Transplantation of adipose derived stem cells overexpressing inducible nitric oxide synthase ameliorates diabetes mellitus induced erectile dysfunction in rats

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Abstract

Background: Erectile dysfunction is a major complication of diabetes mellitus. Adipose derived stem cells (ADSCs) have attracted much attention as a promising tool for the treatment of diabetes mellitus induced erectile dysfunction (DMED). Inducible nitric oxide synthase (iNOS) plays an important role in protecting penile tissues from fibrosis. The aim of this study was to determine the efficacy of ADSCs overexpressing iNOS on DMED in rats.

Methods: ADSCs were isolated and infected with adenovirus overexpressing iNOS (named as ADSCs-iNOS). The expression of iNOS was detected using western blot analysis and real-time PCR. Rats were randomly assigned into five groups: control group, DMED group, ADSCs group, ADSCs-EGFP group and ADSCs-iNOS group. 5×10^5 cells were given once via the intracorporal route. Two weeks after treatment, erectile function was assessed by electrical stimulation of the cavernous nerve. Penile tissues were obtained and evaluated at histology level.

Results: We found that ADSCs-iNOS had significantly higher expression of iNOS at mRNA and protein levels and generated more nitric oxide. ADSCs-iNOS reduced collagen I and collagen IV expression of corpus cavernosum smooth muscle cells (CCSMCs) in cell co-culture model. Transforming growth factor-β1 expression and p-Smad2/3 to Smad2/3 ratio in CCSMCs reduced following co-culture with ADSCs-iNOS. Injection of ADSCs-iNOS significantly ameliorated DMED in rats and decreased collagen/smooth muscle cell ratio of penile tissues. Moreover, elevated nitric oxide and cGMP concentrations were detected in penile tissues of ADSCs-iNOS group.

Conclusion: Taken together, ADSCs-iNOS significantly improved erectile function of DMED rats. The therapeutic effect may be achieved by increased nitric oxide generation and the suppression of collagen I and collagen IV expression in the CCSMCs to decrease penile fibrosis.

Introduction

Erectile dysfunction (ED) is one of the most significant complications in men with diabetes mellitus (DM). Numerous studies have found that more than 50% of men with DM are afflicted with ED (*Thorve et al., 2011*). Men with DM tend to suffer from ED about 10 years earlier than the general population (*Johannes et al., 2000*). Diabetes mellitus induced erectile dysfunction (DMED) is severe and has a negative effect on the quality of life. Phosphodiesterase 5 inhibitors are the predominant medicines to treat ED. However, diabetic male patients are less responsive to phosphodiesterase 5 inhibitors (*Cheng, 2007*). Therefore, developing new therapeutic options for DMED is urgent.

Over the past years, stem cell therapy has produced positive effects on DMED in experimental animals. Adipose derived stem cells (ADSCs) are attracting considerable attention becuase they can be isolated through standardized procedures and vastly expanded, and they secrete a broad range of trophic factors (*Sowa et al., 2012*). Moreover, ADSC transplantation seems to be safe and avoid the risks of tumorigenesis (*Alagesan & Griffin, 2014*). Autologous

60 ADSCs significantly improved erectile function of type-II diabetic rats (*Garcia et al., 2010*).
61 Stem cell therapy has beneficial effects to increase smooth muscle and endothelium, NO-cGMP
62 pathway, neuronal nitric oxide synthase (nNOS)-positive nerve fibers, and decrease fibrosis and
63 apoptosis in the penis (*Chen et al., 2017; Qiu et al., 2011; Ryu et al., 2012; Sun et al., 2012;*
64 *Zhang et al., 2016*).
65   Nitric oxide (NO) is a neurotransmitter which plays an important role in penile erection. NO
66 is synthesized by three nitric oxide synthase (NOS) isoforms, including nNOS, endothelial NOS
67 (eNOS) and inducible NOS (iNOS). Among these synthases, iNOS can produce NO
68 continuously independent of Ca^{2+} (*Eissa et al., 1998*). Recent evidence suggests that the
69 pathogenesis of DMED may be due to impaired NO production, and iNOS acts an essential part
70 in protecting penile tissues from the pro-fibrotic effects of hyperglycemia (*Ferrini et al., 2010*).
71 Continuous NO production by iNOS inhibits collagen synthesis, directly reacts with ROS to
72 produce peroxynitrite, and downregulates transforming growth factor-β1 (TGF-β1)/Smad
73 pathway, thus reducing fibrosis level (*Gonzalez-Cadavid & Rajfer, 2010*). Gene transfer of iNOS
74 DNA decreased TGF-β1 and plasminogen-activator inhibitor-1 expression, and regressed
75 Peyronie's disease-like plaque (*Davila et al., 2004*). Our previous study showed that saRNA
76 mediated iNOS expression improved DMED via endogenously generating NO (*Wang et al.,*
77 *2013*).
78   Given the beneficial effects of ADSCs and iNOS in the treatment of ED, we hypothesized
79 that ADSCs combined with iNOS could improve erectile function of DMED rats. In this study
80 we aimed to determine the efficacy of ADSCs combined with iNOS for improving erectile
81 function in DMED rats.
82

Materials and methods

83

Rat ADSCs isolation and culture

84

85   Rat ADSCs were isolated as previously described (*Wang et al., 2015*). Briefly, adipose
86 tissues were incised from inguen, and digested at 37°C in 0.1% collagenase type I (Sigma-
87 Aldrich, St. Louis, USA) with vigorous shaking for 90 minutes. The digested tissues were mixed
88 with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum
89 (FBS), and centrifuged at 220 g for 10 minutes. The cells were suspended in DMEM containing
90 10% FBS and filtered through a 75-μm cell striner (Solarbio, Beijing, China). Then cells were
91 plated and cultured in DMEM containing 10% FBS at 37°C in humidified incubator with 5%
92 CO_2. Cells were passaged with trypsin/EDTA (Beyotime, Nantong, China) as required. The
93 fourth-passage ADSCs were used in this study.
94

Flow cytometry

95

96   ADSCs were characterized by flow cytometry at passage 4. Briefly, ADSCs were harvested
97 and washed twice with phosphate-buffered saline (PBS). Then, the cells were incubated for 30
98 minutes in PBS containing anti-CD29-FITC(cat. # 555005, BD, San Diego, USA), CD31-PE(cat.
99 # 555027, BD), CD49-FITC(cat. # 557457, BD), CD90-PE(cat. # 551401, BD), CD106-PE(cat.

