Published in final edited form as:

J Sex Med. 2017 February ; 14(2): 187–195. doi:10.1016/j.jsxm.2016.12.012.

M1 Macrophages Are Predominantly Recruited to the Major Pelvic Ganglion of the Rat Following Cavernous Nerve Injury

Hotaka Matsui, MD^{1,2,3,*}, Nikolai A. Sopko, MD, PhD^{1,*}, Johanna L. Hannan, PhD^{1,4}, Allison A. Reinhardt, BS⁵, Max Kates, MD¹, Takahiro Yoshida, MD, PhD¹, Xiaopu Liu, MA¹, Fabio Castiglione, MD, PhD⁶, Petter Hedlund, PhD⁷, Emmanuel Weyne, MD, PhD⁶, Maarten Albersen, MD, PhD⁶, and Trinity J. Bivalacqua, MD, PhD¹

¹The James Buchanan Brady Urological Institute and Department of Urology, The Johns Hopkins School of Medicine, Baltimore, Maryland, USA

²Department of Urology, The University of Tokyo, Tokyo, Japan

³Department of Urology, Doai Memorial Hospital, Tokyo, Japan

⁴Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, North Carolina, USA

⁵Departments of Chemistry, Ripon College, Ripon, Wisconsin, USA

⁶Laboratory for Experimental Urology, Department of Development and Regeneration, University of Leuven, Leuven, Belgium

⁷Department of Clinical and Experimental Pharmacology, Lund University, Sweden

Abstract

Introduction—Neurogenic erectile dysfunction (ED) is a common sequela of radical prostatectomy. The etiology involves injury to the autonomic cavernous nerves (CNs), which arise from the major pelvic ganglion (MPG), and subsequent neuroinflammation, which leads to recruitment of macrophages to the injury site. Currently, two macrophage phenotypes are known: neurotoxic M1 macrophages and neuroprotective M2 macrophages.

Aim—To examine whether bilateral cavernous nerve injury (BCNI) in a rat model of ED would increase recruitment of neurotoxic M1 macrophages to the MPG.

Methods—Male Sprague Dawley rats underwent bilateral cavernous nerve injury (BCNI) and the major pelvic ganglion (MPG) was harvested at various time points post-nerve injury. The corpora cavernosa was used to evaluate tissue myography responses to electrical field stimulation *ex vivo*.

Corresponding Author: Trinity J. Bivalacqua, MD, PhD, The James Buchanan Brady Urological Institute, 600 N. Wolfe Street/ Marburg 409, Baltimore, MD, 21287, USA. Tel: (410)955-0352; Fax: (410)614-0197; tbivala1@jhmi.edu.

*Authors contributed equally to this work

Conflict of Interest: None

Disclosure: The authors have no conflict of interest to this content.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

qPCR was used to examine the gene expression of global macrophage markers, M1 macrophage markers, M2 macrophage markers and cytokines/chemokines in the MPG. Mathematical calculation of M1/M2 index was used to quantify macrophage changes temporally. Western blot of MPG tissues was used to evaluate the protein amount of M1 and M2 macrophages markers quantitatively. Immunohistochemistry staining of MPGs for CD68, CD86 and CD206 was used to characterize M1 and M2 macrophage infiltration.

Main Outcome Measures—Corpora cavernosa responsiveness ex vivo; gene (pPCR) and protein (Western Blot) expression of M1 and M2 markers, cytokines/chemokines; immunohistochemical localization of M1 and M2 macrophages.

Results—BCNI impaired corporal parasympathetic-mediated relaxation response to EFS and enhanced contraction response to EFS. Gene expression of pro-inflammatory (*Il1b*, *Il16*, *Tnfa*, *Tgfb*, *Ccl2*, *Ccr2*) and anti-inflammatory (*Il10*) cytokines was upregulated in the MPG 48 hours following injury. M1 markers (CD86, iNOS, IL1b) and M2 markers (CD206, ARG1, IL-10) was increased following BCNI in the MPG with the M1/M2 index above 1.0 indicating that more M1 macrophages are recruited to the MPG than M2 macrophages. Protein expression of the M1 macrophage markers (iNOS) was increased in MPGs following BCNI. However, the protein amount of M2 macrophage markers (Arginase-1) remained unchanged. Immunohistochemical characterization demonstrated predominant increases in M1 (CD68+CD86+) macrophages in the MPG following BCNI.

