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Treatment of erectile dysfunction in the obese type 2 diabetic ZDF rat with adipose tissue-derived stem cells

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Abstract

Introduction—Impotence, or erectile dysfunction (ED), is a major complication of type-II diabetes, and many diabetic men with ED are refractory to common ED therapies.

Aim—To determine whether autologous adipose tissue derived stem cells (ADSC) injected into the penis of impotent obese type-II diabetic rats survive and improve erectile function.

Main outcome measures—Intracorporal pressure (ICP) increase with cavernous nerve (CN) electrostimulation, immunohistochemistry, real-time PCR, and serum glucose and testosterone assays.

Methods—Twenty-two 10-week old male fatty type-II diabetic ZDF rats underwent weight and blood glucose measurement every 2 weeks. At age 22 weeks, all animals underwent unilateral CN electrostimulation and ICP measurement to confirm impotence, and paragonadal adipose tissue (5 grams) was harvested and digested to yield 1.5 million ADSC. Impotent animals were randomized to ADSC treatment and sham control groups. At age 23 weeks, treatment group animals underwent penile injection of 1.5 million ADSC; control group animals received only PBS. Erectile function studies were repeated at age 26 weeks, followed by harvest of tissue and serum.

Results—Pre- and post-treatment stimulation ICP increase was significantly different between groups ($p < 0.002$). In the control group, mean (SD) pre- and post-treatment stimulation ICP increase was 33.8 (15.9) and 31.4 (24.3) cmH₂O, respectively, whereas in the treatment group they were 27.4 (14.8) and 65.3 (15.4) cmH₂O.

BrdU-labeled ADSC were observed within corporal tissue of the treatment group. TUNEL staining ($p < 0.0001$) and caspase-3 m-RNA expression ($p < 0.05$) were significantly higher within corporal tissue of control group versus treatment group animals.

Conclusion—Autologous ADSCs injected into the penis appear to survive and improve erectile function. Autologous ADSC therapy is a promising approach to treat diabetic impotence.

Introduction

Erectile dysfunction (ED) is a major health problem that has profound effects upon the quality of life of both patients and their partners. Recent advances in our understanding of the pathophysiologic molecular mechanisms involved in ED suggest that it is a disease of predominantly neuro-vascular etiology. First-line therapy for the majority of patients today is use of selective phosphodiesterase type-5 inhibitors (PDE-5I), as these are a convenient oral form of therapy associated with reasonable efficacy (>70%). However, ED in the diabetic

population, is significantly more difficult to manage and oral medications are associated with less efficacy [1,2]. A growing body of evidence suggests that the pre-diabetic state of insulin-resistance is an independent risk factor for many of the morbid effects of type-II diabetes [3–8]. In the U.S. 90–95% of diabetics have type-II versus type-I diabetes [9].

The ZDF obese type-II diabetic rat (Charles River, Wilmington, MA) has non-functional leptin receptors and develops hyperphagia, obesity, metabolic syndrome, and type-II diabetes [10]. In a previous pilot study, we have shown that ~85% of male ZDF fatty type-II diabetic rats first develop impotence, defined as intracorporal pressure (ICP) increase of <60 cmH₂O in response to direct bilateral cavernous nerve (CN) electrostimulation, at age 21–22 weeks.

To our knowledge, use of adipose tissue-derived stem cells (ADSC) for the treatment of diabetes-related ED has not been reported, and the fate of autologous unmodified ADSC injected into the penis has not been described. In this study, we assess whether autologous ADSC injected into the penis of impotent type-II diabetic ZDF rats survive in-vivo, and whether they can restore erectile function.

Methods

Animals

A total of 22 ZDF type-II diabetic male rats (10 weeks old) were generously provided by Charles River Laboratories. All experimental protocols were approved by the Institutional Animal Care and Use Committee at University of California San Francisco. Body weight and glucometer tail-vein blood glucose levels (Bayer HealthCare, Tarrytown, NY) were measured bi-weekly.