100 # 559229, BD), CD34-FITC(cat. # sc-7324, Santa Cruz, Dallas, Texas), CD45-FITC(cat. #
101 MCA43FT, AbD, Oxford, UK), CD73-FITC (cat. # bs-23233R, Bioss, Beijing, China) , CD105-
102 FITC (cat. # bs-10662R, Bioss). The stained cells were then subjected to flow cytometry analysis.
103

Differentiation of ADSCs

105 For adipocyte differentiation, ADSCs were cultured at a density of $1×10^4$ cells/cm^2 in
106 DMEM containing 10% FBS, 50 mg/L indomethacin, 0.5 mmol/L isobutylmethylxanthine, 10
107 mg/L insulin and 1 mmol/L dexamethasone (all from Sigma-Aldrich). After 21 days of induction,
108 the cells were stained using Oil red-O staining solution.
109 For smooth muscle cell differentiation, ADSCs were cultured at a density of $1×10^4$ cells/cm^2
110 in low-glucose DMEM containing 10% FBS, 50 μg/mL platelet-derived growth factor-BB and 5
111 μg/L TGF-β1 (all from PeproTech, Rocky Hill, USA). After 14 days of induction, the cells were
112 stained with the antibody against α-smooth muscle actin (α-SMA, cat. # BM0002, Boster,
113 Wuhan, China). Immunofluorescence staining was performed as previously described (*Zhang et*
114 *al., 2016*).
115

Transfection with adenovirus

117 The **fourth**-passage ADSCs were cultured for 24 h. ADSCs were incubated with adenovirus
118 expressing EGFP or iNOS-EGFP (GenecChem, Shanghai, China) at a multiplicity of infection
119 (MOI) of 30. After incubation for 12 h, the medium was changed into fresh growth medium.
120

Real-time PCR

122 Real-time PCR was performed in cultured cells on the 1st, 3rd, 5th, 7th, 10th and 14th day
123 after infection with adenovirus. RNA was extracted with a multisource RNA miniprep kit
124 (Corning, NY, USA). Total RNA (500 ng) was reversely transcribed into cDNA using the
125 PrimeScript^{TM} RT reagent kit (TaKaRa, Dalian, China). Real-time PCR for each sample was
126 performed on an MX3000P quantitative PCR system (Agilent, Santa Clara, USA) using SYBR
127 Premix Ex Taq (TaKaRa) and the following primers: iNOS_ 5'-
128 AAGCACATTTGGCAATGGAGAC-3', 5'-TGGAGCCCAGGCCAAATAC-3', β-actin_ 5'-
129 GACGGTGTGCACCAACATCTA-3', 5'-TTCTTGGCTTTCAGGATGGAG-3'. PCR condition
130 included 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds
131 and 72°C for 30 seconds. Relative expression level of target genes was calculated with $2^{-\Delta\Delta Ct}$
132 method. β-actin was chosen as normalization control.
133

Western blot analysis

135 Protein extraction from rat penile tissues and cultured cells was performed using NP-40 lysis
136 buffer (Beyotime). Protein concentration was detected with the BCA protein assay kit
137 (Beyotime). 50 μg protein was electrophoresed on 12% sodium dodecyl sulfate/polyacrylamide
138 gels and then blotted on a polyvinylidene difluoride membrane (Millipore, Billerica, USA). The
139 membrane was blotted with primary antibodies against collagen I (cat. # 14695-1-AP,
140 Proteintech, Wuhan, China), collagen IV (cat. # 55131-1-AP, Proteintech), iNOS (cat. # sc-7271,

141 Santa Cruz, Dallas, USA), TGF-β1 (cat. # ab92486, Abcam, Cambridge, UK), Smad2/3 (cat. #
142 ab63672, Abcam) and phospho-Smad2/3 (p-Smad2/3, cat. # ab63399, Abcam) followed by
143 HRP-labelled secondary antibodies. The enhanced chemiluminescence detection system (Pierce;
144 Thermo Fisher, Rockford, USA) was used to detect protein bands. Each band was quantified by
145 densitometry with Image J software.

146

147 **Measurement of NO concentration**
148   Cells were incubated in culture medium containing 10 mmol/L L-Arginine for 24 h on the 1st,
149 3rd, 5th, 7th, 10th and 14th day after infection with adenovirus. Then the supernatant was
150 harvested. NO concentrations in penile tissues or cultured cells were determined with total NO
151 assay kit (Beyotime) according to the manufacturer's instructions.

152

153 **Cyclic guanosine monophosphate (cGMP) concentration determination**
154   The cGMP concentrations in penile tissues or cultured cells were measured using an enzyme-
155 linked immunosorbent assay (ELISA) kit (Westang, Shanghai, China) according to the
156 manufacturer's instructions.

157

158 **Rat corpus cavernosum smooth muscle cell (CCSMC) isolation and culture**
159   Rat CCSMCs were obtained from corpus cavernosum tissues as described previously with
160 minor modifications(*Chen et al., 2011*). Briefly, corpus cavernosum tissues were harvested and
161 cut into fragments of 1 mm^3. These fragments were placed in an overturned 25 cm^2 culture flask.
162 Then, 4 ml DMEM containing 10% FBS was added into culture flask. After 30 minutes, the flask
163 was flipped over. Differential adherence method was used to purify CCSMCs. Immunostaining
164 with an antibody against α-SMA was performed to identify CCSMCs. Immunofluorescence
165 staining was performed as previously described (*Zhang et al., 2016*).