Conclusion—These results suggest that an increase in M1 macrophage infiltration of the MPG following BCNI is associated with impaired neurogenic mediated erectile tissue physiology ex vivo and thus has significant implications for CN axonal repair. Future studies are needed to demonstrate that inhibition of M1 macrophage recruitment prevents ED following CN-injury.

Keywords

Peripheral nerve injury; neuroinflammation; macrophage markers; neuroprotective; major pelvic ganglion; erectile dysfunction

Introduction

The mechanisms of peripheral nerve injury (PNI) induced axonal loss and denervation of target organs are well described [1]. Schwann cells are activated in response to PNI [2]. Inflammatory cytokines and chemokines are secreted by Schwann cells in the process of cleaning myelin or debris of dead neurons. Macrophages are then recruited to induce local inflammation [3]. Two types of macrophages are currently known in the setting of nerve injury: M1 macrophages and M2 macrophages. M1 macrophages, also called classically activated macrophages, are demonstrated to play a detrimental role in nerve regeneration [4]. M2 macrophages, or alternatively activated macrophages, have neuroprotective effects following nerve injury [5].

Neurogenic erectile dysfunction (ED) is a common sequela following radical prostatectomy (RP) for the treatment of clinically localized prostate cancer [6,7]. The etiology of neurogenic ED following RP is damage to the neurovascular bundle or autonomic cavernous nerves (CN) arising from the pelvic ganglia, which innervate the end organ penis [7,8]. It is

now known that BCNI leads to axonal damage, reduced density of nitrergic nerves innervating the corpora cavernosa of the penis with subsequent smooth muscle cell loss and fibrosis [9,10]. Most studies have investigated end organ changes of the penis following BCNI however, the true etiologic mechanisms of BCNI induced axonal loss is lacking at this time.

In the major pelvic ganglion (MPG), a number of pro-inflammatory cytokines and chemokines are increased as early as 24 to 48 hours after bilateral cavernous nerve injury (BCNI) in rodents [11,12]. This increase in pro-inflammatory cytokines and chemokines can alter macrophage phenotypes toward neurotoxic M1 macrophages [13]. Based on these findings, we hypothesized that an increase in pro-inflammatory cytokines and chemokines following BCNI would enhance recruitment of neurotoxic M1 macrophages to the lesion and potentially contribute to the pathogenesis of CN injury induced-ED. To test these hypotheses, we evaluated the effect of BCNI on the terminal organ (i.e. penis) by ex vivo myography, temporal transcriptional changes of key cytokines and chemokines as well as characterizing macrophage phenotype involved in neuroinflammation in the MPG by quantitative real-time polymerase chain reaction (qPCR), and macrophage polarization in the MPG by immunohistochemistry and Western blot.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA, USA; aged 8 weeks, weighing 300–350 g) were utilized in this study. Rats were randomly separated into 4 groups (n=10/group): (i) Sham; (ii) BCNI 48 hours (BCNI-48h); (iii) BCNI 7 days (BCNI-7d); (iv) BCNI 14 days (BCNI-14d). The MPGs and penises of the Sham, BCNI-48h, BCNI-7d and BCNI-14d were harvested after surgery as previously described [14,15]. All the experiments were conducted in accordance with the XXXXX Guidelines for Animal Care and Use, and the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Bilateral Cavernous Nerve Injury Model

Under 3% isoflurane anesthesia, the prostate was exposed by midline laparotomy. The right CN was identified posterolateral to the prostate [14]. To induce BCNI, both CNs (2–3 mm distal to MPG) were crushed with Dumont #5 forceps. The forceps were closed completely 3 times for 15 seconds each [15]. The same surgeon performed all the BCNI surgeries. In sham surgery, abdomens were closed after identifying the MPGs and CNs without crushing.

