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ORIGINAL RESEARCH & REVIEWS

BASIC SCIENCE

Increased Level of Tumor Necrosis Factor-Alpha (TNF-α) Leads to Downregulation of Nitrergic Neurons Following Bilateral Cavernous Nerve Injury and Modulates Penile Smooth Tone

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ABSTRACT

Background: Erectile dysfunction (ED) after injury to peripheral cavernous nerve (CN) is partly a result of inflammation in pelvic ganglia, suggesting that ED may be prevented by inhibiting neuroinflammation.

Aim: The aim of this study is to examine temporal changes of TNF-α, after bilateral CN injury (BCNI), to evaluate effect of exogenous TNF-α on neurite outgrowth from major pelvic ganglion (MPG), and to investigate effect of TNF-α signal inhibition to evaluate effects of TNF-α on penile tone with TNF-α receptor knockout mice (TNFRKO).

Methods: Seventy Sprague-Dawley rats were randomized to undergo BCNI or sham surgery. Sham rats' MPGs were harvested after 48 hours, whereas BCNI groups' MPGs were at 6, 12, 24, 48 hours, 7, or 14 days after surgery. qPCR was used to evaluate gene expression of markers for neuroinflammation in MPGs. Western blot was performed to evaluate TNF-α protein amount in MPGs. MPGs were harvested from healthy rats and cultured in Matrigel with TNF-α. Neurite outgrowth from MPGs was measured after 3 days, and TH and nNOS immunofluorescence was assessed. Wild type (WT) and TNFRKO mice were used to examine effect of TNF-α inhibition on smooth muscle function after BCNI. MPGs were harvested 48 hours after sham or BCNI surgery to evaluate gene expression of nNOS and TH.

Outcomes: Gene expression of TNF-α signaling pathway, Schwann cell and macrophage markers, protein expression of TNF-α in MPGs, and penile smooth muscle function to electrical field stimulation (EFS) were evaluated.

Results: BCNI increased gene and protein expression of TNF-α in MPGs. Exogenous TNF-α inhibited MPG neurite outgrowth. MPGs cultured with TNF-α had decreased gene expression of nNOS ($P < .05$). MPGs cultured with TNF-α had shorter nNOS+ neurites than TH+ neurites ($P < .01$). Gene expression of nNOS was enhanced in TNFRKO mice compared to WT mice ($P < .01$). WT mice showed enhanced smooth muscle contraction of penises of WT mice was enhanced to EFS, compared to TNFKO ($P < .01$). Penile smooth-muscle relaxation to EFS was greater in TNFKO mice compared to WT ($P < .01$).

Clinical Translation: TNF-α inhibition may prevent ED after prostatectomy.

Strength/Limitations: TNF-α inhibition might prevent loss of nitrergic nerve apoptosis after BCNI and preserve corporal smooth muscle function but further investigation is required to evaluate protein expression of nNOS in MPGs of TNFKO mice.

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Conclusions: TNF-α inhibited neurite outgrowth from MPGs by downregulating gene expression of nNOS and TNFRKO mice showed enhanced gene expression of nNOS and enhanced penile smooth-muscle relaxation.
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Key Words: Erectile dysfunction; Prostatectomy; Tumor necrosis factor-alpha; Peripheral nerve injury; Neurite

INTRODUCTION

Erectile dysfunction (ED) remains a common sequela following radical prostatectomy (RP).[1] The most common cause of ED following RP is cavernous nerve injury (CNI) at the time of surgery.[2,3] It is known that CNI leads to axonal damage, reduced density of neuronal nitric oxide synthase (nNOS) in the penis, and penile smooth muscle fibrosis.[4−6] The majority of men experience some degree of ED after RP and may require as long as two to four years to regain potency postoperatively.[2]

In general, Schwann cells are activated in response to peripheral nerve injury (PNI).[7] Various inflammatory cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-α), are released by neurons or Schwann cells to initiate the clearing of the debris. Macrophages are then recruited as part of the local inflammatory response.[8] Subsequently, TNF-α is secreted by these macrophages, and as part of a positive feedback loop, further stimulating inflammation. In a model of sciatic nerve injury, neuronal cell death is induced by this upregulation of TNF-α.[8] In the major pelvic ganglion (MPG), our group has shown that gene expressions of multiple inflammatory cytokines, including TNF-α, are increased 24 hours after bilateral CNI (BCNI).[9]

Based on these findings, we hypothesized that gene and protein expressions of TNF-α would be increased following BCNI and increased TNF-α would impair neurite outgrowth from the MPG. To test these hypotheses, we have performed *in vivo* and *ex vivo* rat studies as well as studies with TNF-α receptor-1/-2 knockout (TNFRKO) mice. From in vivo rat experiments, we evaluated the temporal transcriptional and translational changes of TNF-α following BCNI. From *ex vivo* rat experiments, we examined the effect of exogenous TNF-α on neuritogenesis of nitrergic and sympathetic neurites from the MPG. With TNFRKO mice, we examined the effect of TNF-α inhibition on penile smooth muscle function.

