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 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 ADSCs significantly improved erectile function of type-II diabetic rats (*Garcia et al., 2010*).

60 Stem cell therapy has beneficial effects to increase smooth muscle and endothelium, NO-cGMP
61 pathway, neuronal nitric oxide synthase (nNOS)-positive nerve fibers, and decrease fibrosis and
62 apoptosis in the penis (*Chen et al., 2017; Qiu et al., 2011; Ryu et al., 2012; Sun et al., 2012;*
63 *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
67 continuously independent of Ca^{2+} (*Eissa et al., 1998*). Recent evidence suggests that the
68 pathogenesis of DMED may be due to impaired NO production, and iNOS acts an essential part
69 in protecting penile tissues from the pro-fibrotic effects of hyperglycemia (*Ferrini et al., 2010*).
70 Continuous NO production by iNOS inhibits collagen synthesis, directly reacts with ROS to
71 produce peroxynitrite, and downregulates transforming growth factor-β1 (TGF-β1)/Smad
72 pathway, thus reducing fibrosis level (*Gonzalez-Cadavid & Rajfer, 2010*). Gene transfer of iNOS
73 DNA decreased TGF-β1 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.,*
76 *2013*).
77   Given the beneficial effects of ADSCs and iNOS in the treatment of ED, we hypothesized
78 that ADSCs combined with iNOS could improve erectile function of DMED rats. In this study
79 we aimed to determine the efficacy of ADSCs combined with iNOS for improving erectile
80 function in DMED rats.
81

Materials and methods

82

Rat ADSCs isolation and culture

83

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

Flow cytometry

94

95   ADSCs were characterized by flow cytometry at passage 4. Briefly, ADSCs were harvested
96 and washed twice with phosphate-buffered saline (PBS). Then, the cells were incubated for 30
97 minutes in PBS containing anti-CD29-FITC(cat. # 555005, BD, San Diego, USA), CD31-PE(cat.
98 # 555027, BD), CD49-FITC(cat. # 557457, BD), CD90-PE(cat. # 551401, BD), CD106-PE(cat.
99 # 559229, BD), CD34-FITC(cat. # sc-7324, Santa Cruz, Dallas, Texas), CD45-FITC(cat. #

100   MCA43FT, AbD, Oxford, UK), CD73-FITC (cat. # bs-23233R, Bioss, Beijing, China) , CD105-
101   FITC (cat. # bs-10662R, Bioss). The stained cells were then subjected to flow cytometry analysis.
102

Differentiation of ADSCs

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

Transfection with adenovirus

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

Real-time PCR

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

Western blot analysis

134   Protein extraction from rat penile tissues and cultured cells was performed using NP-40 lysis
135   buffer (Beyotime). Protein concentration was detected with the BCA protein assay kit
136   (Beyotime). 50 µg protein was electrophoresed on 12% sodium dodecyl sulfate/polyacrylamide
137   gels and then blotted on a polyvinylidene difluoride membrane (Millipore, Billerica, USA). The
138   membrane was blotted with primary antibodies against collagen I (cat. # 14695-1-AP,
139   Proteintech, Wuhan, China), collagen IV (cat. # 55131-1-AP, Proteintech), iNOS (cat. # sc-7271,
140   Santa Cruz, Dallas, USA), TGF-β1 (cat. # ab92486, Abcam, Cambridge, UK)) followed by

141 HRP-labelled secondary antibodies. The enhanced chemiluminescence detection system (Pierce;
142 Thermo Fisher, Rockford, USA) was used to detect protein bands. Each band was quantified by
143 densitometry with Image J software.

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 mm^3. These fragments were placed in an overturned 25 cm^2 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 was performed to identify CCSMCs. Immunofluorescence
163 staining was performed as previously described (*Zhang et al., 2016*).

