Comparison Between Subcutaneous Injection of Basic Fibroblast Growth Factor-Hydrogel and Intracavernous Injection of Adipose-derived Stem Cells in a Rat Model of Cavernous Nerve Injury

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OBJECTIVE
To compare the effects of subcutaneous penile injection of basic fibroblast growth factor (bFGF)-hydrogel and intracavernous injection of human adipose-derived stem cells (h-ADSCs) on improving erectile function in a rat model of cavernous nerve injury.

MATERIALS AND METHODS
Adult male Sprague-Dawley rats were randomly divided into 5 groups (n = 10 per group): age-matched control (normal group), bilateral cavernous nerve injury (BCNI group), penile subcutaneous injection of hydrogel after BCNI (hydrogel group), penile subcutaneous injection of bFGF-hydrogel after BCNI (bFGF-hydrogel group) and intracavernous injection of h-ADSCs after BCNI (ADSC group). Four weeks after the treatment, all rats underwent an erectile function test. Then, penile tissue was harvested for immunohistological analysis of bFGF, phalloidin, and cluster of differentiation (CD) 31. The cyclic guanosine monophosphate (cGMP) level of the corpus cavernosum was quantified by cGMP assay.

RESULTS
From the functional test and immunohistological result, we observed that bFGF-hydrogel and h-ADSCs injection significantly elevated intracavernous pressure. The evaluation of filamentous actin content, CD31 expression, and cGMP concentration in the corpus cavernosum were meaningfully increased in the bFGF-hydrogel and ADSC groups compared with BCNI group. The bFGF released from bFGF-hydrogel prevented smooth muscle atrophy. Moreover, bFGF expression was significantly increased in bFGF-hydrogel group.

CONCLUSION
The subcutaneous injection of bFGF-hydrogel prevented smooth muscle atrophy, increased the intracavernous pressure, and improved erectile function like an intracavernous injection of h-ADSCs.

Radical prostatectomy (RP) is considered the standard treatment for localized prostate cancer. Despite the continuous development of technical and anatomic innovations, erectile dysfunction (ED) in postprostatectomy patients still remains a cause for high morbidity. Cavernous nerve (CN) injury is the main reason for postprostatectomy ED. In response to CN injury, the endothelium and smooth muscle undergo structural changes, including smooth muscle loss, apoptosis, and fibrosis, that contribute to the development of venous leaking and corporal venocclusive dysfunction.

During the past several decades, the most common treatment option after surgery was oral phosphodiesterase type 5 inhibitors; however, these drugs have had an average response in postprostectomy patients. Recent studies have been more focused toward cavernous tissue regeneration, which might be a promising treatment for the ED. In this respect, stem cell–based therapy has been regarded as a promising research direction in the improvement of ED after CN injury. Various types of adult stem cells have had encouraging results by preserving the corpus cavernosum microstructure and rehabilitating the injured CN. Previous studies proved that the therapeutic efficacy of intracavernously injected stem cells is due to
stem cells trafficking to the major pelvic ganglia. However, intracavernously injected stem cells rapidly escaped the penis, and finding the injected stem cells in the penile tissues was difficult. Besides stem cell therapy, researchers have demonstrated that angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), have shown therapeutic effect on the ED treatment.

In this study, we compared the subcutaneous injection of bFGF-hydrogel and the intracavernous injection of human adipose-derived stem cells (h-ADSCs) on improving erectile function in a rat model of CN injury.

MATERIALS AND METHODS

All animal experiments were approved by the Institutional Animal Care and Use Committee of Catholic University Medical College, Seoul, Korea.

Rat Model

Adult male Sprague-Dawley rats, 10 weeks old and weighing 250-300 g, were obtained from Orient Bio Company (Gyeonggi, South Korea) and were randomly divided into 5 equal groups (n = 10 per group): age-matched control (normal group), bilateral CN injury (BCNI group), hydrogel penile subcutaneous injection after BCNI (hydrogel group), bFGF-hydrogel penile subcutaneous injection after BCNI (bFGF-hydrogel group) and h-ADSCs intracavernous injection after BCNI (ADSC group). Four weeks after the treatment, rats were anesthetized with an inotraperitoneal injection of tiletamine and zolazepam (30 mg/kg) and xylazine (10 mg/kg) and underwent erectile function test. Then, penile tissue was harvested for immunohistological and cyclic guanosine monophosphate (cGMP) assay.

