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

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Background: Erectile dysfunction is a major complication of diabetes mellitus. Adipose derived stem cells (ADSCs) has 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- β 1 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 improve 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.

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21 Abstract

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

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

33 **Results:** We found that ADSCs-iNOS had significantly higher expression of iNOS at mRNA and
34 protein levels and generated more nitric oxide. ADSCs-iNOS reduced collagen I and collagen IV
35 expression of corpus cavernosum smooth muscle cells (CCSMCs) in cell co-culture model.
36 Transforming growth factor- β 1 expression and p-Smad2/3 to Smad2/3 ratio in CCSMCs reduced
37 following co-culture with ADSCs-iNOS. Injection of ADSCs-iNOS significantly ameliorated
38 DMED in rats and decreased collagen/smooth muscle cell ratio of penile tissues. Moreover,
39 elevated nitric oxide and cGMP concentrations were detected in penile tissues of ADSCs-iNOS
40 group.

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

44

45

46 Introduction

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

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

60 erectile function of type-II diabetic rats (*Garcia et al., 2010*). Stem cell therapy has beneficial
61 effects to increase contents of smooth muscle and endothelium, NO-cGMP pathway, neuronal
62 nitric oxide synthase (nNOS)-positive nerve fibers, and decrease fibrosis and apoptosis in penis
63 (*Chen et al., 2017; Qiu et al., 2011; Ryu et al., 2012; Sun et al., 2012; Zhang et al., 2016*).

64 Nitric oxide (NO) is a neurotransmitter which plays an important role in penile erection. NO
65 is synthesized by three nitric oxide synthase (NOS) isoforms, including nNOS, endothelial NOS
66 (eNOS) and inducible NOS (iNOS). Among these synthases, iNOS can produce NO continuously
67 independent of Ca^{2+} (*Oliveira-Paula, Lacchini & Tanus-Santos, 2014*). Recent evidence suggests
68 that the pathogenesis of DMED may be due to impaired NO production, and iNOS acts an essential
69 part in protecting penile tissues from the pro-fibrotic effects of hyperglycemia (*Ferrini et al.,*
70 *2010*). Continuous NO production by iNOS inhibits collagen synthesis, directly reacts with ROS
71 to produce peroxynitrite, and downregulates transforming growth factor- β 1 (TGF- β 1)/Smad
72 pathway, thus reducing fibrosis level (*Gonzalez-Cadavid & Rajfer, 2010*). Gene transfer of iNOS
73 DNA decreased TGF- β 1 and plasminogen-activator inhibitor-1 expression, and regressed
74 Peyronie's disease-like plaque (*Davila et al., 2004*). Our previous study showed that saRNA
75 mediated iNOS expression improved DMED via endogenously generating NO (*Wang et al., 2013*).

76 Given the beneficial effects of ADSCs and iNOS in the treatment of ED, we hypothesized that
77 ADSCs combined with iNOS could improve erectile function of DMED rats. In this study we
78 aimed to determine the efficacy of ADSCs combined with iNOS for improving erectile function
79 in DMED rats.

80

81 **Materials and methods**

82 **Rat ADSCs isolation and culture**

83 Rat ADSCs were isolated as previously described (*Wang et al., 2015*). Briefly, adipose tissues
84 were incised from inguen, and digested at 37°C in 0.1% collagenase type I (Sigma-Aldrich, St.
85 Louis, USA) with vigorous shaking for 90 minutes. The digested tissues were mixed with
86 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum
87 (FBS), and centrifuged at 220 g for 10 minutes. The cells were suspended in DMEM containing
88 10% FBS and filtered through a 75- μ m cell strainer (Solarbio, Beijing, China). Then cells were
89 plated and cultured in DMEM containing 10% FBS at 37°C in humidified incubator with 5% CO₂.
90 Cells were passaged with trypsin/EDTA (Beyotime, Nantong, China) as required.

91

92 **Flow cytometry**

93 ADSCs were characterized by flow cytometry at passage 4. Briefly, ADSCs were harvested
94 and washed twice with phosphate-buffered saline (PBS). Then, the cells were incubated for 30
95 minutes in PBS containing anti-CD29-FITC, CD34-FITC, CD45-FITC, CD49-FITC, CD73-FITC,
96 CD105-FITC, CD31-PE, CD90-PE, CD106-PE (all from BD Biosciences, San Diego, USA). The
97 stained cells were then subjected to flow cytometry analysis.

