Exosomes from human urine-derived stem cells combined with hyaluronic acid ameliorate erectile dysfunction in rats of type 2 diabetes by topical administration

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Research Article

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Abstract

Background: The high prevalence of erectile dysfunction (ED) in patients with type 2 diabetes mellitus (DM2) is a challenging clinical problem. Researches on exosomes from urinary-derived stem cells (USC-Exo) have shown that they have significant therapeutic effects in a variety of diseases including ED by injection. Hyaluronic acid (HA) is especially useful for delivering bioactive molecules. This study investigated the effects and related mechanisms of local administration of human USC-Exo combined with HA (USC-Exo-HA) on a rat model of DM2ED.

Methods: UCSs were extracted from human urine samples, and identified for preparation of the corresponding USC-Exo. The effects of high glucose and USC-Exo on human umbilical vein endothelial cells (HUVECs) were assessed in vitro using a CCK-8 assay to determine cell proliferation and pick the most appropriate concentration for subsequent experiments. Scratch and tube formation assays were performed to assess the function of HUVECs. qRT-PCR was used to detect the expression of genes such as B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (BAX) and superoxide dismutase-2 (SOD2). HA and USC-Exo-HA were prepared at concentrations and then administered topically to DM2ED rats multiple times. Intracavernous pressure (ICP) and mean arterial pressure (MAP) were measured to assess erectile function in rats. Masson, Tunel, Immunohistochemistry and Western blot analysis were performed to assess the fibrosis and endothelial function in corpus cavernosum, respectively.

Results: Compared with the control group, the proliferation, migration ability, and tube-forming ability of HUVECs decreased in high glucose environment, while USC-Exo could optimize the function of HUVECs, reverse the expression of apoptotic genes, and enhance the antioxidant capacity. USC-Exo-HA showed improvement in erectile dysfunction compared to the HA group, and the 10-dose group was better than the 5-dose group. Histologically, the USC-Exo-HA group significantly improved apoptosis, angiogenesis and smooth muscle regeneration in the corpus cavernosum compared to the HA group.

Conclusions: The topical application of USC-Exo-HA in the treatment of DM2ED rats has been proved effective. The potential mechanism might to promote the proliferation of endothelial cells and smooth muscle in the corpus cavernosum, which leads to the remodeling of erectile function. And multiple dosing at intervals may make the effect more pronounced.

Background

Erectile dysfunction (ED) is a common andrological disease, which refers to the inability of men to achieve a full erection required for sex or to maintain an adequate erection for satisfactory sexual duration[1]. According to the European Male Ageing Study (EMAS), the largest multi-centre population study of elderly men (40-79 years old) in Europe reveals that the prevalence of erectile dysfunction ranges from 6–64%, depending on different age subgroups and increasing with age, with an average prevalence of 30%[2]. In terms of etiology, ED can be divided into organic, psychological and endocrine. Organic ED can be induced by various underlying disorders (such as diabetes, hypertension, atherosclerosis,

Peyronie's disease, etc.) or physical shock (such as trauma, pelvic surgery, etc.)[3, 4]. Compared with the general population, the onset of ED in diabetic patients is earlier. The prevalence of them approximately 3.5 times more than that of non-diabetic people, and the comprehensive prevalence rate of all age groups is more than 35%[5]. Although during the past two decades, therapies such as oral phosphodiesterase-5 inhibitors (PDE5i), intracavernosal injections (ICI) of vasoactive agents and low-intensity shock waves have emerged. The therapeutic effect is limited to the relief of symptoms, and often shows temporary improvement rather than permanent cure or fundamental pathological transformation[6].

Stem cells have been widely reported in the literature on the treatment of various complications of diabetes, which is a promising new field. Urine-derived stem cells (USCs) are a subset of cells isolated from urine, and have similar surface markers to mesenchymal stem cells (MSCs), including CD73, CD90, and CD105. They also demonstrate the biological characteristics of stem cells, which can differentiate into bone, muscle, and fat cells in special induction cultures[7]. There has been reported that significant improvement can induced by intravenous USCs in many kinds of complications of diabetes, including diabetic nephropathy[8], chronic diabetic non-healing wounds[9] and diabetes-induced erectile dysfunction[10].

Numerous studies reported that the restorative function of stem cells is based on paracrine mechanisms[11]. It emphasizes that the improvement is caused by exosomes secreted after stem cell injection. Exosomes are small enveloped vesicles with a diameter of 30-150 nm, which can directly transfer various biologically active molecules derived from donor cells and act on recipient cells, including mRNA, microRNA and proteins[12]. At present, ICI is the mainstream method for the treatment of erectile dysfunction with exosomes, and many experimental results of a variety of exosomes have proven to be effective[13–15]. However, due to the abundant blood flow in the corpus cavernosum, it is difficult for exosomes to remain in place and will soon be cleared, resulting in insufficient recovery of erectile function and short duration of efficacy[16]. In addition, traditional ICI increases the risk of bleeding and arteriovenous fistula, as well as cases of Peyronie's disease[17]. Since the human or rat glans is covered by stratified squamous epithelium without stratum corneum[18, 19], some drugs can be absorbed through this site, such as local anesthetics for premature ejaculation[20]. In this experiment, it was discovered for the first time that hyaluronic acid (HA) loaded with urine-derived stem cell exosomes (USC-Exo) can improve the symptoms of ED in diabetic rats. As a new way of administration, it can avoid various adverse reactions of ICI and improve patient compliance.

Methods

Isolation, culture, and identification of USCs

The isolation and cultivation process of USCs is as described in the reference[21].A total of 13 sterile mid- and last-stream urine samples (at least 200 mL/sample) were collected from 3 healthy young man aged 20-28 years. We centrifuged each sample at 400g for 10 minutes at a room temperature. The supernatant was discarded, and the cell pellet was washed twice with phosphate-buffered saline (PBS; Gibco, USA).

The collected cells were resuspended in special USC medium which consisted of medium A and medium B at a 1:1 ratio. Medium A consisted of high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco) + GlutaMAX (Gibco) + non-essential amino acid (NEAA) solution (Gibco), supplemented with 10 ng/mL human epidermal growth factor (hEGF; PeproTech, USA), 2 ng/mL platelet-derived growth factor (PDGF; PeproTech) and 10% (v/v) fetal bovine serum (FBS; Gibco). Medium B consisted of renal epithelial cell growth medium (REGM BulletKit; Lonza, Switzerland). The cell suspension was seeded into a 6-well plate pre-coated with gelatin and placed in a cell incubator at 37℃ with 5% CO_2, which was considered to be passage 0(P0). The culture medium was changed every two days until the confluence was more than 60% and passaged to a new 6-well plate. USCs of P2-5 were used in following experiments.

