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Adipose Derived Stem Cells Ameliorate Hyperlipidemia-associated Detrusor-overactivity in a Rat Model System

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Abstract

Purpose—It has been previously demonstrated that adipose tissue-derived stem cell (ADSC) can differentiate into muscle and neuron-like cells *in vitro*. In this study, we investigate the utility of ADSC in the treatment of overactive bladder (OAB) in obese hyperlipidemic rats (OHR).

Materials and Methods—Hyperlipidemia was induced in healthy rats by administration of a high fat diet. The resulting OHR were then treated with bladder injection of saline or ADSC or tail vein injection of ADSC. Bladder function was assessed by 24-h voiding behavior study and conscious cystometry. Bladder histology was assessed using immunostaining and trichrome staining followed by image analysis.

Results—Serum total cholesterol and low-density lipoprotein levels were significantly higher in OHR than in normal rats ($p < 0.01$). Micturition intervals were shorter in the saline-treated OHR relative to normal rats, OHR that received ADSC via tail vein, and OHR that received ADSC by bladder injection (143 ± 28.7 vs 407 ± 77.9 vs 281 ± 43.9 vs 368 ± 66.7 seconds respectively, $p = 0.0084$). Smooth muscle content of the bladder wall was significantly lower in OHR than in normal animals ($p = 0.0061$) while there was no significant difference between OHR groups. Nerve content and blood vessel density were lower in control than in ADSC-treated OHR.

Conclusions—Hyperlipidemia is associated with increased urinary frequency and diminished bladder blood vessel and nerve density in rats. Treatment with ADSC ameliorates these adverse effects and holds promise as a potential new therapy for OAB.

Keywords

Adipose tissue-derived stem cells; lower urinary tract symptoms; obese hyperlipidemic rat; overactive bladder

INTRODUCTION

Lower urinary tract symptoms (LUTS) consist of storage, voiding and postmicturition symptoms. Storage symptoms include increased micturition frequency, nocturia, urinary urgency, and urinary incontinence. Voiding symptoms include weak stream, hesitancy, and intermittency. Postmicturition symptoms include the sensation of incomplete emptying and postmicturition dribble.¹

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It is estimated that moderate to severe LUTS (AUA Symptom Score ≥ 8) are present in 36% of men aged 50–59 years, 50% of men aged 60–69 years and 60% of men aged 70–79 years.² LUTS in men are frequently coincident with obesity and benign prostatic hyperplasia (BPH);^{3, 4} however, LUTS may also arise from other disorders, such as detrusor dysfunction. Overactive bladder (OAB), a subset of storage LUTS often associated with detrusor overactivity (DO), is defined as urgency and is often associated with frequency, nocturia, and urinary incontinence.¹

Many treatment options are available for patients with LUTS. Mild LUTS may not be bothersome enough to necessitate treatment; if therapy is desired, oral medication is typically the initial treatment choice. Antimuscarinic drugs are currently the first-line pharmacotherapy of choice for patients with bothersome OAB not thought to be related to BPH. However, antimuscarinics do not produce adequate relief in all patients and they do not resolve the underlying functional disorder.⁵ In addition, these medications have potential adverse effects including dry mouth, constipation, headache and blurred vision.⁶

Surgical interventions such as transurethral resection of the prostate (TURP) are appropriate for men with moderate/severe LUTS associated with BPH who (i) do not improve after medical therapy, (ii) do not want medical therapy, or (iii) present with a BPH-related complications developed.⁷ TURP is considered the gold standard therapy for BPH-associated LUTS. Symptomatic improvement has been reported in up to 88% of patients and the magnitude of reduction in obstructive symptoms may be as great as 85%.⁸ However, it has been reported that TURP may often (20–40% of cases) fail to alleviate storage symptoms especially nocturia.^{9–11} In addition, long-term follow up reveals that OAB symptoms recur in over 60% of the patients who have undergone TURP. Recurrence of symptoms may be secondary to prostate regrowth but may also be attributable to comorbid OAB.¹² Furthermore, TURP is not a therapeutic option for LUTS in women.