166

167 **Transwell co-culture of ADSCs and CCSMCs**
168   ADSCs and CCSMCs were co-cultured in medium containing 30 mmol/L glucose in 0.4 μm
169 pore size Transwell inserts (Corning, NY, USA). CCSMCs were cultured in 12-well plates at
170 1×10^4 cells/well. ADSCs, ADSCs-EGFP or ADSCs-iNOS were seeded on Transwell inner
171 membrane at a density of 1×10^4 cells/well. The co-culture system allowed ADSCs and CCSMCs
172 to grow in the same medium without direct contact between them.

173

174 **Animal experiments**
175   Animal experiments were carried out according to the guidelines and regulations by the
176 Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science
177 and Technology. This study was approved by the Ethical Committee (TJ-A20131213). Sixty
178 male SD rats (8-week old) were purchased from Hunan SJA Laboratory Animal Co., Ltd. After
179 an overnight fast, fifty-one of them were randomly selected and received intraperitoneal injection
180 with streptozotocin (60 mg/kg, Sigma-Aldrich) (*Yang et al., 2013*). The remaining 9 rats were
181 used as controls. Three days after streptozotocin injection, rats with fasting blood glucose level

182 higher than 16.7 mmol/L were selected as DM. After 8 weeks, DM rats were selected by the
183 apomorphine (APO) test. Briefly, APO (80 mg/kg, Sigma-Aldrich) was injected in the loose skin
184 of neck. DMED rats were identified if rats did not exhibit an erectile response within 30 minutes.
185   DMED rats were randomly divided into different treatment groups (9 rats in each group):
186 DMED group received no treatment; ADSCs group received corpus cavernosum injection of 60
187 μl PBS containing 5×10^5 ADSCs; ADSCs-EGFP group received corpus cavernosum injection of
188 60 μl PBS containing 5×10^5 ADSCs-EGFP; ADSCs-iNOS group received corpus cavernosum
189 injection of 60 μl PBS containing 5×10^5 ADSCs-iNOS.
190

Assessment of erectile function

192   After 2 weeks, intracavernous pressure (ICP) and mean arterial pressure (MAP) were
193 detected to evaluate erectile function as described previously (*Yang et al., 2013*). The stimulus
194 parameters were two voltages (2.5, 5.0 volts), frequency 15 Hz, duration 1 minute and pulse
195 width 1.2 ms. Erectile response was measured at 2.5 and 5.0 voltages. Pressure was measured
196 and recorded with a data acquisition system (AD Instruments Powerlab/4SP, NSW, Australia).
197 Then, penile tissues were harvested for measurement of NO and cGMP concentration (n =
198 4/group) and masson trichrome staining (n = 5/group)
199

Masson trichrome staining

201   Penile tissues were embedded in paraffin and cut into 5 μm thick sections. Masson trichrome
202 staining was performed as previously described (*Yang et al., 2013*). Image Pro Plus 6.0 software
203 (Media Cybernetics Inc, Bethesda, USA) was used to quantitatively analyze smooth muscle
204 content and collagen in five randomly selected specimens per group.
205

Statistical Analysis

207   Results were expressed as mean ± standard deviation. Data were analyzed with one-way
208 analysis of variance followed by Tukey-Kramer test for post hoc comparisons using GraphPad
209 Prism 5.0 (GraphPad Software, San Diego, USA). Normal distribution was determined by
210 Kolmogorov-Smirnov test. $P<0.05$ was considered statistically significant.
211

Results

Isolation and characterization of ADSCs

214   The isolated ADSCs exhibited spindle-shaped morphology at passage 1 (Fig. 1A). ADSCs
215 expanded *in vitro* and showed fibroblast-like shape at passage 4 (Fig. 1B). To characterize
216 ADSCs, surface markers of the cultured cells at passage 4 were determined using flow cytometry.
217 As shown in Fig. 1C, most of the cells expressed CD29 and CD90, while few cells were positive
218 for CD34, CD45, CD73, CD105, CD31 and CD106. To identify the multi-lineage differentiation
219 ability of ADSCs, we induced adipogenic and myogenic differentiation of ADSCs for 21 or 14
220 days in appropriate induction medium. Oil red-O staining indicated that ADSCs could
221 differentiate to adipocytes (Fig. 1D). Immunofluorescence staining with an antibody against α-

222 SMA indicated that ADSCs could differentiate to smooth muscle cells (Fig. 1E).

223   After culture for 5 to 7 days primary rat CCSMCs in spindle shape grew out of corpus
224 cavernosum tissues. After about 2 weeks of culture, cells were passaged and purified by the
225 differential adherence method (Fig. 1F). The cells were identified as CCSMCs by
226 immunostaining of α-SMA (Fig. 1G).

227

228 Overexpression of iNOS in ADSCs

229   To overexpress iNOS in ADSCs, we infected the cells with recombinant adenovirus. 3 days
230 after infection, fluorescence microscopy was used to observe EGFP-expressing ADSCs (Fig. 2A).
231 Real-time PCR and Western blot analysis showed that the mRNA and protein expression levels
232 of iNOS were significantly higher in ADSCs-iNOS group than ADSCs-EGFP and ADSCs
233 groups ($P<0.05$, Fig. 2B and 2C). The expression of iNOS in ADSCs could persisted for up to 14
234 days (Fig. 2D).

235

236 Effects of iNOS overexpression on CCSMCs

237   As expected, NO concentration in supernatant of ADSCs-iNOS was significantly higher than
238 that of ADSCs and ADSCs-EGFP ($P<0.05$). Moreover, NO concentration in supernatant of
239 ADSCs-iNOS reached maximum level on the 7th day ($P<0.05$, Fig. 3A). We detected the effect
240 of ADSC-iNOS on CCSMCs using Transwell co-culture model. The expression of collagen I and
241 collagen IV in CCSMCs significantly increased in high glucose concentration ($P<0.05$).
242 However, significant reduction of collagen I and collagen IV expression was observed in
243 CCSMCs co-cultured with ADSCs-iNOS ($P<0.05$, Fig. 3B, 3C and 3D). In addition, we found
244 that TGF-β1 expression and p-Smad2/3 to Smad2/3 ratio in CCSMCs reduced following co-
245 culture with ADSCs-iNOS ($P<0.05$, Fig. 3E and 3F).