Myography Experiments

Contractile response to KCl, electrical field stimulation (EFS), Phenylephrine, and parasympathetic-mediated relaxation of the penis were assessed in a muscle strip myograph (Danish Myograph Technology, 820M, Aarhus, Denmark). The penises were carefully removed (length: 10 mm) and placed in a physiological Krebs solution (130 mM NaCl, 4.7 mM KCl, 1.56 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 14.9 mM NaHCO₃, and 5.6 mM dextrose) [15–17]. Under a dissecting microscope, the urethras including the corpus spongiosum, the dorsal nerves, the dorsal arteries, the dorsal veins, and the glans

were carefully removed from the penises. Excised penises were cut in half along the urethral bed once the urethra and all supporting structures are removed with microscissors [17]. The penile sections were mounted in a muscle-strip myograph and bathed in 5 mL Krebs solution, maintained at 37°C, and bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The penile sections were left untensioned for 30 minutes and stretched to an optimal resting tension of 4 mN for 60 minutes. The penile sections were exposed to KCl 120mM (14.4 mM NaCl, 120 mM KCl, 1.56 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 14.9 mM NaHCO₃, and 5.6 mM dextrose) to evaluate the contractile responses. Once the contraction reached the plateau, the sections were washed with Krebs solution every 15 minutes. Contractile response to EFS was measured with frequencies of 4.0, 8.0, 16.0 and 32 Hz (for 10 seconds at 40 V, 2mS, Grass S88 Stimulator, Grass Medical Instruments, Quincy, MA, USA). Following pre-contraction by phenylephrine (PE; 3.0×10^{-5} M, Sigma Aldrich, St. Louis, MO), relaxations of penile sections to EFS were recorded with frequencies of 2.0, 4.0, 8.0, 16.0, and 32.0 Hz (for 10 seconds, at 40 V, 2 mS) to evaluate parasympathetic-mediated relaxation [17]. The force generation was recorded with AD Instruments PowerLab 8/30 acquisition hardware and LabChart 7 software (ADI Instruments, Colorado Springs, CO, USA). Penile sections were weighed after myograph experiments.

Quantitative Polymerase Chain Reaction

We evaluated the transcriptional changes in the MPGs by real-time quantitative polymerase chain reaction (qPCR). Total RNA was extracted and purified from homogenized MPGs using RNeasy System (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA was reverse transcribed with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Pittsburgh, PA, USA). TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) were used to evaluate the gene expression of CD11b (Rn00709342_m1), CD68 (Rn01495634_m1), CCL2 (Rn00580555_m1), CD86 (Rn00571654_m1), inducible nitric oxide synthase (iNOS: Rn00561646_m1), IL-6 (Rn01410330_m1), IL-1 β (Rn00580432_m1), CD206 (Rn01487342_m1), IL-10 (Rn00563409_m1), and Arginase-1 (ARG1: Rn00691090_m1) and normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT; Rn01527840_m1). All experiments were performed on 5 different whole MPGs from each group with triplicate technical replicate PCR reactions per sample [11]. M1/M2 index was used to evaluate the ratio of gene expression of M1 macrophage markers and M2 macrophage markers. M1/M2 index is defined as follows [18,19]:

$$\text{M1/M2 index} = \frac{\sum (\text{M1 marker fold change/HPRT})}{\sum (\text{M2 marker fold change/HPRT})}$$

Immunohistochemistry

MPGs were fixed in 4% paraformaldehyde for 24 hours and in 70% ethanol before embedded in paraffin. Sections (4 μ m-thick) underwent deparaffinization and antigen retrieval using the Trilogy™ method (Cell Marque, USA). Endogenous peroxidase and alkaline phosphatase activity was quenched. (Dako, Carpinteria, CA, USA). Slides were then incubated for 45 minutes at room temperature with antibodies against CD68 (Serotec,

Kidlington, UK, MCA341R, 1:250) and CD86 (Bioss, Woburn, MA, bs-1035R, 1:100) or CD206 (Abcam, Cambridge, MA ab64693, 1:10,000). Slides were then incubated with anti-mouse and anti-rabbit antibodies conjugated to horse radish peroxidase (Leica, New Castle, UK) followed by incubation with 3,3-diaminobenzidine substrate (DAB, Vector Laboratories, Burlingame, CA 4 minutes), and the sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO). Slides were imaged using a Leica microscope (Leica, Wetzlar, Germany).