Bladder ischemia is a potential factor in the pathogenesis of LUTS.¹³ A clinical model of DO has been reported in rats fed a high-fat diet (obese hyperlipidemic rats).¹⁴ It has been demonstrated that diminution in blood flow may injure the intrinsic nerves of detrusor muscles and induce denervation supersensitivity.¹⁵ Furthermore, in a rabbit model, high-fat diet was demonstrated to induce endothelial denudation and disruption of vascular supply to the bladder.¹⁶

Adipose-derived stem cells (ADSC) are multipotent progenitor cells isolated from the stromal vascular fraction of adipose tissue.¹⁷ Unlike bone marrow stem cells, ADSC can be obtained in large quantities at low risk.¹⁸ In addition to being more abundant and easily accessible, adipose tissue yields far more stem cells than bone marrow on a per-gram basis (5,000 vs 100–1,000).¹⁹ In previous studies, we have demonstrated that ADSC can differentiate into endothelial and neuron-like cells *in vitro*.²⁰ This capacity makes ADSC an attractive potential therapy for conditions known to be associated with disruption of endothelial and neuronal tissues.

In this study, we investigate the efficacy of ADSC in the treatment of DO and bladder histological changes associated with high-fat diet in the rat model animal.

MATERIALS AND METHODS

Animal Groups and Experimental Design

Forty (40) 3-month old male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA). All animal care, treatments and procedures were

approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco (AN080146-01).

Ten rats fed a diet of standard rat chow served as negative controls. The remaining 30 rats (obese hyperlipidemic rats, OHR) were fed a high-fat diet consisting of 2% cholesterol and 10% lard (Zeigler Brothers, Gardner, PA, USA). At five months, all rats in the negative control group and ten rats randomly selected from the OHR were placed in a metabolic cage for 24 hours, to perform voiding behavior studies.

After metabolic cage study, all rats underwent para-testicular fat harvest for procurement of ADSC. After culture and purification of ADSC the 30 OHR were divided into three groups: 1) injection of phosphate buffered saline (PBS) into the detrusor through a laparotomy (OHR+PBS), 2) Injection of ADSC via tail vein (OHR+TV), or 3) Injection of ADSC into the detrusor through a laparotomy (OHR+B). Negative control animals underwent laparotomy and PBS injection into the detrusor (NR+PBS).

One month after these treatments all rats underwent conscious cystometry. Animals were then euthanized and serum samples obtained for assessment of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride and glucose. Bladder tissues were harvested for immunohistochemical examination for Masson's trichrome, 5-ethynyl-2'-deoxyuridine (EdU), α -smooth muscle actin (α -SMA), rat endothelial cell antigen-1 (RECA-1), and rat choline acetyltransferase (ChAT) staining.

Metabolic Cage

All rats undergoing 24 hour voiding behavior studies were placed in a metabolic cage for 24 hours on a 12/12 hour dark/light photocycle.²¹ Each monitoring period started at 16:00. Micturition time and volumes were recorded via a fluid collector connected to a pressure transducer (Utah Medical Products, Midvale, UT, USA). The pressure transducer was connected to a Dell Pentium 4 computer with Laboratory View 6.0 software (National Instruments, Austin, TX, USA) to monitor the cumulative weight of the collected urine. All rats received water and food ad-libitum throughout the duration in the cage. Voiding frequency and volume were recorded for 24 hours.

Harvest and Processing of ADSC and EdU Labeling

Animals were anesthetized with isoflurane. A midline abdominal incision was made to expose the perigonadal fat pad. A specimen of para-testicular fat was harvested and placed in ice cold PBS. The animal was then closed in two layers with absorbable suture and anesthesia was weaned.