MATERIALS AND METHODS

Animals

A total of seventy male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) aged 8 weeks and weighing 300-350g, were used for the *in vivo* studies. The animals had ad libitum access to water and food. These rats were randomly divided into 7 groups (n=10/group, 2 − 3 rats/cage): (i) Sham; (ii) BCNI 6 hours; (iii) BCNI 12 hours; (iv) BCNI 24 hours; (v) BCNI 48 hours; (vi) BCNI 7 days; (vii) BCNI 14 days. Sham rats' MPGs were collected at 48 hours after surgery. MPGs from the respective surgery groups were harvested 6 hours, 12 hours, 24 hours, 48 hours, 7 days, or 14 days after BCNI. No animal died or was excluded during the course of this study.

For the *ex vivo* study, bilateral, whole MPGs were carefully harvested from non-crushed rats (n=6/group) and cultured in Matrigel (Corning, Bedford, MA, USA) for 72 hours as previously described.[10]

Wild type (WT) mice and TNF-α receptor-1 and -2 double knockout (TNFRKO) mice (Jackson Laboratory, Bar Harbor, ME, USA) were used to examine the effect of TNF-α inhibition (n = 5 / group). For TNFRKO model, mice with double knockout of *Tnfrsf1a* and *Tnfrsf1b* were used. C57BL/6J mice was used as WT mice. The mice underwent sham or BCNI surgery (n = 5 / group). MPGs and penises were harvested 48 hours after surgery.

All the experiments were conducted in accordance with the Johns Hopkins University School of Medicine Guidelines for Animal Care and Use, and the National Institute of Health Guide for the Care and Use of Laboratory Animals.

BCNI

The prostate of a rat or a mouse was identified by midline laparotomy under 3% isofluorane anesthesia. The cavernous nerve (CN) was identified posterolateral to the prostate.[11] Bilateral CNs (2-3 mm distal to MPG) were crushed with Dumont #5 forceps to induce BCNI. The forceps were closed completely 3 times for 15 seconds each.[12] All the BCNI and sham surgeries were performed by a single surgeon for both rats and mice. For the sham surgery, CNs were not crushed and the abdomens were closed after identifying the MPGs and CNs.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Real-time qPCR was used to examine the gene expression in the MPGs. We isolated the total RNA from homogenized

MPGs with 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 Healthcore, Pittsburgh, PA, USA). The relative mRNA expression was evaluated with TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA). We evaluated the gene expression of TNF-α (*Tnfa*), TNF-α receptor-1 (*Tnfr1*), myelin protein zero (*Mpz*), peripheral myelin protein 22 (*Pmp22*), glial fibrillary acidic protein (*Gfap*), integrin subunit alpha M (*Itgam*; gene codes CD11b), and CD68. Additionally, tyrosine hydroxylase (*Th*), and nNOS (*Nos1* codes nNOS) were examined in the ex vivo study. As previously described, all values were normalized to *Hprt1* (hypoxanthine phosphoribosyltransferase 1) transcript levels (Applied Biosystems). In the mouse study, gene expression of *Tnfa, Nos1* and *Th* was evaluated and normalized to *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase). All experiments were performed on 5 different whole MPGs from each group with triplicate technical replicate PCR reactions per sample.[10]

WESTERN BLOT ANALYSIS

Tris-HCl buffer (pH 7.5) was used to homogenize the MPGs. Cytosolic fractions for TNF-α, and beta-actin (β-actin) were isolated by Western blot (WB). We loaded the 50μg of protein on 4%-20% tris-HCl gel (Bio-Rad) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isolated proteins were transferred to polyvinylidene fluoride membranes and incubated with primary antibodies (TNF-α (R&D) 1:400, β-actin (Sigma Aldrich) 1:400) overnight at 4° C.[11] In order to visualize the membrane, we used an enhanced chemiluminescence kit with horseradish peroxidase linked secondary antibody (Amersham). Densitometry was quantified with Image J (National Institutes of Health) and normalized to β-actin for TNF-α.

MAJOR PELVIC GANGLION CULTURE AND NEURITE OUTGROWTH ASSESSMENT

Whole MPGs were carefully harvested from non-crushed rats. MPGs were kept on ice in serum-free media (RPMI 1640 with 1% Penicillin-Streptomycin, GIBCO) until they were embedded in growth-factor reduced Matrigel (n=6/group).[13] We placed 200 μL of Matrigel on the bottom of a 24 well plate. After polymerization of Matrigel, whole MPGs were embedded in the center of each well. MPGs were then covered with 300 μL of Matrigel and 1 mL of media. In the TNF-α group, exogenous TNF-α (R&D, Minneapolis, MN) were added to the media (10, 20, 30 ng/mL). Media and TNF-α were changed every 24 hours. We cultured the MPGs at 37°C in a humidified atmosphere with 5% carbon dioxide. With a Nikon TE200 inverted microscope attached to a CCD camera, we took photographs of neurite outgrowths from the MPGs at 48 and 72 hours after culture.

The images were analyzed with Elements software (Nikon Instruments, Melville, NY, USA). We compared the averages of 5 longest neurites in each area to evaluate the effect of exogenous TNF-α on neurite outgrowths (20-25 neurites/MPG) 48 and 72 hours after culture.[10] The growth rate was calculated as follows: ((average neurite length at time point X) − (average neurite length at time point Y)) / (time difference between time point X and time point Y). To compare the neurite outgrowth patterns between the control group and the TNF-α group, neurite lengths of all the neurites were also measured to create histograms of neurite lengths and to calculate the variances of the neurite lengths. MPGs were then frozen in liquid nitrogen at 72 hours after culture to be processed to evaluate gene expression by RT-PCR. Additional MPGs from the control group and the TNF-α 20 ng/mL group were fixed with 4% paraformaldehyde + PBS + 3% Tween at 72 hours after culture for immunofluorescence.