98

99 **Differentiation of ADSCs**

100 For adipocyte differentiation, ADSCs were cultured at a density of 1×10^4 cells/cm² in DMEM
101 containing 10% FBS, 50 mg/L indomethacin, 0.5 mmol/L isobutylmethylxanthine, 10 mg/L
102 insulin and 1 mmol/L dexamethasone (all from Sigma-Aldrich). After 21 days of induction, the
103 cells were stained using Oil red-O staining solution.

104 For smooth muscle cell differentiation, ADSCs were cultured at a density of 1×10^4 cells/cm²
105 in low-glucose DMEM containing 10% FBS, 50 µg/mL platelet-derived growth factor-BB and 5
106 µg/L TGF-β1 (all from PeproTech, Rocky Hill, USA). After 14 days of induction, the cells were
107 stained with the antibody against α-smooth muscle actin (α-SMA, 1:200, Boster, Wuhan, China).

108

109 **Transfection with adenovirus**

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

113

114 **Real-time PCR**

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

126

127 **Western blot analysis**

128 Protein extraction from rat penile tissues and cultured cells was performed using NP-40 lysis
129 buffer (Beyotime). Protein concentration was detected with the BCA protein assay kit (Beyotime).
130 50 µg protein was electrophoresed on 12% sodium dodecyl sulfate/polyacrylamide gels and then
131 blotted on a polyvinylidene difluoride membrane (Millipore, Billerica, USA). The membrane was
132 blotted with primary antibodies against collagen I (1:500, Proteintech, Wuhan, China), collagen
133 IV (1:500, Proteintech), iNOS (1:500, Santa Cruz, CA, USA), TGF-β1(1:1,000, Abcam,
134 Cambridge, UK), Smad2/3 (1:1,000, Abcam) and phospho-Smad2/3 (p-Smad2/3, 1:1,000, Abcam)
135 followed by secondary antibodies. Each band was quantified by densitometry with Image J
136 software.

137

138 **Cell proliferation assay**

139 Proliferation assay was performed with cell counting kit-8 (CCK-8, Dojindo, Shanghai,
140 China). Briefly, ADSCs, ADSCs-EGFP and ADSCs-iNOS were cultured in 96-well plates at

141 3×10^3 cells/well. After 1, 2, 3, 5, 7 and 14 days, 10 μ l CCK-8 solution was added into each well,
142 and incubated at 37°C for 2 hours. The absorbance at 450 nm was detected with a multiscan MK3
143 microplate reader (Thermo Fisher Scientific, Waltham, USA).

144

145 **Measurement of NO concentration**

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

150

151 **Cyclic guanosine monophosphate (cGMP) concentration determination**

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

155

156 **Rat corpus cavernosum smooth muscle cell (CCSMC) isolation and culture**

157 Rat CCSMCs were obtained from corpus cavernosum tissues as described previously with
158 minor modifications(*Chen et al., 2011*). Briefly, corpus cavernosum tissues were harvested and
159 cut into fragments of 1 mm³. These fragments were placed in an overturned 25 cm² culture flask.
160 Then, 4 ml DMEM containing 10% FBS was added into culture flask. After 30 minutes, the flask
161 was flipped over. Differential adherence method was used to purify CCSMCs. Immunostaining
162 with an antibody against α -SMA (1:200, Boster) was performed to identify CCSMCs.

163

164 **Transwell co-culture of ADSCs and CCSMCs**

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

170

171 **Animal experiments**

172 Animal experiments were carried out according to the guidelines and regulations by the Ethical
173 Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and
174 Technology. This study was approved by the Ethical Committee (TJ-A20131213). Sixty male SD
175 rats (8-week old) were purchased from Hunan SJA Laboratory Animal Co., Ltd. After an overnight
176 fast, fifty-one of them were randomly selected and received intraperitoneal injection with
177 streptozotocin (60 mg/kg, Sigma-Aldrich) (*Yang et al., 2013*). The remaining 9 rats were used as
178 controls. Three days after streptozotocin injection, rats with fasting blood glucose level higher than
179 16.7 mmol/L were selected as DM. After 8 weeks, DM rats were selected by the apomorphine
180 (APO) test. Briefly, APO (80 mg/kg, Sigma-Aldrich) was injected in the loose skin of neck.
181 DMED rats were identified if rats did not exhibit an erectile response within 30 minutes. ,

182 DMED rats were randomly divided into different treatment groups (9 rats in each group):
183 DMED group received no treatment; ADSCs group received corpus cavernosum injection of 60
184 μl PBS containing 5×10^5 ADSCs; ADSCs-EGFP group received corpus cavernosum injection of
185 60 μl PBS containing 5×10^5 ADSCs-EGFP; ADSCs-iNOS group received corpus cavernosum
186 injection of 60 μl PBS containing 5×10^5 ADSCs-iNOS.