Determination of Erectile Function

Surgery in all animals was performed under 2% isoflurane anesthesia. Isothermia was maintained at 37°C with a heating pad. Using a low abdominal midline incision, the right major pelvic ganglion was exposed, and the ipsilateral CN was identified. Next, the right base of the penis was exposed and the right corporal body was cannulated with a 23-G butterfly needle primed with 250 U/ml heparin-saline solution and connected to a pressure transducer (Utah Medical Products, Midvale, UT). A bipolar stainless steel hook electrode was used to directly stimulate the right cavernous nerve. A signal generator (National Instruments, Austin, TX) generated monophasic rectangular pulses. Stimulus parameters were 1.5 mA, 20 Hz, pulse-width 0.2 ms, and duration 50 seconds. The ICP was recorded at a rate of 10 samples/s using a sensor input module (model SCXI 1121, National Instruments) connected to a computer with LabView 6.0 software (National Instruments). Maximum ICP was recorded. If ICP increase during electrostimulation was less than 60 cmH₂O (classified as impotent), then ~5 grams of paragonadal adipose tissue was harvested for processing to isolate ADSC and placed in saline at 4°C. All impotent animals were randomly assigned to control or ADSC treatment groups. Aortic blood pressure was measured at time of sacrifice at age 26 weeks and mean arterial pressure was calculated. Animals with baseline ICP increase (Δ) >90 cmH₂O were excluded from the study.

Adipose tissue-derived stem cell (ADSC) isolation, culture, and autologous injection

ADSC were isolated from adipose tissue specimen using a modified version of a previously published protocol [11]. Briefly, within 4 hours of harvest, the tissue was incubated in a solution containing 0.075% collagenase Type IA (Sigma-Aldrich, St. Louis, MO) for 1 hour, at 37°C with vigorous shake for 15 seconds in 20 minute intervals. After centrifugation, the resulting pellet, which is defined as the adipose tissue stromal-vascular-fraction (SVF), was exposed to lysis buffer for 10 minutes to remove red blood cells. The remaining cells were suspended in

DMEM media supplemented with 10% FBS, filtered through 40 μ m cell strainer, and plated at a density of 1×10^6 cells per dish. After reaching approximately 80% confluence, the cells were separated and cultured. Before injection, the cells were labeled with 5 μ M 5-bromo 2'-deoxyuridine (BrdU) (Sigma Chemical, St. Louis, MO) for 12 hours.

Seven days later, at age 23 weeks, 1×10^6 ADSC suspended in 500 μ L sterile PBS were injected autologously into each treatment-group animal using a 27-G needle. Control animals received an equal volume of PBS vehicle (sham). Gentle tourniquet pressure was applied to the proximal base of the penis immediately before injection, and maintained for 90 seconds after injection. A single 6-0 nylon suture was placed to close and mark the injection site.

RNA isolation and real-time polymerase chain reaction

Total RNA from rat penile tissue was isolated using RNeasy Isolation Kit (Qiagen, Valencia, CA.). Total RNA was reverse transcribed into a complementary DNA library. All reagents for real-time PCR, including the primers for rat Caspase-3, TGF- β , and glyceraldehydes-3-phosphate dehydrogenase (GADPH) were purchased from Applied Biosystems (Foster City, CA). Primer sequences are presented in Figure 2. The reactions were run in Applied Biosystems' PRISM 7300HT sequence detection system using the 96-well plate format. The cycling conditions included an initial phase at 95 $^{\circ}$ C for 3 min., 40 cycles at 95 $^{\circ}$ C for 15 sec., and 55 $^{\circ}$ C for 60 sec., followed by a melting curve analysis. The real-time PCR results were analyzed by Applied Biosystems' SDS 7000 software to determine the expression levels of interested genes relative to that of GADPH.

Immunohistochemistry

Freshly dissected tissue was fixed and frozen in OCT compound (Sakura Finetek, Torrance, CA). Sections were cut (5 micron thickness) and treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. Slides were treated with 10% HCl for 30 min at 37 $^{\circ}$ C. After rinsing, sections were washed twice in PBS for 5 min, followed by 30 minutes incubation with 3% horse serum/PBS/0.3% Triton X-100. After draining excess fluid, sections were incubated at 4 $^{\circ}$ C overnight with mouse anti-BrdU (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:500), mouse anti- α -smooth muscle actin (Sigma-Aldrich; 1:2000), mouse anti-nNOS (Santa Cruz Biotechnologies; 1:500), or mouse anti-RECA (Santa Cruz Biotechnologies; 1:500). After washing, sections were immunostained with the avidin-biotin-peroxidase method (Elite ABC, Vector Laboratories, Burlingame, CA), using diaminobenzidine as chromagen. For image analysis, five randomly selected tissue fields per animal were photographed and recorded using a Retiga Q Image digital still camera and ACT-1 software (Nikon Instruments, Melville, NY). Immunostained cells within each image were then quantified at 200X magnification using Image-Pro Plus pixelated image analysis software (Media Cybernetics, Bethesda, MD).

