Nanoparticle Improved Stem Cell Therapy for Erectile Dysfunction in a Rat Model of Cavernous Nerve Injury

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Purpose: Recently intracavernous injection of stem cells has garnered great interest as a potential treatment of erectile dysfunction. However, most stem cells are washed out immediately after intracavernous injection. The goal of this study was to investigate using NanoShuttle™ magnetic nanoparticles to maintain stem cells in the corpus cavernosum after intracavernous injection, thereby improving stem cell therapy of erectile dysfunction in an animal model.

Materials and Methods: Adipose derived stem cells were magnetized with NanoShuttle magnetic nanoparticles to create Nano-adipose derived stem cells. A total of 24 rats underwent bilateral cavernous nerve crush and were randomly assigned to 3 groups, including adipose derived stem cells, Nano-adipose derived stem cells and Nano-adipose derived stem cells plus magnet. Cells were tracked at days 1, 3, 5 and 9 after intracavernous injection. Another 40 rats with bilateral cavernous nerve crush were randomly assigned to 4 groups, including bilateral cavernous nerve crush, bilateral cavernous nerve crush plus adipose derived stem cell intracavernous injection, bilateral cavernous nerve crush plus Nano-adipose derived stem cell intracavernous injection and bilateral cavernous nerve crush plus Nano-adipose derived stem cell intracavernous injection plus magnet. Functional testing and histological analysis were performed 4 weeks after intracavernous injection.

Results: In the in vitro study 1) NanoShuttle magnetic nanoparticles were successfully bound to adipose derived stem cells and 2) Nano-adipose derived stem cells migrated toward the magnet. In the in vivo study 1) cell tracking showed that Nano-adipose derived stem cells were successfully retained in the corpus cavernosum using the magnet for up to 3 days while most adipose derived stem cells were washed out in other groups by day 1 after intracavernous injection, and 2) intracavernous pressure/mean arterial pressure, and αSMA (α-smooth muscle actin) and PECAM-1 (platelet endothelial cell adhesion molecule 1) expression in the Nano-adipose derived stem cell group was significantly higher than in the other groups.

Conclusions: Magnetization of adipose derived stem cells with NanoShuttle magnetic nanoparticles kept adipose derived stem cells in the corpus cavernosum and improved adipose derived stem cell therapy of erectile dysfunction in an animal model.

Key Words: penis, erectile dysfunction, peripheral nerve injuries, magnetite nanoparticles, stem cells
Prostatectomy is the recommended procedure for patients with low and intermediate risk localized prostate cancer. ED is the most common complication with a prevalence of 20% to 90%. For those patients with ED earlier penile rehabilitation has been widely practiced to protect erectile function before the cavernous nerves recover to baseline function, which can take 2 years or even longer. Early intervention with phosphodiesterase type 5 inhibitors, vacuum erectile devices, or intracavernous or transurethral administration of vasodilators is recommended. However, the reported recovery rate varies and patient compliance is low due to cumbersome application and ineffectiveness. Therefore, there is a great need to explore novel rehabilitation modalities.

Recently ICI of stem cells has shown some potential to treat erectile dysfunction in animal models. Many studies have shown functional and structural improvements with ICI of stem cells. However, groups have described difficulty in finding injected stem cells in the corpora cavernosa after ICI. Because most stem cells went to bone marrow and other places in the body, it was proposed that ICI of stem cells is systemic therapy in nature and paracrine factors may have an important role in stem cell therapy. However, ICI is local therapy and systemic therapy is never the aim of ICI of stem cells. It is much more logical that retaining stem cells in the local target area would avoid systemic effects. Thus, there is a demand for methods to retain stem cells in the CC after ICI for ED therapy.

To address this need, we took a novel approach to keeping stem cells in the CC after ICI by magnetizing cells and using magnetic forces to retain them in the CC. Specifically we magnetized cells with NanoShuttle, a biocompatible magnetic nanoparticle assembly (approximately 50 nm) consisting of gold nanoparticles, iron oxide and poly-L-lysine. NanoShuttle magnetizes cells by electrostatically and nonspecifically attaching to cell membranes via poly-L-lysine (approximately 50 pg per cell) with no effect on cell proliferation or viability. It does not interfere with fluorescence imaging. After trypsinization and resuspension in medium, the remaining cells were washed of excess NanoShuttle, trypsinized and incubated with NanoShuttle (1 μl/10,000 cells) overnight to allow for cell binding. The next day the cells were plated on cell culture plates and cultured at 37°C with 5% CO₂ with 95% humidity with medium exchanged daily. ADSCs were used at the third passage for the following study.