Fat tissue was rinsed with PBS in a 50 ml conical tube. Adipose tissue was removed from the upper layer to a fresh tube, and digested in 0.075% collagenase I (Sigma-Aldrich Cp., St Louis, MO, USA) for 1 hr at 37° C with shaking. The top lipid layer was removed and the remaining liquid portion was centrifuged at $220 \times g$ for 10 min. The pellet was then treated with 160 mM NH_4Cl for 10 min to lyse red blood cells. The remaining cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and plated at a density of 1×10^6 cells in a 10-cm dish. The culture dish was placed in a 5% CO_2 incubator for 3–5 days to allow the formation of ADSC colonies. For the purpose of cell tracking, ADSC were treated with 10 μM EdU (Invitrogen, Carlsbad, CA, USA) overnight. A total of 3×10^6 EdU-labeled ADSC were collected into a 2-ml conical tube containing 1 ml PBS; these ADSC were subsequently utilized for injection.

ADSC Injection

All animals were anesthetized with isoflurane and underwent midline laparotomy to expose the bladder. Animals underwent injection of 1 ml PBS (NR+PBS and OHR+PBS group), or 3 million of autologous ADSC in 1 mL PBS (OHR+B) into the bladder. Animals in the OHR+TV group received 3 million of autologous ADSC transplantation via the tail vein. After treatment the laparotomy was closed in two layers.

Placement of Bladder Catheters and Conscious Cystometry

Bladder catheters were implanted under isoflurane anesthesia 24 hours prior to conscious cystometry. The abdomen was opened through a midline incision and a polyethylene catheter (PE-90, Clay-Adams, Becton Dickinson, Parsippany, NJ, USA) was implanted into the bladder through the dome. The catheter was tunneled subcutaneously and brought out through a skin incision on the rat's back.

Conscious cystometry was performed by placing an awake rat in a custom-made tunnel within a metabolic cage (Braintree Scientific, Braintree, MA, USA). Conscious cystometry enables the recording of micturition time and volumes via a fluid collector connected to a pressure transducer connected to surgically implanted bladder catheters (Utah Medical Products, Midvale, UT, USA). The bladder catheter was connected via a T-tube to both a pressure transducer (Utah Medical Products, Midvale, UT, USA) and an infusion pump (Harvard Model 22, KD Scientific, Holliston, MA, USA). After calibration of the pressure transducer to zero, the bladder was filled with room temperature normal saline at a rate of 0.1 mL/min, while recording simultaneous pressure on a sensor input module (model SCXI 1121, National Instruments, Austin, TX, USA) connected to a Dell Pentium 4 computer with Laboratory View 6.0 software (National Instruments). All rats were given around 10 min for the voiding patterns to stabilize. Thereafter micturition were recorded for 30 min [13].

Immunohistochemical Staining

After euthanasia, tissue samples were fixed in cold 2% formaldehyde and 0.002% picric acid in 0.1 M phosphate buffer, PH 8.0, for 4 hours followed by overnight immersion in buffer containing 30% sucrose. The specimens were then embedded in OCT Compound (American Master Tech Scientific, Inc, Lodi, CA, USA) and stored at 70°C until use. Sections were cut at 5 µm, mounted into charged slides and air dried for 5 min. The slides were stained with Masson's trichrome for connective tissue and smooth muscle histology.

EdU staining with immunostaining for α -SMA was performed by placing slides in 0.3% H₂O₂/methanol for 10 min. Slides were then twice-washed in PBS for 5 min and incubated with 3% horse serum in PBS/0.3% Triton X-100 for 30 min at room temperature. After draining this solution from the tissue section, the slides were incubated at room temperature with anti- α -SMA antibody (Abcam Inc., Cambridge, MA, USA) overnight. After rinsing with PBS, sections were incubated with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After three rinses with PBS, the slides were incubated with freshly made Click-iTTM reaction cocktail (Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature without light followed by Dilute Hoechst (Invitrogen, Carlsbad, CA, USA) for nuclear staining.