Terminal dUTP Nick-End Labeling (TUNEL)

To quantify apoptosis in the penile cavernous tissue, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed according to manufacturer's specifications using the TUNEL apoptosis detection kit (Chemicon, Billerica, MD). For image analysis, five randomly selected fields of intracavernous tissue per animal were photographed and recorded at 400X magnification using a Retiga Q Image digital still camera 1300 and Nikon E300. The images were analyzed with ImagePro-Plus 5.1 software to quantify the signal. The number of TUNEL positive cells in control and experimental group animals was determined by photographing at 200X *five* representative corporal tissue areas (2 lateral images per side, and one central) within a cross-section of each animal's penis. The total number of TUNEL-positive cells was analyzed in a double-blinded fashion by two separate reviewers, with the aid of a software-based cell counting program *Image-Pro Plus*.

Serum glucose and testosterone studies

Six randomly selected frozen serum specimens from each group were processed by the UC Davis Veterinary Comparative Pathology Lab (Davis, CA). Serum glucose and testosterone were measured using the Roche Diagnostics Cobas Integra 400 Plus assay system (Roche Diagnostics, Indianapolis, IN). Assays were performed in accordance with the manufacturer's specifications and laboratory protocols.

Statistics

Results were analyzed by comparing group data using the two-tailed Student's *t* test ($\alpha=0.05$) using Prism 4 software (GraphPad, La Jolla, CA). Groups were considered to be significantly different at $p<0.05$. Data are given as the mean (standard deviation [SD]) unless otherwise stated.

Results

Baseline erectile function

Blood glucose levels and body weight steadily increased in all animals between age 10 and 23 weeks (Table 1). At 22 weeks, two of 22 (10%) animals were potent, with stimulation ICP increase > 90 cmH₂O during unilateral CN stimulation, and were excluded from the study. The remaining 20 animals all demonstrated a stimulation ICP increase of less than 60 cmH₂O (Figure 1), and were randomly divided into ADSC treatment (N=10) and sham control (N=10) groups. Mean baseline ICP increase was not significantly different between groups ($p=0.36$) (Table 2).

Erectile function three weeks after treatment

In the control group, three weeks after the sham injection, mean [SD] stimulation ICP increase was 31.4 [24.3] cmH₂O, which was slightly less than pre-treatment value (33.8 [15.9] cmH₂O). In the ADSC treated group, mean stimulation Δ ICP increased significantly to 65.3 [15.4] cmH₂O from the pre-treatment value (27.4 [14.8] cmH₂O; $p<0.002$) (Figure 1; Table 2). Mean arterial pressure (MAP) within control (126.7 [19.6] cmH₂O) and treatment groups (119.4 [9.2] cmH₂O) was not significantly different ($p=0.301$). The mean ratio [SD] of ICP/MAP was significantly higher in ADSC treated animals (0.55 [0.14]) versus control group animals (0.24 [0.18]; $p=0.0004$).

Survival of injected ADSC

BrdU labeled ADSC were visualized in penis tissue sections of all 10 treatment group animals. In all specimens, a few ADSC were visualized within intra- and extra-corporal blood vessels, among corporal sinusoids, and connective tissue superficial to the tunica. No inflammatory changes were evident at the injection site in any of the specimens.

Apoptosis in corpus cavernosum tissue

Cells staining positive for TUNEL were manually counted in a blinded fashion for all samples. The number of cells staining positive for TUNEL was significantly higher in the control group as compared to the ADSC treated group ($p<0.0001$) (Table 2). In addition, real-time-PCR analysis demonstrated significantly higher levels of Caspase-3 expression in control group versus ADSC treated animals ($p<0.05$). Expression of mRNA for TGF- β was not significantly different between groups.

Dorsal penile nerve neuronal nitric oxide synthase (nNOS) staining

Dorsal nerves positive for nNOS immunohistochemical staining were visualized in both groups. The control group specimens demonstrated a significantly lower number of nNOS-positive nerve fibers, as compared to the ADSC treated group ($p<0.0001$) (Figure 3; Table 2). Furthermore, the staining patterns were different: nNOS positive fibers were more diffuse in the treatment group, while in the control their presence was more focal.