Nano-ADSC Preparation for ICI
ADSCs were cultured to 70% to 80% confluence and incubated with NanoShuttle (1 μl/10,000 cells) overnight to allow for cell binding. The next day the cells were washed of excess NanoShuttle, trypsinized and resuspended in fresh medium. To track cells all cells to be used for transplantation were labeled for 30 minutes with CellTracker™ Green CMFDA before ICI. Approximately 1 × 10⁶ labeled cells in 0.2 ml PBS were used for each ICI.

Intracavernous Injection
Seven days after BCNC the rats were anesthetized with isoflurane inhalant. ADSCs or Nano-ADSCs were injected into the CC. In the Nano-ADSC plus magnet group 2 N52 magnets (0.05-inch outer diameter × 0.25-inch high, 100 G) were placed outside the bilateral CC of the penis immediately after injection and removed 6 hours later.

Animal Model and Groups
A total of 24 Sprague Dawley rats underwent BCNC. In this procedure the rat was anesthetized and disinfected as

We hypothesized that magnetization with NanoShuttle would keep ADSCs in the CC for a long time after ICI so that the ADSCs could exert a local therapeutic function and improve erectile function. This hypothesis was tested in the BCNC animal model, a widely used cavernous injury model that mimics ED after radical prostatectomy. The results of this study would greatly expand the current array of stem cell based therapies of ED.

MATERIALS AND METHODS

Animals
Eight-week-old male Sprague Dawley® rats were obtained from Harlan Laboratories, Dublin, Virginia. Care and treatment were approved by the institutional animal care and use committee at our institution. The rats were allowed time to become accustomed to the new environment as required by our institutional animal care and use committee before the study.

ADSC Isolation and Culture
ADSCs were isolated from the inguinal fat of rats. The rats were anesthetized by isoflurane inhalant and a midline abdominal incision was made. The inguinal fat was identified, excised and placed in PBS on ice. The harvested fat was minced into small pieces and incubated in digestion buffer consisting of PBS, bovine serum albumin and collagenase type I for 1 hour at 37°C with shaking every 20 minutes. The digested solution was centrifuged at 1,000 × gravity for 10 minutes at room temperature and the supernatant was removed. The remaining cells were suspended in 10 ml Dulbecco’s modified Eagle’s medium/F-12 with 1% antibiotic-antimycotic solution and 10% fetal bovine serum (Life Technologies®). The cells were plated on cell culture plates and cultured at 37°C in 5% CO₂ with 95% humidity with medium exchanged daily. ADSCs were used at the third passage for the following study.

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required. A midline suprapubic incision was made to expose the bladder and prostate. The MPG and cavernous nerves were identified on both sides of the prostate. Five mm distal to the MPG the bilateral cavernous nerves were crushed using an ultrafine hemostat with full tip closure for 30 seconds, removed for 30 seconds and then reapplied for another 30 seconds. The incision was closed with 3-zero Vicryl® sutures.26

Seven days later the rats were randomized to 3 groups of 8 each, including ADSCs, Nano-ADSCs and Nano-ADSCs plus magnets. In the latter group magnetic forces were applied outside the bilateral CC for 6 hours. Two rats per group were sacrificed 1, 3, 5 and 9 days after ICI. The rat penis was harvested and processed for fluorescent cell tracking.

Another 40 Sprague Dawley rats underwent BCNC and were randomly assigned to 4 groups of 10 each, including BCNC, BCNC plus ADSC ICI with ADSCs administered via ICI, BCNC plus Nano-ADSC ICI with Nano-ADSCs administered via ICI and BCNC plus Nano-ADSC ICI plus magnet with Nano-ADSCs administered via ICI and magnets placed outside the bilateral CC for 6 hours. At 28 days the ICP of all rats was measured during cavernous nerve stimulation. MAP was recorded. The penis was harvested and processed for immunohistochemistry and Western blotting for PECAM-1 and zSMA.

**Intracavernous Pressure/MAP**

ICP was measured under electric stimulation of the cavernous nerve. After the rat was anesthetized the MPG and cavernous nerve were exposed on either side of the prostate. A 25 gauge needle connected to a polyethylene-50 tube was inserted in the CC to measure ICP. The left carotid artery was exposed and cannulated with a polyethylene-50 tube to record MAP. Stimulations were performed at 16 Hz with a duration of 5 milliseconds at 5 V for 60 seconds with 5 minutes between subsequent stimulations. The maximal increase in ICP during nerve electrostimulation was selected for statistical analysis in each animal.27

**Immunohistochemistry**

Immunohistochemistry was performed as previously described.28 Penile sections were incubated with primary antibodies, including mouse anti-zSMA (1:500) and rabbit anti-PECAM-1 (1:100, Santa Cruz Biotechnology®) overnight at 4C, followed by biotinylated secondary antibody working solution (1:200, Vector® Laboratories) for 30 minutes at room temperature.

