Animal Models of Diabetic Complications Consortium
(U01 DK076162)

Annual Report
(2007)

Diabetic Uropathy Pathobiology Site
The Cleveland Clinic

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypothesis- We hypothesize that depletion of manganese superoxide dismutase (MnSOD) specifically in smooth muscle of adult mice will exacerbate accumulation of reactive oxygen species (ROS) in smooth muscle during streptozotocin (STZ)-induced diabetes and accelerate the onset of the decompensation phase of diabetic bladder dysfunction. We further hypothesize that limiting depletion of MnSOD to arterial smooth muscle will have a lesser effect on STZ-induced diabetic bladder dysfunction by limiting exacerbation of STZ-induced ROS accumulation to the vasculature.

Recent Progress and Major Accomplishments:

1- Obtaining and breeding healthy $MnSOD^{lox/lox}$ mice and $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ mice so the breeding of our desired model of $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}$ mice could begin.

In order to determine whether heterozygous or homozygous knockout of MnSOD was preferable for experiments, i.e.- yields sufficient depletion of MnSOD without causing undue distress to the mice, and to optimize the course of OHT treatment to maximize $CreER^{T2}$-mediated excision, a small pilot study was designed to compare $MnSOD^{lox/+}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ (heterozygous floxxed) mice with $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ (homozygous floxxed) mice. We received 4 female adult $MnSOD^{lox/lox}$ mice and 5 male adult $MnSOD^{lox/lox}$ from the laboratory of Dr. Frank Brusias at the University of Michigan. Due to a procedural error, no sentinel mice were shipped with the MnSOD mice, so two of the lox/lox mice had to be sacrificed for testing. Also, while the colony was in quarantine at the Cleveland Clinic, two additional mice died leaving us with three female and two male $MnSOD^{lox/lox}$ mice.

There was an unforeseen problem with the supplier of the $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ mice which delayed their arrival by several months. To maintain the line of $MnSOD^{lox/lox}$ mice, we started inbreeding the mice. Once we started breeding the mice, two of the female mice died during their pregnancy, leaving us with one viable female and two viable male $MnSOD^{lox/lox}$ mice. We then started to breed the two remaining male $MnSOD^{lox/lox}$ mice with wild-type female mice and set-up a breeding strategy to preserve and recreate the $MnSOD^{lox/lox}$ mice model. In addition, while we were genotyping the newborn pups, we decided to verify the genotype of the MnSOD parents shipped to us. It proved that the male mice were $MnSOD^{wt/lox}$ mice and not $MnSOD^{lox/lox}$ mice as assumed which further delayed our pilot experiment.

Once the supplier of the $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ finally resolved their issues, they sent us one male $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ and one female $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ mouse. While they were in quarantine here at the Cleveland Clinic, the male mouse died. Again, we started a breeding strategy using the female $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ mouse with a wild-type male mouse to preserve and recreate the heterozygous Cre/+ mice we desired.

After all of these setbacks, we have finally reached a point where we have 7 healthy $MnSOD^{lox/lox}$ mice and 4 $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ mice. At this point we are ready to start the initial pilot experiment by cross-breeding the two species, with the aid of Jackson Laboratories in Bar Harbor, ME.

2- Continuation of major progress on studies of pathophysiology of diabetic bladder dysfunction (DBD). Our lab continues to be at the forefront of examination of mechanisms of DBD. We have completed the following studies:

External Urethral Sphincter Activity in Diabetic Rats (Submitted- Neurourology and Urodynamics). The objective our study was to examines the temporal effects of diabetes mellitus (DM) on the bladder and the external urethral sphincter (EUS) activity in diabetic rats. In this study, 24 female Sprague Dwaley (SD) rats were divided into 2 groups: streptozotocin-induced diabetics and age, sex-matched controls. Cystometry (CMG) under urethane anesthesia and electromyogram (EMG) of the EUS were evaluated in all rats after 6 and 20 week of diabetes induction. After EMG assessment, the tissues of the-urethra were harvested for morphological examination. Our results showed that diabetes caused reduction of body weight compared to controls, and the bladders of diabetic rats weighed more than the controls after 6- or 20-weeks of diabetes induction. CMG measurements showed diabetes increase threshold volume, contraction duration, high-frequency oscillations (HFO), and higher residue volume. Peak contraction amplitude increased in 6-week but not in 20-week diabetic rats. EUS-EMG measurements showed significantly increased frequency of EUS-EMG bursting discharge during voiding in 6-week (8.1±0.2, 6.9±0.6/sec, respectively) but not in 20-week (5.8±0.3, 6.0±0.2/sec, respectively) diabetic rats compared to controls. EUS-EMG bursting period was also increased in 6-week (6.8±0.3, 4.1±0.6 sec, respectively) and 20-week (7.5±0.6, 4.3±0.4 sec, respectively) in diabetic rats compared to controls. EUS-EMG silent periods were reduced in 6-week (0.072±0.004, 0.100±0.010 sec, respectively), not changed in 20-week (0.135±0.015, 0.115±0.005 sec respectively), but active period did not change in 20-week, increased in 6-week diabetic rats compared to controls. Morphometric analysis showed atrophy of striated muscle in the EUS after 20 week but not 6 week of DM induction. From this study we concluded that diabetes causes marked functional and anatomical abnormalities of the EUS. These abnormalities may contribute to the previously reported time-dependent bladder dysfunction in diabetic rats.