164

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

171

172 **Animal experiments**
173   Animal experiments were carried out according to the guidelines and regulations by the
174 Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science
175 and Technology. This study was approved by the Ethical Committee (TJ-A20131213). Sixty
176 male SD rats (8-week old) were purchased from Hunan SJA Laboratory Animal Co., Ltd. After
177 an overnight fast, fifty-one of them were randomly selected and received intraperitoneal injection
178 with streptozotocin (60 mg/kg, Sigma-Aldrich) (*Yang et al., 2013*). The remaining 9 rats were
179 used as controls. Three days after streptozotocin injection, rats with fasting blood glucose level
180 higher than 16.7 mmol/L were selected as DM. After 8 weeks, DM rats were selected by the
181 apomorphine (APO) test. Briefly, APO (80 mg/kg, Sigma-Aldrich) was injected in the loose skin

182   of neck. DMED rats were identified if rats did not exhibit an erectile response within 30 minutes.

183   DMED rats were randomly divided into different treatment groups (9 rats in each group):

184   DMED group received no treatment; ADSCs group received corpus cavernosum injection of 60

185   μl PBS containing 5×10^5 ADSCs; ADSCs-EGFP group received corpus cavernosum injection of

186   60 μl PBS containing 5×10^5 ADSCs-EGFP; ADSCs-iNOS group received corpus cavernosum

187   injection of 60 μl PBS containing 5×10^5 ADSCs-iNOS.

188

189   **Assessment of erectile function**

190   After 2 weeks, intracavernous pressure (ICP) and mean arterial pressure (MAP) were

191   detected to evaluate erectile function as described previously (*Yang et al., 2013*). The stimulus

192   parameters were two voltages (2.5, 5.0 volts), frequency 15 Hz, duration 1 minute and pulse

193   width 1.2 ms. Erectile response was measured at 2.5 and 5.0 voltages. Pressure was measured

194   and recorded with a data acquisition system (AD Instruments Powerlab/4SP, NSW, Australia).

195   Then, penile tissues were harvested for measurement of NO and cGMP concentration (n =

196   4/group) and masson trichrome staining (n = 5/group)

197

198   **Masson trichrome staining**

199   Penile tissues were embedded in paraffin and cut into 5 μm thick sections. Masson trichrome

200   staining was performed as previously described (*Yang et al., 2013*). Image Pro Plus 6.0 software

201   (Media Cybernetics Inc, Bethesda, USA) was used to quantitatively analyze smooth muscle

202   content and collagen in five randomly selected specimens per group.

203

204   **Statistical Analysis**

205   Results were expressed as mean ± standard deviation. Data were analyzed with one-way

206   analysis of variance followed by Tukey-Kramer test for post hoc comparisons using GraphPad

207   Prism 5.0 (GraphPad Software, San Diego, USA). Normal distribution was determined by

208   Kolmogorov-Smirnov test. $P<0.05$ was considered statistically significant.

209

210   # Results

211   **Isolation and characterization of ADSCs**

212   The isolated ADSCs exhibited spindle-shaped morphology at passage 1 (Fig. 1A). ADSCs

213   expanded *in vitro* and showed fibroblast-like shape at passage 4 (Fig. 1B). To characterize

214   ADSCs, surface markers of the cultured cells at passage 4 were determined using flow cytometry.

215   As shown in Fig. 1C-K, most of the cells expressed CD29 and CD90, while few cells were

216   positive for CD34, CD45, CD73, CD105, CD31 and CD106. To identify the multi-lineage

217   differentiation ability of ADSCs, we induced adipogenic and myogenic differentiation of ADSCs

218   for 21 or 14 days in appropriate induction medium. Oil red-O staining indicated that ADSCs

219   could differentiate to adipocytes (Fig. 1L). Immunofluorescence staining with an antibody

220   against α-SMA indicated that ADSCs could differentiate to smooth muscle cells (Fig. 1M).

221   After culture for 5 to 7 days primary rat CCSMCs in spindle shape grew out of corpus

222 cavernosum tissues. After about 2 weeks of culture, cells were passaged and purified by the
223 differential adherence method (Fig. 1N). The cells were identified as CCSMCs by
224 immunostaining of α-SMA (Fig. 1O).