187

188 **Assessment of erectile function**

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

196

197 **Masson trichrome staining**

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

202

203 **Statistical Analysis**

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

208

209 **Results**

210 **Isolation and characterization of ADSCs**

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

220 After culture for 5 to 7 days primary rat CCSMCs in spindle shape grew out of corpus
221 cavernosum tissues. After about 2 weeks of culture, cells were passaged and purified by the

222 differential adherence method (Fig. 1F). The cells were identified as CCSMCs by immunostaining
223 of α -SMA (Fig. 1G).

224

225 **Overexpression of iNOS in ADSCs**

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

231

232 **Effects of iNOS overexpression on CCSMCs**

233 As shown in Fig. 3A, the growth rate of ADSCs-iNOS did not change significantly compared
234 with ADSCs-EGFP and ADSCs. As expected, NO concentration in supernatant of ADSCs-iNOS
235 was significantly higher than that of ADSCs and ADSCs-EGFP ($P<0.05$). Moreover, NO
236 concentration in supernatant of ADSCs-iNOS reached maximum level on the 7th day ($P<0.05$,
237 Fig. 3B). We detected the effect of ADSC-iNOS on CCSMCs using Transwell co-culture model.
238 The expression of collagen I and collagen IV in CCSMCs significantly increased in high glucose
239 concentration ($P<0.05$). However, significant reduction of collagen I and collagen IV expression
240 was observed in CCSMCs co-cultured with ADSCs-iNOS ($P<0.05$, Fig. 3C, 3D and 3E). In
241 addition, we found that TGF- β 1 expression and p-Smad2/3 to Smad2/3 ratio in CCSMCs reduced
242 following co-culture with ADSCs-iNOS ($P<0.05$, Fig. 3F and 3G).

243

244 **Effects of ADSCs-iNOS on erectile function**

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

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

263 ($P<0.05$) (Fig. 4E and 4F).

264

265 Discussion

266 In this study we demonstrated that infection of ADSCs with adenovirus containing iNOS
267 expression cassette led to significantly high expression of iNOS and increased generation of NO.
268 ADSCs played a positive role in restoring DMED in rats. Furthermore, overexpression of iNOS in
269 ADSCs was proved to achieve a more significant improvement of erectile function. The
270 therapeutic effect may be achieved by increased NO generation and the suppression of collagen I
271 and collagen IV expression in the CCSMCs to decrease penile fibrosis.

272 Corpus cavernosum is composed of a loose trabecular meshwork of smooth muscle and
273 connective tissues, which are structural basis of erectile function. Ryu *et al* found that collagen
274 fiber content was significantly increased in corpus cavernosum of patients with vasculogenic ED
275 (Ryu *et al.*, 2004). Further studies demonstrated that extracellular matrix such as fibronectin,
276 collagen IV and collagen I accumulated and collagen I/collagen III ratio was decreased in the
277 corpus cavernosum tissue of diabetic ED rats (Hirata *et al.*, 2009; Zhou *et al.*, 2012). Abnormal
278 extracellular matrix can cause mechanical alterations of corpus cavernosum, which may provoke
279 penile venous leakage, leading to vasculogenic ED (Li *et al.*, 2013). Increased expression of TGF-
280 β 1 pathway could be involved in collagen fiber accumulation and penile fibrosis (Ryu *et al.*, 2004).
281 CCSMCs are the predominant mesenchymal cell type in the corpus cavernosum. Synthesis of
282 connective tissue proteins and collagen by CCSMCs was significantly increased by exogenous
283 TGF- β 1 (Moreland *et al.*, 1995). In accordance with previous studies, our study showed that
284 collagen I and collagen IV expression in CCSMCs significantly increased under high glucose
285 condition and penile fibrosis significantly increased in DMED rats. Then we co-cultured CCSMCs
286 with ADSCs, and found significantly decreased synthesis of collagen I and collagen IV.
287 Furthermore, we found that TGF- β 1 expression and p-Smad2/3 to Smad2/3 ratio in CCSMCs
288 significantly decreased, indicating the involvement of TGF- β 1/Smad signaling pathway. However,
289 further studies are needed to elucidate precise mechanism of ADSCs' effect on CCSMCs. In *vivo*,
290 ADSCs ameliorated penile fibrosis in ED rats, maybe due to secreting exosomes enriched with
291 miR-132 and miR- let7 which inhibit the expression of tissue inhibitors of metalloproteinases and
292 stimulate the expression and activity of matrix metalloproteinases (Gokce *et al.*, 2014; Zhu *et al.*,
293 2017).