We used RECA-1 as a marker for endothelial cells to study the changes in vascular profile and ChAT in cholinergic nerves profile. The tissue sections were similarly prepared as described above. After draining excess fluid, the sections was incubated overnight at room temperature with the following antibodies: mouse anti-RECA-1 (Abcam Inc, Cambridge, MA, USA) and goat anti-ChAT (Chemicon, Temecula, CA, USA). Immunostaining of the tissue was performed with the avidin-biotin-peroxidase method (Elite ABC, Vector Labs,

Burlingame, CA, USA), with 3,3-diaminobenzidine as chromagen, followed by hematoxylin counterstain.

Image and Statistical Analysis

For image analysis, five randomly selected fields per slide were photographed and recorded at 200x magnification for Masson's trichrome staining and at 400x magnification for RECA-1 and ChAT staining using a digital still camera (Nikon DXM1200) and ACT-1 software (Nikon Instruments Inc., Melville, NY, USA). Image-Pro Plus imaging software was used for quantification of differential staining (Media Cybernetics, Silver Spring, MD, USA). The percentage of smooth muscle was calculated as the sum of the smooth muscle (red stained) areas divided by the sum of all smooth muscle and connective tissue (blue stained) areas.¹⁵

Data was analyzed with Prism 4 (GraphPad Software, Inc., San Diego, CA, USA) and expressed as mean $\pm$ standard error of the mean for continuous variables. The continuous data was compared the groups using Student's t-test and one-way analysis of variance. The Bonferroni test was used for post-hoc comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

The 24-Hour Voiding Behavior Studies

Micturition frequency of animals fed the high-fat diet was significantly higher than that of controls at five months (14.7 ± 1.51 vs 9.9 ± 0.78 time/day, $p = 0.0114$). Mean volume per void was significantly lower in OHR relative to controls (0.57 ± 0.059 mL vs 1.01 ± 0.154 mL, $p = 0.0115$).

Physiological Variables and Biochemistry

Total body, bladder, and prostate weights were significantly higher in OHR than in rats fed a normal diet for 6 months ($p < 0.05$). Serum total cholesterol and LDL levels were significantly higher in OHR than in rats fed a normal diet for six months ($p < 0.05$). There were no significant differences in glucose, triglyceride or HDL levels between the four groups (table 1).

3.3 Conscious cystometry

Representative bladder pressure and voiding measurements from conscious cystometry in NR+PBS and OHR+PBS rats are presented in figure 1. Micturition interval was significantly shorter in the OHR+PBS group (143 ± 28.7 sec) compared to the NR+PBS (407 ± 77.9 sec) and OHR+B (368 ± 66.7 sec) groups (table 2). The OHR+TV group (281 ± 43.9 sec) had longer micturition intervals than the OHR+PBS group but the difference did not reach statistical significance.

The mean volume per void in the OHR+PBS group (0.23 ± 0.048 ml) was significantly smaller than the NR+PBS (0.665 ± 0.131 ml) and OHR+B (0.628 ± 0.122 ml) groups. The OHR+TV group (0.472 ± 0.054 ml) had larger voided volume than the OHR+PBS group but the difference did not reach statistical significance. There was no statistically significant difference in post-void residual urine volume and maximal voiding pressure among the four groups.

Tracking Transplanted ADSC

Autologous EdU-labeled ADSC were transplanted via tail vein (OHR+TV group) or bladder (OHR+B group) and identified by Alexa-594 stain. The histological data indicated the presence of EdU-labeled cells in the submucosal connective tissue and muscular tissue of the bladder. Many more EdU-labeled cells were identified in the OHR+B group relative to the OHR+TV group. A few EdU-positive nuclei appeared to co-localize with α -SMA staining, suggesting the possibility of stem cell differentiation into smooth muscle cells (fig. 2).

Blood Vessel Content

RECA-1 staining for endothelial tissue showed intense positivity in the submucosal layer of bladders from the NR+PBS, OHR+TV and OHR+B groups; there was minimal RECA-1 positivity in the OHR+PBS group (fig. 3). There was no significant difference between the NR+PBS, OHR+TV and OHR+B groups (table 3), suggesting that ADSC transplantation maintained vascular endothelial integrity in the bladder of ADSC-treated OHR.

