Implantation of Autologous Bone Marrow-derived Cells Improves Erectile Dysfunction in Spontaneously Hypertensive Rats

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Purpose : Erectile dysfunction (ED) decreases the quality of life. However, some patients are refractory to phosphodiesterase-5 (PDE-5) inhibitors. This preliminary study investigated the possibility that autologous bone marrow-derived cells could improve the ED of spontaneously hypertensive rats (SHRs).

Materials and Methods : Erectile responses were induced by apomorphine. Bone marrow cells were harvested from femurs of SHRs, and following culturing and labeling, they were implanted autologously into the corpus cavernosum penis. Control SHRs received cell-free injections. At 7 days after the implantation, apomorphine-induced erectile responses were estimated. The presence of implanted bone marrow-derived cells in the corpora cavernosa and the expression of neuronal nitric oxide synthase (nNOS) were determined by microscopy and reverse transcription polymerase chain reaction.

Results : The number of the negative reactions for apomorphine in the SHRs was significantly higher compared to the Wister Kyoto (WKY) rats ($P = 0.045$). Ten of 15 cell-free control SHRs did not respond to apomorphine, while 8 of 9 cell-implanted SHRs responded. Significantly more cell-implanted SHRs responded to apomorphine than did cell-free SHRs ($P < 0.013$). The implanted cells formed clusters within the corpora cavernosa, and some expressed nNOS mRNA and protein.

Conclusions : Bone marrow-derived cells autologously implanted into the corpus cavernosum penis significantly increased the number of SHRs having erectile responses to apomorphine. *Shinshu Med J 65 : 37—44, 2017*

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Key words : apomorphine, autologous bone marrow-derived cells, erectile dysfunction, neuronal nitric oxide synthase, spontaneously hypertensive rats

I Introduction

Erectile dysfunction (ED) has a negative impact on the quality of life[1)2)]over 50 % of men beyond 40 years of age are affected by ED[3)-6)]. Administration of phosphodiesterase-5 (PDE-5) inhibitors improves cyclic guanosine monophosphate (cGMP)-dependent smooth muscle and arteriole relaxation. This widely accepted treatment for ED acts by amplification of the nitric oxide (NO) signaling level within the corpus cavernosum penis. While approximately 70 % of patients benefit from PDE-5 inhibition[7)], many others do not[1)6)]. For those refractory patients, implantation of autologous bone marrow-derived cells, which are capable of differentiating into nerve, muscle, and other tissues, may be an effective alternative treatment[8)9)].

Spontaneously hypertensive rats (SHRs) are affected with ED[10)-13)]. While the mechanism of the hypertensive-related ED remains to be clarified[14)-16)], these animals may serve as a suitable model to test the ability of implanted autologous bone marrow-derived cells to relieve ED. These cells, when implanted into freeze-injured urinary bladders or urethras, differentiate into smooth and skeletal muscle cells and restore physiological functions to these damaged

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organs[17)18)]. Furthermore, considering future clinical applications, autologous cells are superior to allogenic cells because the use of autologous cells is not burdened with immunological rejection or ethical problems. Therefore in this preliminary study, we investigated the ability of implanted autologous bone marrow-derived cells to remediate hypertensive-related erectile dysfunction in SHRs.

Ⅱ Materials and Methods

A Animals

Male Wister Kyoto (WKY) rats and SHRs at postnatal week 25 (Japan SLC Inc., Shizuoka, Japan) were used to assess the erectile responses. In separate experiments, SHRs were used to assess the effects of implanted autologous bone marrow-derived cells. All animals were treated in accordance with National Institutes of Health Animal Care Guidelines and the guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine.

B Drug

This study used apomorphine (Sigma-Aldrich, Inc., St. Louis, MO, USA), which is the central dopamine D1/D2-recepter agonist in the central nervous system to induce erectile responses[19)-21)]. The apomorphine was dissolved to the desired concentration with 0.9 % saline.

C Measurements of intracavernous pressure

The apomorphine-induced erectile responses of untreated SHRs (n = 15) and WKY rats (n = 16) were assessed by intracavernous pressure measurements. As previously described[22)], a 22-gauge needle attached to a polyethylene catheter (PE-50) was inserted into the corpus cavernosum penis of animals anesthetized by inhalation of sevoflurane (Sevofrane®, Abbot Japan Co., Ltd., Tokyo, Japan). The catheter was sealed and brought out subcutaneously to the back of the neck. For measurements of blood pressure, another single catheter was inserted into the right carotid artery and then brought subcutaneously to the back of the neck. The skin incision was closed. After the surgery, each animal was placed without any restraint in a cage to recover for over 2 hours.