294 NO/cGMP signaling pathway plays a crucial role of in erectile function. NO released from
295 nonadrenergic noncholinergic nerves and endothelium is the principal mediator of penile erection.
296 Moreover, this pathway plays an important role in ameliorating tissue fibrosis. Marta *et al* found
297 that NO inhibited TGF- β /Smad induced gene transactivation in a cGMP-dependent manner in
298 endothelial cells, leading to proteasomal degradation of phosphorylated Smad. In addition, NO
299 reduced and delayed nuclear translocation of activated Smad (Saura *et al.*, 2005). cGMP
300 significantly reduced TGF- β induced upregulation of collagen I and collagen II at mRNA and
301 protein levels (Beyer *et al.*, 2015). In this study, we found that iNOS overexpression in ADSCs
302 significantly enhanced NO generation and could last for 14 days. Compared to ADSCs, ADSCs-

303 iNOS significantly increased NO and cGMP concentrations in penile tissues, and decreased
304 collagen I and collagen IV expression in CCSMCs to ameliorate penile fibrosis in DMED rats.
305 These results are consistent with previous studies that the inhibition of iNOS promoted penile
306 fibrosis. For example, Monica *et al* found that more collagen content was deposited in corpus
307 cavernosum of iNOS knockout mouse. In addition, inactivation of iNOS gene led to exacerbated
308 penile fibrosis in DM mouse by increasing oxidative stress and TGF- β 1 expression under
309 hyperglycemia condition (Ferrini *et al.*, 2010). Similarly, L-NIL as an inhibitor of iNOS activity
310 increased oxidative and fibrosis in the media of arteries (Ferrini *et al.*, 2004).

311 However, other studies suggested that erectile function was impaired by iNOS expression.
312 The induction of iNOS accompanying penil fibrosis was demonstrated in rat models of DMED,
313 Peyronie's disease and aging-related ED (Bivalacqua *et al.*, 2000; Ferrini *et al.*, 2001; Usta *et al.*,
314 2003). In addition, iNOS inhibition improved erectile function in Peyronie's disease or DMED rat.
315 However, the application of aminoguanidine as an iNOS inhibitor was not appropriate, because
316 aminoguanidine inhibited not only all three kinds of NOS, but also advanced glycation end
317 products involved in tissue fibrosis. Moreover, further studies are needed to determine if elevated
318 iNOS expression is possibly a compensatory reflection when eNOS and nNOS expression is
319 decreased under pathological conditions.

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

334 Conclusions

335 In conclusion, intracavernous administration of ADSCs-iNOS improved erectile function of
336 DMED rats. Injection of ADSCs-iNOS in a rat model of DM significantly decreased penile
337 fibrosis, mainly due to increased NO generation and suppressed expression of collagen I and
338 collagen IV in the CCSMCs.