Cholinergic Nerve Content

The OHR+PBS group had significantly lower cholinergic nerve content relative to NR+PBS, suggesting that cholesterol may adversely affect nerve content (fig. 4). Cholinergic nerve content in the OHR+B was significantly greater than what was observed in the OHR+PBS group; there was no significant difference between OHR+TV and OHR+PBS groups (table 3).

Smooth Muscle Content

All OHR groups had significantly thicker individual muscle bundles relative to the NR+PBS group (fig. 5). Tissue sections from the NR+PBS group had higher smooth muscle percentage than the OHR+PBS, OHR+TV and OHR+B groups (table 3). However, there was no significant difference between animals fed a high-fat diet.

DISCUSSION

OAB is a complex syndrome attributed to numerous factors. In addition to neurologic disease, bladder outlet obstruction, congenital malformations, dysfunctional behavioral, tissue senescence, myogenic disease, and inflammation, ischemia is a potential cause of OAB.²² The mechanism of ischemia related bladder dysfunction is complex but may be related to ischemic denervation of the detrusor; this may produce supersensitivity of the muscarinic receptors to acetylcholine and OAB.¹⁵ In addition to neurological injury, ischemia may lead to smooth muscle fibrosis, and desquamation of urothelium.¹³ Azadzi *et al.* found that hyperlipidemia-induced chronic ischemia increases transforming growth factor- β 1 (TGF- β 1) expression in the bladder leading to fibrosis and non-compliance. Moreover, increased serum low density lipoprotein (LDL) deposition has been shown to enhance TGF- β 1 production and fibronectin synthesis in various other organs.²³ Frisbee *et al.* demonstrated that obesity led to progressive microvascular rarefaction (a reduction in microvascular density) within multiple tissues/organs with the likely end result of progressive tissue ischemia.²⁴

In the current study, it was determined that micturition frequency over a 24 hours period is higher, and voided volume significantly lower, in rats fed a high-fat diet for five months relative to those fed a normal diet. Four weeks after ADSC transplantation, these functional parameters improved toward normalcy in the OHR+TV and OHR+B groups. It is therefore implied that 1) hyperlipidemia and obesity produces a phenotype similar to OAB in rats, and

2) ADSC may ameliorate obesity related OAB. In addition, application of ADSC by direct injection appears to be more efficacious than application by tail vein injection.

The precise mechanism(s) by which ADSC enhance bladder function remains to be elucidated. ADSC have the capability to differentiate into a variety of cells types such as adipocytes, cardiomyocytes, chondrocytes, endothelial cells, smooth muscle cells, neuronal-like cells, and osteoblasts *in-vitro*.^{20, 25, 26} Furthermore, ADSC secrete cytokines and growth factors such as vascular endothelial growth factor and hepatocyte growth factor that stimulate angiogenic recovery in a paracrine manner.^{27–29} The angiogenic and vascular restorative properties of ADSC by either intravenous or local administration have been demonstrated in multiple *in vivo* studies.^{28–30} In addition to angiogenic mechanisms, ADSC may provide antioxidant chemicals, free radical scavengers and heat shock proteins in ischemic tissues.²⁷ Histological data from our study suggests that ADSC treatment enhances microvessel and neuronal content of the detrusor muscle. However, while it is possible that ADSC may have differentiated into various somatic cells within our study animals, the scant staining for EdU in the bladder tissues suggests that the therapeutic effect of ADSC was likely mediated primarily by the paracrine release of cytokines and growth factors. Further study of the specific cytokines and growth factors secreted by ADSC *in vivo* is required.