Western Blot Analysis

MPGs were homogenized in tris-HCl buffer (pH 7.5). We isolated cytosolic and membrane fractions for inducible nitric oxide synthase (iNOS), ARG1, β -actin by Western blot. The protein was loaded (50 μ g) on 4%-20% tris-HCl gel (Bio-Rad®) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then the proteins were transferred to polyvinylidene fluoride membranes and incubated with primary antibodies iNOS (Abcam, 1:400) and Arg-1 (BD Bioscience, 1:400) overnight at 4°C [16]. An enhanced chemiluminescence kit was used to visualize the membrane incubated with horseradish peroxidase linked secondary antibody (Amersham, GE Healthcare System, Buckinghamshire, UK). We quantified the densitometry results with Image J (National Institutes of Health) and normalized to β -actin (Sigma Aldrich, St. Louis, MO).

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) and analysis was performed using GraphPad Prism 5, San Diego, CA, USA. The differences between multiple groups were compared by two-way analysis of variance and one-way analysis of variance with Tukey's post-hoc test. Student's *t*-test was used to compare the difference between 2 groups. $p < 0.05$ was considered statistically significant.

Results

Effect of BCNI on the Penis

Analysis with 2-way ANOVA demonstrated that BCNI significantly impaired parasympathetic-mediated corporal relaxation response to EFS following pre-contraction by PE (3.0×10^{-5} M; $p = 0.0014$; Figure 1A). Similarly, EFS-mediated contraction was also significantly effected by BCNI ($p < 0.0001$; Figure 1B). Analysis using 1-way ANOVA with Tukey's post-hoc test also demonstrated significant impairment in EFS-mediated relaxation in all time points post-BCNI compared to sham at 4 and 8 Hz frequencies as well as tissue from animals 48 hours post-BCNI at 16 Hz ($p < 0.05$). EFS-mediated contraction was also significantly impaired in tissue from animals 48 hours post-BCNI at 2, 4, 8, and 16 Hz frequencies ($p < 0.05$). Although initially decreased compared to sham penile tissues at 48 hours and 7 days, the contractile response of penile tissue harvested from rats 14 days following BCNI was significantly greater than sham animals at 8 and 16 Hz ($p < 0.05$). These *ex vivo* physiologic results highlight the impaired neurogenic mediated relaxation, which contributed to heightened corporal contractile response in the corporal vascular bed.

Relative mRNA Expression of Cytokines and Chemokines

BCNI significantly increased both pro-inflammatory (*Il1b*, *Il16*, *Tnfa*, *Tgfb*, *Ccl2*, *Ccr2*) and anti-inflammatory (*Il10*) cytokines and chemokines in the MPG (Figure 2). The maximal increase in gene expression of all the cytokines and chemokines examined in the MPG was observed at the early time point 48hrs post-CN injury ($p<0.05$; Figure 2).

Relative mRNA Expression of the Markers of M1 Macrophages and M2 Macrophages

qPCR demonstrated BCNI significantly increased mRNA expression of macrophage markers (CD11b, CD68, and CCL2; Figure 3A) in the MPG. The maximal increase of CD68 and CCL2 in the MPG was observed at 48 hours following injury ($p<0.05$), while the maximal increase in the gene expression of CD11b was at 7 days after BCNI ($p<0.05$). M1 markers were also significantly increased following BCNI in the MPG (Figure 3B). The maximal increase in the gene expression of CD86 and IL1b was at 48 hours following injury ($p<0.05$). INOS showed its peak at 7 days after injury ($p<0.05$). BCNI-48h showed the maximal increase in the gene expression of all the M2 markers examined in the MPG ($p<0.05$; Figure 3C). Based on the gene expression of these markers, M1/M2 index was calculated (Figure 3D). M1/M2 index was below 1.0 in the MPG of the Sham rats (0.29) but M1/M2 index was constantly above 1.0 following BCNI (BCNI-48h: 1.75, BCNI-7d: 1.17, BCNI-14d: 1.02). This increase in M1/M2 index suggests that BCNI altered the macrophage polarization toward M1 macrophages.

Western Blot of M1 and M2 Macrophage Markers

To evaluate the M1 and M2 macrophage populations in the MPG quantitatively, Western blot was used to examine the relative protein expression of iNOS (M1 marker) and ARG1 (M2 marker) (Figure 4A). BCNI significantly increased the MPG protein expression of iNOS ($p<0.05$) while ARG1 remained unchanged following BCNI. The ratio of iNOS and ARG1 expression was calculated by dividing the relative protein expression of iNOS by ARG1. The ratio of iNOS and ARG1 was significantly increased and above 1.0 following BCNI ($p<0.05$; Figure 4B), which suggests greater protein expression of M1 markers, which is consistent with immunohistochemistry.