The expression levels of cell surface markers and specific genes were analyzed by flow cytometry to detect the characteristics of USCs. The P3 USCs were digested with 0.25% trypsin(Gibco) and centrifuged to prepare 1 X 10^5/ml single cell suspension after incubated with 3% bovine serum albumin (BSA; Gibco) in PBS for 30 min. Then, the cells were incubated with fluorescence-conjugated antibodies against CD29-PE-Cy7, CD44-PE, CD73-APC, CD90-PE, CD146-FITC, CD31-APC-Cy7, CD45-FITC and HLA-DR-APC-Cy7 (BD Biosciences, USA)for 30 min at 4℃ and no-light conditions. The surface markers of USCs were evaluated by flow cytometry analysis (FACSCalibur; BD Biosciences) after washed three times to remove unbound antibodies.

Isolation and identification of USC-Exo

USC-Exo were isolated from the culture medium as previously described[22]. Briefly, USCs of P2-5 in logarithmic growth phase, with 80-90% confluence, were changed into complete medium with exosome-free FBS for 48 h, and the conditioned medium was harvested. To isolate exosomes, it was centrifuged at 300 x g for 10 min, then 2000 x g for 20 min at 4 ℃. The collected supernatant was filtered using a 0.22um sterile filter to remove residual cells and debris and ultra-centrifuged at 100,000 g for 70 min at 4 ℃, and the supernatant was discarded. The pelleted USC-Exo was resuspended in PBS and stored at -80 ℃.

Tecnai G2 Spirit transmission electron microscope (TEM; FEI, Netherlands) was adopted to visualize the structures of USC-Exo. The USC-Exo was fixed in 2.5% glutaraldehyde-paraformaldehyde mixed fixative solution, and 10uL was dropped onto a special coated copper grid for TEM. After standing for 2 min, the liquid was sucked, 2% uranyl acetate was added dropwise for 5 min, and then the liquid was evaporated. The specimens were transferred to the microscope and analyzed at 120 kV. The particle size distribution and concentration of USC-Exo were detected by Nanoparticle Tracking Analysis (NTA; NanoSight NS300, China). The surface markers of USC-Exo, including CD9 (20597-1-AP, 1:500; ProteinTech), CD63 (25682-1-AP, 1:1000; ProteinTech), and TSG 101 (28283-1-AP, 1:1000; ProteinTech), were analysed by Western blot.

Cell viability assay

Briefly, 3000 HUVECs/well suspended in 100uL of endothelial cell growth medium-2 (EGM-2; Lonza) were plated in a 96-well plate at 37°C and 5% CO^2. After the cells adhered for 2 hours, the initial medium was

discarded and replaced with the medium corresponding to each group. According to reference[22] and our pre-experiments, cells were divided into 6 groups in different conditional medium: normal glucose (NG, 5.5 mM), NG + USC-Exo (10^8 or 10^9 particles/ml), high glucose (HG, 33mM) and HG + USC-Exo (10^8 or 10^9 particles/ml). Each group of 5 wells was cultured at 37°C and 5% CO^2 for 48 hours. Then, 10uL of cell counting kit-8 (CCK-8; Dojindo, Japan) solution was added to each well and incubated for 2h, and the absorbance at 450 nm of each well was measured using a a microplate reader. All the experiments were repeated for three times.

Scratch assay

To investigate the effect of USC-Exo on migration of HUVECs in high glucose, in vitro scratch experiments were performed as follow. 1×10^5 cells/well were seeded into a 24-well plate and adhered for 24h. After 48 h of treatment in each group, the cell monolayer was scratched from top to bottom using a sterile 200 uL pipette tip, and washed with PBS to remove cell debris. Photographs were taken using an inverted optical microscope (Nikon, Japan) at 0h, 4h, and 6h after scratching. And five randomly choosed fields of each well were analyzed by imagej to record the migration area. The migration rate was calculated as follows: Migration area (%) = $(A_0 - A_T)/A_0 \times 100\%$, where A_0 represents area of the initial wound and A_T represents the remaining area of the wound at that time point.

Tube formation assay

Three groups of HUVECs (NG; HG; HG+USC-Exo) were pretreated in 6-well plates for 48 hours. Matrigel (Corning, USA) was added to a 24-well plate at 200uL per well, which is operated on ice using pre-cooled pipette tips and then incubated at 37 ℃ for 30 min. 1×10^5 cells from different treatment groups were suspended in 500 uL medium, and then covered on Matrigel in a well. After culturing for 16 h at 37 ℃ and 5% CO_2, the tubular structure was observed using an inverted optical microscope.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted using TRIzol® reagent (Invitrogen, USA). cDNA was synthesised from mRNA using GoScript™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. qRT-PCR was conducted to determine mRNA levels using GoScript™ qPCR Master Mix (Promega). The thermal cycling parameters were 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. GAPDH was used as the internal reference for other target genes. The genes used for real-time qPCR analysis are as follow: BAX (human): forward sequence (5'-3') CCCGAGAGGTCTTTTTCCGAG, reverse sequence (5'-3') CCAGCCCATGATGGTTCTGAT. Bcl-2 (human): forward sequence (5'-3') GGTGGGGTCATGTGTGTGG, reverse sequence (5'-3') CGGTTCAGGTACTCAGTCATCC. SOD2 (human) forward sequence (5'-3') GCTCCGGTTTTGGGGTATCTG, reverse sequence (5'-3') GCGTTGATGTGAGGTTCCAG. GAPDH (human) forward sequence (5'-3') CTGGGCTACACTGAGCACC, reverse sequence

(5'-3') AAGTGGTCGTTGAGGGCAATG.

Animals

Six-week-old male Sprague-Dawley (SD) rats were purchased from Shanghai Jihui Experimental Animal Breeding Co., Ltd. (production license number: SCXK (Shanghai) 2017-0012). Animals were maintained on a 12-hour light-dark cycle, with temperature of 22±3 ℃ and humidity of 60 ± 5%. Four rats were fed in one cage free of specific pathogens and allowed to eat and drink freely. All the animal experiments were performed according to protocols approved by the institutional review board of Department of Laboratory Animal Science, Fudan University (Shanghai, China).