CN Injury

In anesthetic conditions, the urinary bladder and prostate were exposed by a lower abdominal incision. The CN below the major pelvic ganglion (MPG) on both sides of the prostate was identified, and then compressed with a clamp for 2 minutes.11

Preparation of bFGF-Hydrogel

Park et al12 reported in situ cross-linkable gelatin-poly(ethylene glycol)-tyramine (GPT) hydrogel by enzyme-mediated reaction. Briefly, bFGF incorporated with GPT hydrogel was prepared as follows: two 3 wt% GPT solutions (1 mL), each containing horseradish peroxidase (HRP; 0.013 mg/mL) and hydrogen peroxide (H2O2; 0.013 wt%), were prepared. bFGF (200 ng/mL; R&D Systems, Minneapolis, MN) was mixed with the GPT solution containing HRP. The hydrogel was formed rapidly using HRP and H2O2. GPT hydrogel has various mechanical and biological advantages, including in situ gelatinization, minimal invasive injection, and biocompatibility. Before being injected into the target site, each solution was loaded into a dual-syringe kit. bFGF was continuously released from GPT hydrogels for 3 weeks (up to 88%), as illustrated by our previous study.13

Injection of bFGF-Hydrogel and h-ADSCs

For the stem cell culture experiments, h-ADSCs were provided by RNL Bio Company, Seoul, Korea. Passage 4 h-ADSCs were used for this experiment. In anesthetic conditions, in the h-ADSCs group, 1 × 10⁶ h-ADSCs diluted in phosphate-buffered saline were injected into both sides of the corpus cavernosum using a 25-gauge syringe. Immediately after drainage through the dorsal vein, it was stopped using an elastic band. The compression was released 1 minute after injection of h-ADSCs (Fig. 1A). In the hydrogel group, only hydrogel was subcutaneously injected into penis. In the bFGF-hydrogel group, bFGF-hydrogel (200 μL) was injected in both sides of the penile subcutaneous ring site (Fig. 1B).

Erectile Function Evaluation

Four weeks after the treatment with h-ADSCs, growth factor, and hydrogel, rats were anesthetized with an intraperitoneal injection of tiletamine and zolazepam (30 mg/kg) and xylazine (10 mg/kg) and placed supine on a table. The carotid artery and CN were exposed for detection of mean arterial pressure (MAP) and intracavernosal pressure (ICP), respectively. The carotid artery was inserted with PE-50 tubing for measurement of MAP. At the same time, a 23-gauge butterfly needle filled with heparin (250 U/mL) was inserted into the corpus cavernosum and then connected to a pressure transducer (Grass model S48 K, Grass Instrument Division, Astro-Med Inc, West Warwick, RI) for measurement of ICP. A bipolar stainless steel electrical stimulator was used for stimulation of the CN at 10 V for 50 seconds and 2.4 mA with a pulse width of 0.5 ms. CN stimulations were conducted at least 3 times, and the interval between stimulations was maintained for more than 10 minutes. The maximum increase of ICP was selected for statistical analysis. The peak
ICP and MAP were recorded on a computer with the PowerLab data acquisition system (ADInstruments). The ratio of ICP to MAP during electrostimulation of the CN was used to evaluate erectile function.

**Immunohistochemistry**

Immediately after the erectile function evaluation, the penis was excised and half of the penis sample was used for immunohistochemistry study. The corpus cavernosum specimens were fixed in 4% paraformaldehyde for 24 hours at 4°C and then embedded in paraffin. The penile midshaft was sectioned at 5 μm on a Microtome (Leica, Wetzlar, Germany) for histologic analysis. The primary antibodies used were against bFGF (1:200; Santa Cruz Biotechnology, Dallas, TX) and CD31 (1:200; Abcam, Cambridge, United Kingdom). Immunoreactivity was visualized using the antibodies Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (1:500; Invitrogen, Carlsbad, CA) and Alexa Fluor 568-conjugated anti-rabbit immunoglobulin G (1:500; Invitrogen). Tissue specimens were counterstained with 4, 6-diamino-2-phenyl-indole (Vector Labs, Burlingame, CA) to visualize nuclei. Digital images were obtained using an Olympus BX51 fluorescence microscope (Olympus Corp, Tokyo, Japan). Quantitative morphometric analysis was performed using Adobe Photoshop 7.0.

**Measurement of cGMP Concentration**

After detection of MAP and ICP, the remaining half portion of the penis was used for cGMP assay. The corpus cavernosum tissue from the penis was harvested and stored at −70°C until use. Frozen cavernosal tissue was homogenized, and the cGMP direct immunoassay kit (K372-100, BioVision, Milpitas, CA) was used for measurement of cavernous cGMP concentration. Specified procedures were performed according to our previous report.

