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S-Nitrosoglutathione Protects the Spinal Bladder: Novel Therapeutic Approach to Post-Spinal Cord Injury Bladder Remodeling

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Abstract

Aims—Bladder and renal dysfunction are secondary events of the inflammatory processes induced by spinal cord injury (SCI). S-Nitrosoglutathione (GSNO), an endogenous nitrosylating agent is pleiotropic and has anti-inflammatory property. Hence, GSNO ameliorates inflammatory sequelae observed in bladder and renal tissues after SCI. Thus, we postulate that GSNO will improve the recovery of micturition dysfunction by quenching the bladder tissue inflammation associated with SCI.

Methods—Contusion-based mild SCI was induced in female Sprague–Dawley rats. Sham operated rats served as the controls. SCI rats were gavaged daily with GSNO (50 µg/kg) or vehicle. Bladder function was assessed by urodynamics at 2 and 14 days following SCI. Urine protein concentration and osmolality were measured. Bladder and kidney tissues were analyzed by histology and immunofluorescence for a variety of endpoints related to inflammation.

Results—Two days after SCI, urodynamics demonstrated a hyperreflexive bladder with overflow and no clear micturition events. By Day 14, vehicle animals regained a semblance of a voiding cycle but with no definite intercontraction intervals. GSNO-treated SCI-rats showed nearly normal cystometrograms. Vehicle-treated SCI rats had increased bladder wet weight, proteinuria, and urine osmolality at Day 14, which was reversed by GSNO treatment. In addition,

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the SCI-induced increase in immune cell infiltration, collagen deposition, iNOS, and ICAM-1 expression and apoptosis were attenuated by GSNO.

Conclusions—These results indicate that oral administration of GSNO hastens the recovery of bladder function after mild contusion-induced SCI through dampening the inflammation sequelae. These findings also suggest that GSNO-mediated redox modulation may be a novel therapeutic target for the treatment of mild SCI-induced renal and bladder dysfunction.

Keywords

bladder dysfunction; GSNO; ICAM-1; inflammation; iNOS; spinal cord injury

INTRODUCTION

Neurogenic bladder (bladder dysfunction due to injured or diseased neurons) has been observed in 70–84% of more than 200,000 spinal cord injury (SCI) patients in the United States.¹ It is also known as spinal bladder.² Impaired supraspinal control of the bladder and bladder dysfunction are present from days to months after SCI in humans and from 1 to 3 weeks in rats.³ Normal urinary bladder function comprises the storage and subsequent emptying of urine, which is highly coordinated by a variety of neuronal inputs.⁴ For example, micturition is the outcome of controlled detrusor contraction with internal and external urinary sphincter relaxation. During micturition the bladder and external urethral sphincter are regulated by supraspinal neurons from the pontine micturition center.⁵ In addition, the spinal interneurons found in the medial cord from T13 to S1 coordinate afferent and efferent inputs.⁶

SCI between T7 and T11 in rats interrupts all direct supraspinal input⁴ leading to neurogenic bladder dysfunction, abnormal urodynamics,⁴ and a wide range of pathological events.^{1,7} For example, bladder areflexia, hyperreflexia, and detrusor–sphincter dyssynergia are encountered with SCI rostral to the lumbar region.⁸ In addition, altered collagen homeostasis also leads to decreased bladder wall compliance, bladder hypertrophy, and fibrosis.^{9,10} The change in tissue collagen composition and mechanical properties of the bladder eventually leads to the development of hydronephrosis and renal dysfunction.¹¹ Several pharmacological interventions including tricyclic anti-depressants, anti-cholinergic drugs, adrenergic agonists, benzodiazepines, GABA agonists, botulinum toxin, opioids, and vanilloids have been used to treat spinal bladder with limited success.¹ In addition to the direct effects of loss or interruption of neuronal input, SCI also triggers a significant inflammatory response in the bladder.¹² Further, the inflammation is mediated both by the vascular and non-vascular inflammatory components of the immune system¹³ and leads to apoptosis-mediated tissue loss and subsequent organ dysfunction.³ Recently, we have documented caspase-3 mediated apoptosis both in the bladder and kidney of rats following SCI.¹² Logically, dampening this inflammatory damage to the urinary system after SCI will increase the rate of recovery. Thus, targeting the inflammatory response may be an important therapeutic target in the treatment of spinal bladder.

We have previously demonstrated that S-nitrosoglutathione (GSNO) possesses anti-inflammatory property in addition to anti-apoptotic, antioxidant and neuroprotective effects

and thus may be beneficial in numerous CNS pathologies.^{14,15} GSNO is a physiological metabolite of glutathione and nitric oxide (NO), which serves as an NO carrier to regulate the physiological functions of several target proteins through S-nitrosylation.¹⁶ It is well-known that normal bladder function is controlled by the NO signaling pathways.¹⁷ Hence, in the present study, we postulated that the anti-inflammatory and anti-apoptotic properties of GSNO could improve the SCI-induced bladder pathologies in rats.