Corporal body smooth-muscle to collagen ratio

The ratio of intracorporal smooth-muscle (α -actin-positive) to surrounding inner-tunica tissue (circularly oriented fibers) revealed no statistically significant difference ($p=0.223$).

Corporal body endothelial cell (RECA) staining

RECA-positive endothelial cells were visualized within both corporal bodies of all control and treatment group animals. ADSC-treated animals demonstrated a significantly greater number of RECA-positive endothelial cells as compared to control group animals ($p=0.025$) (Table 2).

Serum glucose and testosterone studies

Mean serum glucose and testosterone levels were not significantly different between control and treatment groups ($p=0.489$; $p=0.470$, respectively) (Table 3).

Discussion

Adipose tissue-derived stem cells (ADSC) represent a large sub-population of cells isolated from the stromal vascular fraction of collagenase-digested adipose tissue [11,12]. ADSC are comparable to bone marrow and umbilical cord blood derived mesenchymal stem cells with respect to differentiation capacity, immune phenotype, and morphology [11,13,14]. ADSC possess multi-lineage potential when induced under lineage-specific conditions in-vitro [11, 15–18]. Their pluripotency has been utilized to repair muscle tissues and improve wound vascularization [19,20].

In our present work, we have shown that unmodified autologous ADSC injected into the penis of impotent type-II diabetic rats are associated with significant functional improvement in erectile function, as compared to untreated animals ($p<0.002$). Intracorporal pressures improved and approached, but did not equal, normal values. This could be due to a variety of factors: erectile function was assessed only 3 weeks after injection, and this may have been insufficient time for maximal treatment effect. Also, the diabetic state remained uncontrolled throughout, and this could have directly damaged and/or countered the effects of the ADSC following transplantation. Results of immunohistochemistry and real-time PCR studies together suggest that the treatment effect may be mediated at least in part by decreased intracorporal tissue apoptosis of the treated animals and increased number of sinusoidal endothelial cells. Interestingly, only relatively few BrdU labeled ADSC were visualized within the penis, suggesting that the principal mechanism of effect from the ADSC may not be through direct transformation into local cell types, but through the elaboration of cytokines, growth factors affecting cell surface receptors, and indirect changes within the extracellular compartment of local tissue.

Real-time PCR showed significantly lower expression of Caspase-3 in treated versus control group animals ($p<0.05$). Immunohistochemical staining for TUNEL was similarly lower in ADSC treated animals versus control group animals ($p<0.05$). While the exact mechanisms for these findings are unclear, it is possible that increased local neovascularity and intracorporal endothelial cell count, mediated by ADSC, improved local tissue health and function. Paracrine secretion of cytokines and growth factors has been observed in other studies with mesenchymal

stem cells, and it is possible that ADSC secretion of such factors improved tissue health in treated animals [21].

Staining for nNOS was compared using the dorsal penile nerves, rather than a sampling of intracorporal tissue, because quantification of nNOS staining within intracorporal cavernosal nerves is less reliable [22]. Dorsal nerve nNOS is an acceptable surrogate for presence of cavernosal nNOS for two reasons: first, cavernous nerves travel with the dorsal nerve bundles to reach the distal-most extremities of the penis. Second, it has been shown that selective damage to the cavernous nerves results in decreased nNOS within both the intracorporal cavernous nerves and within the dorsal penile nerves [22]. Celtek et al. showed that uncontrolled type-I diabetes results in progressive loss of nNOS in dorsal penile nerves [23]. Early-phase decrease in axon (not cell body) nNOS occurred. This was reversible with administration of insulin and resulted in decreased apoptosis. Axon and cell body structural damage occurred together in a later, non-reversible phase [23]. Because ADSC treatment was associated with increased nNOS, it is possible that ADSC mediated a rehabilitative effect on nitrergic neuron axons and ganglia. Also, it is possible that improved physiologic erectile function provides improved inflow of oxygenated blood and growth factors, which would improve corporal tissue and dorsal nerve nNOS and decrease corporal tissue apoptosis. Serum testosterone and blood glucose levels were similar between groups, suggesting that treatment effect was not mediated by these factors.