Immunohistochemistry of MPGs

CD68 positive cells were increased in the MPGs of BCNI-48h, BCNI-14d, compared to Sham (Figure 5). This finding indicates that BCNI induced recruitment of macrophages to the MPGs. To elucidate the phenotypes of these macrophages, we co-stained the MPGs with CD68 (general macrophage marker) and CD86 (M1 marker) or CD206 (M2 marker). CD68 positive cells in BCNI-48h, BCNI-14d, were predominantly CD86 positive (Figure 5), suggesting that these macrophages are mostly M1 macrophages, which is consistent with gene expression profiling of M1 and M2 macrophage markers.

Discussion

The present study demonstrated that BCNI impaired corporal parasympathetic-mediated relaxation response to EFS and enhanced contraction response to EFS at 14 days after injury. We also found that the gene expression of a diverse set of cytokines and chemokines was

upregulated in the MPG at 48 hours following BCNI. The gene expression of M1 markers (CD86, iNOS, IL1b) and M2 markers (CD206, ARG1, IL-10) was increased following BCNI in the MPG with the M1/M2 index above 1.0 indicating that more M1 macrophages are recruited to the MPG than M2 macrophages. In fact, immunohistochemical characterization demonstrated predominant increases in M1 (CD68+CD86+) macrophages in the MPG following BCNI. Western blot also showed a significant increase of an M1 marker (iNOS), which was consistent with the findings from the macrophage gene expression profiles.

Martinez-Salamanca *et al.* reported that the corporal tissue from the patients who underwent radical prostatectomy and from BCNI rats had impaired corporal parasympathetic mediated relaxation and increased sympathetic nerve mediated contraction [20,21]. In the present study, using a pre-clinical rodent model to study the pathobiology of radical prostatectomy impaired BCNI, we also found that the corporal parasympathetic-mediated relaxation was also impaired at 48 hours, 7 days, and 14 days post-injury. The most significant impairment was observed at 48 hours following BCNI. Although the relaxation response to EFS was not fully recovered at 14 days, the relaxation response to EFS was improved at 14 days when compared to the 48 hour time point. In addition, the corpora cavernosa of BCNI rats had hyper-contractile response to the EFS at 14 days following BCNI. This is also consistent with the findings from human corporal tissue after radical prostatectomy [20]. Notably, the contractile response was also impaired at 48 hours and 7 days after BCNI, which indicates that both parasympathetic and sympathetic nerves undergo neuropraxia at earlier time points. However, the corpus cavernosum of the rats had hyper-contractile response at 14 days after BCNI, which may suggest that the corporal tissue is primarily re-innervated with sympathetic innervation following BCNI. Therefore, the penis is in a sympathetic-nerve dominant state that leads to impaired relaxation of the smooth muscle cells and highlights a potential additional mechanism of neurogenic ED found in this animal model.

Our group has previously demonstrated an increase in gene expression of a variety of cytokines and chemokines in the MPG following BCNI [9,11,13]. In this study, we found that the gene expression of multiple pro-inflammatory cytokines and chemokines (*IL-1B*, *Il6*, *Tnfa*, *Tgfb*, *Ccl2*, *Ccr2*) are up-regulated at 48 hours, 7 days, and 14 days after injury with maximal increase at 48 hours following BCNI. The increase in inflammatory cytokines and chemokines is known to recruit macrophages to the injury site [13]. Hence, we looked at the gene expression of general macrophage markers, CD11b, CD68, and IL1b. All macrophage markers were significantly increased following BCNI in the MPG, indicating that macrophages are recruited to the injury site. Currently, two different macrophage phenotypes are known: M1 and M2 macrophages. M1 macrophages or classically activated macrophages are shown to inhibit nerve regeneration following peripheral nerve injury [4]. Meanwhile, M2 macrophages or alternatively activated macrophages are known to promote nerve regeneration following peripheral nerve injury [5,18,19]. Gene expression associated with the two macrophage phenotypes was up-regulated following BCNI but the M1/M2 index was constantly above 1.0 at all-time points measured in the present study, suggesting that the macrophages recruited to the injury site are mostly neurotoxic M1 macrophages and may be involved in CN axonal degeneration. A similar phenomenon has been shown in the CNS following traumatic spinal cord injury [22].