225

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

233

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

244

245 **Effects of ADSCs-iNOS on erectile function**
246 Finally we evaluated the effects of ADSCs-iNOS on erectile function of DMED rats. There
247 was no significant difference in initial body weight or serum glucose concentration among the 5
248 groups of rats. 14 days after the induction of diabetes, body weight was significantly lower and
249 fasting glucose concentration was significantly higher in the diabetic rats than the age-matched
250 control rats ($P<0.05$). However, there were no significant differences in body weight or serum
251 glucose concentration among DMED, ADSCs, ADSCs-EGFP and ADSCs-iNOS groups (Table
252 1).
253 Erectile function was measured 2 weeks after ADSCs transplantation. The ICP/MAP ratio was
254 significantly lower in DMED group than that in control group. Intracavernous injection of
255 ADSCs or ADSCs-EGFP significantly improved erectile function of DMED rats. The ICP/MAP
256 ratio of ADSCs-iNOS group was markedly elevated compared to DMED, ADSCs and ADSCs-
257 EGFP groups (all $P<0.05$, Fig. 4A and 4B). In addition, NO and cGMP concentrations in penile
258 tissues of ADSCs and ADSCs-EGFP groups were significantly elevated compared to DMED
259 group, although they were still significant lower compared to ADSCs-iNOS group ($P<0.05$, Fig.
260 4C and 4D). The mean collagen/smooth muscle cell ratio significantly increased in the DMED
261 rats compared to the control rats ($P<0.05$). Intracavernous injection of ADSCs or ADSCs-EGFP
262 attenuated collagen content ($P<0.05$). Furthermore, mean collagen/smooth muscle cell ratio in

263 ADSCs-iNOS treated rats was significantly lower than that in ADSCs or ADSCs-EGFP group
264 ($P<0.05$) (Fig. 4E-I and 4J).
265

Discussion

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

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

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

324   There are some limitations in the current study. First, we did not investigate the effect of
325 iNOS expression on the stemness of ADSCs. In our study, more NO was released from ADSCs-
326 iNOS than ADSCs. Tapia-Limonchi *et al* found that NO maintained embryonic stem cells (ESC)
327 pluripotency and delayed ESC differentiation by regulating Gsk3-β/β-catenin and PI3K/Akt
328 signaling pathways (*Tapia-Limonchi et al., 2016*). The effect of NO released from ADSCs iNOS
329 on ADSC stemness required further investigation. Second, ADSCs were only transplanted into
330 corpus cavernosum once. Repeated injection of ADSCs may achieve better therapeutic effects
331 but need to be confirmed in future studies. Third, we did not compare therapeutic effect of
332 ADSCs-iNOS to that of iNOS alone. Although adenovirus act as efficient agents for gene
333 transfer, they can activate cellular and humoral immune response in the hosts, which limits the
334 safety and efficacy *in vivo*. The innate immune response is mediated by the adenovirus particle,
335 but not viral transcription (*Muruve, 2004*). In contrast, transplanted stem cells could suppress
336 excessive immune response (*Lin, Lin & Lue, 2012*). ADSCs overexpressing iNOS may be safer
337 and more efficient than iNOS alone. Fourth, the statistical power for this study was relatively
338 low (49.9%) which may be due to the small group size, and more experimental rats will be
339 included to validate these results in our further study. Fifth, our study did not detect distinct
340 downstream pathways of TGF-β1 to determine the precise mechanism of ADSCs-iNOS' effect
341 on CCSMCs. A separate but more extensive experiment on this topic will be carried out.