After confirming that each rat was alert, each catheter was attached to a pressure transducer (DX-100, Nihon Kohden, Tokyo, Japan). Intracavernous pressure signals were passed through a carrier amplifier (AP-601G, Nihon Kohden) and recorded by a multichannel pen-recorder (Nihon Kohden). After the pressures demonstrated stable traces, 0.1 mg/kg apomorphine was administered subcutaneously just below the back of the neck. Our previous studies confirmed that the dose was able to induce erectile responses in healthy rats[22)]. We quantified the following parameters : blood pressure (cmH_2O), time to first response (TFR ; minutes), number of peak pressures per 30 minutes (PP30), tonic peak pressure (TPP ; cmH_2O), and burst peak pressure (BPP ; cmH_2O).

D Harvest and culture of autologous bone marrow-derived cells

Twenty-four SHRs were anesthetized by inhalation of sevoflurane (Abbot Japan Co., Ltd.). In each animal, two pediatric bone marrow needles were inserted approximately 1 cm apart into a femur. The bone marrow cells were flushed out with saline and collected through the other needle[11)23)]. After centrifugation, the cells were suspended with culture medium composed of Dulbecco's modified eagle medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 15 % fetal bovine serum (Biowest, Paris, France) and 0.1 % penicillin-streptomycin (Invitrogen). The harvested bone marrow cells were cultured on type I collagen-coated culture dishes (Asahi Glass Co., Ltd., Tokyo, Japan). During the culture, the medium was completely replaced every day, and non-attached cells were discarded. At 5 days, the Qtracker 525 Cell Labeling Kit (Invitrogen) was used for 24 hours to label the cytoplasm with nanocrystals. The cells taking the marker during the culture period were easily detected within the recipient tissues by a Leica DAS Microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 488 nm excitation. At two days after labeling, the adherent, proliferating, labeled bone marrow-derived cells were collected after brief incubation with a 0.25 % trypsin solution[17)18)] and then suspended in culture

medium at 1.0×10^7 cells/ml (viability > 80 %).

E Implantation of autologous bone marrow-derived cells

Seven days after the culture, the SHRs from which the bone marrow cells were collected were randomly divided into cell implantation (n = 9) and cell-free injection control (n = 15) groups. After re-anesthetizing the SHRs, the corpus cavernosum penis was exposed. For the cell implantation group, the suspended autologous bone marrow-derived cells (1.0×10^6 cells/100 μl) were autologously implanted with a 29-gauge syringe into the corpus cavernosum penis at the 3- and 9-o'clock positions. For the cell-free injection control group, 100 μl of cell-free culture medium was similarly injected at the same positions. The implantation cell number and volume were chosen to avoid further damaging the host tissues or the implanted cells with shear stress[17)18)]. At each operation, the retention of small swellings that included the implanted cells or control media was visually confirmed.

At 7 days after these operations, the apomorphine-induced erectile responses of the cell-implanted and control injected rats were estimated by intracavernous pressure measurements (described above). Following the measurement, the rats were euthanized, and the corpus cavernosum penis was removed for immunohistochemical investigations and real-time reverse transcription polymerase chain reaction (RT-PCR) as described below.

F Immunohistochemistry

Tissue samples were fixed in 4 % paraformaldehyde and 4 % sucrose in 0.1 M phosphate buffer (PB), pH 7.4, for 12 hr at 4 ℃, and then embedded in paraffin. The sections (3 μm) were cut on a microtome and then deparaffinized, rehydrated, rinsed three times with 0.01 M phosphate buffer saline (PBS), and immersed in 10 mM sodium citrate, pH 6.0. For antigen retrieval, they were then microwaved at 100 ℃ for 5 min. The specimens were treated with 1.5 % normal donkey serum (Chemicon International Inc., Temecula, CA, USA) and 1.5 % non-fat milk in PBS for 1 hr at 4 ℃. The sections were incubated for 12 hr at 4 ℃ with antibody for neuronal nitric oxide synthase (nNOS, 1 : 300, goat polyclonal, Osenses Pty Ltd, Flagstaff Hill, SA, Australia). The sections were rinsed with PBS at 4 ℃, and then incubated with secondary antibody consisting of donkey anti-goat IgG conjugated with Alexa Fluor 594 (1 : 250, Molecular Probes, Eugene, OR, USA) for 1 hr at 4 ℃. Negative controls were performed without the primary antibody, and positive controls were performed with rat lungs. Following rinsing, the nuclei were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, 5 μg/ml, Molecular Probes), and then coated with Fluorescent Mounting Medium (Dako Cytomation, Carpinteria, CA, USA). The slides were observed and photographed with a Leica DAS Microscope (Leica Microsystems GmbH). The other sections from each sample were stained with hematoxylin and eosin (H&E).