MATERIALS AND METHODS

Drugs and Chemicals

GSNO was purchased from World Precision Instruments, Inc., (Sarasota, FL). The TUNEL assay kit was obtained from Serological Corp., (Norcross, GA). iNOS, ICAM-1, and β -actin antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA). All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO).

Animal Preparation, SCI, and Drug Treatment

Female Sprague–Dawley rats (225–250 g) purchased from Harlan Laboratories (Durham, NC) were housed with free access to food and water. Experiments were performed in compliance with, and with the permission of the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina. A total of 32 rats were used in the present study. Rats were anesthetized with ketamine and xylazine (80 and 10 mg/kg body wt., respectively) and a T9–T10 laminectomy and contusion-based SCI were performed as previously described¹⁸ with the modification that the contusion depth was set to 1.5 mm. Controls were sham operated. Following injury, the overlying muscle and skin were closed with polysorb-4 and the body temperature was maintained (37°C) using a heating pad until the animals regained consciousness. All animals became paraplegic following recovery from surgery. During the rest of the experiments, the bladder was manually emptied three times a day during the first week following SCI, and twice a day thereafter. Urine protein and osmolality were measured as previously described.¹² Animals were gavaged with GSNO (0.05 mg/kg in H₂O; 1,000 μ l/kg) or vehicle (H₂O; 1,000 μ l/kg) 1 h after SCI and every 24 h thereafter till the end of the experiment. Sham-operated animals also received vehicle. Previous studies from our lab have shown that this dosing regime augmented the recovery of locomotor function in SCI rats¹⁸ and was also neuroprotective in other CNS pathologies.^{19,20} We used 17 rats for cystometry studies (control 7: vehicle 5: GSNO 5). The remaining 15 rats were involved in bladder weight and urine characteristics and histology and immunofluorescence studies (control 5: vehicle 5: GSNO 5).

Preparation for Cystometry

For animals to be analyzed on Day 2, catheter implantation occurred concomitantly with SCI, while for Day 14 rats, catheter implantation occurred on Day 12 post-SCI. Rats were anesthetized with ketamine–xylazine (80 and 10 mg/kg, respectively) cocktail and the bladder dome was exposed through a midline abdominal incision. A catheter (PE50 tubing with a flared end) was inserted and secured in place with a 5–0 nylon purse string suture. The distal end of the tubing was tunneled subcutaneously and externalized through a small incision at the back of the neck and cauterized. The animals were fitted with an infusion

harness (SAI Infusion Technologies, Libertyville, IL) and allowed to recover for 48 h before the cystometry was performed.

Cystometry

Conscious rats were placed unrestrained in a Small Animal Cystometry Lab Station (Catamount Research and Development Company, St. Albans, VT). The distal end of the catheter was connected to a syringe pump with an in-line pressure transducer. The animals were given 30 min to acclimate to the cystometry lab station. Saline was then infused (80 μ l/min) for 90–120 min until at least four cystometrograms (CMGs) were measured. A CMG was defined as the period from which the intravesicular pressure returned to baseline following a previous voiding contraction, through the filling cycle, until just after the next voiding contraction. Non-voiding contractions were defined as contractions generating pressures in excess of 7 cm of H₂O without concomitant urine release. Cystometric parameters were analyzed with CMG Analysis software (version 1.06; Catamount Research and Development Company). Micturition pressure, intercontraction interval and number of voidings, and the volume of urine were determined.

Histology and Immunofluorescence

On Day 14 after SCI, the rats were sacrificed with excess Nembutal and transcardially perfused with phosphate buffered saline followed by 10% formalin. Bladder and kidney tissue were extracted and further fixed for 48 h in 10% formalin and dehydrated through the ascending series of ethanol using a Leica TP-1020 automatic tissue processor. Paraffin-embedded tissue blocks were sectioned (5–7 μ m) with a Leica HM 325 rotary microtome. Staining of sections with hematoxylin and eosin (H&E) and Masson's trichrome stain (MTS), as well as immunofluorescence, were performed as described previously.^{12,21} Bladder sections under 40 $\times$ objective view were used for collagen quantification using Image Pro Plus 5.1 software. Multiple classifications were used to segment and compare values of different color groups. The threshold and measure of percent area of collagen was determined based on the proportion of blue color area (collagen) to all other different color groups (muscle and cytoplasm and background). Five sections from three animals of each group were randomly selected and studied by two observers blinded to the experimental groups. Anti-iNOS and ICAM-1 antibodies were used at 1:100 dilution and the immunofluorescence was detected with Alexafluor-488 conjugated secondary antibody. Immunofluorescence data were quantitated with Image Pro-Plus 5.1 software as we described previously.¹⁹

TUNEL Assay

Paraffin embedded tissue sections of bladder and kidney were analyzed using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay as we described previously (ApopTag fluorescein in situ apoptosis kit; EMD Millipore, Billerica, MA).¹⁹ In this assay, apoptotic DNA strand breaks are detected by labeling the free terminus with modified nucleotides, which are subsequently visualized by light microscopy. Images were captured with an epifluorescence microscope equipped with a digital camera device and analyzed with Image Pro Plus 5.1 software. Three microscopic fields (20 $\times$) of TUNEL-positive cells in three different sections of bladder and kidney were

chosen and imaged. Cell numbers were counted in the images captured from these areas using NIH ImageJ software. The number of positive cells/tubules was calculated as the mean of the number of positive cells in the bladder and renal tubules and the percentage change over the control were calculated.