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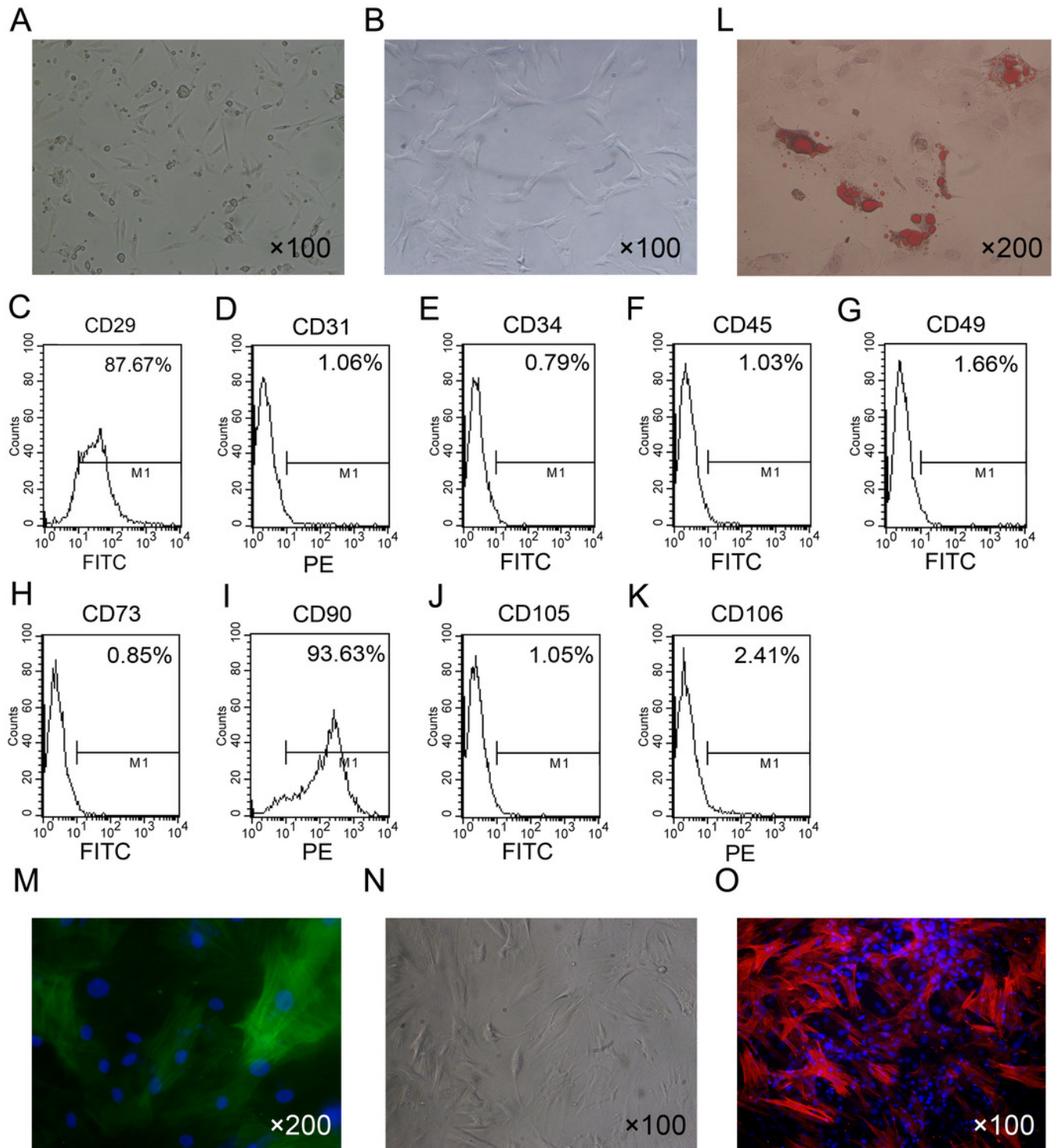
Figure 2

Figure 2. iNOS expression in ADSCs.

