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Spinal Cord Injury Induced Arrest in Estrous Cycle of Rats Is Ameliorated by S-nitrosoglutathione: Novel Therapeutic Agent to Treat Amenorrhea

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Abstract

Introduction—Amenorrhea following spinal cord injury (SCI) has been well documented. There has been little research on the underlying molecular mechanisms and therapeutics.

Aim—The purpose of the present study was to investigate the effect of GSNO in ameliorating SCI-induced amenorrhea through affecting the expression of CX43, NFkB, and ER protein.

Methods—SCI was induced in female SD rats at the T9-T10 level. Estrous stage was determined by vaginal smear. GSNO (50 µg/kg body weight) was gavage fed daily. Animals were sacrificed on day 7 and 14 post SCI. Ovaries were fixed for histological and biochemical studies. Expression levels of ER, CX-43, and NFkB were analyzed by Western blot and immunofluorescence.

Main Outcome Measures—GSNO hastens resumption of the estrous cycle following SCI-induced transient arrest.

Results—Resumption of estrous cycle was hastened by GSNO. Atretic and degenerating follicles seen in the ovary of SCI rats on day 14 post-SCI were decreased in GSNO treated animals. The increased CX43 expression observed with SCI ovary was decreased by GSNO. ER expression decreased significantly on day 7 and 14 post-SCI and was restored with GSNO treatment. Following SCI, NFkB expression was increased in the ovarian follicles and the expression was reduced with GSNO administration. The number of terminal deoxynucleotidyl transferase-mediated biotinylated uridine triphosphate (UTP) nick end labeling positive follicular and luteal cells was increased after SCI. GSNO-treated animals had significantly fewer apoptotic cells in the ovary.

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Conclusion—SCI-induced amenorrhea is accompanied by an increase in CX43 expression and a decrease in ER expression. SCI animals treated with GSNO resumed the estrous cycle significantly earlier. These results indicate a potential therapeutic value for GSNO in treating amenorrhea among SCI patients.

Keywords

Spinal Cord Injury; Amenorrhea; Sexual Function; Estrous Cycle

Introduction

Regaining sexual function is among the highest priorities of spinal cord injury (SCI)-induced paraplegics [1]. In 2009, 262,000 people were diagnosed with SCI (<http://www.miamiproject.miami.edu/Document.Doc?id=197>). Since most SCI patients are men, research has focused on their recovery or on issues common to both males and females. Unfortunately, issues particular to female patients have not been investigated to the same degree [2]. However, each year, more than 3,000 women of childbearing age in the United States are afflicted with SCI [3]. In addition, 59% of SCI patients of either sex manifest at least one form of sexual dysfunction [4]. Hence, assessment and management of sexual dysfunction need more research on the pathophysiology and optimal treatment of sexual dysfunction associated with chronic illness like SCI [5,6].

The spinal nerves (T10-L2) [7] and the abdominal branches of the vagus nerve [8] enervate the ovaries. However, anterograde signaling occurs only through the spinal cord [9]. Hence, interruptions in signaling due to SCI alter ovarian physiology through inflammation and apoptosis. Activated nuclear NFkB is often found at the sites of inflammation in the central nervous system [10]. NF-kB is a ubiquitous heterodimeric transcription factor that is sequestered in the cytoplasm by the inhibitor of kappa B (IκB) proteins. Degradation of IκB allows translocation of NFkB into the nucleus and subsequent activation of its target genes.

Amenorrhea may either stem from normal physiological conditions like pregnancy, menopause, and lactation, or develops as a consequence of pathological conditions like SCI. Sleep disturbances (43%), night sweats (40%), decreased sex drive (38%), and amenorrhea (60%) are common consequences of SCI [11]. This interruption in the menstrual cycle is commonly experienced for 8–12 months after SCI [12]. Through interrupted anterograde signaling, SCI affects the hypothalamus–pituitary–ovary (HPO) axis crucial to normal hormone regulation [12]. Estrogen is produced primarily by developing follicles in the ovary in response to gonadotropic hormones, with the peak of production occurring at ovulation. Decreases in estrogen level result in infrequent menstrual cycles [13]. In addition to controlling reproductive physiology, estrogen also targets the brain [14], significantly affecting individuals' cognitive, psycho-emotional, and depressive status [15]. Estrogen acts through estrogen receptors, a DNA binding transcription factor. Cellular sensitivity to estrogen is determined by the receptor level, and its down-regulation will significantly decrease estrogenic effects [16]. In addition, the changes in steroid concentration is also reflected in the expression level of connexin 43 (CX-43) at tissue level [17]. CX-43 is widely expressed in a variety of cell types including the ovary and involved in the formation of gap junctions.

Gap junctional intercellular connections (GJIC) are necessary for the growth, development, and differentiation of the ovary [18]. GJIC control the coordinated development of somatic granulosa cells, theca cells, and germinal oocytes [19]. GJIC are comprised of connexins, which regulate the spread of intercellular messengers through gap junctions and mediate the paracrine communication of molecules [20]. Crucial to these GJIC, CX-43 is widely

expressed in a variety of cells types, including the ovary. In healthy cells, the GJIC are tightly regulated [21]. However, this control is lost under pathological conditions [22–24] and the altered expression of CX-43 in follicles has also been reported to affect ovulation [25].