246

247 Effects of ADSCs-iNOS on erectile function

248   Finally we evaluated the effects of ADSCs-iNOS on erectile function of DMED rats. There
249 was no significant difference in initial body weight or serum glucose concentration among the 5
250 groups of rats. 14 days after the induction of diabetes, body weight was significantly lower and
251 fasting glucose concentration was significantly higher in the diabetic rats than the age-matched
252 control rats ($P<0.05$). However, there were no significant differences in body weight or serum
253 glucose concentration among DMED, ADSCs, ADSCs-EGFP and ADSCs-iNOS groups (Table
254 1).

255   Erectile function was measured 2 weeks after ADSCs transplantation. The ICP/MAP ratio was
256 significantly lower in DMED group than that in control group. Intracavernous injection of
257 ADSCs or ADSCs-EGFP significantly improved erectile function of DMED rats. The ICP/MAP
258 ratio of ADSCs-iNOS group was markedly elevated compared to DMED, ADSCs and ADSCs-
259 EGFP groups (all $P<0.05$, Fig. 4A and 4B). In addition, NO and cGMP concentrations in penile
260 tissues of ADSCs and ADSCs-EGFP groups were significantly elevated compared to DMED
261 group, although they were still significant lower compared to ADSCs-iNOS group ($P<0.05$, Fig.
262 4C and 4D). The mean collagen/smooth muscle cell ratio significantly increased in the DMED

263 rats compared to the control rats ($P<0.05$). Intracavernous injection of ADSCs or ADSCs-EGFP
264 attenuated collagen content ($P<0.05$). Furthermore, mean collagen/smooth muscle cell ratio in
265 ADSCs-iNOS treated rats was significantly lower than that in ADSCs or ADSCs-EGFP group
266 ($P<0.05$) (Fig. 4E and 4F).

267

Discussion

269   In this study we demonstrated that infection of ADSCs with adenovirus containing iNOS
270 expression cassette led to significantly high expression of iNOS and increased generation of NO.
271 ADSCs played a positive role in restoring DMED in rats. Furthermore, overexpression of iNOS
272 in ADSCs was shown to achieve a significantly larger improvement of erectile function. The
273 therapeutic effect may be achieved by increased NO generation and the suppression of collagen I
274 and collagen IV expression in the CCSMCs to decrease penile fibrosis.
275   Corpus cavernosum is composed of a loose trabecular meshwork of smooth muscle and
276 connective tissues, which are structural basis of erectile function. Ryu *et al* found that collagen
277 fiber content was significantly increased in corpus cavernosum of patients with vasculogenic ED
278 (*Ryu et al., 2004*). Further studies demonstrated that extracellular matrix such as fibronectin,
279 collagen IV and collagen I accumulated and collagen I/collagen III ratio was decreased in the
280 corpus cavernosum tissue of diabetic ED rats (*Hirata et al., 2009; Zhou et al., 2012*). Abnormal
281 extracellular matrix can cause mechanical alterations of corpus cavernosum, which may provoke
282 penile venous leakage, leading to vasculogenic ED (*Li et al., 2013*). Increased expression of
283 TGF-β1 pathway could be involved in collagen fiber accumulation and penile fibrosis (*Ryu et al.,
284 2004*). CCSMCs are the prdominant mesenchymal cell type in the corpus cavernosum. Synthesis
285 of connective tissue proteins and collagen by CCSMCs was significantly increased by exogenous
286 TGF-β1 (*Moreland et al., 1995*). In accordance with previous studies, our study showed that
287 collagen I and collagen IV expression in CCSMCs significantly increased under high glucose
288 condition and penile fibrosis significantly increased in DMED rats. Then we co-cultured
289 CCSMCs with ADSCs, and found significantly decreased synthesis of collagen I and collagen IV.
290 Furthermore, we found that TGF-β1 expression and p-Smad2/3 to Smad2/3 ratio in CCSMCs
291 significantly decreased, indicating the involvement of TGF-β1/Smad signaling pathway.
292 However, further studies are needed to elucidate precise mechanism of ADSCs' effect on
293 CCSMCs. In *vivo*, ADSCs ameliorated penile fibrosis in ED rats, and the possible mechanism is
294 still needed to be elucidated precisely. ADSCs significantly prevented upregulation of collagen
295 III and elastin in the tunica albuginea of Peyronie's disease rats (*Castiglione et al., 2013*).
296 Intratunical injection of ADSCs decreased the expression of tissue inhibitors of
297 metalloproteinases, and promoted the expression and activity of matrix metalloproteinases
298 (*Gokce et al., 2014*). In addition, exosomes from ADSCs were enriched with miR-132 and miR-
299 let7 which played antifibrotic role through regulating TGF-β1 pathway (*Zhu et al., 2017*).
300   NO/cGMP signaling pathway plays a crucial role of in erectile function. NO released from
301 nonadrenergic noncholinergic nerves and endothelium is the principal mediator of penile
302 erection. Moreover, this pathway plays an important role in ameliorating tissue fibrosis. Marta *et*

303 *al* found that NO inhibited TGF-β/Smad induced gene transactivation in a cGMP-dependent
304 manner in endothelial cells, leading to proteasomal degradation of phosphorylated Smad. In
305 addition, NO reduced and delayed nuclear translocation of activated Smad (*Saura et al., 2005*).
306 cGMP significantly reduced TGF-β induced upregulation of collagen I and collagen II at mRNA
307 and protein levels (*Beyer et al., 2015*). In this study, we found that iNOS overexpression in
308 ADSCs significantly enhanced NO generation and could last for 14 days. Compared to ADSCs,
309 ADSCs-iNOS significantly increased NO and cGMP concentrations in penile tissues, and
310 decreased collagen I and collagen IV expression in CCSMCs to ameliorate penile fibrosis in
311 DMED rats. These results are consistent with previous studies that the inhibition of iNOS
312 promoted penile fibrosis. For example, Monica *et al* found that more collagen was deposited in
313 corpus cavernosum of iNOS knockout mouse. In addition, inactivation of iNOS gene led to
314 exacerbated penile fibrosis in DM mouse by increasing oxidative stress and TGF-β1 expression
315 under hyperglycemia condition (*Ferrini et al., 2010*). Similarly, L-NIL as an inhibitor of iNOS
316 activity increased oxidative stress and fibrosis in the media of arteries (*Ferrini et al., 2004*).