(A-C) EGFP-expressing ADSCs were observed by fluorescence microscopy 3 days after infection (primary magnification: ×100). (D) 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. (E) iNOS expression at protein level was detected by Western blot analysis. Data are the mean of 3 independent experiments. [*]$P<0.05$ *vs.* ADSCs, [&]$P<0.05$ *vs.* ADSCs-EGFP
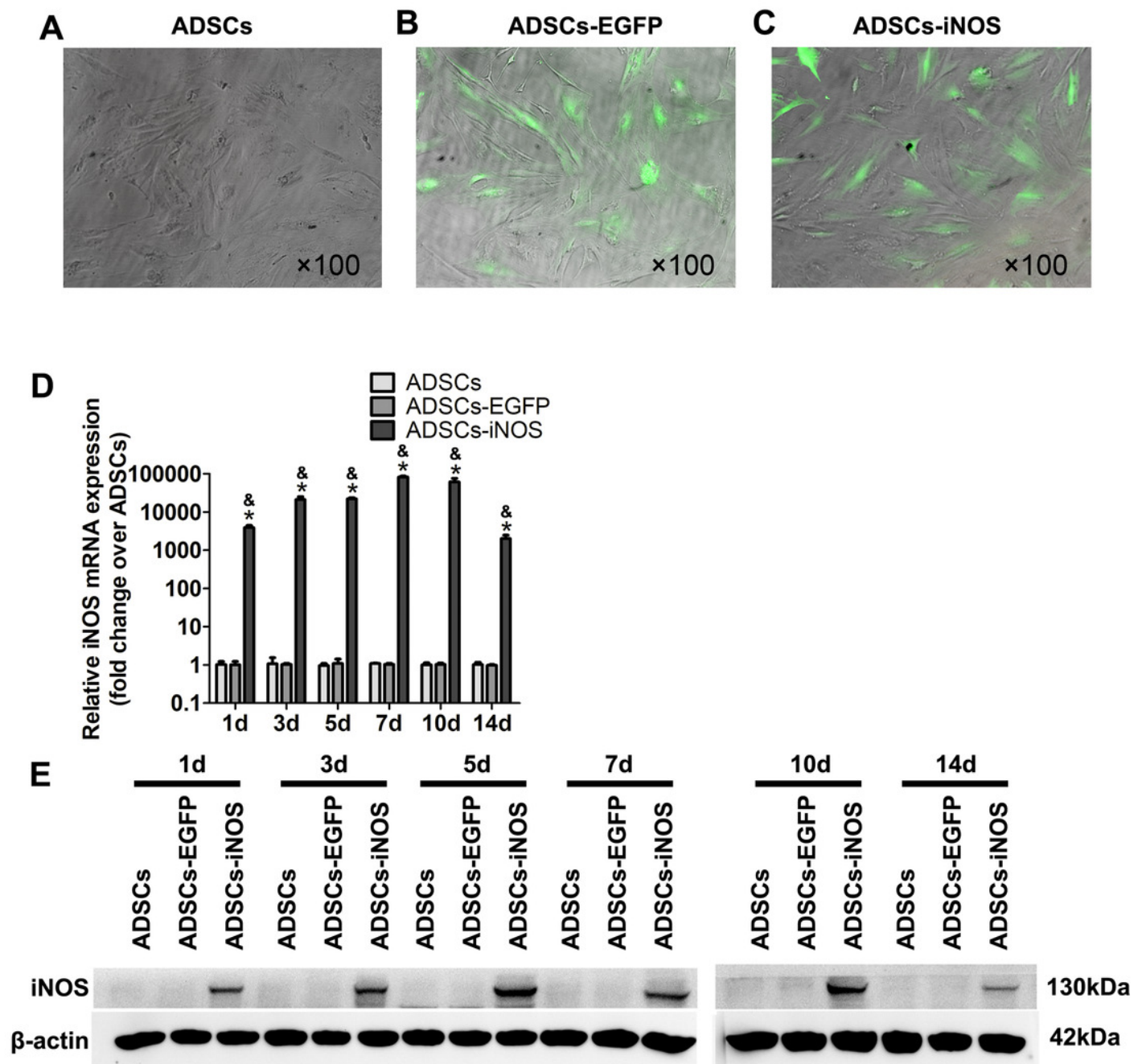
Figure 3