IMMUNOFLUORESCENCE

For the histologic analysis, we fixed the MPGs harvested from Sham, BCNI 48 hours, BCNI 7 days, and BCNI 14 days in 4% paraformaldehyde for 24 hours and then in 70% ethanol before embedding them in paraffin. Immunofluorescence staining was performed as previously described. Slides were incubated with primary antibodies against TNF-α (R&D 1:400), and β-Tubulin (1:500, Abcam). Secondary antibodies were conjugated with Alexa-488 and Alexa-594 (1:200; Invitrogen). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen). Slides were imaged with Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

For the immunofluorescence of the *ex vivo* study, MPGs were incubated with primary antibodies against tyrosine hydroxylase (TH; 1:200 Santa Cruz) and neuronal nitric oxide synthase (nNOS; 1:200 Santa Cruz) for 24 hours. The same secondary antibodies were used for the ex vivo study. MPGs were imaged with Zeiss LSM 700 confocal microscope and the images were processed with Zen Software (Carl Zeiss).

CORPUS CAVERNOSUM(OR PENILE) VASOREACTIVITY

Contractile response to electrical field stimulation (EFS) and parasympathetic-mediated relaxation of the penises were evaluated with a muscle-strip organ bath (820M, Danish Myograph Technology, Aarhus, Denmark). Mouse penises were harvested and placed in Krebs solution (NaCl 130 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 1.56 mmol/L, $MgSO_4$ 1.18 mmol/L, KH_2PO_4 1.18 mmol/L, $NaHCO_3$ 14.9 mmol/L, dextrose 5.6 mmol/L).[12,13] Urethras and glans were carefully removed from the penises under a dissecting microscope. The penile sections were placed in a muscle-strip myograph. These sections were bathed in Krebs solution 5mL (maintained at 37°C), and bubbled with a mixture of 5% carbon dioxide and 95% oxygen. We

measured contractile response to EFS with frequencies of 4.0, 8.0, 16, and 32 Hz for 10 seconds at 40 V, 2 mS (Grass S88 Stimulator, Grass Medical Instruments, Quincy, MA, USA). Parasympathetic-mediated relaxation response of penile tissue was evaluated after pre-contraction by phenylephrine (3.0×10^{-5} mol/L; Sigma-Aldrich, St Louis, MO, USA) with frequencies of 2.0, 4.0, 8.0, 16 and 32 Hz for 10 seconds at 40 V, 2mS.[13]

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (SEM). Unpaired t test was used to evaluate the difference between two groups. We compared the differences between the multiple groups by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (GraphPad Prism 5, San Diego, CA, USA). A *P*value of less than .05 was used as criteria for statistical significance.

RESULTS

Effect of BCNI on TNF-α Signaling Pathway, Schwann Cell Markers, and Macrophage Markers

There was an increase in gene expression of *Tnfa, Tnfr1,* and *Tnfr2* in the rat MPGs after BCNI (n=5/group, Figure 1). The maximal increase in gene expression was 48 hours after surgery ($P < .01$ vs Sham). By 7 days post-BCNI, the gene expression of TNF-α, TNFR1, and TNFR2 was decreased. Schwann cell markers (MPZ and PMP22) were also increased following BCNI (Figure 1). These Schwann cell markers remained elevated 7 and 14 days after BCNI. Macrophage markers (CD11b and CD68) were increased following BCNI with maximal expression at 48 hours (Figure 1).

Protein Amount of TNF-α in the MPG Following BCNI

Western blot demonstrated increased protein expressions of TNF-α in rat BCNI MPGs (Figure 2A, $P < .05$). The maximal increase in TNF-α protein was observed 7 days after BCNI. Immunofluorescence of MPGs demonstrated a qualitative increase in TNF-α protein expression after BCNI. TNF-α was primarily detected in the perivascular area at 48 hours, around the cell bodies of the neurons, and cytoplasm of the cell bodies at 14 days (Figure 2B).

Effect of Exogenous TNF-α on Parasympathetic and Sympathetic Neurites Outgrowth from the MPG Ex Vivo

MPGs were cultured in Matrigel with TNF-α at the concentrations of 0, 10, 20, and 30 ng/dL (Figure 3A-D). The average lengths of the longest neurites in the TNF-α group were similar amongst all groups at 48 hours after culture (Figure 3E). However, after 72 hours in culture, we observed that TNF-α impaired neurite outgrowth in a dose-dependent fashion ($P < .05$) (Figure 3F). Similarly, the growth rates of the neurites were also significantly downregulated by exogenous TNF-α in a dose-dependent manner (Figure 3G).