339

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

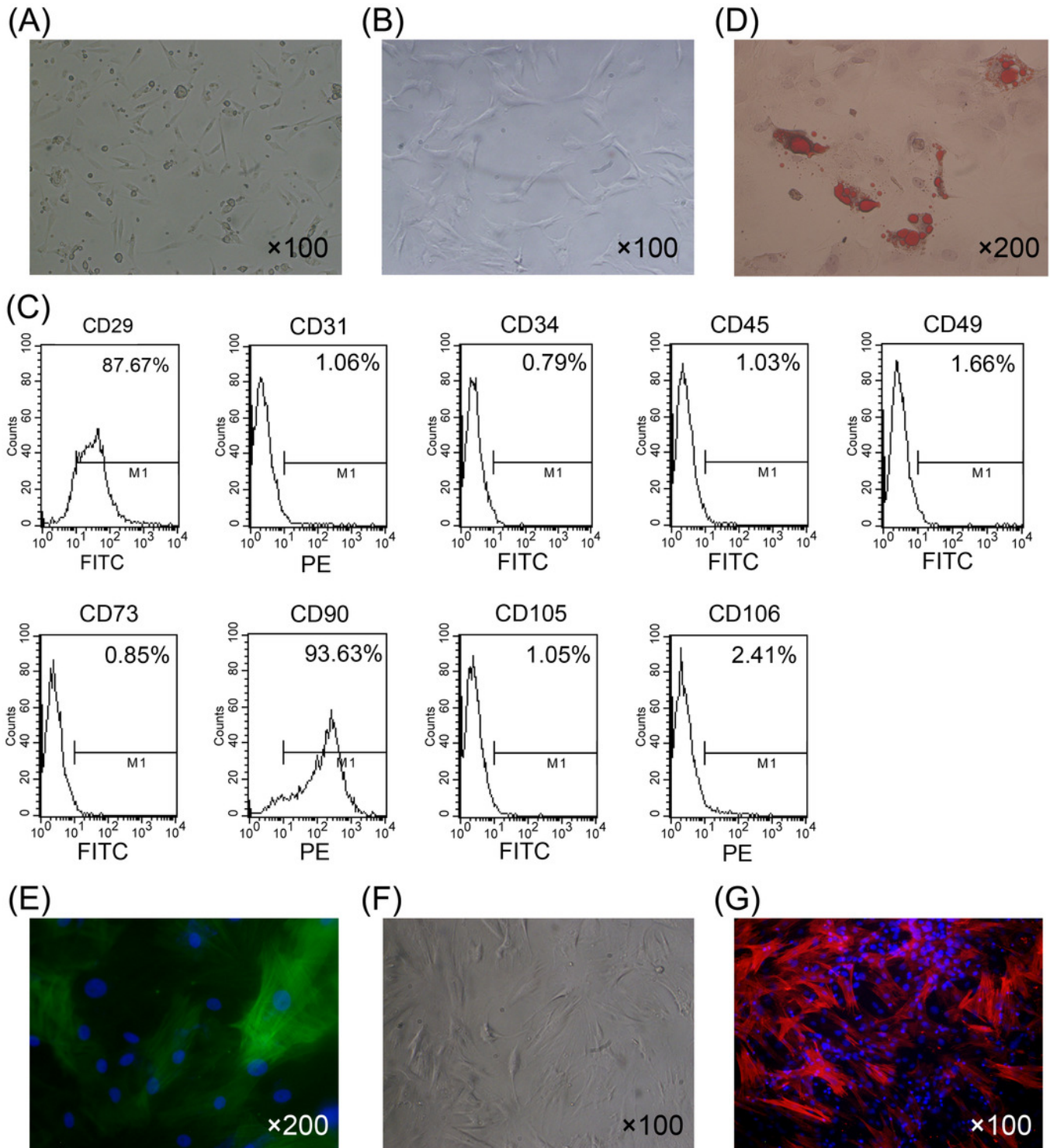
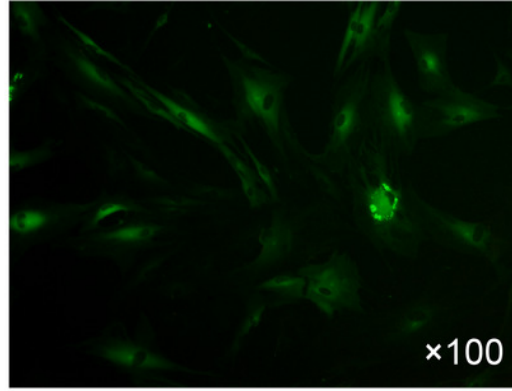
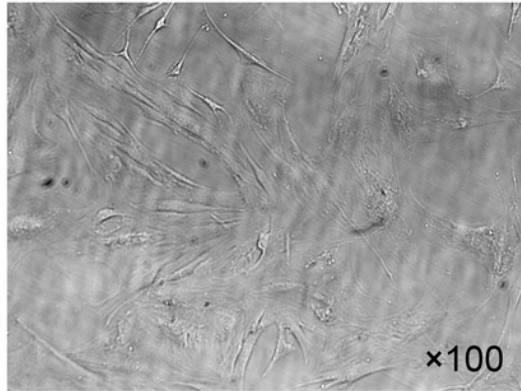