Rat model and drug administration

The rats were adaptively fed for 7 days and then randomly divided into four groups (n=10): normal control group (NC), DM2ED + HA group (HA), DM2ED + USC-Exo combined with HA for 5 times group (Exo X 5) and for 10 times group (Exo X10). DM2ED rats were modeled and validated according to Albersen's method[23]. Briefly, SD rats were fed with a high-fat diet (D12451; 45% calorie from fat; FBSH Biotechnology Co.,Ltd., China) for 2 weeks and then received 2 intraperitoneal injections of streptozotocin (STZ, 30 mg/kg; Sigma-Aldrich, USA) 3 days apart (NC rats were given vehicle citrate buffer in the same volume). We measured random blood glucose levels by clipping the tail tip with a glucose meter (ONETOUCH, USA) and recorded body weights from the first day of STZ injection. One week after STZ injection, rats with blood glucose levels higher than 16.7 mmol/L were identified as DM. After another 6 weeks, as previously reported, rats positive for the insulin challenge assay were identified as DM2. DM2ED rats were screened with apomorphine (APO; 100 mg/kg; Sigma-Aldrich) 8 weeks after STZ injection. The drug for topical application was prepared as follows: sodium hyaluronate (Sigma-Aldrich) powder was uniformly dissolved in PBS at 1% (w/v) to form a hydrogel. Based on the results of experiments in vitro, the prepared hydrogel was compounded with 8 X 10^{10} particles/ml USC-Exo in a ratio of 3:1 and shaken by vortexing to form USC-Exo + HA mixture. For DM2ED rats, after isoflurane inhalation anesthesia, 100ul of HA hydrogel or USC-Exo + HA mixture was applied to the glans every three days. The hydrogel is very sticky, so no extra steps were taken to fix it, just re-covering the foreskin to the glans after a period of administration. At four weeks after administration, the rats were subjected to functional evaluation and tissue collection.

Evaluation of erectile function

The erectile function was evaluated as previously described by measuring the intracavernous pressure (ICP) and mean arterial pressure (MAP)[13]. Briefly, Animals were anesthetized, and bilateral major pelvic ganglia and cavernous nerves were exposed from the ventral midline. A 23-gauge needle was inserted into the proximal left corpus cavernosum, filled with 250 U/mL heparin solution, and the other end was connected to a pressure transducer for ICP measurement. At the same time, a PE-50 tube full of heparin solution was inserted into the left femoral artery to continuously monitor MAP. The cavernous nerve was electrically stimulated by a signal generator using stainless-steel bipolar electrodes (0.2 mm in diameter, 1 mm apart) to generate monophasic rectangular pulses (15 Hz; 5.0 V; duration 60s). The three installations was connected to a signal acquisition system (AD Instruments PowerLab, Australia). Three stimulations were performed on each side, and the maximum ICP in six times was used for statistical analysis in each animal. The max ICP and the ratio of maximal ICP to MAP (ICP/MAP) was recorded

individually. ICP integral (area under the curve) is represented by the product of ICP (mmHg) and time (s) in 1 min of stimulation. After euthanasia, the penis was divided into two parts for liquid nitrogen cryopreservation and fixation for histologic analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

According to the manufacturer's protocol, the penis sections were stained using the In Situ Cell Death Detection Kit (Roche, Germany). The apoptotic index was quantitatively analyzed by the number of TUNEL and DAPI-positive cells in five randomly selected fields of the corpus cavernosum in each animal, obtained as digital images using a fluorescence microscope.

Immunohistochemistry and Masson trichrome staining

The middle part of the penile tissue samples were harvested and fixed in 4% paraformaldehyde for Masson trichrome staining and. The samples were embedded in paraffin, and cut into 4 mm thickness. These sections were stained with Masson trichrome (Sigma) and photographed using a light microscopy. The paraffin sections for immunohistochemistry were incubated with ethylene diamine tetraacetic acid antigen retrieval buffer (EDTA; Thermo, USA). After serum blocking, sections were incubated with primary antibody against α-SMA (1:200; 14395-1-AP, proteintech, USA), eNOS (1:50; 27120-1-AP, proteintech) or CD31 (1:50; 38365, SAB, USA) at 37°C for 1 hour. The sections were then incubated with biotinylated secondary antibodies for 30 min at 25 ℃.

Western blot analysis

The collected corpus cavernosum tissue was homogenized using ice-cold RIPA Lysis Buffer (Beyotime, China) with 1 mM phenylmethanesulfonyl fluoride (PMSF, Beyotime). Protein concentrations of the lysates were determined by the bicinchoninic acid assay (Beyotime). Particulate mass was removed by centrifugation at 15000 X g for 15 min. Samples were boiled for denaturation within 5 min and then loaded. Equal amounts of protein (15 μg) were separated by 10% SDS-PAGE and subsequently transferred onto PVDF membranes. After blocking with 5% skimmed milk in tris buffered saline tween (TBST) for 2 h, the membranes were incubated overnight at 4 ℃ with primary antibodies including α-SMA (1:1,000; CST, USA), TGF-β1 (1:1,000; CST), nNOS (1:1,000; CST), eNOS (1:1,000; CST) and GAPDH (1:5000; CST). After hybridization of secondary antibodies, the positive bands were analysed using Fiji software, and GAPDH was used as a loading control.

Statistical Analysis

All results are expressed as the mean ± standard deviation (SD). The data were analyzed using GraphPad Prism 9.0.0 (GraphPad Software, USA), and multiple groups were compared using one-way analysis of variance analysis. The images were analyzed using Fiji (ImageJ) software. A $P<0.05$ was considered statistically significant.

Result

Characterization of USCs and USC-Exo

The cell colonies were observed around 3-5 days after initial seeding of cells isolated from urine samples (Fig. 1A). The culture medium was changed regularly, and the USCs were confirmed with a fibroblast-like morphology after contact inhibition under light microscopy (Fig. 1A). After 14 days, cell colonies contained an increasing number of cells and reached more than 70% confluence. Flow cytometry analysis showed that USCs were positive for CD29, CD44, CD73, and CD90 and negative for CD31, CD45, and HLA-DR (Fig. 1B), which are common characteristics of pluripotent MSCs. In addition, CD146, a marker of renal pericytes, was highly expressed. These demonstrates that the characteristics of the cells used in this experiment meet the current main identification standards of USCs.

The morphology of the negatively stained USC-Exo sample was observed by TEM, which showed that USC-Exos were spherical vesicles with uneven sizes and a complete membrane structure (Fig. 1C). The diameter of USC-Exo was measured to be about 40–130 nm by NTA analysis (Fig. 1D). In addition, Western blot analysis demonstrated that the collected USC-Exo expressed exosomal specific surface markers CD9, CD63 and TSG-101 (Fig. 1E).

USC-Exo enhanced the proliferation, migration and tube formation of HUVECs in vitro

In order to explore the in vitro pro-angiogenic potential of USC-Exo under high glucose and the most suitable dosage for in vivo experiments, we designed a series of experiments using HUEVCs. HUVECs were treated with normal (5.5 mM, NG) or high (33 mM, HG) concentrations of glucose in combination with different concentrations (10^8 or 10^9 particles/ml) of USC-Exo. After 48 h, the absorbance of high glucose-treated HUVECs were significantly reduced compared with the normal group ($P<0.01$; Fig. 2A). With the normal glucose, 10^9 group had a certain proliferation effect on cells ($P<0.001$; Fig. 2A) while 10^8 group did not. In addition, USC-Exo significantly reversed the deleterious effects of high glucose on HUVECs ($P<0.001$; Fig. 2A), and 10^9 group were better than 10^8 group. Therefore, 10^9 particles/ml USC-Exo was subsequently selected as the experimental group.