Statistics

For cystometry, Mann–Whitney test was applied as the comparison involved two groups of non-parametric data. For all other analysis, one way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used. In all the tests, *P* values < 0.05 were considered significant.

RESULTS

GSNO Ameliorates the Negative Urodynamic Effect of SCI

To determine if GSNO treatment would have a favorable therapeutic value on the bladder physiology of SCI rats, we compared the urodynamics of SCI animals treated with this drug for 14 days to uninjured controls and to vehicle-treated SCI rats 2 and 14 days after injury. Figure 1A shows a typical tracing from an untreated control animal. The large peaks in pressure correspond to voids and the maximum pressure in these peaks is recorded as the voiding pressure. A tracing 2 days after SCI in a vehicle-treated animal is provided to demonstrate the immediate effect of SCI on the bladder functioning (Fig. 1B). As shown, there are constantly oscillating pressures and no clear CMG cycles or even micturition events (Fig. 1B). Parameters considered indicative of a hyperreflexive bladder. There was no difference between vehicle and GSNO-treated rats on this day (data not shown). In animals that continue to receive only vehicle for 14 days, some semblance of a voiding cycle has returned, although with irregular micturition (i.e., not on a regular time scale), frequent dribbling (tracings from scale not shown) and CMGs that possessed numerous non-voiding contractions (Fig. 1C). The pattern was variable making data quantification unreliable. In contrast, SCI rats treated for 14 days with GSNO regained remarkable bladder control (Fig. 1D) with normal voiding pressures and reduced number of non-voiding contractions, although the intercontraction intervals were extended when compared to the control group.

Several of the urodynamic parameters in the control and SCI Day 14 GSNO-treated rats, including intercontraction interval, voiding pressure, and voiding volume were quantitated and presented in Table I. As stated above, the SCI Day 14 vehicle-treated group was variable making data quantification difficult. Significant difference was still detected in the intercontraction interval and the number of micturitions per hour between the GSNO treated and control groups.

GSNO Attenuates SCI-Induced Bladder Hypertrophy and Normalizes Urine Characteristics

As shown in Figure 2A and B, rats 14 days post-SCI showed a ninefold increase in the bladder biomass compared to control rats. GSNO significantly attenuated the SCI-induced bladder hypertrophy with the bladder weighting only threefold over control. SCI also increased proteinuria (Fig. 2C) with the vehicle group excreting significantly more protein through the urine compared with the sham-operated control group. In contrast, GSNO

significantly decreased proteinuria in the SCI rats. Urine osmolality also increased following SCI but was significantly decreased by GSNO treatment (Fig. 2D).

GSNO Improves Spinal Bladder and Renal Histomorphology

Bladders from SCI rats are characterized by a very thin and disorganized detrusor muscle layer compared to sham-operated controls (Fig. 3A upper row). GSNO treatment for 14 days improved the detrusor morphology. In the vehicle-treated SCI rats kidney glomerular degeneration was apparent (Fig. 3A lower row: arrow). In addition, tubular disintegration with an exudate accumulation was also apparent. (Fig. 3A lower row: arrowhead). When bladders were stained with MTS, increased collagen deposition was apparent in the lamina propria of vehicle-treated SCI bladder compared with control (Fig. 3B arrows). The collagen deposition level was significantly decreased in the bladder of GSNO-treated SCI rats. Also apparent in Figure 3B is the urothelial hyperplasia that accompanies SCI (arrow heads), which was also attenuated by the GSNO treatment. Relative collagen staining intensity was measured with Image Pro Plus 5.1 software and expressed as percentage of the section that stains positive for collagen. As shown in Figure 3C, the vehicle group had significantly higher levels of collagen in the lamina propria compared to sham operated controls. GSNO treatment significantly decreased the level of collagen when compared with the vehicle group of rats (Fig. 3C).

GSNO Reduces Apoptosis in Spinal Bladder and Kidney

It is known that SCI increases apoptosis in the bladder and kidney of rats.¹² Likewise, in the present study, we have also detected an increase in the apoptotic cell death both in the lamina propria of the bladder as well as the renal tubules of rats (Fig. 4A). The percent increase of TUNEL positive cells over sham-operated controls was $60.67 \pm 15.49\%$ in bladder (Fig. 4B) and $20.00 \pm 5.79\%$ in kidney (Fig. 4C). GSNO treatment significantly attenuated the cell death seen in both the tissues following SCI.