Neurite Outgrowth Patterns from the MPGs and Effect of Exogenous TNF-α on Sympathetic and Parasympathetic Neurites

Although there was a reduction in length and growth rate, neurite outgrowth was not completely impaired by exogenous TNF-α (Figure 3 A-D). To visualize this difference, we made a histogram of neurite lengths at 72 hours (Figure 4A). The TNF-α group showed bimodal distribution in the histograms of the neurite lengths of all the neurites, while the control group demonstrated a normal distribution. One of the peaks of the TNF-α

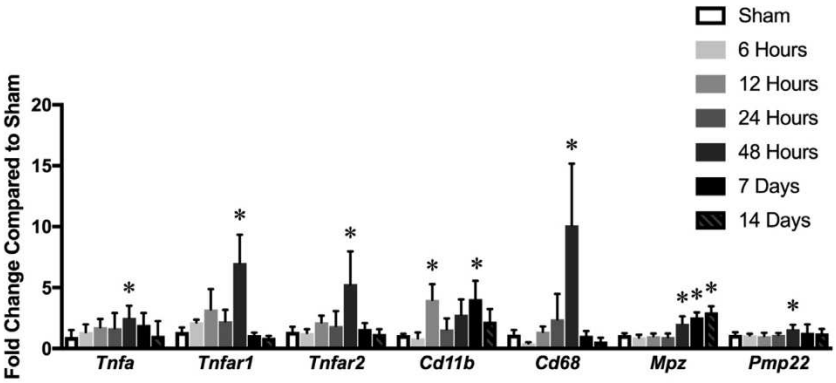

Figure 1. Bar graphs demonstrate the relative gene expression to sham of *Tnfa, Tnfar1, Tnfar2, Cd11b, Cd68, Mpz,* and *Pmp22* in the rat MPGs after BCNI. The times indicate when MPGs were harvested. One-way ANOVA followed by Tukey's multiple comparisons test was used to calculate *P*values. Unpaired *t* test was used to evaluate the difference of two groups. * indicates $P < .05$ compared with sham group (n = 5/group).

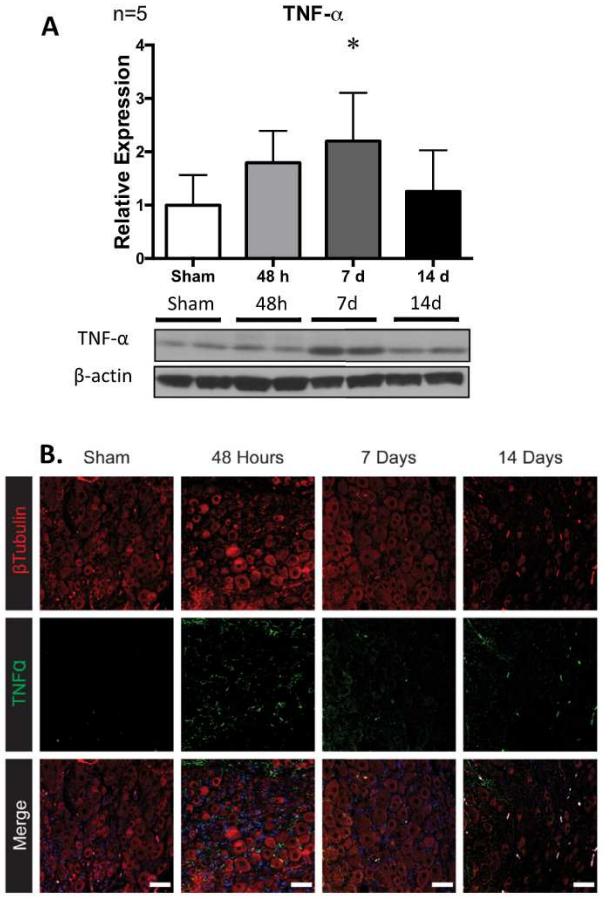

Figure 2. Panel A shows WB analysis of TNF-α and β-actin in MPGs at 48 hours, 7 days, and 14 days after BCNI. Relative protein amount of TNF-α to β-actin is indicated in the bar graphs. One-way ANOVA followed by Tukey's multiple comparisons test was used to calculate p values. Unpaired t test was used to evaluate the difference of 2 groups. * indicates $P < .05$ compared to Sham. Representative WB of TNF-α and β-actin is also demonstrated. Panel B indicates representative immunofluorescences of TNF-α and β-tubulin in the MPGs of sham rats and MPGs harvested from BCNI rats at 48 hours, 7 days, and 14 days after surgery. TNF-α was primarily detected in the perivascular area at 48 hours, around the cell bodies of the neurons, and cytoplasm of the cell bodies at 14 days

group was at the average length of the control group and the other peak was at much shorter length (Figure 4A). To demonstrate this difference quantitatively, we compared the variances of the 2 groups because a variance must be small if a histogram shows normal distribution. The variance of the control group was significantly smaller than the TNF-α group (Figure 4B), suggesting a differential distribution between the two group. Therefore, we assessed gene expression and immunofluorescence staining of nNOS and TH was performed to examine the type of neurites impaired by TNF-α (Figure 4 C, D). The gene expression of *Nos1* was significantly downregulated by exogenous TNF-α. Meanwhile, the gene expression of *Th* remained unchanged by addition of exogenous TNF-α. Immunofluorescence demonstrated that nNOS neurites were significantly shorter in the MPGs cultured with TNF-α. In fact, the average length of nNOS positive neurites were significantly shorter than that of TH positive neurites in the TNF-α group ($p<0.05$) but the average lengths of nNOS positive neurites and TH positive neurites were similar in the control group (Figure 4E).