Effects of Diabetes on Urinary Incontinence- (Kim JH, Huang X, Liu G, Moore CK, Bena J, Damaser MS, Daneshgari F. Diabetes slows the recovery from urinary incontinence due to simulated childbirth in female rats. Am J Physiol Regul Integr Comp Physiol. 2007 May 9- This study was done to test the hypothesis that simulated vaginal birth by vaginal distension (VD) causes more severe urinary incontinence and slower recovery in diabetic rats. After measuring baseline leak point pressure (LPP) in 16 diabetic mellitus (DM) and 16 age and weight-matched control (Ct) female Sprague-Dawley rats, these animals underwent either VD or sham VD (sham). Four and ten days after the procedures, LPP and conscious cystometry were assessed. Tissues were then harvested and examined by light microscopy. LPP at baseline was equal among all four groups. Four days after VD, LPP in both VD groups dropped to significantly lower levels than in sham rats ($p<0.001$). Moreover, LPP in the DM+VD group was significantly lower than in the Ct+VD group. At 10 days, LPP in the Ct+VD group had recovered to its baseline value, whereas the LPP in the DM+VD group remained significantly reduced. DM rats had larger bladder capacity and longer voiding intervals

than Ct rats. Histological findings included more severe damage to the external sphincter striated musculature of the urethra in DM+VD group compared to Ct+VD. In conclusion, these findings suggest that DM causes increased severity and delayed functional recovery from the effects of simulated childbirth.

Measurement of Bladder Afferent Sensation- (Submitted: Journal of Urology) We have developed an implantable device (Bladder Sensory perception Threshold or BST Device) and successfully integrated the BST device with the Neurometer® CPT/C diagnostic neurostimulator (Neurotron, Inc., Baltimore, MD) for examination of afferent sensation of the bladder. For sensory testing, we used this battery powered neurostimulator capable of generating constant AC stimuli between 1 and 10000 μA with quartz crystal calibrated sinusoid waveform stimuli at frequencies of 5 Hz, 250 Hz and 2000 Hz. In a series of experiments, we have demonstrated the feasibility of measuring BST in rats, generated data on inter- and intra-operator variability of BST measurements, and tested the fiber selectivity of the BST values by performing the measurements in rats treated with afferent fiber specific stimuli or vehicle:

We found that implantation of our BST device into the bladder of female Sprague-Dawley rats led to a significantly decreased mean voided volume and increased number of voids in 24 hours (p-values of 0.003 and 0.006, respectively), however those parameters returned to normal after 4 days. After implantation of our device, sine-wave stimuli at 2000, 250, and 5 Hz were applied to the bladder mucosa at increasing intensities until a startle response of the animal was observed. The minimum intensity at which this response was seen was defined as the Bladder Sensory perception Threshold (BST). To determine the inter-observer uniformity of the BST measurements, two investigators, each blinded to the results of the other, obtained BST values from the same six rats. No significant differences in BST values were found between the two investigators (Table 1). In a second set of experiments, the day-to-day and week-to-week consistency of our technique was determined. BST measurements were repeated in the same rats daily over 3 days (Fig.1A) or weekly over 3 weeks (Fig. 1B), showing that the BST values at all three frequencies were stable over those times**.**