GSNO Quenches iNOS and ICAM-1 Expression in the Spinal Bladder and Kidney

ICAM-1 and iNOS are important mediators of inflammation known to be elevated in the bladder and kidney during inflammatory diseases.^{22,23} In the present study, we have shown that SCI induces the expression of iNOS and ICAM-1 in the bladder and kidney tissues of rats. Representative immunofluorescence images revealing the expression of iNOS and ICAM-1 proteins in control, SCI, and GSNO treated SCI rats are given in Figure 5A. Figure 5B and C represents the mean number of iNOS and ICAM-1 positive cells, respectively, in the bladder and kidney of control SCI and GSNO treated SCI rats. As shown, significantly increased number of iNOS positive cells and tubules were present in the lamina propria of the bladder as well as the renal tubules of the kidney. GSNO treatment significantly decreased the number of iNOS positive cells in the bladder and renal tubules of SCI rats. A similar trend was seen with ICAM-1 positive cells in the bladder and kidney. The vehicle-treated group showed significantly higher number of ICAM-1 positive cells both in the lamina propria of the bladder as well as the renal tubules of the kidney, while GSNO significantly reduced the number of cells expressing ICAM-1. The results clearly show that GSNO inhibits secondary inflammation through the inhibition of iNOS and ICAM-1 expression.

DISCUSSION

Traumatic SCI is well known to negatively affect the bladder function resulting in a condition known as spinal bladder.²⁴ Recent studies from our laboratory support the therapeutic potential of GSNO in several CNS pathologies including SCI.^{14–16,18,20,21,25,26} The present study was designed to evaluate the therapeutic potential of GSNO on spinal bladder. Contusion based traumatic SCI was inflicted to rats at T9–T10 level and the bladder functional recovery was studied using conscious cystometry. Post-mortem tissue analysis was done to explore the molecular mechanisms involved in the process. Data from the present study show that GSNO augments the micturition functional recovery in rats after SCI through its anti-inflammatory property.

SCI-induced bladder dysfunction is a complex response involving tissue remodeling and inflammation³ that results in bladder hypertrophy⁹ and fibrosis.¹⁰ Increase in bladder mass is dependent on the nature and accumulation of connective tissue.²⁷ Hypertrophied bladder exerts decreased pressure per smooth muscle cell.²⁸ In addition, the increased collagen deposition in the bladder also impairs the contractile property of the bladder.²⁹ In the present study, the cystometrogram obtained from rats on Day 2 and 14 post-SCI did not show regular voiding contractions. Increased collagen deposition following SCI is known to induce bladder hypertrophy.³⁰ We have also observed a significantly increased collagen content in the hypertrophying spinal bladder. On the other hand, GSNO treatment significantly decreased the bladder collagen level. Hence, it may be presumed that the GSNO-induced decrease in collagen level may be responsible for the augmented contractile functional recovery of the bladder. However, the collagen content of the GSNO bladder was still higher than that of the control group. Hence, a temporal study revealing the time required for a complete reversal to normal collagen level and the molecular mechanisms involved in the GSNO-induced inhibition of collagen synthesis and accumulation remains to be performed. In the present study we have also shown SCI to induce urothelial hyperplasia (Fig. 3A). The present observation is also in corroboration with the earlier report.³¹ Urothelium plays several important roles including passive barrier to antigen presentation and modulating the bladder afferent output.³² Urothelial hyperplasia observed in the spinal bladder was decreased in the bladder of SCI rats treated with GSNO (Fig. 3B). Hence, it can be presumed that the GSNO-mediated functional recovery of the bladder may also be due to the decrease in urothelial hyperplasia.

The urinary bladder stores urine in a low-pressure system. Therefore, the one-way valve mechanism of the ureter, which prevents the retrograde reflux of urine into the kidney, will be in effect as long as the oblique course of the ureter to the bladder wall is maintained. This orientation of ureter to the bladder is altered in spinal bladder due to bladder hypertrophy, increased vesicular pressure, and detrusor dysfunction. This results in an increase in the intravesicular pressure to the point that the ureteral valves cannot prevent the retrograde reflux of urine into kidneys resulting in hydronephrosis.¹¹ Hydronephrosis causes glomerular atrophy as has been presented in Figure 3A. Further, we have already shown an increase in the retention volume of urine in SCI rats.¹² Increased urinary retention, through the enhanced renal sympathetic activity, results in an increased vasoconstriction.³³ GSNO is

known for its vasodilatory effect and hence will help negate the renal vasoconstriction resulting from bladder dysfunction.³⁴

SCI significantly increase the cellular apoptosis in the bladder and kidney of rats.¹² Inhibiting cell death in the bladder of SCI rats has also been shown to recover bladder function at a much faster rate.³⁵ GSNO modulates cellular apoptosis through modulating the S-nitrosylation/denitrosylation redox switch.³⁶ A recent report from our lab also confirms the anti-apoptotic property of GSNO in the spinal cord of rat animal model of spinal stenosis.³⁷ The present observation supports the anti-apoptotic property of GSNO also in the bladder and renal tissues of SCI rats.

