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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

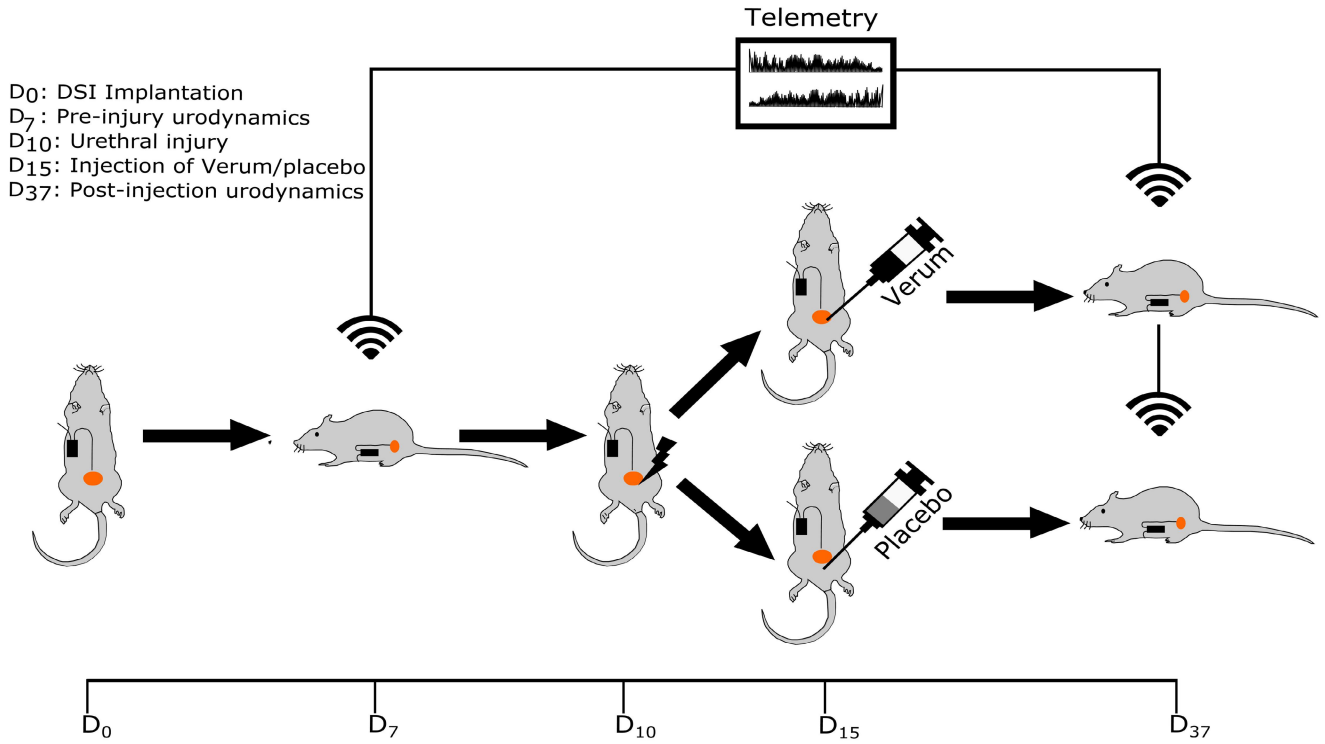

FIGURE 1 Experimental design. DSI was surgically implanted (D_0) followed by the first urodynamic measurement (pre-injury, D_7). On day 10 (D_{10}) the urethral sphincter was injured using electrocauterization followed on day 15 (D_{15}) by the injection of either MuSCs or placebo into the urethral sphincter.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

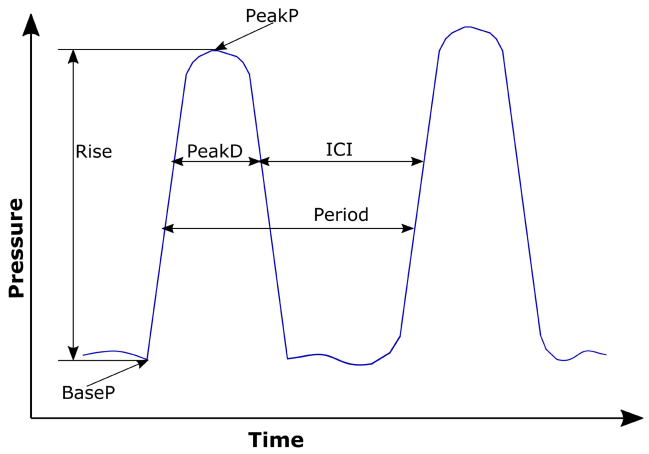

FIGURE 2 Graphic description of telemetry parameters. BaseP, base pressure; ICI, inter-contraction interval; PeakD, peak duration; PeakP, peak pressure

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.