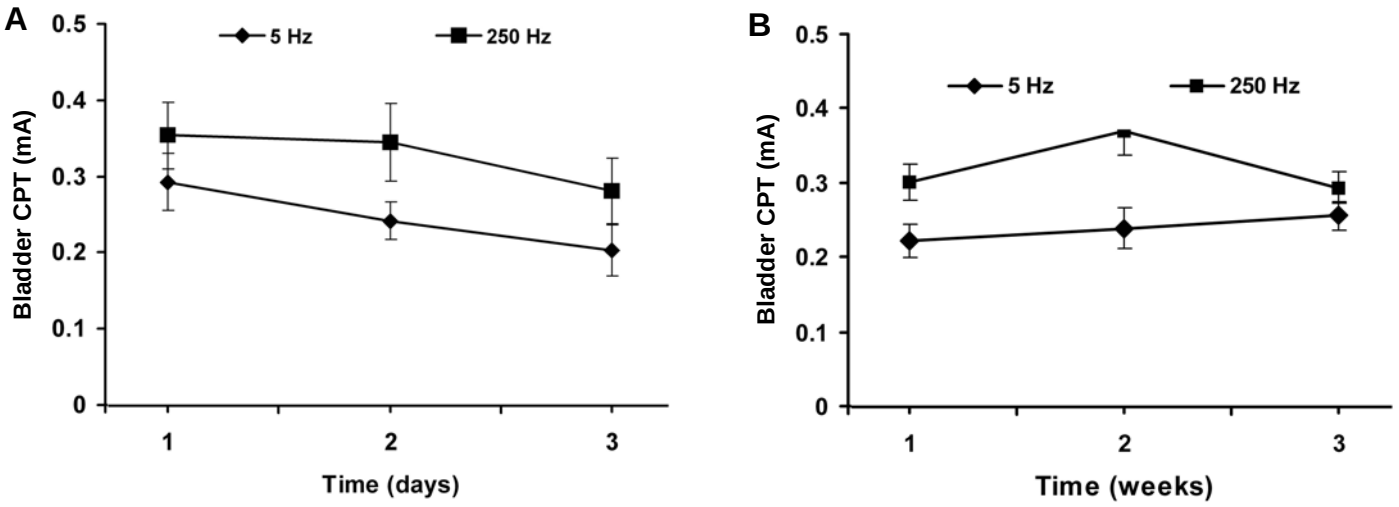

Figure 1 . BST values in rats were stable over periods of 3 days (A, n=6) and 3 weeks (B, n=8).

Table 1. Inter-observer reliability of bladder CPT values obtained from 6 rats by 2 investigators

Rat	Frequency (Hz)	Investigator #1 SPT value (mA)	Investigator #2 SPT value (mA)	Difference SPT value (mA)
1	250	0.28	0.27	0.01
	5	0.21	0.22	0.01
2	250	0.29	0.30	0.01
	5	0.36	0.32	0.04
3	250	0.27	0.27	0
	5	0.21	0.22	0.01
4	250	0.26	0.27	0.01
	5	0.16	0.17	0.01
5	250	0.36	0.35	0.01
	5	0.20	0.18	0.02
6	250	0.27	0.28	0.01
	5	0.17	0.18	0.01

The differences of SPT values between two investigators were analyzed by using Wilcoxon matched pairs signed rank sum tests: $p = 0.41$

Specificity of spinal c-Fos expression induced by Neurometer®-bladder CPT electrostimulation (submitted: Journal of Urology). The neuroselectivity of Neurometer® electrostimulation of bladder afferent pathways was assessed using expression in different spinal cord regions of the protooncogene c-Fos, known to be induced by increased neuronal activity, as a marker. Using the Neurometer® with our newly developed BST device, sine-wave electrical stimulation was applied for 90 minutes to the bladder in rats. Following Neurometer® stimulation at 5 Hz with a current of 2.0 mA, the distribution of immunocytochemically-detected c-Fos positive cells in the spinal cord segments (L1 to S1) that contribute axons to the pelvic and hypogastric nerves was measured (Fig. 2). The distribution of a major peak of c-Fos expression in L6 and minor peaks in L1 and S1 was very similar to that found in rats that received a 30 minute intravesical injection of capsaicin instead of Neurometer® stimulation (Fig. 2). Since capsaicin stimulates predominantly C-fibers that experiment provides evidence for C-fiber selectivity of Neurometer® stimulation at 5 Hz.