A limitation of our work is that we could not assess the local retention or net survival of the injected ADSC. Emerging experimental imaging modalities that allow labeled cells to be followed in-vivo are crucial to our understanding of the stem cell molecular mechanisms and for the development of appropriate therapies and clinical trials. Another challenge to cellular therapy is how to improve local retention of the transplanted ADSC. Improved local retention could lead to greater improvement in erectile function after treatment. Use of bioabsorbable PLGA microspheres is a well-established means by which to improve local retention, survival, and possibly therapeutic effect of transplanted cells [24]. Lastly, given that mesenchymal stem cells appear to spontaneously migrate to areas of injury [25,26], it is essential that we study the behavior of ADSC not only in healthy animal models, but also in injured and diseased animal models, as homing factors, survival, dispersion, and differentiation characteristics may vary by disease status.

An additional limitation is the use of the BrdU label to identify the ADSC post-transplantation. Despite good cell labeling efficiency with BrdU, the longevity and specificity of the BrdU signal decreases substantially over time [27]. More efficient and reliable stem cell labeling techniques are needed.

As a source of stem cells, adipose tissue has several key advantages: it is accessible by minimally invasive approaches (e.g. liposuction); harvest is generally associated with minimal morbidity; adipose tissue is self-replenishing; and, given its abundance in most people, it is likely that sufficient quantity for therapeutic applications could be harvested within a single procedure, thus precluding the need for cell culture. Freedom from reliance upon cell-culture has two important advantages. 