Figure 2

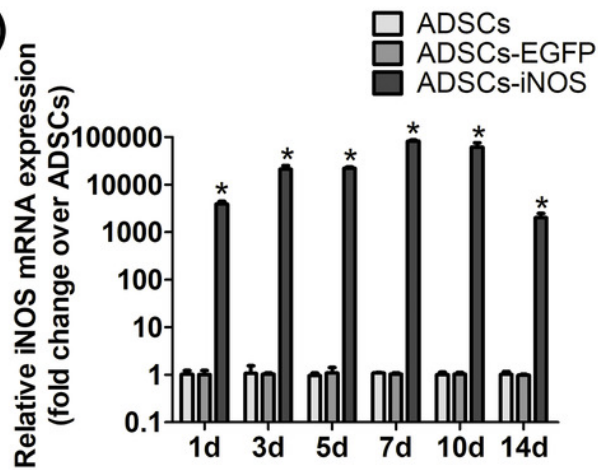
Figure 2. iNOS expression in ADSCs.

(A) EGFP-expressing ADSCs were observed by fluorescence microscopy 3 days after infection (primary magnification: $\times 100$). (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. (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. * $P < 0.05$ vs. ADSCs, # $P < 0.05$ vs. 1d

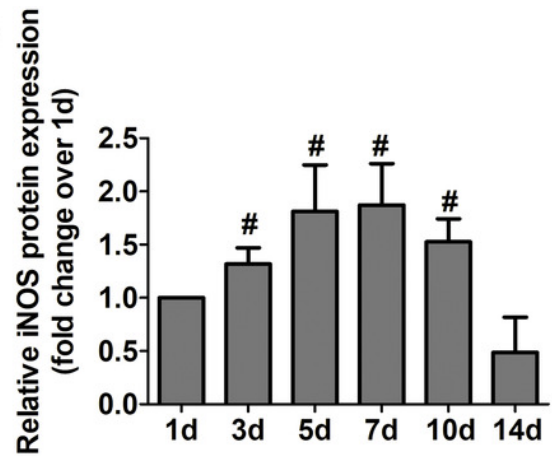
(A)



(B)



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(C)

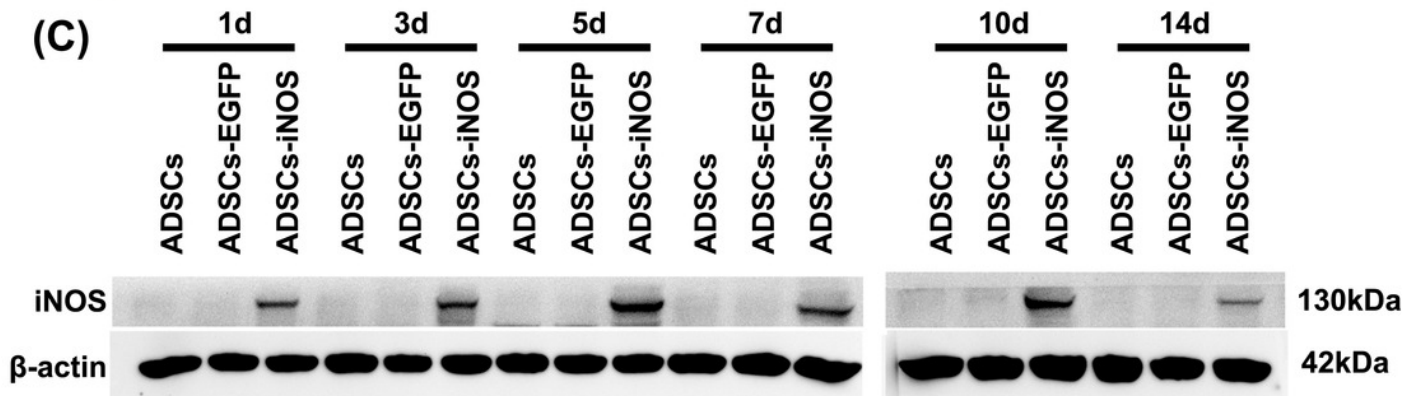


Figure 3

Figure 3. Characterization of ADSCs-iNOS.

(A) CCK-8 assay was performed to detect the proliferation of ADSCs-iNOS. (B) NO concentration in supernatant of ADSCs was measured after incubation in 10 mmol/L L-Arginine for 24 hours. (C) The protein expression of collagen I and collagen IV in CCSMCs was detected by Western blot analysis after 7 days of co-culture. (D) Data were shown as the relative density values of collagen I to β -actin as loading control. (E) Data were shown as the relative density values of collagen IV to β -actin as loading control. (F) Data were shown as the relative density values of TGF- β 1 to β -actin as loading control. (G) Data were shown as the relative density values of p-Smad2/3 to Smad2/3. * $P < 0.05$ vs. ADSCs, # $P < 0.05$ vs. CCSMCs, $^{\&}$ $P < 0.05$ vs. CCSMCs cultured in DMEM containing 30mM glucose, $^{\$}$ $P < 0.05$ vs. CCSMCs co-cultured with ADSCs.