317   However, other studies suggested that erectile function was impaired by iNOS expression.
318 The induction of iNOS accompanying penile fibrosis was demonstrated in rat models of DMED,
319 Peyronie's disease and aging-related ED (*Bivalacqua et al., 2000; Ferrini et al., 2001; Usta et
320 al., 2003*). In addition, iNOS inhibition improved erectile function in Peyronie's disease or
321 DMED rat. However, the application of aminoguanidine as an iNOS inhibitor was not
322 appropriate, because aminoguanidine inhibited not only all three kinds of NOS, but also
323 advanced glycation end products involved in tissue fibrosis. Moreover, further studies are needed
324 to determine if elevated iNOS expression is possibly a compensatory reflection when eNOS and
325 nNOS expression is decreased under pathological conditions.

326   There are some limitations in the current study. First, we did not investigate the effect of
327 iNOS expression on the stemness of ADSCs. In our study, more NO was released from ADSCs-
328 iNOS than ADSCs. Tapia-Limonchi *et al* found that NO maintained embryonic stem cells (ESC)
329 pluripotency and delayed ESC differentiation by regulating Gsk3-β/β-catenin and PI3K/Akt
330 signaling pathways (*Tapia-Limonchi et al., 2016*). The effect of NO released from ADSCs iNOS
331 on ADSC stemness required further investigation. Second, ADSCs were only transplanted into
332 corpus cavernosum once. Repeated injection of ADSCs may achieve better therapeutic effects
333 but need to be confirmed in future studies. Third, we did not compare therapeutic effect of
334 ADSCs-iNOS to that of iNOS alone. Although adenovirus act as efficient agents for gene
335 transfer, they can activate cellular and humoral immune response in the hosts, which limits the
336 safety and efficacy *in vivo*. The innate immune response is mediated by the adenovirus particle,
337 but not viral transcription (*Muruve, 2004*). In contrast, transplanted stem cells could suppress
338 excessive immune response (*Lin, Lin & Lue, 2012*). ADSCs overexpressing iNOS may be safer
339 and more efficient than iNOS alone. Fourth, the statistical power for this study is relatively low
340 (49.9%) which may be due to the small group size, and more experimental rats will be included
341 to validate these results in our further study.

342 **Conclusions**

343   In conclusion, intracavernous administration of ADSCs-iNOS improved erectile function of
344   DMED rats. Injection of ADSCs-iNOS in a rat model of DM significantly decreased penile
345   fibrosis, possibly due to increased NO generation and suppressed expression of collagen I and
346   collagen IV in the CCSMCs.

347

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Figure 1

Figure 1. Primary culture and characterization of rat ADSCs and CCSCMs.

(A) Morphological features of ADSCs at passage 1 (primary magnification: ×100) . (B) Morphological features of ADSCs at passage 4 (primary magnification: ×100). (C) ADSCs were identified by flow cytometry at passage 4. (D) Adipogenic differentiation of ADSCs assessed by oil red-O staining (primary magnification: ×200). (E) Myogenic differentiation of ADSCs assessed by immunofluorescence staining with α-SMA antibody (green, primary magnification: ×200). (F) Morphological features of CCSMCs after purification (primary magnification: ×100) . (G) CCSMCs were assessed by immunostaining with α-SMA antibody (red, primary magnification: ×100). The nuclei were labeled with DAPI (blue).
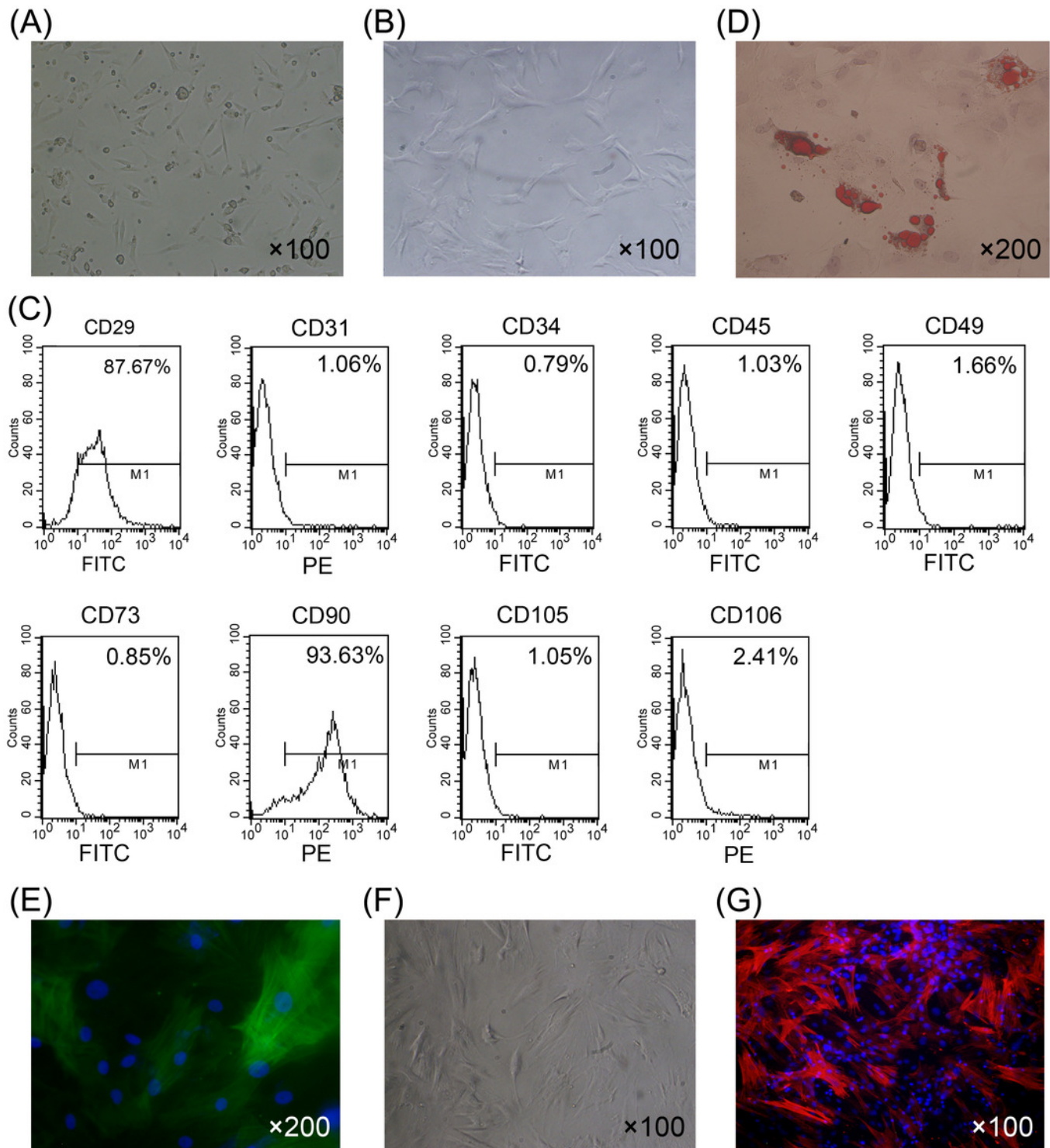
Figure 2