G Real-time RT-PCR

For real-time RT-PCR, the corpus cavernosum of SHRs were homogenized, and total RNA was extracted with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Complementary DNA (cDNA) was synthesized from 0.2 μg of total RNA with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real time RT-PCR of the cDNA was performed at 50 ℃ for 2 minutes followed by 95 ℃ for 10 minutes. These were followed by 40 cycles at 95 ℃ for 15 seconds and 60 ℃ for 1 minute. The primer of neuronal nitric oxide synthase 1 (Rn00583793_m1, Applied Biosystems) was used to measure the nNOS mRNA expression level. Gene activity was expressed as the ratio to the internal standard gene beta-actin (Rn00667869_m1).

H Statistical analysis

Results were expressed as means ± standard deviation. We determined statistical differences with Fisher's exact probablility test. Non-repeated measures ANOVA followed by the Tukey procedure was used among experimental groups having the apomorphine-induced erectile responses. Also, unpaired t-tests were used to compare between the cell-implantation and control injection group. P-values less than 0.05 were considered significant. These tests were performed with GraphPad InStat®

Table 1 Apomorphine-induced erectile responses in normal WKY rats and SHRs

	Apomorphine-induced Erectile Response	
	Positive Response	Negative Response
WKY rats (n = 16)	14	2
SHRs (n = 15)	7	9

Fisher's exact probability test. P = 0.045, erectile response for WKY rats vs SHRs.

Table 2 Intracavernous pressure measurements of rats having apomorphine-induced erectile responses

	TFR (minutes)	PP30	TPP (cmH_2O)	BPP (cmH_2O)
Untreated				
WKY rats (n = 14)	9.6 ± 5.6	2.4 ± 1.3	76.4 ± 47.8	159.6 ± 97.0
SHRs (n = 7)	8.5 ± 3.2	2.7 ± 2.1	49.2 ± 30.0	93.5 ± 63.1

TFR ; time to first response, PP30 ; number of peak pressures per 30 minutes,
TPP ; tonic peak pressure, BPP ; burst peak pressure.

(GraphPad Software, Inc. San Diego, CA, USA).

Ⅲ Results

The blood pressure in normal SHRs, 236.4 ± 26.4 cmH_2O, was significantly higher than that of the normal WKY rats, 154.4 ± 21.7 cmH_2O ($P < 0.01$).

A Erectile functions of normal WKY rats and SHRs

Apomorphine induced an erectile response in 14 of 16 WKY rats (**Table 1**). In contrast, 7 of 15 of the untreated SHRs responded to the apomorphine, while 8 did not. Thus, the negative responses by the normal SHRs were significantly greater than that by the normal WKY rats ($P < 0.05$). For the 14 responsive WKY rats and the 7 responsive SHRs, there were no differences in TFR, PP30, TPP, or BPP (**Table 2**).

B Cultured autologous bone marrow-derived cells

Immediately after seeding, the harvested bone marrow cells consisted of heterogeneous, spindle-shaped, round, and polygonal cells, along with red blood cells. After 7 days of culture, the adhered proliferating cells reached approximately 90 % confluence and were relatively homogenous in spindle-shaped appearance. The cultured autologous bone marrow-derived cells did not stain with antibodies for nNOS just prior to implantation. Thus, this finding suggested that the cells might not differentiate into nNOS-produced cells during the culture period.

C Effects of autologous bone marrow-derived cells

Blood pressure of the cell-implanted SHRs, 225.3 ± 16.9 cmH_2O, was not significantly different from the cell-free injected control SHRs, 234.0 ± 47.4 cmH_2O ($P = 0.623$). Seven days after implantation, 8 of 9 cell-implanted SHRs developed apomorphine-induced erectile responses. In the cell-free control SHRs, only 5 of 15 rats ($P = 0.013$) responded (**Table 3**). Neither TFR nor PP30 for the cell-implanted SHRs differed significantly from the cell-free controls. While TPP and BPP in the cell-implanted SHRs tended to be greater than in cell-free controls, the difference was not significant (**Table 4**).