The scratch wound healing assay showed that compared with NG group, HG group had an inhibitory effect on migration of HUVECs, and the difference appeared in a short time (4h, 6h; $P<0.001$; Fig. 2B, C and D). In HG group, the simultaneous addition of USC-Exo reversed this phenomenon and promoted the healing process ($P<0.001$; Fig. 2C and D). As shown in Figure 2E, after incubation with matrigel for 16 h, the lumen of the control group was uniform, with many vascular connection nodes and abundant long branches. In contrast, the HG group formed less intact lumina which were thinner and irregular with more unconnected areas ($P<0.05$; Fig. 2F and G). The addition of USC-Exo to the HG group restored the impaired angiogenesis ability, formed more complete and uniform lumen, and returned to normal levels in the number of junctions and branches ($P<0.01$; Fig. 2F and G). The results of qRT-PCR in Figure 2H, I and J showed that compared with NG group, the expression of BAX in HUVECs was up-regulated and the expression of Bcl-2 and SOD2 was down-regulated after HG treatment ($P<0.001$). However, adding USC-Exo at the same time in HG group can down-regulate the expression of BAX and up-regulate that of Bcl-2

and SOD2 (P<0.01 or P<0.001). These results indicated that USC-Exo could improve high glucose-induced oxidation of HUVECs, and promote their proliferation and angiogenesis in vitro.

Modeling and administration workflow for DM2ED rats

Before the injection of STZ and the assessment of erectile function, we analyzed the body weight and fasting blood glucose of all the rats, respectively. After modeling, the body weight of rats in the HA group and the two Exo groups was significantly lower than that in the NC group (P<0.001; Fig. 3A). In addition, the fasting blood glucose levels in the HA group and the two Exo groups were significantly higher than those in the NC group (P<0.01; Fig. 3B). In the insulin challenge test, blood glucose levels in NC rats decreased significantly at 15, 30, 45, 60, 90, and 120 minutes after intraperitoneal insulin injection. DM2 rats were less responsive to insulin compared to NC rats, which means the modeling was successful (P<0.01; Fig. 3C). We performed APO testing 8 weeks after STZ injection and excluded individuals in the diabetic group which still had erectile responses without stimulation, as shown in Fig. 3D. The topical administration of the hydrogel (Fig. 3F and G) was carried out with the aid of a 1 ml syringe. The foreskin is turned over and the hydrogel is applied in a circle around the glans. After topical absorption for 15 minutes, the foreskin is re-covered with the glans. Fig. 3E shows the workflow of the whole animal research study.

USC-Exo-HA restored erectile function in DM2ED rats and reduced apoptosis in corpus cavernosum

Four weeks after topical administration, to evaluate erectile function in each group of rats, erection stiffness was measured by recording ICP and MAP during electrical stimulation of the cavernous nerve under anesthesia (Fig. 3H). As shown in Fig. 4A to D, max ICP, max ICP/MAP and ICP integral were used to evaluate erectile function in each rat. The NC group exhibited high max ICP (91.35±11.35) and max ICP/MAP (0.79±0.11) with large area under the curve (AUC). After diabetes modeled, the HA group consistently resulted in ED, with lower max ICP (26.13±3.61) and ICP/MAP (0.26±0.09). These metrics were significantly increased after administration of USC-Exo-HA (P<0.01), and the 10 treatments group improved erectile function more effectively than 5 treatments group (P < 0.05).

Apoptosis in corpus cavernosum of each rat was detected by TUNEL. Fluorescence images were taken according to the site in Fig. 4F. As shown in Fig. 4E, apoptotic cells in 4 groups of corpus cavernosum exhibited green fluorescence. Apparently, apoptosis was reduced after USC-Exo-HA. Fig. 4G indicated the apoptotic index for each group. After USC-Exo-HA treatment, cell apoptosis was less than that in HA group (P<0.01).

USC-Exo-HA alleviates fibrosis in the corpus cavernosum of DM2ED rats

As shown in Fig. 5A, the corpus cavernosum of the rats in the DM2ED group exhibited severe fibrosis. According to the results of Masson trichrome staining, Western blot analysis and immunohistochemistry (Fig. 5A, B and 6A), smooth muscle content was reduced in the HA group compared to the NC group. Exosome-treated groups exhibited partial inhibition of fibrosis. Significantly, USC-Exo-HA increased

smooth muscle content to a greater extent than the HA group ($P < 0.05$; Fig. 5B and C). TGF-β1 is an important profibrotic factor in the corpus cavernosum, and was significantly increased in the HA group. However, the levels of TGF-β1 in the Exo X5 and Exo X10 group were significantly lower than in the HA group ($P < 0.01$; Fig. 5B and D). The above results indicated that USC-Exo-HA could inhibit the fibrosis of corpus cavernosum in DM2ED rats.

Improvement from USC-Exo-HA in endothelium of diabetic penis tissues

To determine the effect of topical treatment on the endothelium of the rat corpus cavernosum, we observed the expression of endothelial markers eNOS and CD31 by immunohistochemistry and Western blot analysis (Fig. 6A and C). According to the schematic screenshot in Figure 6B, the expressions of eNOS and CD31 in the NC and Exo X10 groups were significantly higher than those in the other two groups, indicating improved vascular function. Consistent with the immunohistochemical results, Western blot analysis of eNOS and nNOS also showed that the expression was decreased in the HA group and partially increased after USC-Exo-HA treatment ($P < 0.05$; Figure 6D, E). All above findings demonstrated that the treatment of USC-Exo-HA improved endothelium function, which may be better with prolonged use.

Discussion

Despite its high incidence, diabetic erectile dysfunction is often overlooked compared to other complications of diabetes. Physiologically, penile erection requires nitric oxide (NO) released by parasympathetic nerve endings or endothelial cells to trigger smooth muscle relaxation, increasing arterial blood flow[24]. The long-term hyperglycemia environment in diabetic patients induces considerable damage to various cells of the corpus cavernosum, including endothelial dysfunction, neurodegeneration and fibrosis, which result in a sharp decrease in NO production, coupled with decreased smooth muscle volume, ultimately leading to erectile dysfunction[25]. Moreover, there are few breakthroughs in the treatment of severe ED, such as diabetic ED and post-traumatic ED (including iatrogenic) that are more common and respond badly to traditional treatments. Recently, exosomes derived from stem cells have been found to have therapeutic effects on ED in models with diabetes and cavernous nerve injury. However, obtaining urine-derived cells is much simpler than obtaining other types of stem cells. The main step getting the primary USCs is to centrifuge the urine sample, and then resuspend the precipitate in the culture medium after washing, without digestion. After a few days of cultivation, clones can be observed. In three generations, more than 1×10^8 USCs can be obtained, which will be sufficient for clinical applications[26]. As a practical wound dressing, the combination of HA with other components such as exosomes and silver particles has been shown to enhance wound healing[27].Compared with some previous studies focusing on the application of HA hydrogel to open wounds, we mainly focus on the perineal absorption of HA hydrogel and exploit its particle-releasing properties. In order to simulate the clinically more common type 2 diabetic ED patients, we designed experiments to clarify the effect of USC-Exo-HA on the DM2ED rat model.