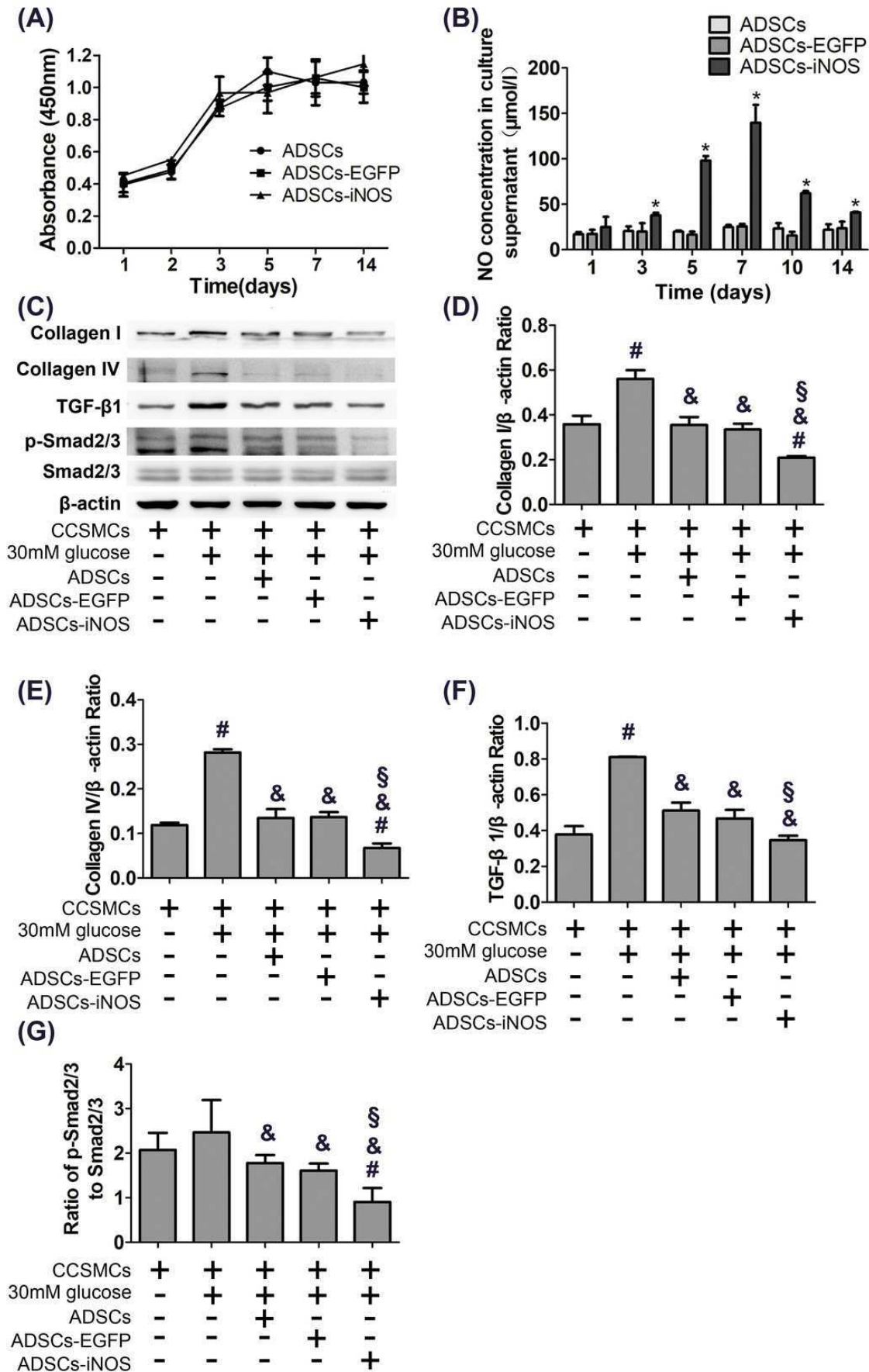


Figure 4

Figure 4. 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. (D) ELISA assay of cGMP concentration in penile tissues of each 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: $\times 100$). (F) The collagen to smooth muscle ratio in penile tissues of each group. * $P < 0.05$ vs. control group, # $P < 0.05$ vs. DMED group, & $P < 0.05$ vs. ADSCs group.