Gene Expression of Tnfa, Nos1 and Th in TNFRKO mice, and Penile Relaxation/Contraction Response to EFS

qPCR demonstrated that the gene expression of *Tnfa* was significantly upregulated both in WT and TNFRKO 48 hours after BCNI, though the difference was not significant. (WT Sham: 1.0, WT BCNI 30.8, TNFRKO Sham 13.2, TNFRKO BCNI 39.3). In order to determine the influence of genetically knockout at TNF-α signaling on penile smooth muscle, we compared the gene expression of Nos1 and penile smooth muscle tone of WT mice and TNFRKO mice. MPGs of WT and TNFRKO mice were harvested 48 hours after sham or BCNI surgery (n = 5/group). Gene expression of *Nos1* was significantly enhanced in sham TNFRKO mice compared to sham WT mice ($P < .05$; Figure 5A). BCNI did not affect gene expression of *Nos1* in WT or in TNFRKO mice. We examined gene expression of *Th* in WT and TNFRKO mice after sham or BCNI. However, no difference in *Th* gene expression was evident in WT or in TNFRKO mice after sham or BCNI (Figure 5A).

Two-way ANOVA analysis showed that TNFRKO mice demonstrated significantly enhanced relaxation response to EFS after pre-contraction with phenylephrine (3.0×10^{-5} M), compared to WT mice ($P < .05$) (Figure 5B). Significant impairment of relaxation response to EFS was found in WT mice after BCNI at 32 Hz with one-way ANOVA with Tukey post hoc test. However, this difference was not detected in TNFRKO mice.

Contractile response to EFS was significantly enhanced in WT mice compared to TNFRKO mice (Figure 5C, $P < .01$). Penises from mice after BCNI demonstrated enhanced contraction compared to sham both in WT and TNFRKO groups (Figure 5C). However, Tukey's multiple comparisons test did not show statistically significant difference among these groups.

DISCUSSION

Both inflammatory cytokines and chemokines are overexpressed and secreted after peripheral nerve injury (PNI). Specifically, TNF-α, has been shown to induce neuronal cell death after sciatic nerve injury.[8,14,15] Previously, we have demonstrated that the recruitment of macrophages is enhanced after BCNI and that during this inflammatory response, these phagocytes excrete TNF-α to recruit additional macrophages to the lesion.[10,13] Prior studies on ED following BCNI evaluated responses at 7 days or 14 days after surgery. The goal of this

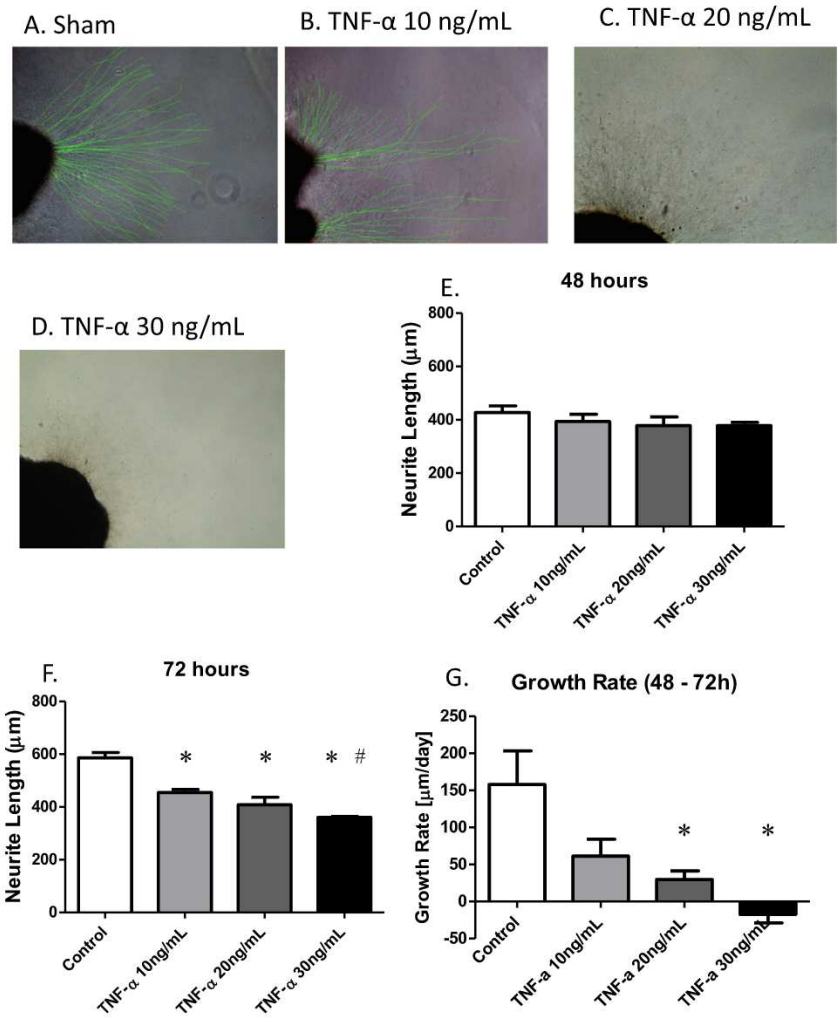

Figure 3. Panels A-D show representative images of neurite outgrowth from MPGs of control, MPGs cultured with exogenous TNF-α at concentrations of 10, 20, and 30 ng/mL. Panel E shows bar graphs of neurite lengths 48 hour after culture and panel F shows bar graphs of neurite lengths 72 hours after culture. One-way ANOVA followed by Tukey's multiple comparisons test was used to calculate p values. Unpaired t test was used to evaluate the difference of two groups. * indicates $P < .05$ compared to control. Panel G demonstrates growth rates at 48-72 hour after culture at each concentration of exogenous TNF-α. * indicates $P < .05$ compared to control.