**Western Blotting**

Western blotting was performed according to the standard manufacturer protocol (Bio-Rad®). The polyvinylidene fluoride membrane was incubated with primary antibodies, including mouse anti-zSMA (1:2,000) and rabbit anti-PECAM-1 (1:1,000), at 4C overnight. Bound antibodies were detected using anti-mouse and anti-rabbit IgG-horseradish peroxidase (incubated at room temperature for 1 hour) and the echochemiluminescence system. Results were quantified by densitometry and normalized to GAPDH.

**Imaging and Statistical Analysis**

Stained tissues were photographed using an Eclipse Ti fluorescent microscope (Nikon Instruments, Melville, New York). Western blot results were analyzed by ImageJ, version 1.48 (http://imagej.nih.gov/ij/). All measurements are expressed as the mean ± SD. Data were analyzed by 1-way ANOVA with post hoc testing and p <0.05 considered statistically significant.

**RESULTS**

**Studies**

**In Vitro.** After co-culture with ADSCs overnight NanoShuttle was successfully bound to the ADSCs (fig. 1). To test the binding timeline we observed Nano-ADSCs for 25 days. NanoShuttle slowly dissociated from ADSCs with time. However, NanoShuttle nanoparticles were still found in ADSCs at day 25 and ADSCs were still growing (fig. 1).

We then placed a magnet underneath the cell culture plate after cells were resuspended to study the effect of magnetic forces on Nano-ADSCs. One day later we observed that Nano-ADSCs aggregated to the magnet with the application of magnetic force (fig. 2).

**In Vivo.** To test the efficacy of keeping Nano-ADSCs in the CC after ICI with magnets we placed 2 magnets at the bilateral CC for 6 hours. Cells were tracked in the long term with CellTracker. Our results showed that Nano-ADSCs were successfully retained in the CC by magnets compared to the other 2 groups on days 1 and 3 after ICI (fig. 3). The CellTracker signal began to biodegrade at day 5 and was lost at day 9.

**ICP/MAP Erectile Function Assessment**

The mean ICP/MAP ratio in the BCNC plus Nano-ADSCs plus magnet group (52.22 ± 5.36) was significantly higher than in the BCNC group (24.35 ± 4.90, p <0.001), the BCNC plus ADSC group (37.19 ± 4.63, p <0.001) and the BCNC plus Nano-ADSC group (37.06 ± 3.94, p <0.01). The BCNC plus ADSC and the BCNC plus Nano-ADSC groups showed higher ICP/MAP ratios than the BCNC group (p <0.05). There was no significant difference between the BCNC plus ADSC and the BCNC plus Nano-ADSC groups (fig. 4).

**Analysis**

**Immunohistochemistry.** On immunohistochemical staining NanoShuttle could still be found in the CC at day 28 after ICI. Some could even be identified in PECAM-1 and zSMA positive areas (fig. 5).

**Western Blot.** Western blot results demonstrated that the BCNC plus Nano-ADSCs plus magnet group had
significantly increased expression of PECAM-1 and αSMA in the CC than the BCNC, BCNC plus ADSC and BCNC plus Nano-ADSC groups (p < 0.05, fig. 6). The BCNC plus ADSC and BCNC plus Nano-ADSC groups showed significantly increased PECAM-1 and αSMA expression in the CC compared with the BCNC group (p < 0.05). There was no significant difference between the BCNC plus ADSC and BCNC plus Nano-ADSC groups.

DISCUSSION
Currently ICI of stem cells to treat ED has shown some effectiveness in experimental studies. However, it is believed that ICI of stem cells is essentially a systemic treatment rather than local therapy as there has been difficulty in holding and then finding the transplanted stem cells in penile tissues. In fact the majority of stem cells exit the CC 1 day after ICI. Typically cells in vitro need 4 to 6 hours to acclimate to a new environment and attach to a surface. However, due to the communication between the CC and the blood circulation system stem cells are washed from the CC by blood flow immediately after ICI, making it almost impossible for stem cells to settle in the CC. This has been a major technical limitation of therapies based on ICI of stem cells.