**Statistical Analysis**

Statistical analysis was performed with SPSS 15.0 software (SPSS Inc, Chicago, IL). Data are expressed as the mean ± standard deviation. Differences between groups were evaluated by analysis of variance tests with the Tukey post-test. The differences were considered statistically significant at P < .05.
RESULTS

Erectile Function Test
Figure 2A shows graphical representations of ICP tracings during the duration of the nerve stimulation. ICP/MAP ratios in the normal, BCNI, hydrogel, bFGF-hydrogel, and ADSC groups were 0.66 ± 0.06, 0.25 ± 0.02, 0.25 ± 0.03, 0.42 ± 0.05, and 0.44 ± 0.08, respectively. Improvement of erectile function was shown in the bFGF-hydrogel and ADSC groups, with increased ICP/MAP ratios compared with the BCNI group (P < .05). There was no significant difference between the bFGF-hydrogel group and ADSC group (Fig. 2B).

bFGF Expression in the Corpus Cavernosum
We quantified bFGF expression as the relative percentage in area stain for the entire corpus cavernosum. bFGF distribution of the corpus cavernosum in the normal, BCNI, hydrogel, bFGF-hydrogel, and ADSC groups was 14.8% ± 3.2%, 5.0% ± 1.6%, 5.6% ± 1.1%, 10.6% ± 2.5%, and 6.5% ± 1.7%, respectively. Significantly increased bFGF content in the bFGF-hydrogel group compared with the BCNI group revealed that the bFGF-hydrogel penile injection could increase bFGF protein expression in the corpus cavernosum. However, bFGF content was not significant in ADSC group compared with the BCNI group (Fig. 3B).

Evaluation of F-Actin Content in the Corpus Cavernosum
The percentages of phalloidin staining area in the normal, BCNI, hydrogel, bFGF-hydrogel, and ADSC groups were 24.8% ± 2.5%, 9.0% ± 1.3%, 9.6% ± 1.7%, 16.4% ± 2.3%, and 16.9% ± 1.4%, respectively. Smooth muscle density was significantly decreased in the BCNI group compared with the normal group (P < .05). The bFGF released from the bFGF-hydrogel injection prevented smooth muscle atrophy and thus increased the smooth muscle content. Moreover, cavernous smooth muscle density was significantly higher in the
bFGF-hydrogel and ADSC groups ($P < .05$) but not in the hydrogel group (Fig. 3C).

**CD31 Expression in the Corpus Cavernosum**
Immunohistochemical staining of red fluorescent CD31 in the BCNI group was dramatically reduced compared with the normal group ($P < .05$). CD31 staining in the bFGF-hydrogel and ADSC groups was significantly elevated compared with the BCNI group ($P < .05$); however, no difference was observed in these 2 groups (Fig. 3D).

cGMP Concentration in the Corpus Cavernosum
The cGMP concentration in the corpus cavernosum in the normal, BCNI, hydrogel, bFGF-hydrogel, and ADSC groups was, in pmol/g, 255.0 ± 26.9, 69.7 ± 14.5, 72.7 ± 9.5, 162.3 ± 9.3, and 167.7 ± 15.0, respectively (Fig. 4). The concentration of cGMP in the corpus cavernosum was significantly elevated in the bFGF-hydrogel and ADSC groups compared with BCNI group. No significant difference was observed between the bFGF-hydrogel group and the ADSC group.

**COMMENT**
About 25%-90% of men undergoing RP experience postoperative ED, as illustrated by research data.14-17 ED is defined as the persistent incapability to achieve or sustain an erection of necessary rigidity to enable successful sexual intercourse.18 Compromised function of the CN after RP is a leading cause for atrophy of the corpus cavernosum, loss of neurotransmitters, and fibrosis, which results in postprostatectomy ED.19

Stem cells hold great promise for regenerative medicine due to their ability for self-renewal and differentiation. Human stem cells do not demonstrate any immunologic rejection when administered in the rats. The absence of an immune recognition may be due to the low level of cell surface class I and the absence of class II human leukocyte antigen and costimulatory molecule expression in h-ADSCs. In addition human stem cells posses immunosuppressive effects mediated by prostaglandin.20 Castiglione et al21 reported that the injection of h-ADSCs improved erectile function in a CN injury rat model. Shuyu et al,11 in our laboratory, used human ADSCs in the rat for the recovery of erectile function in a CN injury. However, intracavernously injected stem cells rapidly escaped the penis, thus presenting difficulties in finding the injected stem cells in the penile tissues. Apart from stem cell therapy, researchers have demonstrated that angiogenic growth factors, such as VEGF and bFGF, have shown therapeutic effect in the treatment of ED.9