In this study, we successfully isolated USCs from human urine, extracted and purified USC-Exo. In addition to demonstrating that USC-Exo can promote the proliferation of HUVECs and reverse the inhibition of cells by high-glucose environment. USC-Exo also increased capillary network formation in vitro and reversed the tendency of slowing down in endothelial cell migration resulting from high glucose. These results suggest that USC-Exo is a positive regulator of angiogenesis. In addition, from the level of gene expression, USC-Exo down-regulated the pro-apoptotic gene BAX under the influence of high glucose, and up-regulated the expression of the antioxidant gene SOD2. From these results, it can be speculated that USC-Exo has an effect of improving vascular oxidative stress in diabetes. We prepared USC-Exo-HA according to the effective concentration in in vitro experiments, and formulated a strict treatment plan. We prepared USC-Exo-HA according to the effective concentration in in vitro experiments, and formulated a strict treatment plan. In the treatment of ED, cavernous smooth muscle plays an important role in restoring erectile function. The results showed that USC-Exo-HA treatment significantly increased the proportion of corpus cavernosum smooth muscle, increased the expression of α-SMA, and decreased apoptotic cells in DM2ED rats. Endothelial function is determined by examining levels of CD31, nNOS and eNOS, which are key factors in erectile function. Compared with the NC group, the expression levels of endothelial markers were significantly decreased in the HA group. On the other hand, USC-Exo-HA treatment resulted in significantly elevated expression of eNOS and nNOS in DM2ED rats. This series of results suggest that topical application of USC-Exo-HA has a therapeutic effect on erectile dysfunction in DM2ED rats.

This research is the first report to combine stem cell-derived exosomes with a smearable dosage form for topical treatment of erectile function. We conducted a series of experiments to demonstrate the efficacy of USC-Exo-HA as a new dosage form for erectile dysfunction. In addition, the drug is simple to use and convenient to produce, and multiple use may have better effects on symptoms. But there are still many aspects that we need to explore, such as the absorption rate of the drug, the specific duration of action and possible side effects. These will be further explored as the follow-up research directions of this topic.

Conclusion

The topical application of USC-Exo-HA in the treatment of DM2ED rats has been proved effective. The potential mechanism might to promote the proliferation of endothelial cells and smooth muscle in the corpus cavernosum, which leads to the remodeling of erectile function. And multiple dosing at intervals may make the effect more pronounced.

Authors' contributions JGH conceived the study and designed the experiments. JGH and DQZ provided funding for the study and revised the manuscript. JMZ and PG performed the research, data analysis, and manuscript writing. HRC, ZJF and JZ contributed to the analysis and interpretation of data. All authors read and approved the final manuscript for publication. **Funding** This study was supported by the national natural science foundation of China (81771683).

Declarations

Authors' contributions JGH conceived the study and designed the experiments. JGH and DQZ provided funding for the study and revised the manuscript. JMZ and PG performed the research, data analysis, and manuscript writing. HRC, ZJF and JZ contributed to the analysis and interpretation of data. All authors read and approved the final manuscript for publication. **Funding** This study was supported by the national natural science foundation of China (81771683).

Availability of data and materials The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate The study was conducted according to the guidelines set by the Ethics Committee of Huashan Hospital.

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Competing interests The authors declare that they have no competing interests.