In order to confirm the recruitment of macrophages and to characterize the macrophage phenotypes, we co-stained the MPGs with anti-CD68 (general macrophage marker) antibody and CD86 (M1 marker) or CD206 (M2 marker) antibodies. The number of macrophages recruited to the MPG was significantly increased following BCNI. Most of these macrophages were CD86 positive M1 macrophages. Although there were some M2 macrophages in the MPG of sham rats, few M2 macrophages were found in the MPG of the rats that underwent BCNI. Western blot was used to evaluate the protein amount of iNOS (M1) marker and ARG1 (M2 marker) to examine the macrophage phenotype quantitatively. The protein amount of iNOS was significantly increased following BCNI. However, the protein amount of ARG1 was not increased following BCNI. The ratio of iNOS and ARG1, which reflects the M1/M2 ratio in the MPG, was constantly above 1.0 following BCNI. This increased iNOS/ARG1 ratio indicates M1 dominant state in the MPG following BCNI, which is consistent with the qPCR results that showed increased M1/M2 index. Alternative mechanisms which activate transcriptional regulation of iNOS could come from resident microglia in the MPG and other inflammatory cytokines released following peripheral CN-injury.

This current study has some limitations. First, we did not evaluate the number of macrophages quantitatively. The increase in macrophages and M1 macrophages was obtained by qPCR gene expression and immunohistochemistry. However, the actual numbers of macrophages, M1 macrophages and M2 macrophages were not annotated in the present study. Ideally, flow-cytometry should be used to evaluate the number of macrophages in the MPGs. Second, we did not demonstrate a direct effect of M1 macrophages on parasympathetic nerve function. Additional studies are necessary to delineate the direct effect of M1 and M2 macrophages on MPG and CN function both *in vitro* and *in vivo*.

Conclusion

Corporal relaxation response to EFS was significantly impaired by BCNI, while the penises showed hyper-contraction to EFS at 14 days. BCNI significantly up-regulated the gene expression of a variety of cytokines and chemokines, leading to enhanced recruitment of neurotoxic M1 macrophages to the MPG. These results may be interpreted to suggest that post-prostatectomy ED may be prevented by inhibiting enhanced recruitment of neurotoxic M1 macrophages to the pelvic ganglion. However, further studies are clearly warranted to show direct causation of M1 polarization on CN axonal degeneration.

Acknowledgments

This work was supported by the National Institute of Health (K08DK090370, TJB) and of the Urology Care Foundation (NAS, JLH, MK, and TJB Neuroinflammation following cavernous nerve injury recruits neurotoxic macrophages in an experimental model of post-prostatectomy erectile dysfunction. Next generation therapies should focus on inhibiting macrophage (M1) polarization to improve cavernous nerve regeneration

Abbreviations

BCNI	bilateral cavernous nerve injury
CN	cavernous nerve

ED	erectile dysfunction
EFS	electrical field stimulation
iNOS	inducible nitric oxide synthase
MPG	major pelvic ganglion
PNI	peripheral nerve injury
qPCR	quantitative real-time polymerase chain reaction
RP	radical prostatectomy