Figure 2. iNOS expression in ADSCs.

(A) EGFP-expressing ADSCs were observed by fluorescence microscopy 3 days after infection (primary magnification: ×100). (B) iNOS expression at mRNA level was detected by real-time PCR and the relative ratio of iNOS/β-actin measured in ADSCs was arbitrarily presented as 1. Data are the mean of 3 independent experiments. (C) iNOS expression at protein level was detected by Western blot analysis. (D) Densitometry analysis of relative ratio of iNOS/β-actin in ADSCs and the relative ratio of iNOS/β-actin measured on 1st day was arbitrarily presented as 1. Data are the mean of 3 independent experiments. $^{*}P<0.05$ *vs.* ADSCs, $^{\&}P<0.05$ *vs.* ADSCs-EGFP, $^{\#}P<0.05$ *vs.* 1d
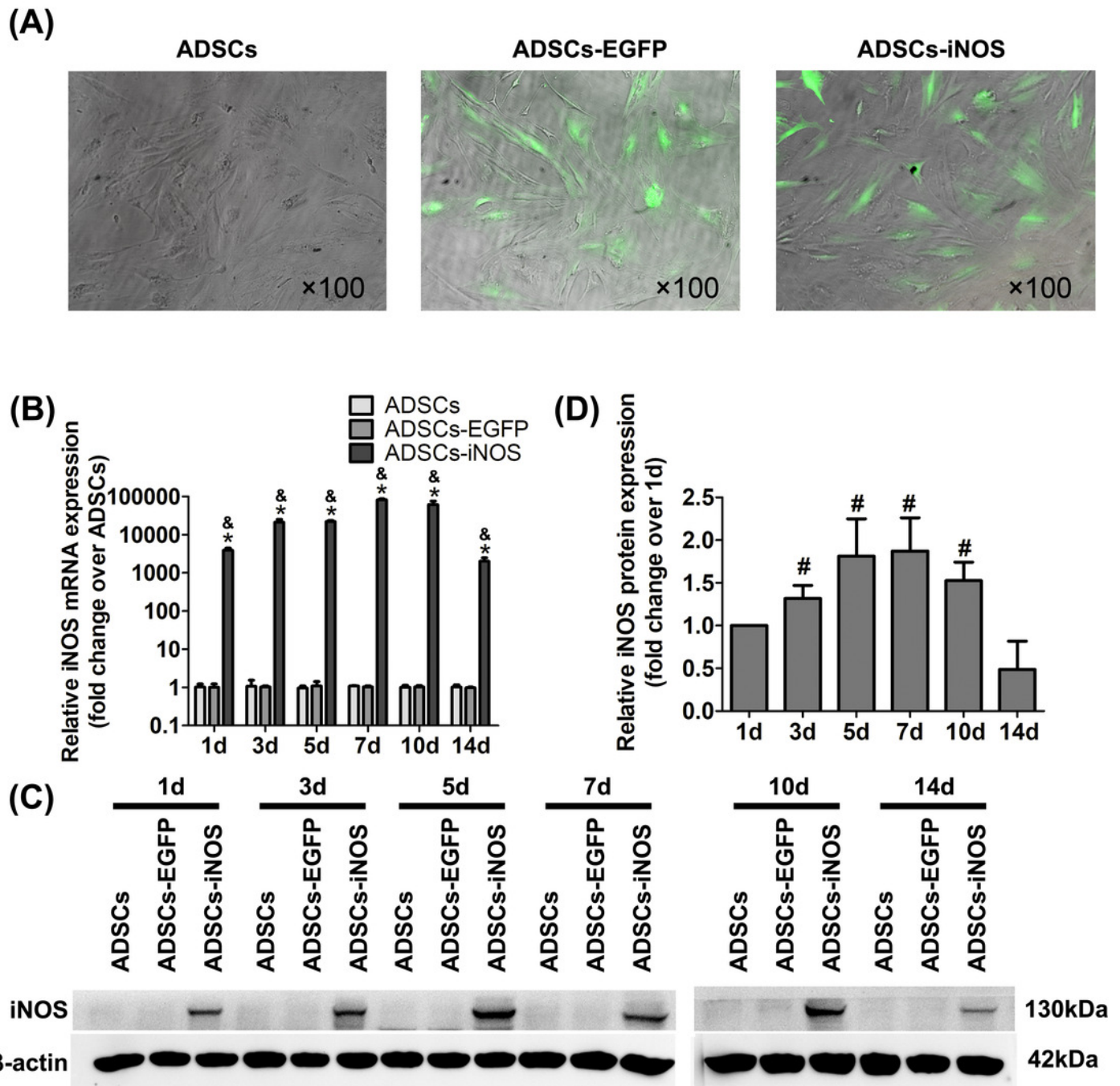
Figure 3

Figure 3. Characterization of ADSCs-iNOS.