study was to better understand the early inflammatory process following peripheral nerve crush injury and the role of TNF-α. In this study, we observed that increased gene expression of TNF-α, TNFR1 and TNFR2 occurred earlier after BCNI with the maximal increase at 48 hours after injury (Figure 1). This increase in gene expression of both TNF-α and its receptor paralleled the increase in macrophage marker (CD11b and D68) upregulation. This early rise in TNF-α signaling indicates the importance of studying the acute inflammatory phase following BCNI in order to elucidate the pathogenesis of CNI induced ED and underscores the importance of early intervention to optimize nerve regeneration in the setting of peripheral nerve crush injuries after pelvic surgery.

Interestingly, the gene expression of Schwann cell markers was significantly increased following BCNI (Figure 1). The increase in gene expression was sustained out to 14 days after BCNI. Schwann cells are integral for nerve regeneration following PNI[16] and the upregulation of these markers confirms the importance of Schwann cell activation and dedifferentiation to promote nerve regeneration after BCNI.

In our previous study, we demonstrated that global macrophage markers are upregulated following BCNI[13] and our current findings confirm that this gene expression upregulated early, during the acute phase (Figure 1). Macrophages play an important role in nerve recovery immediately after injury, and their peak role appears to be early, but may be sustained over time. Further studies are necessary to determine the functional consequences of sustained macrophage induction on neurovascular control of the penis.

Although the gene expression of TNF-α peaked at 48 hours after BCNI, protein expression of TNF-α was persistently elevated following BCNI to 7 days (Figure 2). We also observed a