D Histological and gene expression evaluation of the corpus cavernosum penis

Seven days after implantation in the penile corpora cavernosa of SHRs, the bone marrow-derived cells formed clusters (**Fig. 1A**) that contained numerous Qtracker 525 nanocrystal-labeled cells (**Fig. 1B**). In contrast, the cell-free control injected SHRs did not have the cell clusters (**Fig. 1C**) or nanocrystal-labeled cells (**Fig. 1D**). Some of the labeled implanted cells were positive for nNOS antibody (**Fig. 2**). The nNOS mRNA expression level of cell-implanted SHRs, 1.18 ± 0.43, tended to be higher than the controls, 0.93 ± 0.25, but the difference was not significant ($P = 0.296$).

Table 3 Apomorphine-induced erectile responses in cell-implanted and cell-free control SHRs

	Erectile Response	
	Positive Response	Negative Response
Cell-free control SHRs (n = 15)	5	10
Cell-implanted SHRs (n = 9)	8	1

Fisher's exact probability test. P = 0.013, erectile response for cell-free control SHRs vs cell-implanted SHRs.

Table 4 Intracavernous pressure measurements of rats having apomorphine-induced erectile responses

	TFR (minutes)	PP30	TPP (cmH_2O)	BPP (cmH_2O)
Cell-treatment				
Cell-free control group (n = 5)	8.7 ± 7.5	2.2 ± 1.3	85.3 ± 46.3	206.8 ± 125.4
Cell-implantation group (n = 8)	8.3 ± 4.9	2.5 ± 1.4	130.3 ± 83.4	317.2 ± 185.6

TFR ; time to first response, PP30 : number of peak pressures per 30 numites,
TPP ; tonic peak pressure, BPP ; burst peak pressure.

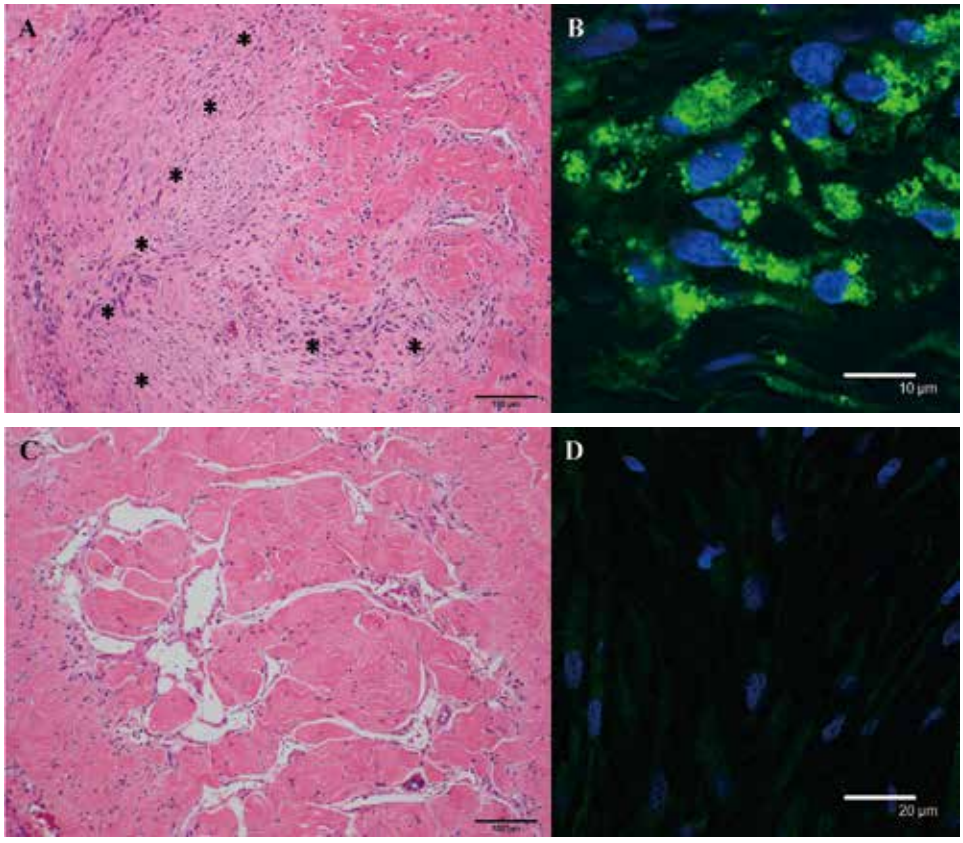

Fig. 1 Histological evaluation of the penile corpus cavernosum. (A) The cell-implanted corpus cavernosum contained clusters of cells (asterisks). (B) These cells contained the green fluorescent-labeled nanocrystals (green) taken up while the cells were in culture before implantation. (C) The cell-free control injected corpora cavernosa did not have any clusters or (D) nanocrystal-labeled cells. Blue, DAPI-labeled nuclei.