In this study we used what is to our knowledge a novel nanotechnology to overcome this challenge. ADSCs were first magnetized by NanoShuttle.
A magnetic force was then used to hold Nano-ADSCs (magnetized ADSCs) for 6 hours against the blood flow in the CC after ICI. Within the 6-hour period Nano-ADSCs were able to firmly attach to the interior of the CC. After the Nano-ADSCs settled they became difficult to wash away by blood flow and, thus, they could grow freely with nutrients from the surrounding blood circulation.

The *in vitro* experiment of this study demonstrated that ADSCs could be magnetized for up to 25 days by a single incubation with Nanoshuttle, although the NanoShuttle nanoparticles slowly disassociated from cells with time in this study. The *in vitro* results also showed that Nano-ADSCs aggregated to the magnet by attraction to the magnetic force. This foreshadowed the possibility that a magnet placed outside the bilateral CC would aggregate and retain Nano-ADSCs in the CC, as we found in the *in vivo* experiment. Using Cell-Tracker ADSCs were barely present in CCs at days 1 and 3, which matched findings in previous studies. In contrast, most Nano-ADSCs were

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**Figure 3.** Tracking ADSCs *in vivo* in the CC by CellTracker. All cells were marked with CellTracker (green areas) before injection and counterstained after harvest with DAPI (4',6-diamidino-2-phenylindole) (blue areas). Rat penes were harvested at days 1 (a), 3 (b), 5 (c) and 9 (d) after ICI. Immunohistochemical results revealed that marked cells remained in CC in Nano-ADSC ICI plus magnet group while few marked cells were observed in ADSC and Nano-ADSCs ICI groups at days 1 and 3. CellTracker signal began to weaken due to biodegradation at day 5 and was lost at day 9. Arrows indicate NanoShuttle. Reduced from ×40.
successfully retained in the CC for at least 3 days with the application of magnetic force in the Nano-ADSC ICI plus magnet group. To our knowledge this is the first study in which most stem cells were retained in the CC 3 days after ICI.

Tracing stem cells through nanoparticle in vivo has been reported previously. NanoShuttle in the CC observed at day 28 indicated that Nano-ADSCs may have still remained in the CC. There is the possibility that these Nano-ADSCs had been differentiating into endothelial or smooth muscle cells as the NanoShuttle was co-localized with positive immunohistochemical stains of PECAM-1 and $\alpha$SMA. Moreover, after quantitative analysis of Western blotting it was determined that PECAM-1 and $\alpha$SMA in the BCNC plus Nano-ADSC plus magnet group were significantly increased compared with the BCNC and BCNC plus ADSCs groups, suggesting that Nano-ADSCs may repair damaged endothelial cells or smooth muscle cells.

Eventually we found that this new approach could effectively improve erectile function compared with that of controls by ICP/MAP testing. When compared with ADSCs alone, the novel approach showed significant advantages in keeping stem cells in the CC and an improvement in erectile function as well as in endothelial and smooth muscle function. This novel approach may provide a window for stem cell therapy of erectile dysfunction or in other fields in which it has been difficult to localize injected stem cells.

Figure 4. Erectile function was assessed by ICP (a) and compared by ICP/MAP ratios (b). Pound sign indicates $p < 0.05$ vs BCNC..

$s$, seconds. Dollar sign indicates $p < 0.05$ vs BCNC plus ADSCs. Yen sign indicates $p < 0.05$ vs BCNC plus Nano-ADSCs.

Figure 5. Immunohistochemistry shows $\alpha$SMA (a) and PECAM-1 (b) expression in CC. In BCNC plus Nano-ADSCs plus magnet group NanoShuttle was still noted in CC at day 28 after ICI (arrows). Some were found on PECAM-1 and $\alpha$SMA positive areas. Scale bars indicate 100 $\mu$m.
There are some limitations to this study. 1) The rats in this study were relatively young and our findings may not mimic results when older rats are studied. 2) The ICI dose of Nano-ADSCs was the same as the traditional dose used for stem cell ICI, which may be too much for this new approach. Most stem cells would exit the CC after ICI using traditional methods, while the new approach in this study can keep the majority of cells in the CC. In the next phase we will optimize the best ICI dose of stem cells for our approach. 3) We did not determine the mechanism of how these stem cells grow, coordinate with resident cells and improve erectile function. Further research will determine these underlying mechanisms.

**CONCLUSIONS**

This novel approach using nanotechnology can effectively keep ADSCs in the CC after ICI, which is a major technical limitation of previous ICI and stem cell therapies for ED. We found that this approach has a better effect than traditional ICI of ADSCs on improving erectile function in ED animal models of cavernous nerve injury.

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**REFERENCES**