References

1. Scheib J, Höke A. Advances in peripheral nerve regeneration. *Nat Rev Neurol*. 2013; 9:668–76. DOI: 10.1038/nrneurol.2013.227 [PubMed: 24217518]
2. Smith ME, van der Maesen K, Somera FP. Macrophage and microglial responses to cytokines in vitro: Phagocytic activity, proteolytic enzyme release, and free radical production. *J Neurosci Res*. 1998; 54:68–78. DOI: 10.1002/(sici)1097-4547(19981001)54:1<68::aid-jnr8>3.0.co;2-f [PubMed: 9778151]
3. Shamash S, Reichert F, Rotshenker S. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. *J Neurosci*. 2002; 22:3052–60. 20026249. [PubMed: 11943808]
4. Kroner A, Greenhalgh AD, Zarruk JG, Passos dos Santos R, Gaestel M, David S. TNF and Increased Intracellular Iron Alter Macrophage Polarization to a Detrimental M1 Phenotype in the Injured Spinal Cord. *Neuron*. 2014; 83:1098–116. DOI: 10.1016/j.neuron.2014.07.027 [PubMed: 25132469]
5. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. 2012; 122:787–95. DOI: 10.1172/jci59643 [PubMed: 22378047]
6. Burnett AL, Aus G, Canby-Hagino ED, Cookson MS, D'Amico AV, Dmochowski RR, et al. Erectile Function Outcome Reporting After Clinically Localized Prostate Cancer Treatment. *J Urol*. 2007; 178:597–601. DOI: 10.1016/j.juro.2007.03.140 [PubMed: 17570435]
7. Walsh PC, Marschke P, Ricker D, Burnett AL. Patient-reported urinary continence and sexual function after anatomic radical prostatectomy. *Urology*. 2000; 55:58–61.
8. Walsh PC. The Discovery of the Cavernous Nerves and Development of Nerve Sparing Radical Retropubic Prostatectomy. *J Urol*. 2007; 177:1632–5. DOI: 10.1016/j.juro.2007.01.012 [PubMed: 17437775]
9. Podlasek CA, Meroz CL, Tang Y, McKenna KE, McVary KT. Regulation of Cavernous Nerve Injury-Induced Apoptosis by Sonic Hedgehog. *Biol Reprod*. 2006; 76:19–28. DOI: 10.1095/biolreprod.106.053926 [PubMed: 16988214]
10. User HM, Hairston JH, Zelner DJ, McKenna KE, McVary KT. Penile weight and cell subtype specific changes in a post-radical prostatectomy model of erectile dysfunction. *J Urol*. 2003; 169:1175–9. DOI: 10.1097/01.ju.0000048974.47461.50 [PubMed: 12576876]
11. Hannan JL, Albersen M, Stopak BL, Liu X, Burnett AL, Hoke A, et al. Temporal changes in neurotrophic factors and neurite outgrowth in the major pelvic ganglion following cavernous nerve injury. *J Neurosci Res*. 2015; 93:954–63. DOI: 10.1002/jnr.23553 [PubMed: 25644064]
12. Calenda G, Strong TD, Pavlovich CP, Schaeffer EM, Burnett AL, Yu W, et al. Whole genome microarray of the major pelvic ganglion after cavernous nerve injury: new insights into molecular profile changes after nerve injury. *BJU Int*. 2012; 109:1552–64. DOI: 10.1111/j.1464-410x.2011.10705.x [PubMed: 22300381]
13. Albersen M, Berkers J, Dekoninck P, Deprest J, Lue TF, Hedlund P, et al. Expression of a Distinct Set of Chemokine Receptors in Adipose Tissue-Derived Stem Cells is Responsible for In Vitro

Migration Toward Chemokines Appearing in the Major Pelvic Ganglion Following Cavernous Nerve Injury. *J Sex Med.* 2013; 1:3–15. DOI: 10.1002/sm2.1