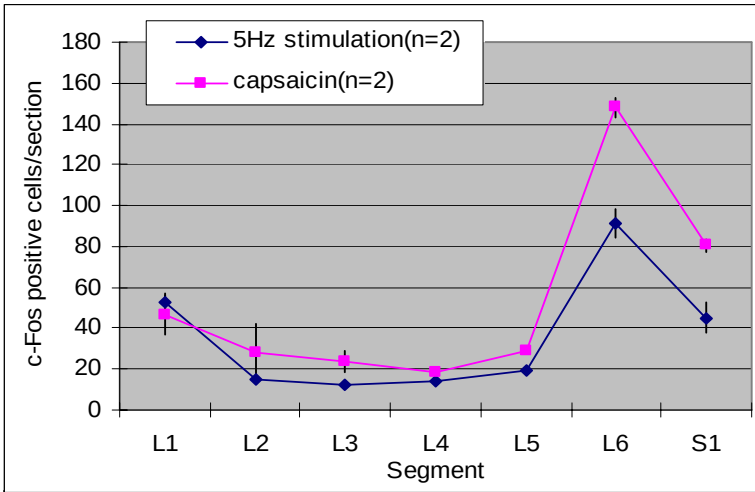

Figure 2. c-Fos expression in L1 to S1 spinal cord after stimulation by Neurometer® or capsaicin. After 5 Hz electrical stimulation (2.0 mA, 90 min, n=2) or intravesical capsaicin (1 mM, 30 min, n=2), c-Fos was detected by immunocytochemistry and the numbers of c-Fos positive cells/section were counted.

Plans for the Upcoming Year- our plans for the next year are along two parallel pathways: a) continue our investiagation of pathophysiology of diabetic bladder dysfunction; b) upon successful creation of our $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}$ mice, begin the experiments related to the following specific aims. For all the experimental studies, we will use the following groups of mice:

1. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ treated with OHT to activate $CreER^{T2}$ to abolish MnSOD expression.
2. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ treated with OHT and with STZ to induce diabetes.
3. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$, sham treated.
4. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ treated with STZ.

Specific aim #1: To examine the temporal alterations in the in-vivo bladder function by evaluation of 24 hours micturition habits and conscious cystometry in the above groups of mice at two time points of 8 and 12 weeks after induction of diabetes.

Specific aim #2: To examine the temporal course of morphological changes in neurogenic and myogenic components of the bladder remodeling in in the above groups of mice by:

2.1 Examination the changes of bladder tissue components and their contribution to remodeling of the wall and chamber of the bladder
2.1. Examination of the changes in bladder innervations markers.

We anticipate that the following experiments will be done after 2008:

Specific aim #3: To examine the temporal alterations in the contractile function of the detrusor in the above groups of mice by:

3.1. Examination of the contractile responses of the detrusor.
3.2. Examination of the contractile and regulatory proteins of the detrusor.
3.3. Examining the alterations of the L-type Ca^{2+} channel.
3.4. Examining the alterations in the capacitive calcium entry (CCE).
3.5. Examining the IP3- and RyR-induced calcium release.
3.6. Examining the Ca^{2+} sensitivity in permeabilized detrusor strips.

Specific aim #4: To examine the temporal alterations induced by STZ in afferent and efferent autonomic pathways innervating the bladder in the in the above groups of mice by:

4.1. Assessment of afferent autonomic function by measurement of Current Perception Threshold (CPT)
4.2. Examining the relative contribution of cholinergic and purinergic components to the contractile response to transmural electrical stimulation.
4.3. Examining the alterations in ATP-P2X3, VR-1 afferent pathway in the bladder.
4.4. Examining the alterations in muscarinic receptors (M2, M3) and/or purinergic receptors (P2X1, P2X2).
4.5. Examining the connexin 43-containing gap junctions in the bladder.

Preliminary Milestones for 2009 and Beyond- With moving along our experiments, and availability of the created mice from other sites of AMDCC, we will begin phenotyping of some of the mice created by other groups to examine the presence of diabetic uropathy in those mice. We would particularly be interested in mice models of neuropathy.

2. Collaboration:

With other AMDCC PIs- We obtained the $MnSOD^{lox/lox}$ from the laboratory of Dr. Frank Brusios at the University of Michigan.

With Jax- We held couple of meetings with JAX scientists and personnel during the March 2007 SC meeting in Baltimore and since then have communicated with Dr. Lietter at Jax in regard to our breeding strategy. During the next few weeks, we will be shipping

our healthy $MnSOD^{lox/lox}$ mice and $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ mice to Jackson lab for cross-breeding the two species. The PI and a co-investigator will participate in the Jax 2007 Summer conference on mice models.

With the MMPCs- We responded to the MMPC intternal RFA for development of Bladder Sensory perception Threshold (BST) device in June 2007. The BST device would allow us to assess the bladder afferent sensory function as a phenotyping measure in diabetic mice. We are awaiting the results of the review of the RFA.