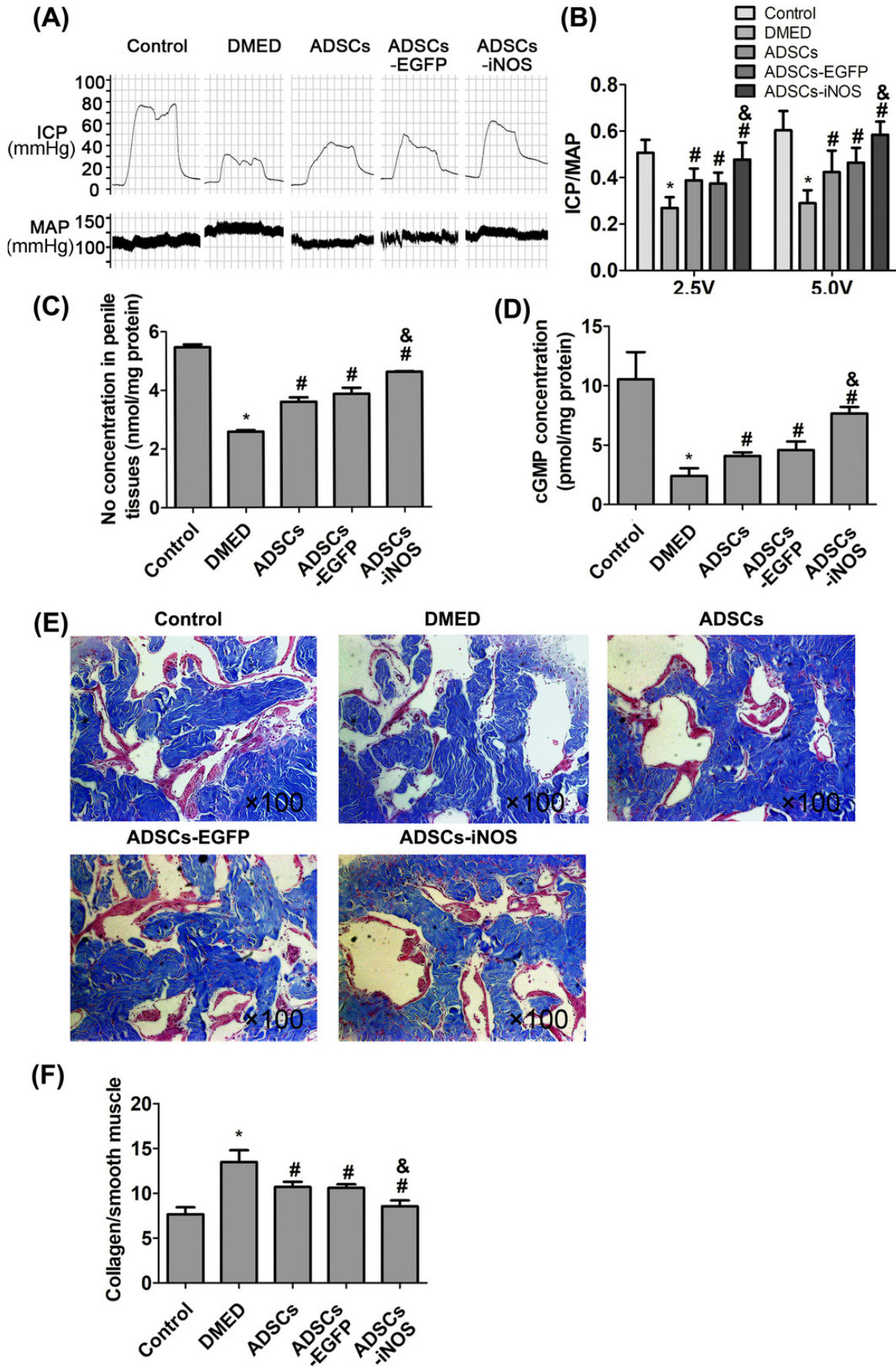


Table 1 (on next page)

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 ± 18.1*	284.5 ± 22.3*	281.8 ± 17.3*	285.1 ± 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 ± 2.0*	29.4 ± 2.1*	30.3 ± 1.6*	30.0 ± 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.
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