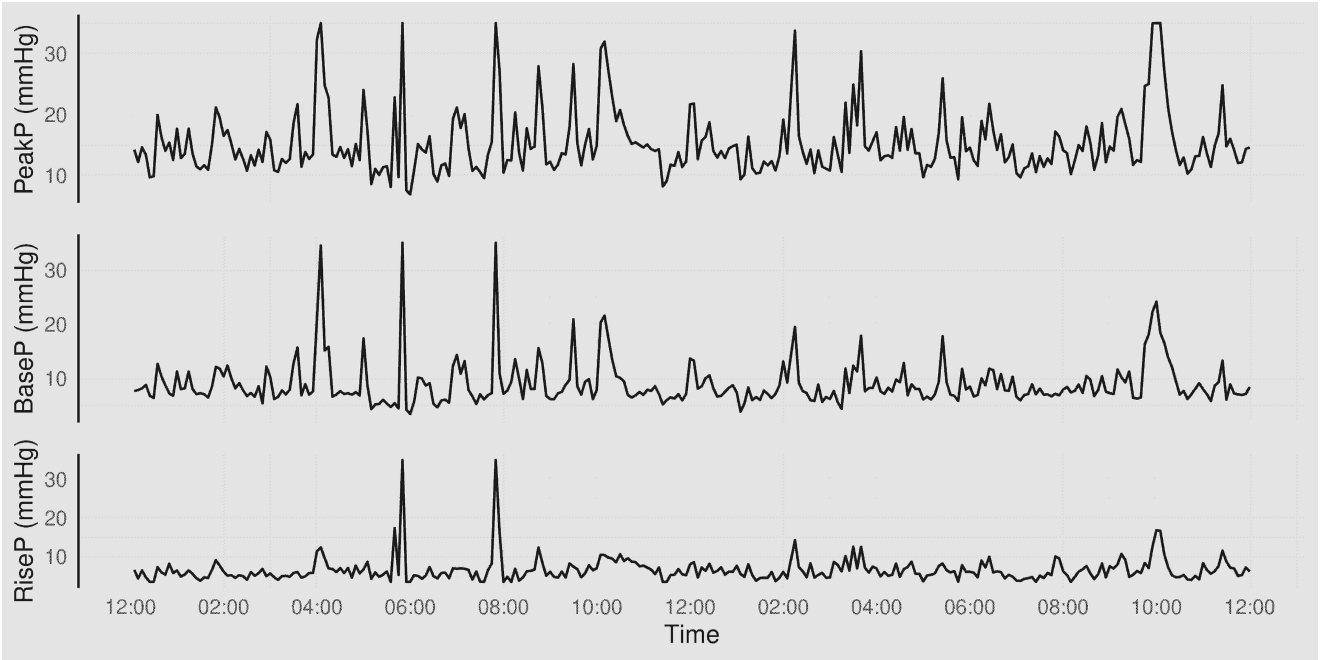

FIGURE 3 A full 24-h monitoring trace of conscious rats using bladder telemetry. These traces were recorded pre-injury. Animals were kept in a 12-h light/dark cycle. The first 12 h represent the light hours