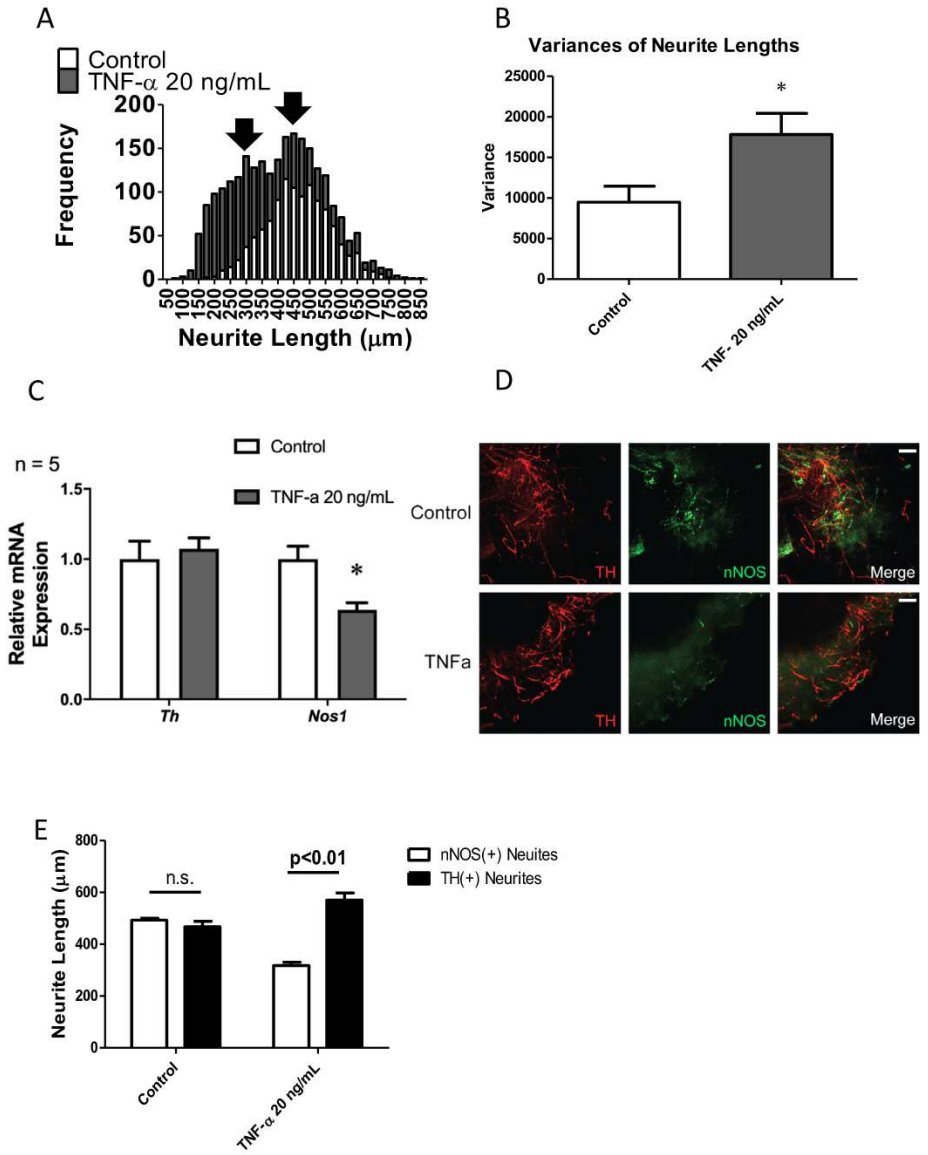

Figure 4. Panel A is the histogram of neurite lengths. Arrows indicate two different peaks of the TNF-α 20 ng/mL group. Panel B demonstrates variances of neurite lengths of control and TNF-α 20 ng/mL groups.c Unpaired t test was used to evaluate the difference of the two groups * indicates $P < .05$ compared to control. Panel C shows relative mRNA expression of *Th* and *Nos1* to control in MPGs 72 hours after culture. * indicates $P < .05$ compared to control. Panel D indicates representative immunofluorescences of TH (stained in red) and nNOS (stained in green). Panel E demonstrates bar graphs of neurite lengths of nNOS positive neurons and TH-positive neurons in control and TNF-α 20 ng/mL groups. N.s. indicates not significant.

temporal change in the localization of TNF-α in the MPGs. TNF-α had strong signal in the perivascular area at 48 hours, in the periphery of the cell bodies at 7 days, and in the cytoplasm of the cell bodies at 14 days. Hence, we hypothesize that TNF-α recruited hematogenous monocytes to the site of nerve injury and then localized them in the periphery of the cell bodies to induce neuronal cell death and/or cleaning of the myelin debris. We have previously shown significant neuronal cell death at these time points, supporting this hypothesis.[10]

Through *ex vivo* experiments, we found that exogenous TNF-α inhibits neurite outgrowth from the MPG (Figure 3) in a dose-dependent manner. TNF-α impaired the neurite outgrowth and reduced growth rate after 72 hours of culture. This decrease in neurite outgrowth coincides with the peak of TNF-α at 48 hours and supports the pathophysiological role of this cytokine.

Studies exploring autonomic nerves from other organ systems have shown variable response to TNF-α depending on the nerve type. Kisiswa et al. demonstrated that TNF-α promoted axonal growth in iris and nasal tissue[17] and Wei et al. showed that TNF-α upregulated sympathetic nerve activity of subfornical organ.[18] In addition, a study by Kondo *et al.* reported that TNF-α decreased the expression of acetylcholine receptors[19] and we

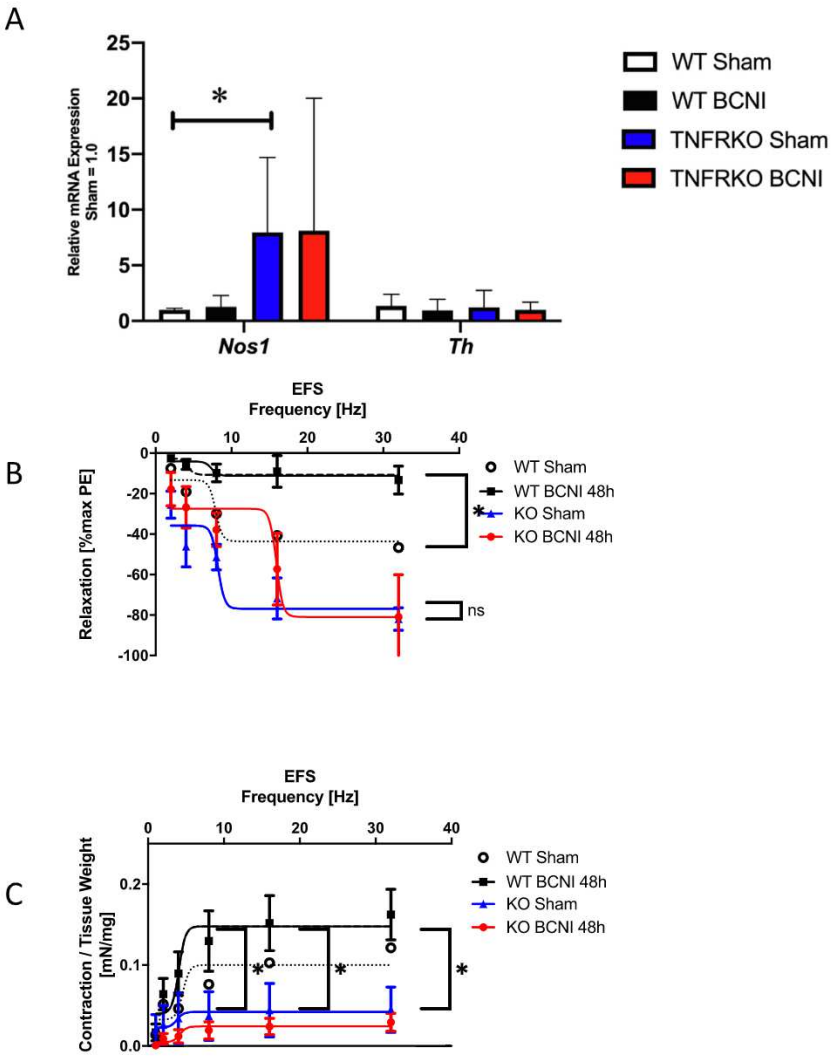

Figure 5. Panel A shows bar graphs of the relative gene expression of *Nos1 and Th* to WT sham in WT BCNI, TNFRKO sham, and TNFRKO BCNI (n = 5/group) One-way ANOVA followed by Tukey's multiple comparisons test was used to calculate p values. * indicates $P < .05$ compared to WT sham group. Panel B and Panel C demonstrate smooth muscle relaxation response and contractile response to EFS, respectively. Pre-contraction was induced with phenylephrine in panel B. * indicates $P < .05$ compared to WT sham group.