Systemic inflammatory response syndrome (SIRS), a series of events initiated by SCI, is characterized by the infiltration of inflammatory cells into peripheral organs such as bladder and kidney. In response to the proinflammatory stimuli, the immune cells interact with endothelial cells through ICAM-1 altering the vascular permeability and thereby increasing the extravasation of immune cells.³⁸ This infiltration and the ensuing inflammation damage the tissues and lead to possible organ failure.³⁹ GSNO has been reported to decrease the SIRS through the modulation of survival and development of T lymphocytes⁴⁰ and expression of ICAM-1 during the inflammatory process.⁴¹ We have shown GSNO to ameliorate CNS pathology also through decreasing the expression of cell adhesion molecules.¹⁴

During normal bladder function NO transduces the mechanical bladder stretch into afferent potentials positively modulating the micturition response.⁴² However, under pathological conditions such as inflammation, NO exerts the opposite effect and negatively modulates micturition.^{43,44} Although, the mechanism for this switch is unknown, SCI represents a model of negative modulation of micturition. Interestingly, in a bladder function study following SCI, a selective iNOS inhibitor (ONO-1714) was found to significantly improve bladder function,²² suggesting that iNOS is capable of modulating urinary dysfunction after SCI. In the present study we have shown that GSNO attenuates the dramatic increase in iNOS expression following SCI in rats. This result suggests the intriguing hypothesis that GSNO may be uroprotective during SCI in part by preventing the increase in iNOS, thus relieving the inhibitory effect NO on the micturition response. However, this is unlikely to be the sole effect of GSNO on the recovery of bladder function from SCI. For example, NF- κ B is well-established to mediate many pro-inflammatory processes in the bladder and we have previously shown GSNO to down regulate the SCI-induced overexpression of NF- κ B in other tissues.²¹ GSNO is also an endogenous nitrosylating agent and, S-nitrosylation is an important post-translational modification modulating the functional status of many proteins of physiological importance⁴⁵ that may be involved in normal micturition process.

CONCLUSIONS

In conclusion, the results of cystometry, histology, immunofluorescence, and TUNEL assay have revealed that GSNO has a therapeutic potential in combating the inflammation-associated bladder and renal degeneration following SCI and suggest that the GSNO-

mediated redox modulation could be a novel therapeutic target to treat bladder dysfunction after mild SCI.

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References

1. Dorsher PT, McIntosh PM. Neurogenic bladder. *Adv Urol.* 2012; 2012:816274. [PubMed: 22400020]
2. Manack A, Motsko SP, Haag-Molkenteller C, et al. Epidemiology and healthcare utilization of neurogenic bladder patients in a US claims database. *Neurourol Urodyn.* 2011; 30:395–401. [PubMed: 20882676]
3. Wognum S, Lagoa CE, Nagatomi J, et al. An exploratory pathways analysis of temporal changes induced by spinal cord injury in the rat bladder wall: Insights on remodeling and inflammation. *PLoS ONE.* 2009; 4:e5852. [PubMed: 19513121]
4. Horst M, Heutschi J, den Brand R, et al. Multi-system neuroprosthetic training improves bladder function after severe spinal cord injury. *J Urol.* 2012;747–53. [PubMed: 22982431]
5. Kruse MN, Noto H, Roppolo JR, et al. Pontine control of the urinary bladder and external urethral sphincter in the rat. *Brain Res.* 1990; 532:182–90. [PubMed: 2282512]
6. Marson L. Identification of central nervous system neurons that innervate the bladder body, bladder base, or external urethral sphincter of female rats: A transneuronal tracing study using pseudorabies virus. *J Comp Neurol.* 1997; 389:584–602. [PubMed: 9421141]
7. Cameron AP, Rodriguez GM, Schomer KG. Systematic review of urological followup after spinal cord injury. *J Urol.* 2012; 187:391–7. [PubMed: 22177149]
8. Watanabe T, Rivas DA, Chancellor MB. Urodynamics of spinal cord injury. *Urol Clin North Am.* 1996; 23:459–73. [PubMed: 8701559]
9. Mimata H, Satoh F, Tanigawa T, et al. Changes of rat urinary bladder during acute phase of spinal cord injury. *Urol Int.* 1993; 51:89–93. [PubMed: 8351761]
10. Deveaud CM, Macarak EJ, Kucich U, et al. Molecular analysis of collagens in bladder fibrosis. *J Urol.* 1998; 160:1518–27. [PubMed: 9751406]
11. Staskin DR. Hydroureteronephrosis after spinal cord injury. Effects of lower urinary tract dysfunction on upper tract anatomy. *Urol Clin North Am.* 1991; 18:309–16. [PubMed: 2017812]
12. Shunmugavel A, Khan M, Te Chou PC, et al. Simvastatin protects bladder and renal functions following spinal cord injury in rats. *J Inflamm (Lond).* 2010; 7:17. [PubMed: 20403180]
13. Geppetti P, Nassini R, Materazzi S, et al. The concept of neurogenic inflammation. *BJU Int.* 2008; 101:2–6. [PubMed: 18307678]
14. Khan M, Sakakima H, Dhammu TS, et al. S-Nitrosoglutathione reduces oxidative injury and promotes mechanisms of neurorepair following traumatic brain injury in rats. *J Neuroinflammation.* 2011; 8:78. [PubMed: 21733162]
15. Khan M, Dhammu TS, Sakakima H, et al. The inhibitory effect of S-nitrosoglutathione on blood–brain barrier disruption and peroxynitrite formation in a rat model of experimental stroke. *J Neurochem.* 2012; 123:86–97. [PubMed: 23050646]
16. Nath N, Morinaga O, Singh I. S-Nitrosoglutathione a physiologic nitric oxide carrier attenuates experimental autoimmune encephalomyelitis. *J Neuro-immune Pharmacol.* 2010; 5:240–51.