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Figures

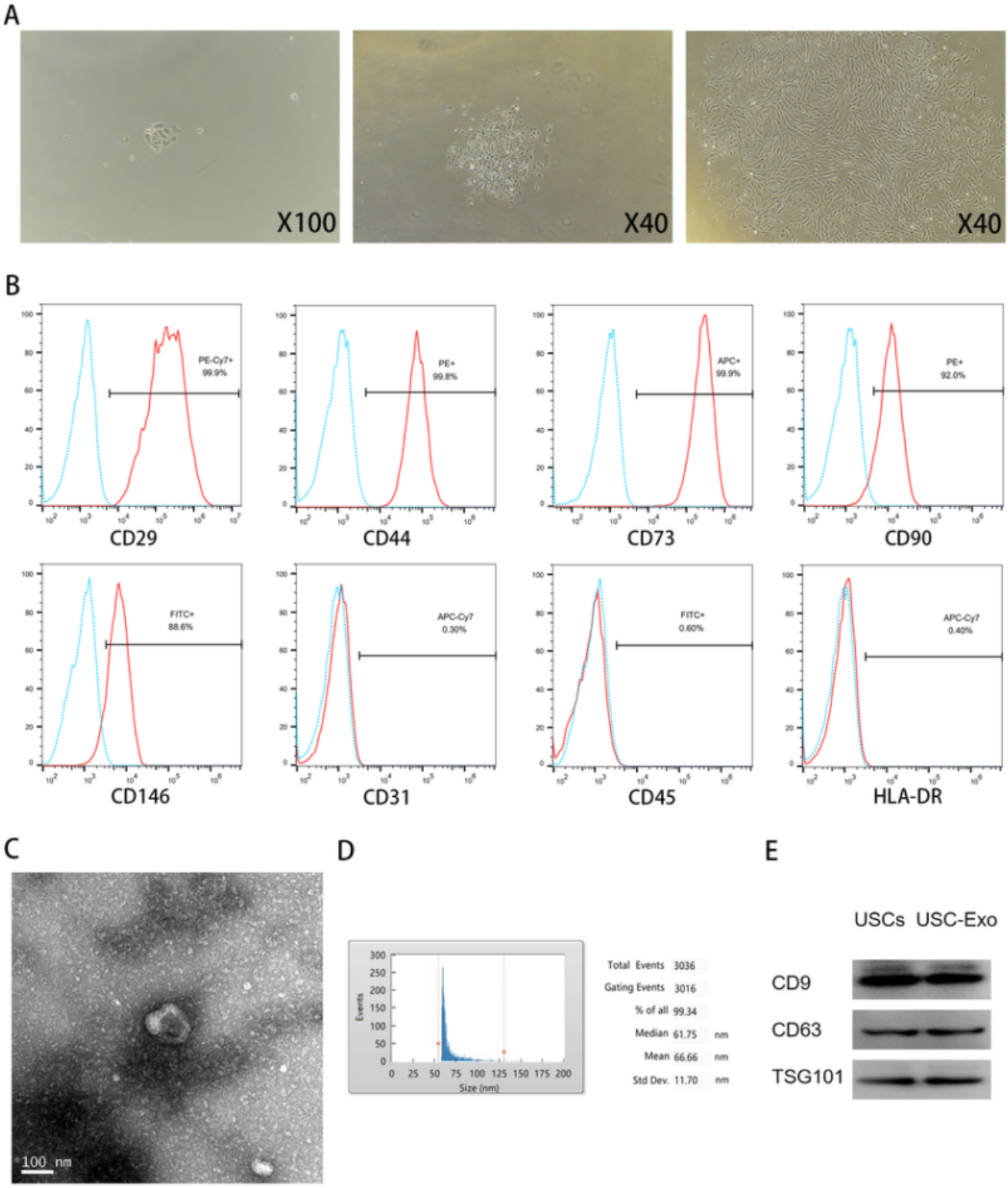

Figure 1

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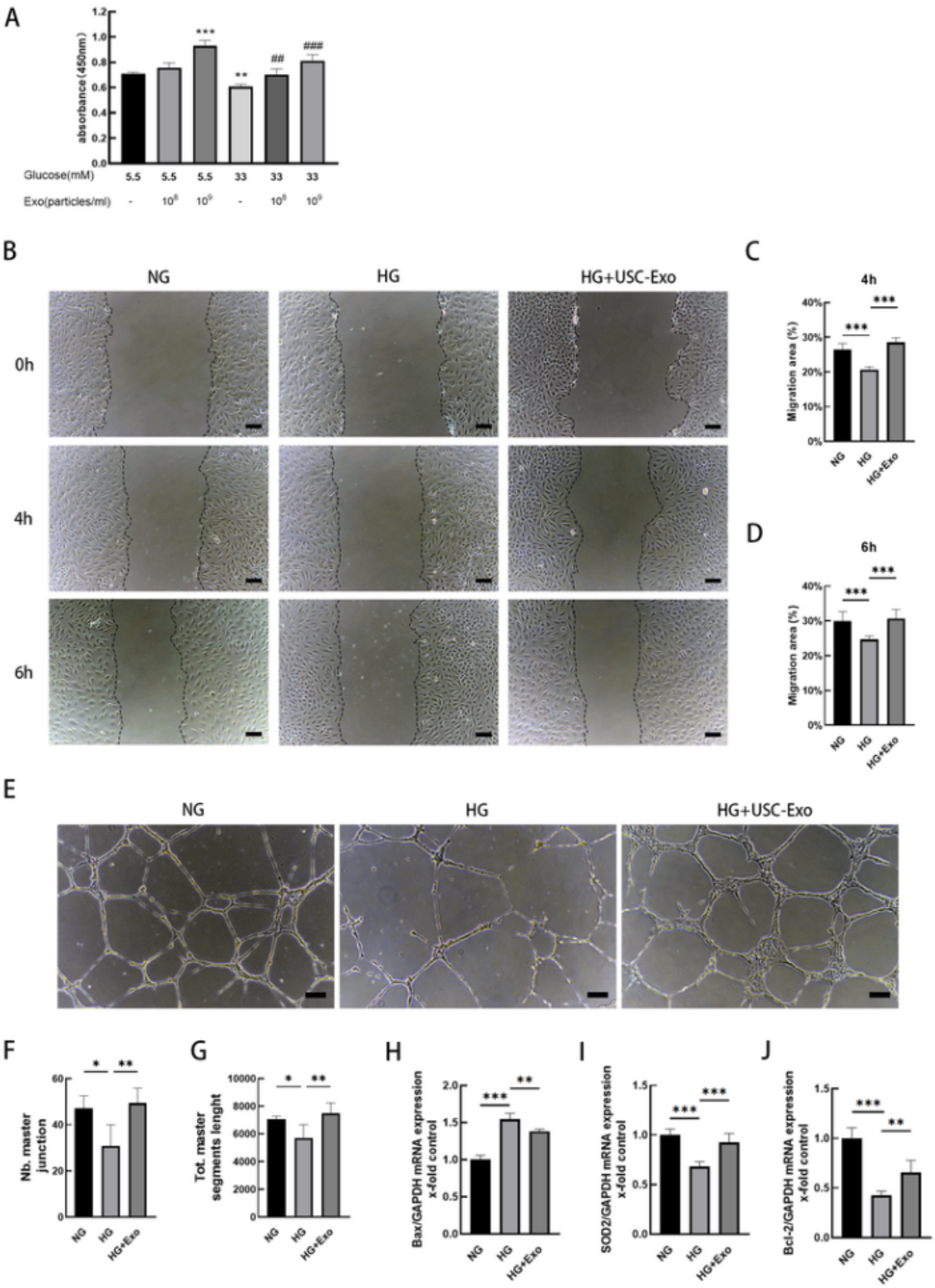

Figure 2

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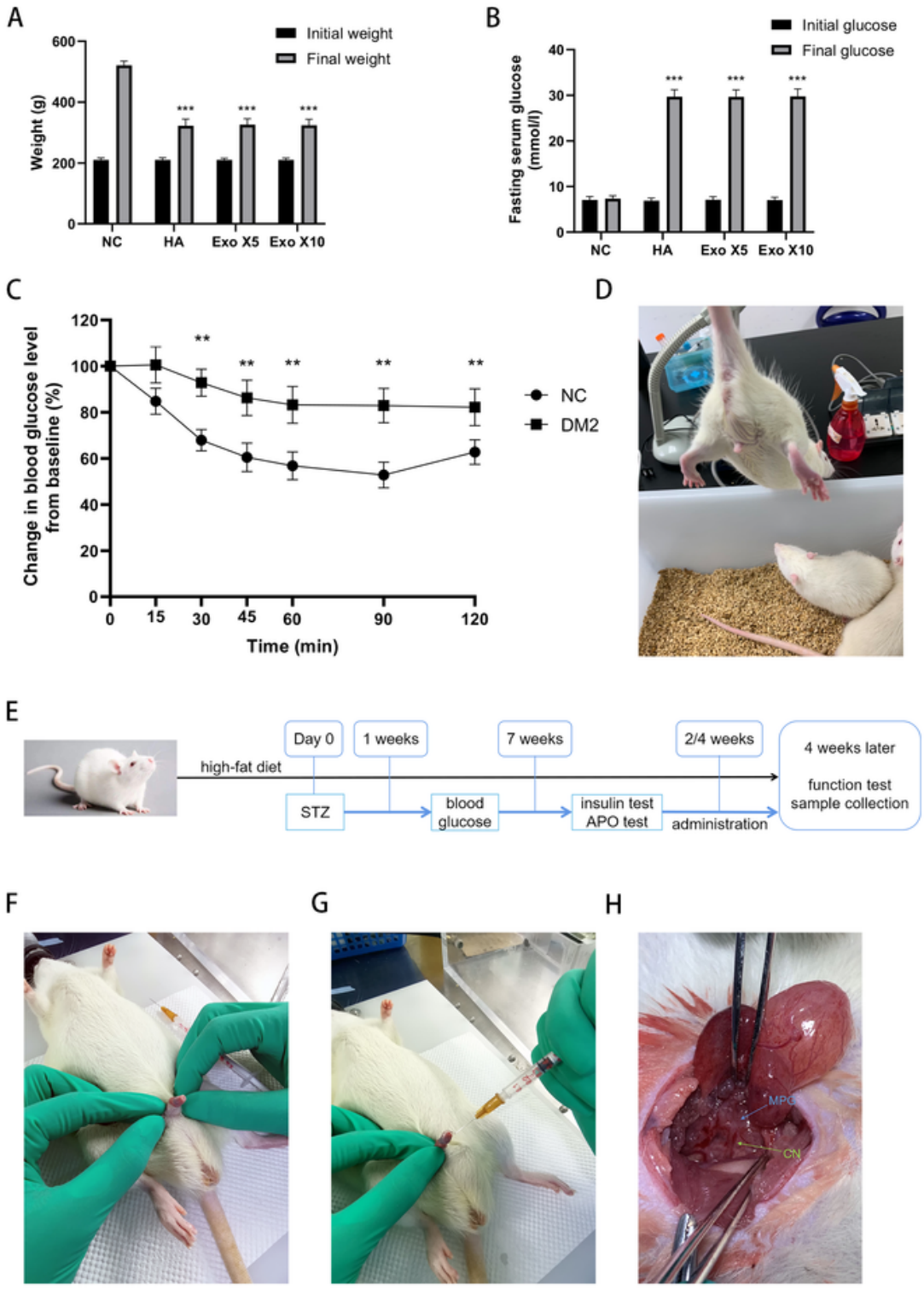