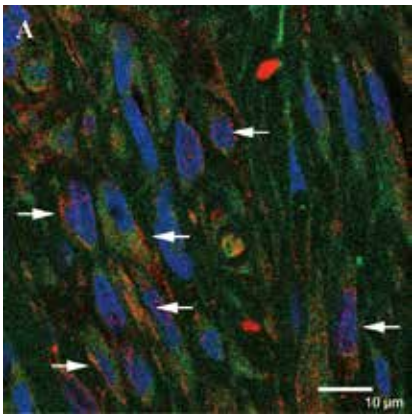

Fig. 2 Immunohistochemistry for nNOS antibodies. The Qtracker-labeled implanted cells (green) differentiated into nNOS-expressing cells (red, arrows) within the penile corpora cavernosa.

Ⅳ Discussion

Along with aging, hypertension, diabetes mellitus, and hyperlipemia are considered to be risk factors for ED[1)5)]. The SHRs are used as an animal model of hypertension as well as models for lower urinary tract symptoms such as C-fiber related detrusor overactivity[24)-26)], benign prostatic hypertrophy[27)], and hypertension-related ED[12)-15)]. In our study, the blood pressure of the sham-operated SHRs was significantly higher than that of the normal sham-operated WKY rats. The frequency of erectile response to apomorphine in SHRs was significantly less than that in WKY rats. These results are consistent with damage to the nervous system, microcirculation, and/or smooth muscle of the corpus cavernosum in SHRs.

The percent of SHRs having apomorphine-induced erections was significantly greater in the cell-implanted rats compared to the cell-free injected ones. The erectile response is initiated by NO produced by nNOS and liberated from nonadrenergic and noncholinergic neurons in the corpora cavernosa[28)]. We showed that the implanted bone marrow-derived cells formed clusters within the corpora cavernosa. Prior to implantation, these cells did not express nNOS, but one week afterwards, some of them did. The implanted cells differentiated into nerve-like cells that produced nNOS, while this study did not identify these cell types. However, the overall level of nNOS mRNA expression was similar between the cell-free injected tissues and the cell-implanted ones. Thus, our results suggest that the apomorphine-induced erectile responses of SHRs might be mediated by not only the nNOS-related NO-dependent mechanisms, but also by other neurogenic and/or muscular systems to induce smooth muscle and arteriole relaxations.

Bone marrow-derived cells are capable of differentiating both in vitro and in vivo along multiple pathways that include nerve, muscle, bone, cartilage, adipose tissue, tendon, and connective tissue[8)9)]. These cells, when situated in areas of tissue damage, have paracrine effects that produce cytokines and growth factors based on available signals from the surrounding tissues[29)30)]. Thus, the paracrine effects of the implanted bone marrow-derived cells might contribute to the reconstruction of healthy peripheral nervous system elements, microcirculation, and/or smooth muscle systems within the penile corpora cavernosa.

This study had some limitations. While our results showed that the implantation of bone marrow-derived cells improved the apomorphine-induced erections, we have not thoroughly demonstrated the mechanisms of the recovery. In this study, we performed cell direct-injection methods to implant the cells. While we detected the implanted cells within the penile corpora cavernosa, the cell numbers were very small. Thus, we need to improve the survival rates of the implanted cells in the penile corpora cavernosa by using the novel techniques, such as cell sheet engineering[31)]. In our next study, we will test these effects and the safety over a long period.

In this study, we conducted autologous cell implantation by harvesting bone marrow cells from a

femur of each anesthetized animal. The cultured autologous cells were similar to the cells harvested from femurs of the euthanized SHRs according to our previous methods[10]. The majority of patients with ED are elderly or have other diseases that may affect the developmental potential of bone marrow cells. However, our results suggest that bone marrow-derived cells harvested from the SHRs behave with the same potential as those from healthy animals. Therefore, the bone marrow-derived cells harvested from aging and/or diseased patients also might retain their developmental potential and be available in treatments for ED.

Ⅴ Conclusions

The proportion of normal SHRs with an erectile response to apomorphine was significantly lower than that of normal WKY rats. SHRs implanted with autologous cultured bone marrow-derived cells had a higher frequency of response than did cell-free injected control SHRs. Therefore, the implantation of autologous bone marrow-derived cells has the potential for development into a treatment for refractory ED.

Ⅵ Conflict of Interest

There are no conflicts of interest to declare.

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