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, collagen IV and TGF-β1 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. $^*P<0.05$ *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.
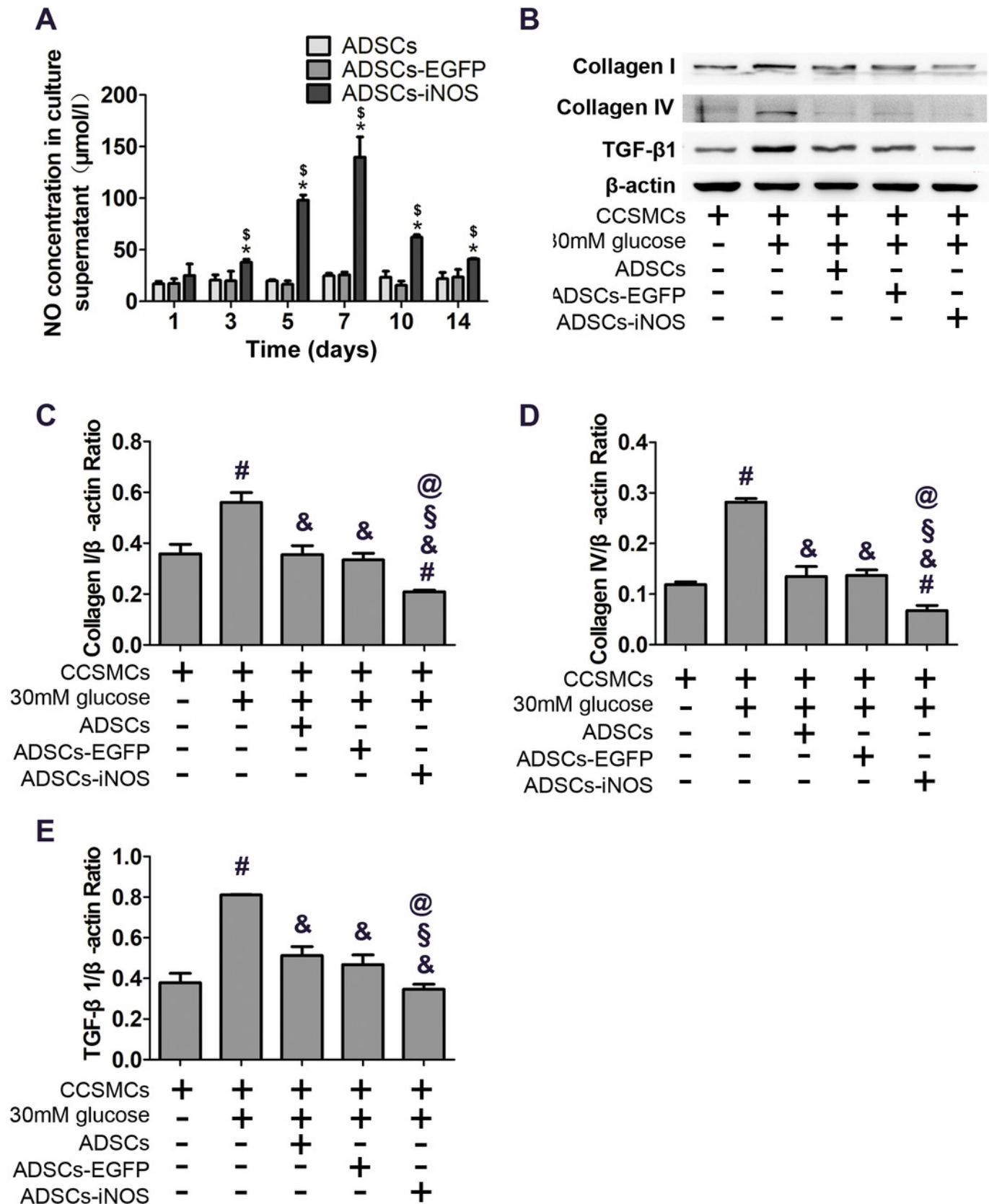
Figure 4