Estrogen in addition to acting through estrogen receptor, stimulate the endogenous nitrosothiol formation [26]. One of the most abundant endogenous S-nitrosothiols is GSNO, which governs many important physiological functions in vivo and is responsible for nitros(yl)ation of several important proteins [27]. Depletion or reductions in the levels of endogenous nitrosothiols under various neuropathological conditions makes GSNO an important therapeutic agent [28].

Hence, we hypothesized that exogenous administration of GSNO would hasten rats' recovery from SCI-induced transient arrest in estrous cycle and that this positive effect was mediated through the down regulation of CX-43 and up regulation of ER , in addition to the inhibition of NFkB.

Materials and Methods

Experimental Animals and Drug

Virgin female Sprague-Dawley rats (225–250 g) were housed individually under a 12-hour light/dark cycle. Food and water were available at all times. The animal procedures for the study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina. Animals exhibiting the same stage of the estrous cycle were subjected to SCI. Following SCI, each animal's estrous stage was determined every day until the animal showed at least two regular 4-day cycles.

Spinal Cord Injury

Contusion-induced SCI was performed under aseptic conditions by following the method described by Bilgen [29]. Animals were anesthetized with a ketamine and xylazine (90 and 10 mg/kg respectively) cocktail injected intraperitoneally. After induction, laminectomy was done at the level of the T8–10 vertebrae. To stabilize the spinal cord during the impact, the T8 and T10 vertebrae were held by stabilizing forceps. SCI had the following parameters: impactor tip—3 mm; duration—85 ms; depth—1 mm. After the injury, the muscles were closed in layers, and the incision was closed with polysorb 4.0. SCI animals were assigned to two groups: one (GSNO) received 0.05 mg/kg GSNO, through gavage 1 hour after SCI and every 24 hours thereafter until the end of the experiment; the other group (Veh) received phosphate buffered saline (PBS). The dose of GSNO used and method of administration followed in the present study is based on our previous studies on the neuroprotective effect of GSNO in rat animal model [30] In the present study, we only included animals that showed estrous arrest after SCI. Obviously some animals did not show estrous arrest following SCI and those were excluded from the study. Sham animals underwent all the procedures except spinal injury.

Estrous Stage Determination

Estrous stage was determined by following the method of Marcondes et al. [31]. Vaginal smear was taken every day between 11am and 12pm. A sample of cells was obtained from the vaginal canal by injecting 50 µl sterile saline solution and with-drawing the same with the help of a 100-µl pipette. The sample containing vaginal cells was placed on a glass slide and observed under the microscope (Olympus DP70). The estrous stage of the animal was determined based on the preponderance of epithelial cell types.

Histology

On post-SCI days 7 and 14, the animals were anesthetized with an excess dose of Nembutal (100 mg/kg). Transcardial perfusion was done with PBS, followed by 10% formalin. The ovaries of sham, vehicle, and GSNO-treated SCI animals were extracted and preserved in 10% formalin for further fixation. Sections for morphological studies were processed and stained with hematoxylin and eosin (H&E), as described previously [32].

Immunofluorescence

Following antigen unmasking as per the procedure of the supplier (Vector Laboratories, Burlingame, CA, USA), endogenous peroxidase activity was eliminated by treating the section with 3% hydrogen peroxide. Sections were blocked in TNT buffer with 0.5% blocking reagent (TNB, supplied with TSA-Direct kit; NEN Life Sciences, Boston, MA, USA) for 30 minutes and incubated overnight with anti ER α , ER β , CX43, and NF κ B antibodies (Sigma, St. Louis, MO, USA; 1:200) at 4°C. After washing with PBS, the sections were stained with Alexafluor 488 (Molecular Probes, Invitrogen, CA, USA) flurophore conjugated secondary antibody. The tissue fluorescence pattern was observed and recorded with a Leica TCS SP5 Laser scanning microscope (Leica Microsystems, Mannheim, Germany).

Western Blot

Western blot analysis was performed using standard procedures. Tissue was harvested and lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% NP-40) containing protease inhibitor cocktail (Boehringer, Mannheim, Germany). The lysate was centrifuged at $14,000 \times g$ for 20 minutes at 4°C. Total protein content was estimated by following BCA assay (Pierce, Rockford, IL, USA). Equal quantity of protein was resolved on 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham LifeSciences, Arlington Heights, IL). The membrane was blocked with 5% nonfat milk in Tween 20-Tris-buffered saline for 1 hour at room temperature. The membranes were incubated overnight at 4°C with primary antibodies at 1:1,000 dilution in blocking buffer (TTBS with 2% nonfat milk). After washing, the membranes were incubated with 1:10,000 diluted horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature, again washed, reacted with ECL reagent (Amersham Life Science), and subjected to autoradiography. β -actin expression level was used as an internal control for equal protein loading. Blots were scanned for densitometry using an Epson perfection 1,200 U scanner, transferred to Adobe Photoshop CS2, and the images were analyzed using the NIH Image J software.