17. Ozawa H, Chancellor MB, Jung SY, et al. Effect of intravesical nitric oxide therapy on cyclophosphamide-induced cystitis. *J Urol*. 1999; 162:2211–6. [PubMed: 10569621]
18. Chou PC, Shunmugavel A, El Sayed H, et al. Preclinical use of longitudinal MRI for screening the efficacy of S-nitrosoglutathione in treating spinal cord injury. *J Magn Reson Imaging*. 2011; 33:1301–11. [PubMed: 21590998]
19. Shunmugavel A, Martin MM, Khan M, et al. Simvastatin ameliorates cauda equina compression injury in a rat model of lumbar spinal stenosis. *J Neuroimmune Pharmacol*. 2012;274–86. [PubMed: 23188522]
20. Khan M, Im YB, Shunmugavel A, et al. Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. *J Neuro-inflammation*. 2009; 6:32.
21. Shunmugavel A, Khan M, Chou PC, et al. Spinal cord injury induced arrest in estrous cycle of rats is ameliorated by S-nitrosoglutathione: Novel therapeutic agent to treat amenorrhea. *J Sex Med*. 2012; 9:148–58. [PubMed: 22024253]
22. Yu Y, Matsuyama Y, Nakashima S, et al. Effects of MPSS and a potent iNOS inhibitor on traumatic spinal cord injury. *Neuroreport*. 2004; 15:2103–7. [PubMed: 15486490]
23. Green M, Filippou A, Sant G, et al. Expression of intercellular adhesion molecules in the bladder of patients with interstitial cystitis. *Urology*. 2004; 63:688–93. [PubMed: 15072882]
24. David BT, Steward O. Deficits in bladder function following spinal cord injury vary depending on the level of the injury. *Exp Neurol*. 2010; 226:128–35. [PubMed: 20713043]
25. Sakakima H, Khan M, Dhammu TS, et al. Stimulation of functional recovery via the mechanisms of neurorepair by S-nitrosoglutathione and motor exercise in a rat model of transient cerebral ischemia and reperfusion. *Restor Neurol Neurosci*. 2012; 30:383–96. [PubMed: 22717646]
26. Pannu R, Singh I. Pharmacological strategies for the regulation of inducible nitric oxide synthase: Neurodegenerative versus neuroprotective mechanisms. *Neurochem Int*. 2006; 49:170–82. [PubMed: 16765486]
27. Brent L, Stephens FD. The response of smooth muscle cells in the rabbit urinary bladder to outflow obstruction. *Invest Urol*. 1975; 12:494–502. [PubMed: 1120643]
28. Mattiasson A, Uvelius B. Changes in contractile properties in hypertrophic rat urinary bladder. *J Urol*. 1982; 128:1340–2. [PubMed: 7154206]
29. Lee HJ, Won JH, Doo SH, et al. Inhibition of collagen deposit in obstructed rat bladder outlet by transplantation of superparamagnetic iron oxide-labeled human mesenchymal stem cells as monitored by molecular magnetic resonance imaging (MRI). *Cell Transplant*. 2012; 21:959–70. [PubMed: 22449414]
30. Heise RL, Parekh A, Joyce EM, et al. Strain history and TGF-beta1 induce urinary bladder wall smooth muscle remodeling and elastogenesis. *Biomech Model Mechanobiol*. 2012; 11:131–45. [PubMed: 21384200]
31. Hess MJ, Hess PE, Sullivan MR, et al. Evaluation of cranberry tablets for the prevention of urinary tract infections in spinal cord injured patients with neurogenic bladder. *Spinal Cord*. 2008; 46:622–6. [PubMed: 18392039]
32. Moore CK, Goldman HB. The bladder epithelium and overactive bladder: What we know. *Curr Urol Rep*. 2006; 7:447–9. [PubMed: 17052439]
33. Chen SS, Chen WC, Hayakawa S, et al. Acute urinary bladder distension triggers ICAM-1-mediated renal oxidative injury via the norepinephrine–renin–angiotensin II system in rats. *J Formos Med Assoc*. 2009; 108:627–35. [PubMed: 19666350]
34. Steinbicker AU, Liu H, Jiramongkolchai K, et al. Nitric oxide regulates pulmonary vascular smooth muscle cell expression of the inducible cAMP early repressor gene. *Nitric Oxide*. 2011; 25:294–302. [PubMed: 21642009]
35. Hamarat M, Yenilmez A, Erkasap N, et al. Protective effects of leptin on ischemia/reperfusion injury in rat bladder. *Chin J Physiol*. 2010; 53:145–50. [PubMed: 21793322]
36. Duan S, Chen C. S-Nitrosylation/denitrosylation and apoptosis of immune cells. *Cell Mol Immunol*. 2007; 4:353–8. [PubMed: 17976315]