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

(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-I) Penile tissues were stained with Masson trichrome in all groups. Collagen fibers were stained blue, while smooth muscle was stained red (primary magnification: ×100). (J) 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, [§]$P<0.05$ *vs.* ADSCs-EGFP.
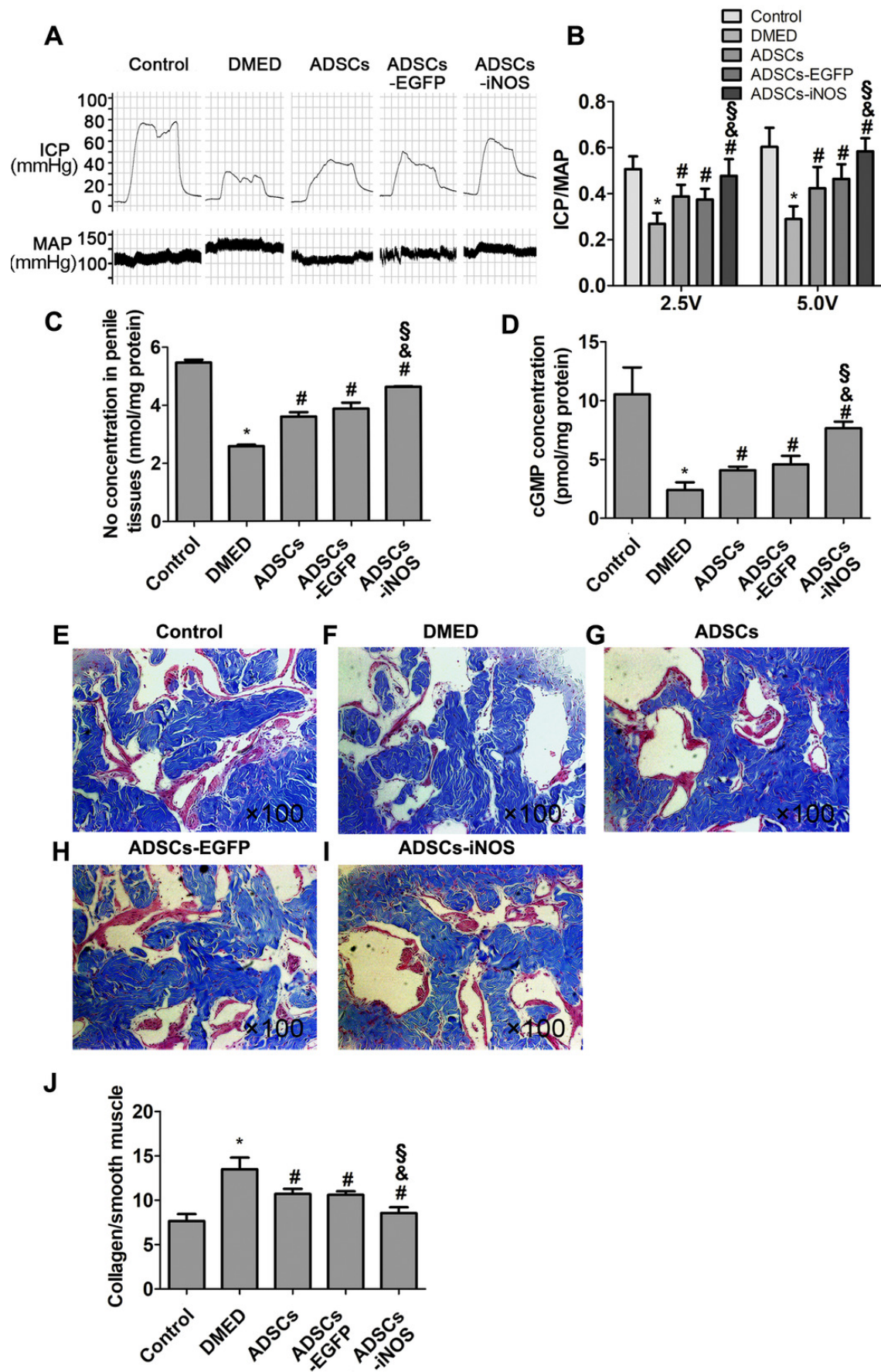
Table 1. Metabolic and physiological variables of experimental rats

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 ± 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.