection. Thus, the interpretation of efficacy was limited by the effect of interindividual variability, as conclusions were drawn based on comparison among animals. We were able to address this limitation in our study by using bladder telemetry; a continuous monitoring in freely moving animals that allows repeated measurements.

Restoration of the urethral sphincter injury is also corroborated by histologic findings. Injury related tissue structural changes were only seen in the placebo group whereas the verum group exhibited normal urethral sphincter tissue post-injection. This is in line with previous studies that showed integration of the injected MuSCs into the urethral sphincter muscle and intact tissue architecture weeks after injection.[5,6,23,24]

This study has a few drawbacks. A larger sample size could help generate more data. Information regarding bladder capacity and micturition pattern were not gathered. Thus, we are unable to comment on the relationship between the changes observed and micturition. Furthermore, whether the changes seen in the verum group are purely due to the regenerative nature of the transplanted cells or the transient release of regenerative cytokines remains an open discussion.[25] These factors should be carefully considered when interpreting the data presented in our study.

In conclusion, functional measurements that allow objective outcome assessment and yield robust data play a pivotal role in bridging the translational gap to the clinic. In this study, we show that periurethral injection of MuSCs restores urethral sphincter function using bladder telemetry in freely moving animals. Translation into clinical practice is foreseeable.

AUTHOR CONTRIBUTIONS

Study concept and design: SS, VS, JK, BB, AM; Acquisition of data: SD, GN; Analysis and interpretation of data: BB, VS, SS, AE, AM; Preparation of the manuscript: BB, SS, VS; Critical revision of the manuscript for important intellectual content: SS, AE, VS, AB; Statistical analysis: AB, BB.

ACKNOWLEDGMENTS

The data presented here are part of the preclinical safety and efficacy study required to perform a first-in-human phase 1/2a clinical trial to test safety and efficacy of primary human muscle stem cells in isolated epispadias. We thank the donors for contributing tissue and cells to this project. The study was supported by the SPARK program of the Berlin Institute of Health. The subsequent clinical trial and Dr. Bekele's position is being financed by the German Ministry for Education and Research (BMBF), Grant number 01EN2002.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, BB, upon reasonable request.

ETHICS STATEMENT

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Muscle biopsy was approved by the Institutional Review Board of Charité – Universitätsmedizin Berlin (EA1/203/08, Charité Berlin). Written informed consent was obtained.

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publication list

No.	Publication	Impact factor
1.	Bungert AD, Urbantat RM, Jelgersma C, **Bekele BM**, Mueller S, Mueller A, Felsenstein M, Dusatko S, Blank A, Ghori A, Boehm-Sturm P, Koch SP, Vajkoczy P, Brandenburg S. Myeloid cell subpopulations compensate each other for Ccr2-deficiency in glioblastoma. Neuropathol Appl Neurobiol. 2022 Nov 8:e12863. doi: 10.1111/nan.12863. Epub ahead of print. PMID: 36346010	6.250
2.	**Bekele, BM**, Schöwel-Wolf, V, Kieshauer, J, et al. Human primary muscle stem cells regenerate injured urethral sphincter in athymic rats. Anim Models Exp Med. 2022; 00: 1- 8. doi: 10.1002/ame2.12280	N/A
3.	**Bekele BM**, Luijendijk M, Schagen SB, de Ruiter M, Douw L. Fatigue and resting-state functional brain networks in breast cancer patients treated with chemotherapy. Breast Cancer Res Treat. 2021 Oct;189(3):787-796. doi: 10.1007/s10549-021-06326-0. Epub 2021 Jul 14. PMID: 34259949; PMCID: PMC8505321	4.624
4.	**Melese, B**., Bayu, B., Wondwossen, F. et al. Prevalence of mental distress and associated factors among Hawassa University medical students, Southern Ethiopia: a cross-sectional study. BMC Res Notes 9, 485 (2016). https://doi.org/10.1186/s13104-016-2289-7	2.150

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