Figure 3

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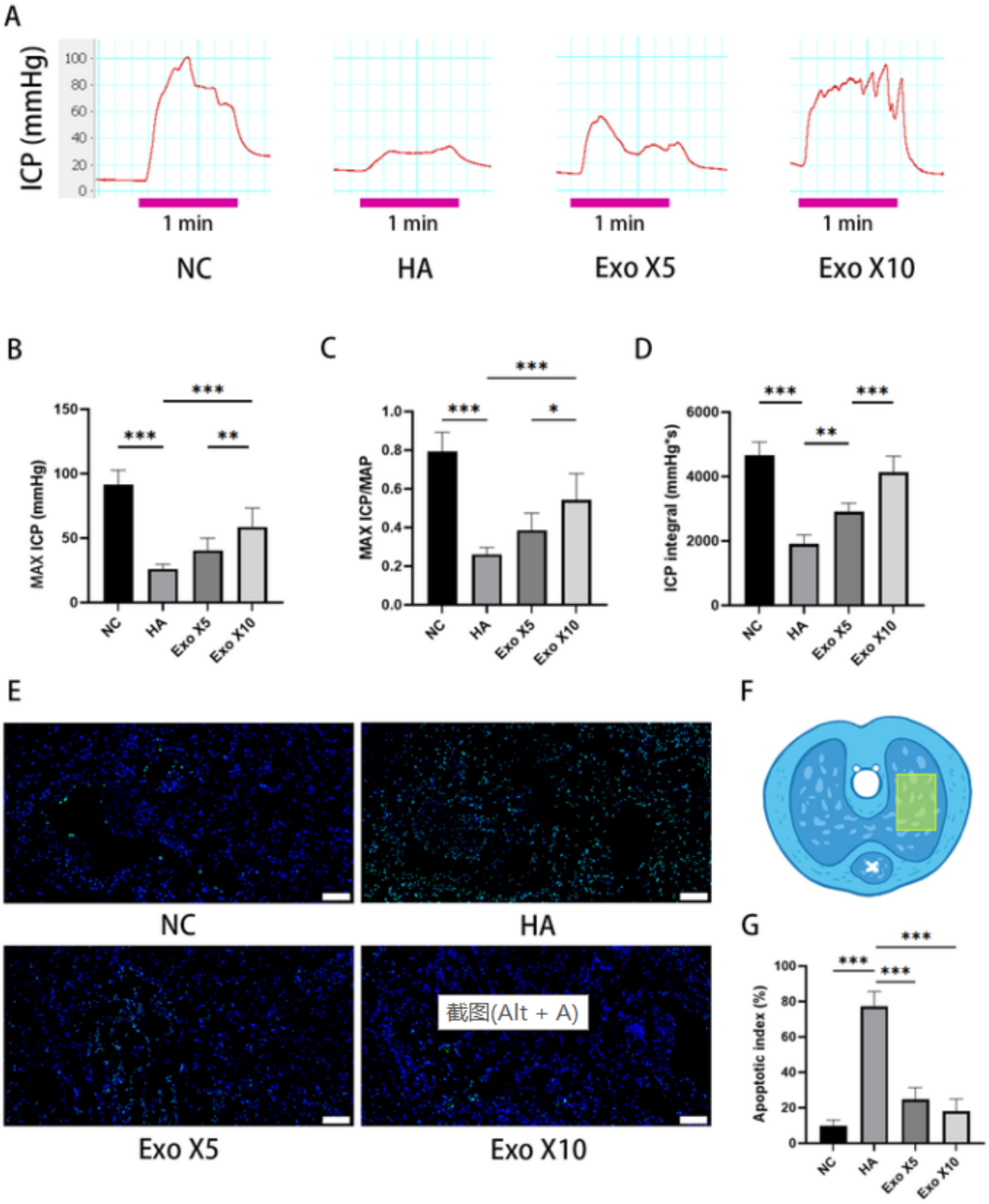