37. Shunmugavel A, Khan M, Martin MM, et al. S-Nitrosoglutathione administration ameliorates cauda equina compression injury in rats. *Neuroscience & medicine*. 2012; 3:294–305. [PubMed: 23997981]
38. Chikawa T, Ikata T, Katoh S, et al. Preventive effects of lecithinized superoxide dismutase and methylprednisolone on spinal cord injury in rats: Transcriptional regulation of inflammatory and neurotrophic genes. *J Neurotrauma*. 2001; 18:93–103. [PubMed: 11200253]
39. Bao F, Brown A, Dekaban GA, et al. CD11d integrin blockade reduces the systemic inflammatory response syndrome after spinal cord injury. *Exp Neurol*. 2011; 231:272–83. [PubMed: 21784069]
40. Duan S, Wan L, Fu WJ, et al. Nonlinear cooperation of p53-ING1-induced bax expression and protein S-nitrosylation in GSNO-induced thymocyte apoptosis: A quantitative approach with cross-platform validation. *Apoptosis*. 2009; 14:236–45. [PubMed: 19082896]
41. Li Q, Sarna SK. Nitric oxide modifies chromatin to suppress ICAM-1 expression during colonic inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2012; 303:G103–10. [PubMed: 22517771]
42. Burnett AL, Calvin DC, Chamness SL, et al. Urinary bladder-urethral sphincter dysfunction in mice with targeted disruption of neuronal nitric oxide synthase models idiopathic voiding disorders in humans. *Nat Med*. 1997; 3:571–4. [PubMed: 9142130]
43. Sasatomi K, Hiragata S, Miyazato M, et al. Nitric oxide-mediated suppression of detrusor overactivity by arginase inhibitor in rats with chronic spinal cord injury. *Urology*. 2008; 72:696–700. [PubMed: 18358516]
44. Caremel R, Oger-Roussel S, Behr-Roussel D, et al. Nitric oxide/cyclic guanosine monophosphate signalling mediates an inhibitory action on sensory pathways of the micturition reflex in the rat. *Eur Urol*. 2010; 58:616–25. [PubMed: 20708839]
45. Murray CI, Uhrigshardt H, O'Meally RN, et al. Identification and quantification of S-nitrosylation by cysteine reactive tandem mass tag switch assay. *Mol Cell Proteomics*. 2012; 11:M111013441.

Fig. 1.

Effect of GSNO on cystometrograms of SCI rats. **A:** Control rats voided at regular intervals and did not display any non-voiding contractions. **B:** Detrusor areflexia in SCI rats on Day 0 (2 days after SCI) show numerous non-voiding contractions with decreased intercontraction interval. Cysto-metrograms of SCI + GSNO rats on Day 2 after SCI were not significantly different from the SCI rats (data not shown). **C:** SCI rats spontaneously regained micturition control on post-SCI Day 14; however, they also displayed a moderate number of non-voiding contractions. **D:** GSNO treated SCI rats on post-SCI Day 14 show contraction frequency and amplitude characteristic of control animals. However, few non-voiding contractions were also seen (n: control 7; SCI 5; SCI + GSNO 5).

Fig. 2.