14. Hannan JL, Albersen M, Kutlu O, Gratzke C, Stief CG, Burnett AL, et al. Inhibition of Rho-kinase improves erectile function, increases nitric oxide signaling and decreases penile apoptosis in a rat model of cavernous nerve injury. *J Urol.* 2013; 189:1155–61. DOI: 10.1016/j.juro.2012.09.104 [PubMed: 23021998]
15. Hannan JL, Matsui H, Sopko NA, Liu X, Weyne E, Albersen M, et al. Caspase-3 dependent nitroergic neuronal apoptosis following cavernous nerve injury is mediated via RhoA and ROCK activation in major pelvic ganglion. *Sci Rep.* 2016; 6:29416.doi: 10.1038/srep29416 [PubMed: 27388816]
16. Hannan JL, Kutlu O, Stopak BL, Liu X, Castiglione F, Hedlund P, et al. Valproic acid prevents penile fibrosis and erectile dysfunction in cavernous nerve-injured rats. *J Sex Med.* 2014; 11:1442–51. DOI: 10.1111/jsm.12522 [PubMed: 24636283]
17. Luttrell IP, Swee M, Starcher B, Parks WC, Chitaley K. Erectile dysfunction in the type II diabetic db/db mouse: impaired venoocclusion with altered cavernosal vasoreactivity and matrix. *Am J Physiol Heart Circ Physiol.* 2008; 294:H2204–11. DOI: 10.1152/ajpheart.00027.2008 [PubMed: 18326798]
18. Yang J, Su J, Owens L, Ibraguimov A, Tang L. A computational model of fibroblast and macrophage spatial/temporal dynamics in foreign body reactions. *J Immunol Methods.* 2013; 397:37–46. DOI: 10.1016/j.jim.2013.08.013 [PubMed: 24001881]
19. Spiller KL, Nassiri S, Witherel CE, Anfang RR, Ng J, Nakazawa KR, et al. Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. *Biomaterials.* 2015; 37:194–207. DOI: 10.1016/j.biomaterials.2014.10.017 [PubMed: 25453950]
20. Martínez-Salamanca JI, La Fuente JM, Fernández A, Martínez-Salamanca E, Pepe-Cardoso AJ, Carballido J, et al. Nitroergic function is lost but endothelial function is preserved in the corpus cavernosum and penile resistance arteries of men after radical prostatectomy. *J Sex Med.* 2015; 12:590–9. DOI: 10.1111/jsm.12801 [PubMed: 25529966]
21. Martínez-Salamanca JI, Zurita M, Costa C, Martínez-Salamanca E, Fernández A, Castela A, et al. Dual Strategy With Oral Phosphodiesterase Type 5 Inhibition and Intracavernosal Implantation of Mesenchymal Stem Cells Is Superior to Individual Approaches in the Recovery of Erectile and Cavernosal Functions After Cavernous Nerve Injury in Rats. *J Sex Med.* 2016; 13:1–11. DOI: 10.1016/j.jsxm.2015.12.001 [PubMed: 26755080]
22. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord. *J Neurosci.* 2009; 29:13435–44. DOI: 10.1523/JNEUROSCI.3257-09.2009 [PubMed: 19864556]

Figure 1.

Cavernous nerve (CN) injury impairs electrical field stimulation (EFS) induced nerve-mediated cavernous tissue contraction and relaxation. Tissue myography was performed on corpora cavernosa obtained from sham and bilateral cavernous nerve injured penises (BCNI). **(A)** BCNI injured penises demonstrated impaired nerve-mediated corporal smooth muscle relaxation induced by EFS following maximal precontraction with phenylephrine. The impairment in relaxation was most pronounced 48 hours following BCNI. **(B)** BCNI injured penises demonstrated impaired corporal smooth muscle contraction 48 hours and 7 days following injury. However, 14 days following BCNI, corporal smooth muscle contraction was significantly increased. These data suggest BCNI significantly impairs both corporal smooth muscle relaxation and contraction immediately following injury with hypercontraction 14 days following BCNI. N=4 per group.

Figure 2.

Bar graph showing the relative gene expression of pro-inflammatory and anti-inflammatory cytokines in the major pelvic ganglion (MPG) following bilateral cavernous nerve injury (BCNI) in the rat. * $p < 0.05$ compared to sham. $N = 5$ per group.

Figure 3.

Bar graph demonstrating the relative gene expression of (A) global macrophage, (B) M1 macrophages, and (C) M2 macrophages in the major pelvic ganglion (MPG) following bilateral cavernous nerve injury (BCNI) in the rat. (D) M1/M2 index was calculated from the gene expression of M1 and M2 macrophages in the MPG. *p<0.05 compared to sham. N=5 per group.

Figure 4.

(A) Western blot analysis of inducible nitric oxide synthase (iNOS, M1) and arginase-1 (Arg1, M2) protein expression in the major pelvic ganglion (MPG) following bilateral cavernous nerve injury (BCNI) in the rat. Bar graph demonstrates the relative expression of iNOS and ARG1 in the MPG following BCNI normalized to Sham. (B) Bar graph demonstrating the ratio of iNOS/ARG1 protein expression following BCNI in the MPG 48 hours to 14 days following injury. (C) Representative western blots of iNOS, Arg1, and beta-actin. * $p < 0.05$ compared to sham; # $p < 0.05$ compared to BCNI 48hrs. N=6 per group.

Figure 5. Representative immunohistochemical staining of M1 (CD68+CD86+) and M2 (CD68+CD206+) macrophages in sham and 48 hours and 14 days following BCNI. There is a qualitative increase in M1 macrophages with increasing time following BCNI, whereas M2 macrophages remain unchanged. Arrowheads represent double positive macrophages. Bar represents 100 μM.