Specific limitations of our study include the lack of pre-obesity conscious cystometry and the unknown durability of ADSC treatment effect. While it would be more convincing to demonstrate that DO is a direct result of high fat diet and obesity by performing pre-obesity conscious cystometry, this would necessitate multiple surgeries and catheter placements, thus increasing the risk of iatrogenic damages. For future studies, it would be useful to know the durability of ADSC effects and whether coupling ADSC therapy with dietary modification could enhance the therapeutic effects. Above all, the greatest need will be the assessment of the long term safety of this form of stem cell therapy before clinical trials in humans can be considered.

CONCLUSIONS

To date, no treatment has been proven capable of repairing the tissue alterations resulting from bladder ischemia. The present study suggests that ADSC administered by local injection enhance angiogenesis and cholinergic nerve innervations after induction of ischemic injury by hyperlipidemic diet. The mechanism of action is likely via cytokine secretion although stem cell differentiation may also play a role. ADSC may hold promise in the treatment of overactive bladder induced by obesity; further studies are warranted.

Abbreviations and Acronyms

α-SMA	α -smooth muscle actin
ADSC	adipose-derived stem cells
BPH	benign prostatic hyperplasia
ChAT	rat choline acetyltransferase
DMEM	Dulbecco's Modified Eagle's Medium
DO	detrusor overactivity
EdU	5-ethynyl-2'-deoxyuridine
FBS	fetal bovine serum
HDL	high density lipoprotein

LDL	low density lipoprotein
LUTS	lower urinary tract symptoms
NR+PBS	rats fed a normal diet received PBS
OAB	Overactive bladder
OHR	obese hyperlipidemic rats
OHR+B	rats fed a high-fat diet received ADSC injection into bladder
OHR+PBS	rats fed a high-fat diet received PBS
OHR+TV	rats fed a high-fat diet received ADSC transplantation via tail vein
PBS	phosphate buffered saline
RECA-1	rat endothelial cell antigen-1
TGF-β1	transforming growth factor- β 1
TURP	transurethral resection of the prostate

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Figure 1.

Representative cystometry curves in (A) normal bladder cystometry and in (B) OAB. The blue upper, red middle and green lower panel present bladder, intra-abdominal and detrusor contraction pressure, respectively. The light blue, lowest panel presents the accumulated micturition volume. The micturition pattern was recorded for 30 min. OAB was defined by shorter micturition intervals and smaller micturition volume per void.

Figure 2.

Tracking transplanted ADSC. The bladder was stained with Alexa-594 (red fluorescence), DAPI (blue fluorescence) and anti- α -SMA antibody (green fluorescence). Only EdU-positive cells show red fluorescence (arrow). No EdU-positive cell were identified in normal (A,B) and OHR (C,D) that received PBS by injection. Several EdU-positive cells were identified in the muscular layer of the rats that received ADSC transplantation via tail vein injection (E, F). A greater concentration of EdU-positive cells was noted in the rats that received ADSC injection directly into bladder relative to those that received ADSC by tail vein injection. (G, H). Magnification is X 100 for Fig. A, C, E and G, and x 400 for Fig. B, D, F and H.

Figure 3.

Blood vessel content of bladder wall. RECA-1 staining of bladder wall from a rats fed a normal diet that received PBS (A), a high-fat diet that received PBS (B), a high-fat diet with ADSC transplantation via tail vein (C) and a high-fat diet with ADSC injection into bladder (D). The endothelium of blood vessels is stained brown (arrow). The number of RECA-1 positive cells was higher in figures A, C and D relative to B. Magnification is X 100.

Figure 4.

Nerve content of bladder wall. ChAT staining of bladder wall from a rat fed a normal diet that received bladder injection of PBS (A), a rat fed a high-fat diet that received bladder injection of PBS (B), a rat fed a high-fat diet that received tail vein injection of ADSC (C) and a rat fed a high-fat diet that received bladder injection of ADSC (D). Choline acetyltransferase positive tissue is stained brown (arrow). The number of ChAT staining cholinergic nerves in the bladder wall was high in A, C and D, but not in B. Magnification is X 400.

Figure 5.