In our present study, we observed that the subcutaneous injection of bFGF-hydrogel had an effect similar to that of an intracavernous injection of h-ADSCs of improving erectile function. We immobilized bFGF into hydrogel to make bFGF stable and to maintain its sustained release. Then, we injected bFGF-hydrogel in the penis subcutaneously and evaluated its efficacy compared with the intracavernous injection of h-ADSCs in the BCNI rat model. Results of the physiologic tests confirmed that the bFGF-hydrogel group and the ADSC group had a higher ICP/MAP ratio than the other experimental groups.

Expression of bFGF, F-actin, and CD31 in the corpus cavernosum decreased significantly in the BCNI group compared with the normal group. The normal content of smooth muscle cells and collagen fibers in the corpus cavernosum is very important for erectile function. Furthermore, the pathogenesis of some kinds of ED is associated with smooth muscle fibers and collagen fibers alteration, and a decrease in the endothelium, bFGF content, and smooth muscle.25 The bFGF released from bFGF-hydrogel prevented smooth muscle atrophy. Staining of F-actin by phalloidin was significantly increased in
the ADSC and bFGF-hydrogel groups compared with the BCNI group. CD31 is a transmembrane glycoprotein that is present on the surface of platelets, monocytes, macrophages, and neutrophils and is a constituent of the endothelial intercellular junction. CD31 plays a crucial role in the adhesion cascade between endothelial cells and inflammatory cells during inflammation in facilitating leukocyte migration and between endothelial cells during angiogenesis. CD31 expression was significantly increased in the ADSC and bFGF-hydrogel groups.

The concentration of cGMP was significantly higher in the bFGF-hydrogel and ADSC groups compared with the other groups (Fig. 4). Nitric oxide-cGMP interaction in the corpus cavernosum mediates the vital pathway for penile erection. Nitric oxide stimulates a soluble guanylyl cyclase, which increases the intracellular concentration of cGMP within the muscle. The increased concentration of cGMP results in the relaxation of the smooth muscle in the arteries and arterioles supplying the erectile tissue. The treatment with h-ADSCs slightly increased bFGF expression but significantly increased the cGMP level. Furthermore, the bFGF-hydrogel and ADSC groups showed remarkable improvement in bFGF expression and in the cGMP level compared with the hydrogel group. In a model of ED, the intracavernosal injection of bFGF also improved vasoreactivity and increased neuronal nitric oxide synthase and VEGF expression, suggesting that some of the valuable effects of bFGF may be facilitated through VEGF.

Our study demonstrated successful elevation of erectile function with administration of bFGF-hydrogel and with h-ADSCs. Thus, bFGF hydrogel can be used instead of ADSCs for the improvement of erectile function. We performed the study during a 4-week interval after bFGF-hydrogel and stem cell injection. The question remains whether the effectiveness of bFGF-hydrogel as well as h-ADSCs will persist as long as 6 months to 1 year. Further investigation will therefore be required in this field. In addition, future studies could be conducted in which ADSCs and growth factors at various doses are compared. Studies can also be conducted by comparing bFGF-hydrogel with other widely used stem cells to examine the result without any adverse events.

CONCLUSION

This study suggests that the subcutaneous bFGF-hydrogel injection had a similar effect of improving erectile function as that of an h-ADSCs injection in the corpus cavernosum. Therefore, the subcutaneous injection of bFGF-hydrogel prevented smooth muscle atrophy, increased the ICP, and improved erectile function like an intracavernous injection of h-ADSCs.

References


17. Albersen M, Fandel TM, Lin G, et al. Injections of adipose tissue-derived stem and stem cell lysate improve recovery of erectile function with administration of bFGF-hydrogel and with h-ADSCs. Thus, bFGF hydrogel can be used instead of ADSCs for the improvement of erectile function. We performed the study during a 4-week interval after bFGF-hydrogel and stem cell injection. The question remains whether the effectiveness of bFGF-hydrogel as well as h-ADSCs will persist as long as 6 months to 1 year. Further investigation will therefore be required in this field. In addition, future studies could be conducted in which ADSCs and growth factors at various doses are compared. Studies can also be conducted by comparing bFGF-hydrogel with other widely used stem cells to examine the result without any adverse events.