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

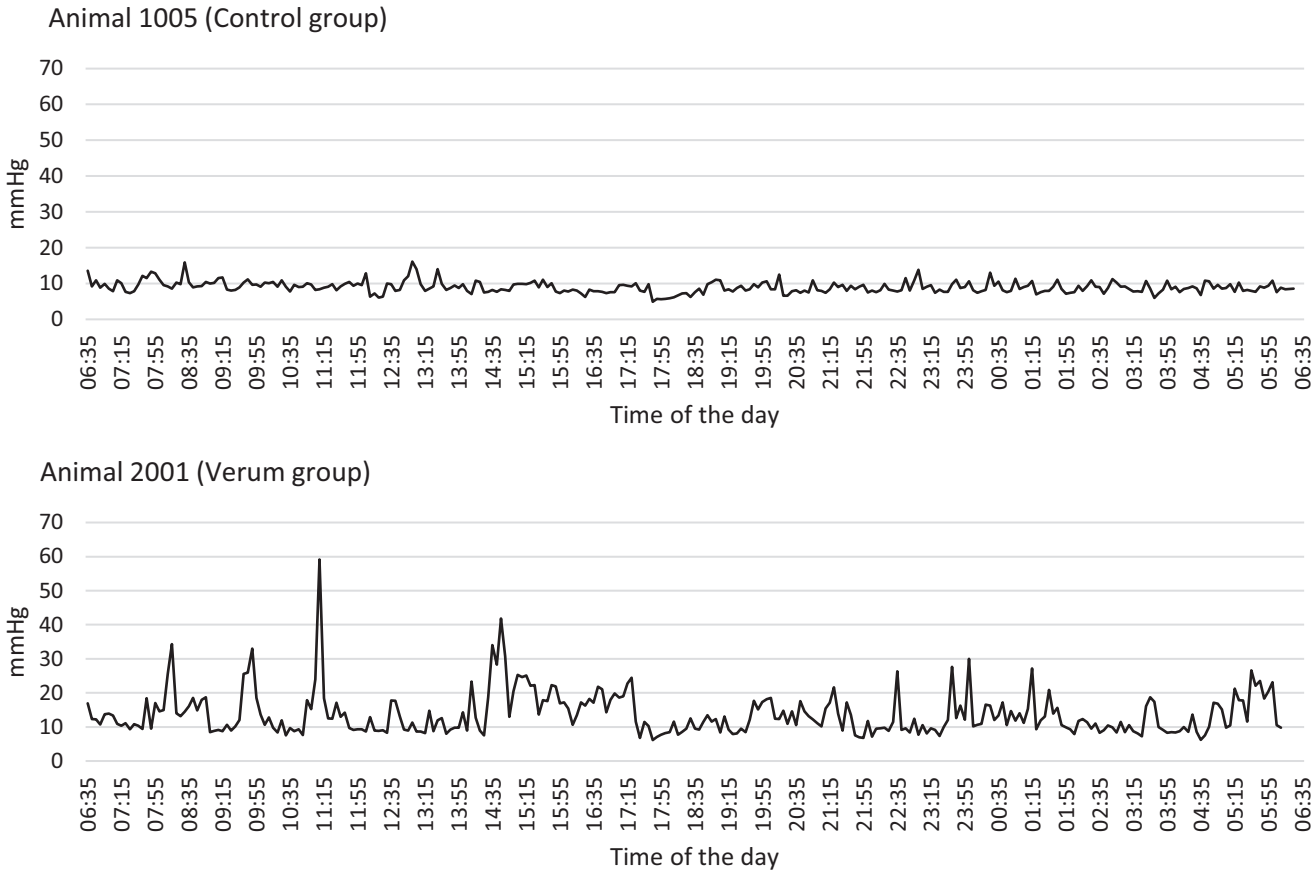

FIGURE 4 Representative post-injection bladder pressure tracings during 24 h of measurement. Pressure is measured in mmHg and corresponding time of the day is indicated in the x-axis

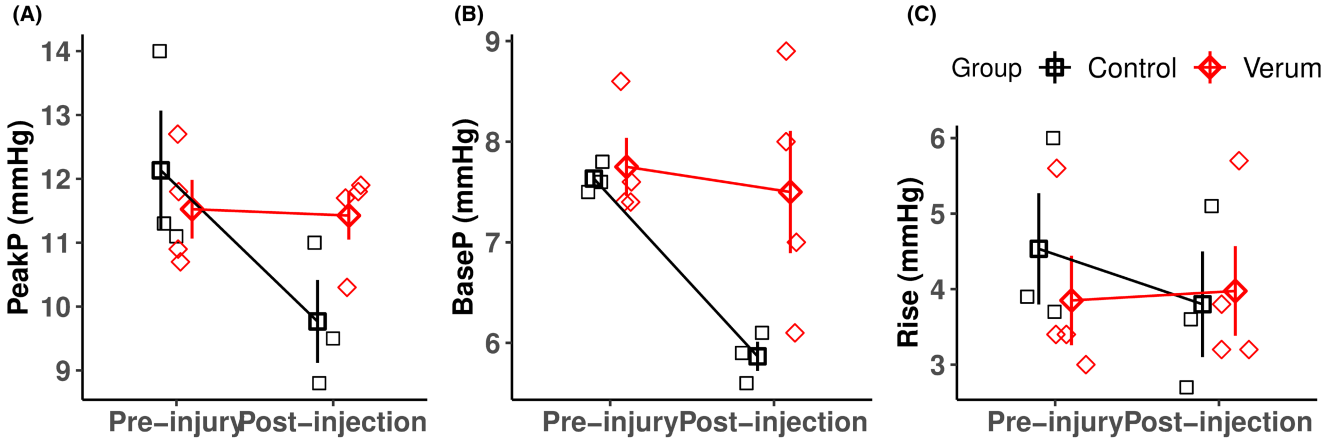

FIGURE 5 Change in urodynamic parameters between pre-injury and post-injection. Each point represents the 24 h median value of each animal. Control $n = 3$, verum $n = 4$. BaseP, base pressure; PeakP, peak pressure; Rise, difference between PeakP and BaseP