Smooth muscle content of the bladder wall. Masson's trichrome staining of bladder wall from a rat fed a normal diet that received bladder injection of PBS (A), a rat fed a high-fat diet that received bladder injection of PBS (B), a rat fed a high-fat diet that received tail vein injection of ADSC (C) and a rat fed a high-fat diet that received bladder injection of ADSC (D). Smooth muscle is stained red and connective tissue blue. The smooth muscle content was higher in A than in B, C and D. Magnification is X 100.

Table 1

Physiological variable and serum biochemistry

Groups	NR+PBS	OHR+PBS	OHR+TV	OHR+B	P value
Body weight (g)	660 ± 16.4	757 ± 20.6 [†]	780 ± 25.8 [†]	739 ± 24.2	0.0048
Bladder weight (g)	0.202 ± 0.008 *	0.278 ± 0.009	0.278 ± 0.013	0.275 ± 0.011	< 0.0001
Prostate weight (g)	1.384 ± 0.017	1.618 ± 0.036	1.728 ± 0.110 [†]	1.734 ± 0.125 [†]	0.0183
Total cholesterol (mg/dL)	77.9 ± 4.04 *	148.5 ± 16.99	148.2 ± 16.08	157.0 ± 17.61	0.0037
HDL (mg/dL)	44.6 ± 3.33	41.6 ± 3.65	42.7 ± 4.84	35.5 ± 2.73	0.3666
LDL (mg/dL)	13.1 ± 0.60 *	104.9 ± 16.49	91.8 ± 14.04	106.4 ± 21.27	0.0007
Triglyceride (mg/dL)	53.0 ± 14.59	50.27 ± 7.76	63.4 ± 14.67	81.5 ± 19.52	0.5198
Glucose (mg/dL)	205.3 ± 9.59	249.3 ± 15.31	230.0 ± 16.70	262.7 ± 20.60	0.1049

One rat, in the NR+PBS group, suffered from tumor in the leg and was excluded.

Six rats were randomly selected for biochemistry analysis from each group.

* Versus other groups $p < 0.05$,

[†] Significantly different versus NR+PBS.

Table 2

Conscious cystometric parameters

Groups	NR+PBS	OHR+PBS	OHR+TV	OHR+B	P value
Micturition interval (sec)	407 ± 77.9 [‡]	143 ± 28.7	281 ± 43.9	368 ± 66.7 [‡]	0.0084
Voided volume (ml)	0.665 ± 0.131 [‡]	0.230 ± 0.048	0.472 ± 0.054	0.628 ± 0.122 [‡]	0.0072
Maximal voiding pressure (cm-H ₂ O)	53.7 ± 6.39	43.3 ± 3.42	57.6 ± 6.58	59.8 ± 6.59	0.1889
Postvoid residual urine (ml)	0.134 ± 0.050	0.116 ± 0.027	0.103 ± 0.020	0.150 ± 0.055	0.854

One rat, in the NR+PBS group, suffered from tumor in the leg and was excluded.

One rat in the NR+PBS group, one rat in the OHR+TV group and one rat in the OHR+B group had mild cystostomy leakage; they were excluded.

[‡] Significantly different versus OHR+PBS.

Table 3

RECA-1, ChAT and Masson's Trichrome stain data in the experimental animals

Groups	NR+PBS	OHR+PBS	OHR+IV	OHR+B	P value
RECA-1 (number/HPF)	51.9 ± 4.29	31.0 ± 3.23 *	51.8 ± 2.17	60.3 ± 3.79	< 0.0001
ChAT (number/HPF)	124.9 ± 15.76 ‡	37.2 ± 12.35	88.1 ± 12.08	104.1 ± 20.22 ‡	0.0049
Bladder smooth muscle (%)	73.4 ± 3.24	57.8 ± 2.09 †	61.7 ± 3.63	59.7 ± 2.78 †	0.0061

HPF = high-power field (x400).

Six rats were randomly selected from each group for immunohistochemical study.

* Versus other groups $p < 0.05$,

† Significantly different versus NR+PBS,

‡ Significantly different versus OHR+PBS.