With other non-AMDCC PIs- We continue to have active collaboration with internal (Cleveland Clinic) and external investigators in the Cleveland Area. The followings are some of our active collaborators:

1. Margot Damaser, Ph.D.- Lerner Research Institute (LRI) of the Cleveland Clinic- Dept of BME-we have the most extensive collaboration with Dr. Damaser's research team. Our collaboration extents from sharing joint lab space, joint experiments, joint mentoring of trainees; joint weekly lab meetings; and submission of several research proposals.
2. Timothy Kern, Ph.D.- Case- Department of Medicine and Ophthalmology- We have extensive collaboration with Dr. Kern extending from sharing animals for joint experiments to monthly joint lab meetings that are alternatively held at Case or CCF campus.
3. Vincent Monnier, M.D.- Case- Department of Pathology- to study the role of Advanced Glycation Endproduct in Diabetic Bladder Dysfunction.
4. Manju Bhat, Ph.D.- LRI- Center for Anesthesia Research- to study the mechanisms of calcium influx into the neuron and detrusor muscle cells.
5. Fernando Casas, Ph.D.- LRI- BME- to study the integration of vocalization of animal models into the assessment of afferent function of the bladder.
6. Lori Birder, Ph.D. and Anthony Kanai, Ph.D. from Departments of Medicine and Pharmacology of the University of Pittsburgh- Our collaboration stated from studies of role of urothelium and reactive oxidative stress products in mechanisms of diabetic bladder dysfunction and led to our joint project funded by JDRF for 2006-2008.
7. Stanley Hazen, M.D. Ph.D.- LRI- to study the role of oxidative stress in Diabetic Bladder Dysfunction
8. Jianguo Cheng, M.D. Ph.D.- Department of Anesthesia and Pain Management- to study the innervation of the lower urinary tract.

3. <u>Address previous EAC comments:</u>

NOT APPLICABLE THIS YEAR

4. <u>Publications:</u>

1. Liu G, Lin Yi-Hao, Daneshgari F: External Urethral Sphincter Activity in Diabetic Rats **(Submitted- Neurourology and Urodynamics)**.
2. Kim JH, Huang X, Liu G, Moore CK, Bena J, Damaser MS, **Daneshgari F**. Diabetes slows the recovery from urinary incontinence due to simulated childbirth in female rats. Am J Physiol Regul Integr Comp Physiol. 2007 May 9
3. Kefer J, Liu G, **Daneshgari F**: Pubo-Urethral Ligament Injury Causes Stress Urinary Incontinence in Female Rat. Accepted for publication at Journal of Urology- July 2007
4. Lin YH, Liu G, **Daneshgari F:** A mouse model of simulated birth trauma induced stress urinary incontinence. Accepted for publication at Neururology & Urodynamics July 2007
5. Lee U, Baskin L, Schaefer W, Lemack G, Wein A, **Daneshgari F**: Highlights of the urethral dysfunction sessions at the Society of Female Urology and Urodynmics. Bladder Dysfunction 2007 June 71(2): 71-16.
6. Liu G, **Daneshgari F**, Li M, Lin D, Lee U, Li T, Damaser MS: Bladder and urethral function in pelvic organ prolapsed lysyl oxidase like-1 knockout mice. BJU Int. 2007 June 6
7. **Daneshgari, F**., Huang, X., Liu, G., Bena, J., Saffore, L., Powell, C. T.: Temporal differences in bladder dysfunction caused by diabetes, diuresis, and treated diabetes in mice. Am J Physiol Regul Integr Comp Physiol 2006 Jun;290(6):R1728-35. Epub 2006 Jan 26
8. **Daneshgari F**, Liu G, Imrey PB: Time dependent changes in diabetic cystopathy in rats include compensated and decompensated bladder function. Journal of Urology. 2006 Jul;176(1):380-6
   This article won the "Honorable Mention" of the Lapdis Essay Competition Award 2005.
9. **Daneshgari, F**. and Moore, C.: Diabetic uropathy. Semin Nephrol, **26:** 182, 2006
10. Liu, G. and **Daneshgari, F.:** Effects of cold storage on the function and morphology of isolated urinary bladder in rat. Neurourology & Urodynamics, 2006 May 18
11. Liu, G. and **Daneshgari, F**.: Temporal diabetes- and diuresis-induced remodeling of the urinary bladder in the rat. Am J Physiol Regul Integr Comp Physiol, 2006 March 2
12. Moore C and **Daneshgari F**: Urological Complications of Diabetes Mellitus. Seminars in Nephrology, 2006 March 26(2):182-5

Respectfully Submitted,

Firouz Daneshgari, M.D.