Figure 4

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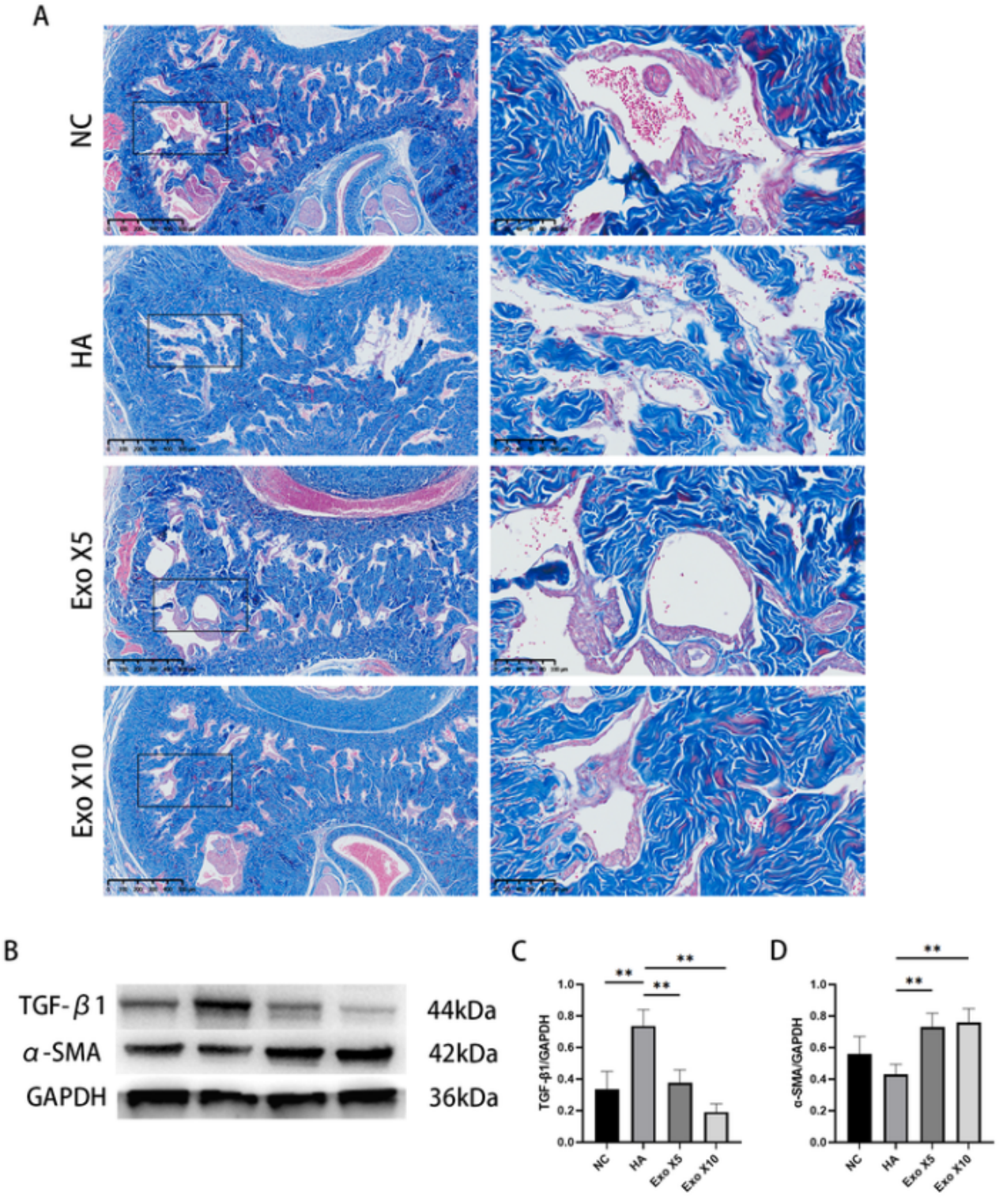

Figure 5

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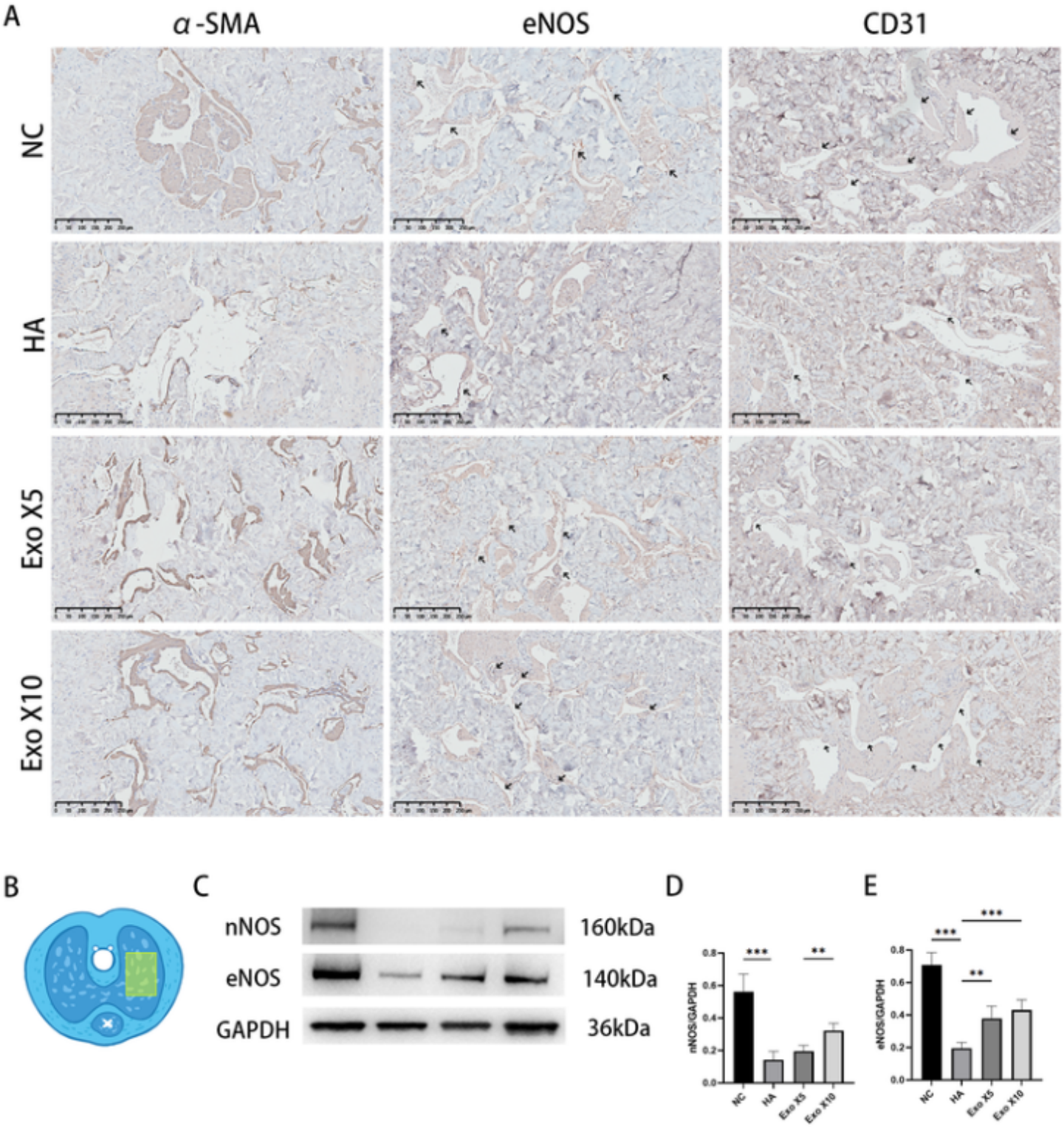

Figure 6

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