1. There is intense debate concerning the perceived significant hazards associated with use of animal by-products in common culture media and cell-expansion protocols [28]. Risk of transmission of viral, prion disease, and, other proteins that could initiate xenogenic immune responses has been reported [29,30]. 2. From a regulatory perspective, omission of ex-vivo cell culture, which is considered a “modification” of the natural cell product, may facilitate regulatory approval for future clinical trials [31].

Conclusion

Autologous ADSC injected into the penis are associated with improved erectile function, decreased intracorporal tissue apoptosis, and increased number of sinusoid endothelial cells and nerve nNOS. While the exact mechanism remains unclear, our findings suggest that the treatment effect from ADSC may not be through *direct transformation* of the ADSC into local cell types, but rather, via a more “indirect” mechanism, whereby ADSC improve the extracellular environment and improve local tissue function within the treatment area. ADSC are an excellent and practical source of mesenchymal stem cells, and present a promising regenerative medicine-based approach to treatment of erectile dysfunction. Further studies that address long-term net survival of transplanted cells, and their spectrum of differentiation and migration patterns, are warranted.

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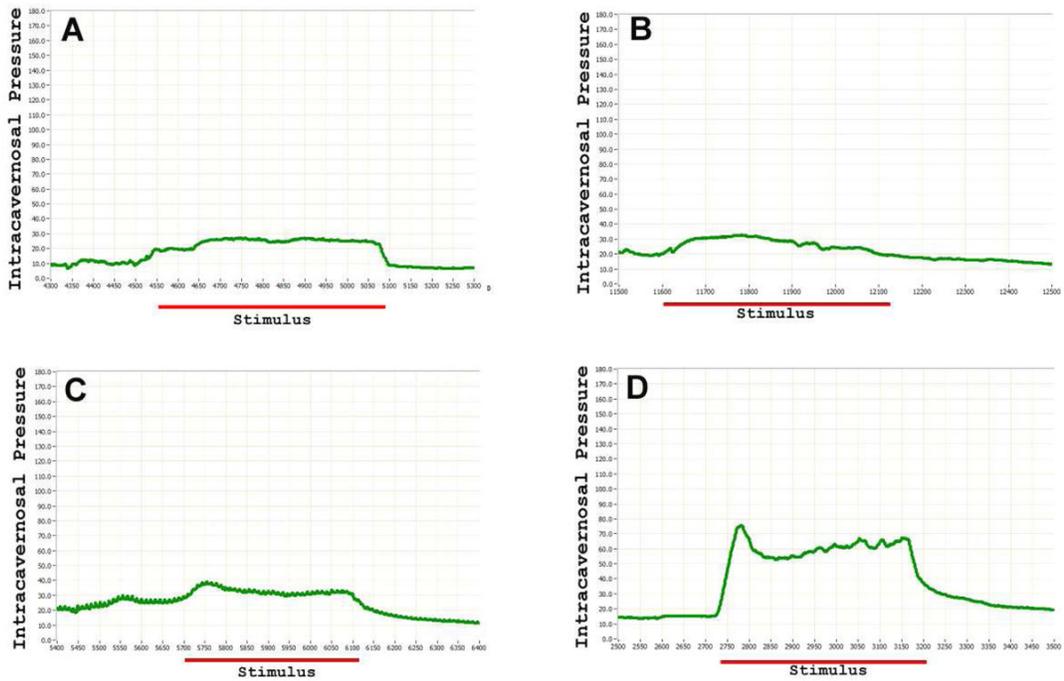
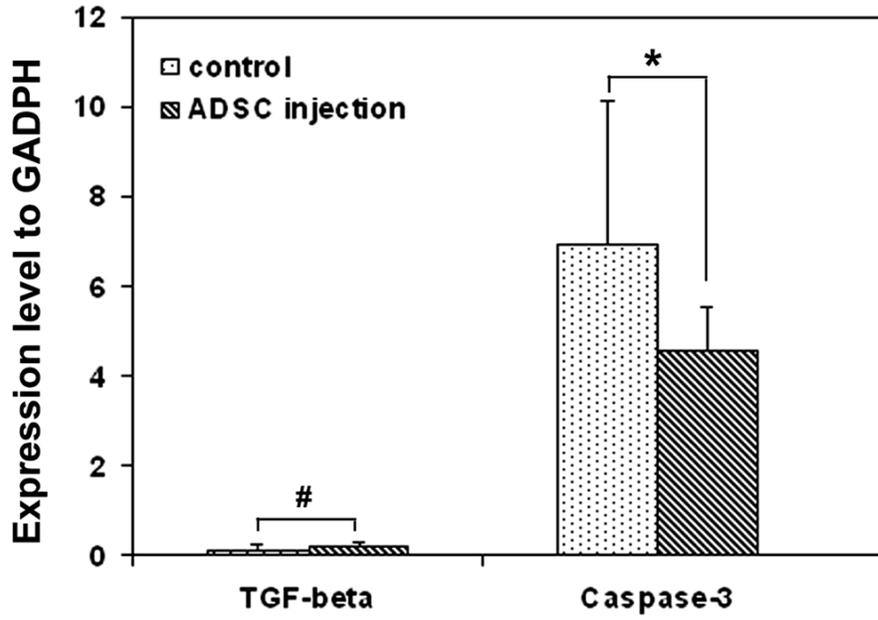