(A) NO concentration in supernatant of ADSCs was measured after incubation in 10 mmol/L L-Arginine for 24 hours. Data are the mean of 3 independent experiments. (B) The protein expression of collagen I and collagen IV in CCSMCs was detected by Western blot analysis after 7 days of co-culture. (C) Data were shown as the relative density values of collagen I to β-actin as loading control. Data are the mean of 3 independent experiments. (D) Data were shown as the relative density values of collagen IV to β-actin as loading control. Data are the mean of 3 independent experiments. (E) Data were shown as the relative density values of TGF-β1 to β-actin as loading control. Data are the mean of 3 independent experiments. (F) Data were shown as the relative density values of p-Smad2/3 to Smad2/3. Data are the mean of 3 independent experiments. $^{*}P<0.05$ *vs.* ADSCs, $^{\$}P<0.05$ *vs.* ADSCs-EGFP, $^{\#}P<0.05$ *vs.* CCSMCs, $^{\&}P<0.05$ *vs.* CCSMCs cultured in DMEM containing 30mM glucose, $^{\$}P<0.05$ *vs.* CCSMCs co-cultured with ADSCs, $^{@}P<0.05$ *vs.* CCSMCs co-cultured with ADSCs-EGFP.
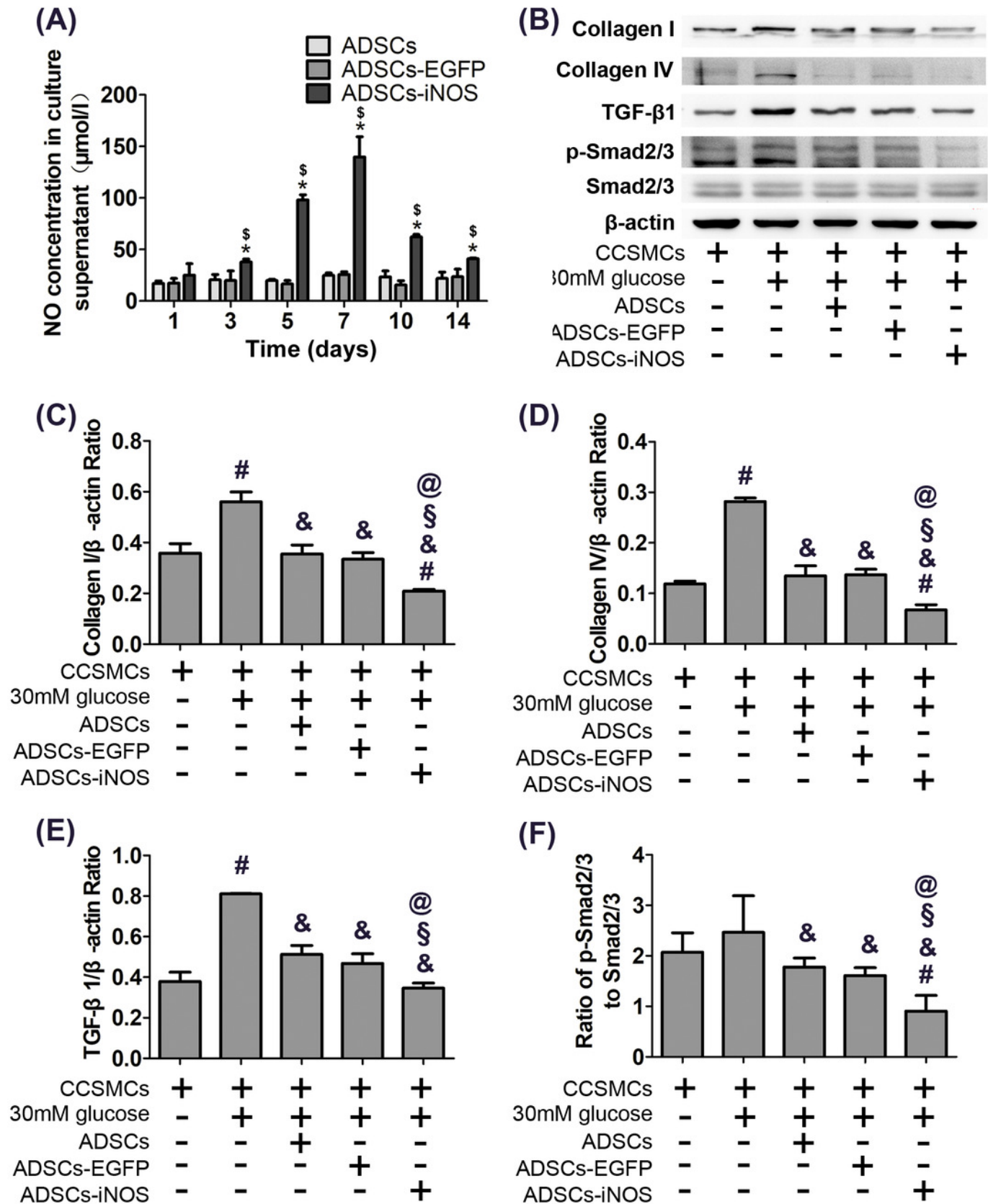
Figure 4

Figure 4. The transplantation of ADSCs-iNOS improved erectile function of DMED rats.