Effect of GSNO on bladder biomass and urine characteristics of SCI rats. SCI rats were divided into two groups. One group received 50 µg/kg body weight GSNO and the other an equal volume of vehicle. Urine was collected using metabolic cages. After 2 weeks, the rats were sacrificed and the bladder was extracted and weighed. **A:** Representative photomicrograph of bladders of control, vehicle, and GSNO treated SCI rats. **B:** Bladder biomass of SCI rats. Vehicle group had significantly higher bladder biomass compared with other groups. GSNO treatment significantly decreased the increase in SCI-induced bladder biomass. *** $P < 0.001$ versus control, ### $P < 0.001$ versus vehicle. **C:** Protein excreted through urine in SCI rats. Significant proteinuria was recorded with vehicle group. There was no significant difference in proteinuria of control and GSNO group. ** $P < 0.01$ versus control and GSNO. **D:** Urine osmolality of SCI rats. Vehicle rats excreted highly concentrated urine compared with other groups. Data are expressed as mean $\pm$ SD for $n = 5$ in each group. ** $P > 0.01$ versus control; * $P < 0.05$ versus control, # $P < 0.05$ versus vehicle.

Fig. 3.

Effect of GSNO on bladder and renal histomorphology of SCI rats. **A:** Representative photomicrographs showing H&E staining of bladder (A: top row) and kidney (A: bottom row) of control and SCI rats. Bladder wall muscular degeneration was seen in vehicle group. Kidney from the vehicle group showed degenerating renal tubules and glomerulus. GSNO treatment decreased the tubular and glomerular degeneration. **B:** Masson's trichrome staining (MTS) of bladder showing collagen (in blue) deposition. Urothelial hyperplasia and deformation (arrow head) was seen in the vehicle group in addition to excess deposition of collagen (arrows). GSNO treatment decreased the urothelial hyperplasia and disintegration and also the collagen deposition in spinal bladder. Photomicrographs are representative of $n = 5$ in each group (H&E bladder: magnification 200 $\times$; H&E kidney: magnification 400 $\times$; MTS: magnification 400 $\times$). **C:** Percentage area of the bladder section occupied with collagen. Vehicle group had significantly higher collagen deposition in the bladder wall. GSNO group had collagen content significantly lesser than vehicle group but equal to control group. Relative area of the section occupied by the collagen was determined using Image Pro Plus 5.1 software. Five sections from three rats from each group were used for collagen quantification. *** $P < 0.001$ versus control, ++ $P < 0.005$ versus GSNO, @ $P < 0.05$ versus control quantification. *** $P < 0.001$ versus control, ++ $P < 0.005$ versus GSNO, @ $P < 0.05$ versus control.

Fig. 4.

Effect of GSNO on apoptosis in bladder and kidney. **A:** TUNEL positive cells are stained and counted in the bladder and kidney sections of control, vehicle, and GSNO treated SCI rats. Vehicle group had significantly higher number of TUNEL positive cells both in the lamina propria of bladder and renal tubules of kidney. GSNO treatment significantly decreased the TUNEL positive cells both in the SCI bladder and the kidney. **B:** Histogram representing the percentage of TUNEL positive cells in the bladder of different experimental groups. Vehicle group showed significantly higher percentage of TUNEL positive cells when compared to control and GSNO group. **C:** Percentage change in the number of TUNEL positive renal tubules over control. TUNEL positive cells were counted in 10 different regions of the sections from $n = 5$ in each group (bladder: magnification 200 $\times$; H&E kidney: magnification 400 $\times$). *** $P < 0.001$ versus vehicle and GSNO.

Fig. 5.

Effect of GSNO on iNOS and ICAM-1 expression. **A:** Representative immunofluorescence image of bladder and kidney of experimental animals showing iNOS and ICAM-1 expression pattern. Vehicle-group had increased expression of both iNOS and ICAM-1 in both the bladder and the kidney (magnification: 200 $\times$). **B:** Histogram showing the number of iNOS and ICAM-1 positive cells in bladder of experimental groups. Cells were counted in 10 different regions of the sections from $n = 5$ in each group. **C:** Histogram showing the number of iNOS and ICAM-1 positive cells in the kidney of experimental animals. Cells were counted in 10 different regions of the sections from $n = 5$ in each group. *** $P < 0.001$ over control and GSNO groups. ### $P < 0.001$ versus control.

TABLE I

Micturition Behavior in Control, SCI, and SCI + GSNO Rats

	Intercontraction interval (sec)	Voiding pressure (cmH ₂ O)	Number of micturitions/h	Voiding volume (μl)
Control	693.4 ± 32.2	62.3 ± 4.2	5.8 ± 0.8	864 ± 26
SCI-14 days	NA	NA	NA	NA
SCI + GSNO-14 days	810.0 ± 151.5*	69.1 ± 7.3	4.4 ± 0.6*	956 ± 38*

Control rats showed normal micturition parameters. Micturition parameters could not be measured with SCI rats, which showed numerous non-voiding contractions, making the measurements difficult. SCI + GSNO rats regained significant micturition control over SCI rats. However, the intercontraction interval was significantly higher than the control. Also the SCI + GSNO rats had significantly less micturitions/h than the control rats. Cystometry data obtained with seven control, five SCI, and five SCI + GSNO rats were analyzed using CMG analysis software. Statistical analysis was done using graph pad prism software. Data are represented as Mean ± SD.

* $P < 0.05$. NA, not applicable.