Figure 1. Stimulation ICP before and after treatment in control and ADSC treated animals
 Erectile function study with unilateral left cavernosal nerve electrostimulation *before* treatment in sham-control group (A) and ADSC treated group animals (B). Erectile function studies *after* treatment in the sham-control group (C) and ADSC treated group animals (D). The *x*-axis is in seconds, and the horizontal bar represents the duration of cavernous nerve stimulation (50 seconds).



Gene Name	Primer Sequence
TGF-β	Forward: 5'-TGAGTGGCTGTCTTTTGACG-3' Reverse: 5'-TTCTCTGTGGAGCTGAAGCA-3'
Caspase-3	Forward: 5'-AATTCAAGGGACGGGTCA-3' Reverse: 5'-GCTTGTGCGCGTACAGTTTC-3
GADPH	Forward: 5'-ATGATTCTACCCACGGCAAG-3' Reverse: 5'-CTGGAAGATGGTGATGGGTT-3'

Figure 2. mRNA expression of TGF-beta and Caspase-3 in corpus cavernosum tissue
 While there is no significant difference for TGF-beta between control and the ADSC treated animals (# $p > 0.05$), Caspase-3 was significantly decreased in ADSC treated animals (* $p < 0.05$). The box shows the corresponding primer sequences.

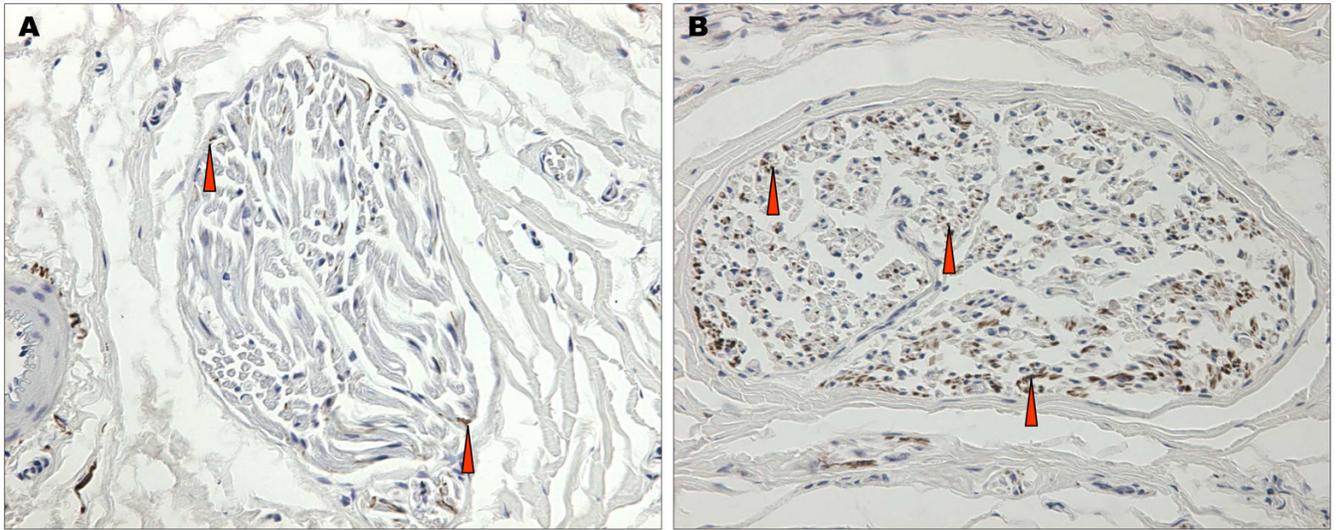


Figure 3. Neuronal nitric oxide synthase (nNOS) staining of the dorsal nerve in a penile midshaft specimen

Cross sections represent untreated control animals (A) and ADSC-treated animals (B). Arrows point to dark brown nNOS positive dorsal nerve neurons. Please note that nNOS-positive stained area was significantly greater in the treatment group versus the control group ($p < 0.0001$).

Table 1

Progression of blood glucose levels and body weight in ZDF rats over an observation period from 10 to 23 weeks of age. Values are based on N = 10 animals and are given as mean \pm SD.

Age (weeks)	Blood glucose (mg/dl)	Body weight (g)
10	363.7 \pm 102.5	350.3 \pm 8.57
12	439.2 \pm 159.5	393.5 \pm 25.5
14	445.6 \pm 99.7	368.7 \pm 30.6
16	498.9 \pm 68.9	396.3 \pm 36.7
18	457.7 \pm 59.6	398.3 \pm 35.3
20	492.0 \pm 102.8	396.4 \pm 37.9
22	539.6 \pm 63.8	407.0 \pm 37.6
23	all > 600	409.3 \pm 39.3

Intracorporal pressure (ICP) measurements and tissue immunohistochemistry studies. ICP increase was measured before treatment at 23 weeks of age and after treatment at 26 weeks of age (A). ICP increase/mean arterial pressure (MAP) was assessed at 26 weeks of age (B). Histological changes were determined in a midshaft penile sample (C-F). Values are given as mean \pm SD.

Table 2

	Group	N	ICP Increase (cmH ₂ O)		p
			Pre-treatment	Post-treatment	
A.	Control	10	33.8 \pm 15.9	31.4 \pm 24.3	p<0.002
	Treatment	10	27.4 \pm 14.8	65.3 \pm 15.4	
B.	Group	N	ICP Increase (cmH ₂ O)/MAP (cmH ₂ O)		p
	Control	10	0.24 \pm 0.18		
	Treatment	10	0.55 \pm 0.14		p=0.0004
C.	Group	N	TUNEL Positive cells		p
	Control	10	156.4 \pm 19.0		
	Treatment	10	36.7 \pm 7.1		p<0.0001
D.	Group	N	Positive nNOS staining area (pixel)		p
	Control	10	301.8 \pm 87.4		
	Treatment	10	484.0 \pm 52.3		p<0.0001
E.	Group	N	RECA positive cells		p
	Control	10	88.0 \pm 18.1		
	Treatment	10	146.3 \pm 73.2		p=0.025
F.	Group	N	Smooth muscle/Collagen ratio		p
	Control	10	7.7 \pm 2.2		
	Treatment	10	6.6 \pm 2.2		p>0.05

Table 3

Serum glucose and testosterone levels in control animals and ADSC treated animals at 26 weeks of age. Each group represents a random selection of 6 animals and values are given as mean \pm SD.

Group (n=6)	Glucose (mg/dl)	Testosterone (pg/ml)
Control	1038.0 \pm 178.3	1128.0 \pm 790.3
Treatment	963.0 \pm 183.6	872.6 \pm 265.1
p-value	0.489	0.470