The transplantation of ADSCs-iNOS improved erectile function of DMED rats. (A) MAP and ICP response to electrostimulation of cavernous nerves (5 Volts, 1 minute). (B) ICP/MAP ratio in control, DMED, ADSCs, ADSCs-EGFP and ADSCs-iNOS groups. (C) NO concentration in penile tissues of each group. Data are the mean of 4 rats per group. (D) ELISA assay of cGMP concentration in penile tissues of each group. Data are the mean of 4 rats per group. (E) Penile tissues were stained with Masson trichrome in all groups. Collagen fibers were stained blue, while smooth muscle was stained red (primary magnification: ×100). (F) The collagen to smooth muscle ratio in penile tissues of each group. Data are the mean of 5 rats per group. $^{*}P<0.05$ *vs.* control group, $^{\#}P<0.05$ *vs.* DMED group, $^{\&}P<0.05$ *vs.* ADSCs group, $^{\S}P<0.05$ *vs.* ADSCs-EGFP.
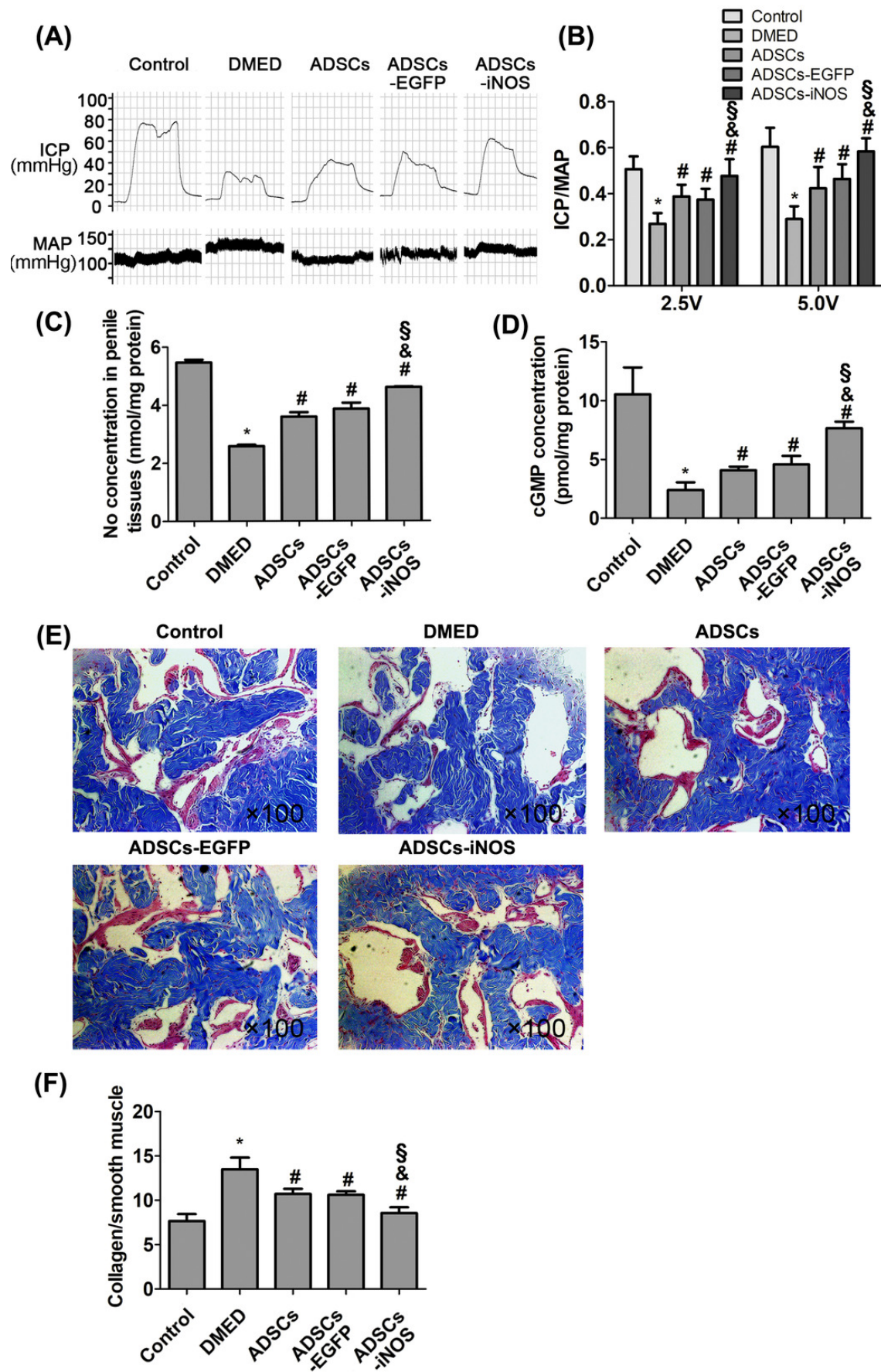
Table 1. Metabolic and physiological variables of experimental rats

Variable	Control	DMED	ADSCs	ADSCs-EGFP	ADSCs-iNOS
Initial weight (g)	268.0 ± 12.7	268.9 ± 8.6	268.4 ± 7.9	266.6 ± 12.9	270.7 ± 11.3
Final weight (g)	596.0 ± 54.5	$274.8 \pm 18.1^*$	$284.5 \pm 22.3^*$	$281.8 \pm 17.3^*$	$285.1 \pm 18.8^*$
Initial fasting glucose (mmol/L)	6.3 ± 0.5	6.2 ± 0.3	6.2 ± 0.4	6.2 ± 0.5	6.2 ± 0.4
Final fasting glucose (mmol/L)	6.2 ± 0.3	$30.8 \pm 2.0^*$	$29.4 \pm 2.1^*$	$30.3 \pm 1.6^*$	$30.0 \pm 2.8^*$
MAP (mmHg)	104.4 ± 5.7	105.1 ± 7.7	105.8 ± 6.3	102.0 ± 7.7	108.4 ± 6.4

1   **Note**: DMED, diabetes mellitus induced erectile dysfunction; ADSCs, adipose derived stem cells; iNOS,
2   inducible nitric oxide synthase; MAP, mean arterial pressure. $^*P<0.05$ *vs.* control group. Data expressed as
3   mean ± standard deviation.