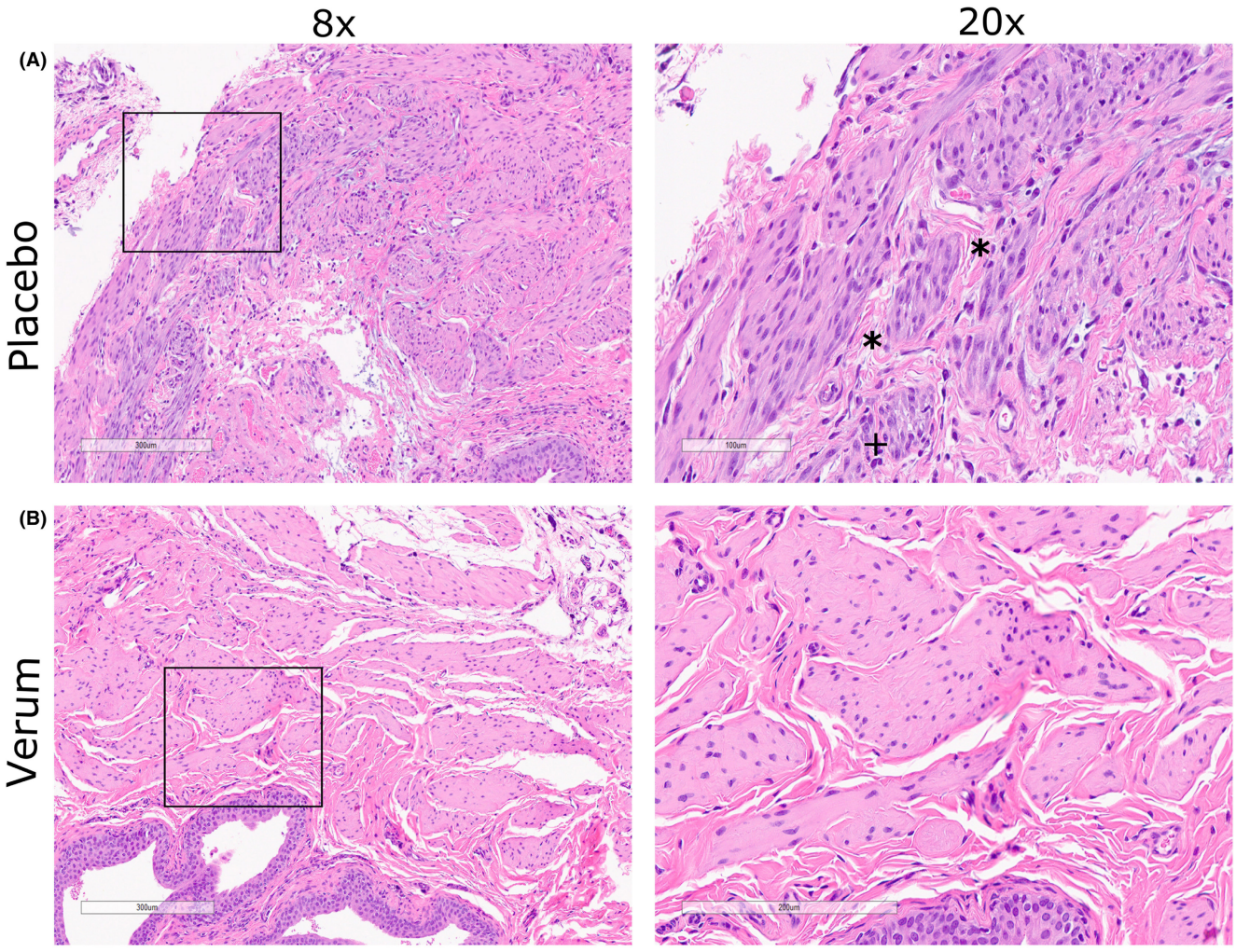

FIGURE 6 Histologic findings in urethral sphincter post-injection. (A), Hematoxylin & Eosin staining showing injury related tissue changes in the placebo group (*, scarring; +, degenerative changes) (B), Hematoxylin & Eosin staining showing normal urethral sphincter tissue architecture in the verum group

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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