therefore hypothesized that TNF-α should selectively inhibit the neurite outgrowth of nitrergic neurons. To test this hypothesis, we first looked at the gene expression of TH and nNOS. When we evaluate the neurite outgrowths from the MPG, we observed that TNF-α does not inhibit global neuritogenesis but has selective population of neurite outgrowth which are influenced by TNF-α. To visually differentiate the type of neurites inhibited by TNF-α and to examine the protein expression of TH and nNOS, we performed the immunofluorescence in the MPGs after culture. The gene expression of sympathetic neurons (TH) remained unchanged with TNF-α treatment, but the gene expression in nitrergic neurons (nNOS) was significantly downregulated by TNF-α. To further examine the difference in the neurite outgrowth pattern, lengths of the neurites were measured. A histogram of neurite lengths of the control MPGs demonstrated a normal distribution, while a histogram of neurite lengths of the TNF-α group had a bimodal distribution. The first peak was at about the same peak length of the control group, whereas the second peak was at a much shorter length (Figure 4A). These data suggest the bimodal distribution represents the two different nerve types. The nNOS stained nerves have a higher frequency at shorter lengths, whereas the TH-stained nerves are stimulated by TNF-α and thus grow longer.

Variances of the groups were calculated to examine the difference in neurite outgrowth pattern quantitatively (Figure 4B). The variance of TNF-α group was significantly larger than the variance of the control group, indicating the inhibition of nNOS fiber growth, but the promotion of TH nerve growth. This finding coincides with the fact that there is a reduction in length of nNOS nerves and therefore a large difference between parasympathetic and sympathetic neurites.

The above findings suggested that TNF-α inhibition might prevent loss of nitrergic nerve apoptosis after BCNI and preserve corporal smooth muscle function. To confirm the effect of TNF-α inhibition, we used TNFRKO mice. TNFRKO resulted in significant upregulation of gene expression of nNOS in the penises (Figure 5A), which suggests that blockade of TNF-α signaling enhances innervation of nitrergic neurons to corporal tissue, though it is possible that this may be due to increased nNOS content in the neurons of TNFRKO mice. On the other hand, TNF-α signal inhibition did not affect gene expression TH (Figure 5A). As a result of enhanced innervation of nitrergic neurons, TNFRKO mice demonstrated enhanced relaxation response to EFS following maximal contraction induced by phenylephrine (Figure 5B). This finding was consistent with the study by Carneiro, *et al.,* which showed increased relaxation of corpora cavernosa of TNF-α knockout mice.[20] Previously, we reported that BCNI increased sympathetic re-innervation and caused enhanced contractile response to EFS.[13] Because of enhanced nitrergic innervation, BCNI did not increase sympathetic innervation in TNFRKO mice and, thus, contractile response to EFS remained unchanged between sham and BCNI groups in TNFRKO mice. Alexander *et al.* reported that chronic infusion of TNF-α decreased nNOS in the kidney, which supports results of this study that TNF-α inhibition can preserve nNOS innervation in the corporal smooth muscle.[21]

The present study has some limitations. First, we did not examine at the protein expression of Schwann cell markers. Second, we did not look at TNFRs on the TH-positive and nNOS-positive neurites, which would have helped clarify the mechanism underlying the selective inhibition of TNF-α on nitrergic nerves. Third, we used TNFRKO mice to evaluate the effect of TNF-α signal inhibition. Genetic knockout of TNFRKO resulted in enhanced innervation of nitrergic neurons to corporal tissue and, therefore, we were not able to evaluate direct effect of TNF-α inhibition on nitrergic nerve regeneration following BCNI. Moreover, TNFRKO mice used in this study were double knockout mice of TNFR-1 and TNFR-2. TNFR-1 is reported to be neurotoxic,[22] while TNFR-2 is shown to be neuroprotective following nerve injury model.[23] Thus, selective inhibition of TNFR-1 may facilitate cavernous nerve regeneration following BCNI. Finally, we only evaluated gene expression of nNOS in the mouse study and did not examined protein amount of nNOS in TNFRKO mice and WT mice. To more adequately explore the role of TNF-α of nitrergic and sympathetic neurons, we would ideally need to perform dissociation of MPG with selective growth of sympathetic and nitrergic selective neurons in culture and measure growth patterns, but that was beyond the scope of this experimental design.

CONCLUSION

The gene expression and protein expression of TNF-α were significantly increased following BCNI. Neurite outgrowth of nitrergic neurons were selectively inhibited by TNF-α. TNFRKO mice demonstrated enhanced gene expression of nNOS and enhanced penile smooth muscle relaxation. These findings suggest that TNF-α inhibition may prevent nerve degeneration following peripheral nerve injury that occurs at time of RP by preserving nitrergic axonal innervation.

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