Apoptosis

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay was performed by using an Apoptag fluorescein in situ apoptosis detection kit (Serological Corporation, Norcross, GA, USA) according to the manufacturer's protocol. The number of TUNEL-positive cells were counted after converting the images to 8-bit gray scale using the NIH Image J software.

Statistical Evaluation

Statistical analysis was performed using Graphpad prism 3.0 software. Values were expressed as mean $\pm$ SD of N determinations. *P* value less than 0.05 was considered statistically significant.

Results

The effect of GSNO in restoring the normal estrous cycle was determined by following the estrous cycle of SCI animals until the animals showed at least two regular 4-day cycles. Sham animals (N = 4) exhibited regular uninterrupted 4-day cycles (data not given). SCI animals treated with vehicle (N = 6) exhibited estrous arrest for 21 ± 1.41 days. GSNO (N = 7) treated animals restored a normal cycle in (12.86 ± 1.35 days). The difference between the groups was significant at 0.005%. The data are presented in Figure 1.

Representative ovarian histomorphology slides of SCI animals with or without GSNO treatment are given in Figure 2. Sham operated animals showed corpus luteum in the formation of corpus albicans (Figure 2A), and normal healthy ovarian follicles at different stages of development (Figure 2D). The vehicle group showed corpus luteum with varying degrees of degeneration and tissue sparing (Figure 2B). In addition, atretic follicles (Figure 2E) were also detected. GSNO treatment greatly reduced the degeneration and tissue sparing of corpus luteum (Figure 2C). The ovarian follicles were normal and healthy in the GSNO group (Figure 2F).

Both developing follicles (Figure 3A) and corpus luteum (Figure 3B) were observed for the incidence of TUNEL-positive cells on day 14 after SCI in all three groups (sham, vehicle, GSNO). Sham animals showed no apoptotic cells in either the developing follicles or the luteal cells. The Vehicle group showed a higher incidence of TUNEL-positive cells both in follicular and luteal cells. The GSNO group showed no TUNEL-positive cells in the follicular cells. However, a few TUNEL-positive cells were recorded among the luteal cells. The histogram (Figure 3C) represents the number of TUNEL-positive cells counted in the corpus luteum of different experimental groups. The vehicle group had significantly higher number of TUNEL-positive cells when compared to sham and GSNO groups. The results show that GSNO is anti apoptotic in the ovary of SCI animals.

Expression of NFkB increased in the ovary of SCI rats is given in Figure 4. Moderate level of NFkB in the ovary of sham animals (Figure 4A, B) was evident. The Vehicle group showed increased NFkB expression in the ovarian cells (Figure 4C, D). GSNO treatment reduced the expression of NFkB. (Figure 4E, F).

Results of immunofluorescence (day 14 after SCI) and Western blot analysis (day 7 and 14 after SCI) are represented in Figure 5A, B. We found increased CX-43 expression in the ovary of vehicle treated SCI animals by both immunofluorescence (Figure 5A) and Western blot assay (Figure 5B). Graphic presentation of the result is shown in Figure 5C. Expression of CX-43 was significantly higher in the vehicle group both on day 7 and 14 following SCI when compared to sham and GSNO groups. GSNO treatment greatly reduced the ovarian CX43 expression of SCI animals.

ERB protein expression level was determined by both immunofluorescence (day 14) and Western blot analysis (day 7 and 14), and the results are represented in Figure 6. Expression of ER- α was also tested. But the expression of the protein was not found to be affected by SCI (data not given). Immunofluorescence showed a decreased level of ER α expression on day 14 after SCI in the vehicle group. GSNO treatment (0.05 mg/kg body weight) significantly increased the expression of the protein (Figure 6A). Western blot analysis also shows the same trend of ER α protein expression. The vehicle group showed a significantly decreased level of ER α expression on both the days tested. GSNO treatment significantly increased the expression of ER α protein on day 7 and 14 after SCI (Figure 6B). From the results, it is evident that GSNO reduced the decrease in the level of ER α protein expression induced by SCI.

Discussion

In this study, we have demonstrated the therapeutic potential of GSNO in treating transient arrest in the estrous cycle following SCI in a rat animal model. GSNO hastened the recovery of the normal estrous cycle after SCI. The SCI-induced increase in ovarian CX-43 expression was decreased on GSNO treatment. At the same time, ovarian ER expression was increased by GSNO treatment. Furthermore, we showed that GSNO treatment decreased the level of apoptosis in the ovary of SCI rats. These results imply that GSNO has potential as treatment for SCI-induced transient amenorrhea.

Follicle development and ovulation is complex. Several factors along the HPO axis are involved in this process. Although transient amenorrhea following SCI subsides after a few months, even without the intervention of any drug, the potential longer-term impact of amenorrhea on the HPO axis merits investigation. Currently, the molecular mechanisms involved in transient amenorrhea have not been well characterized.

Despite the difference in duration, the rat estrous cycle resembles the human menstrual cycle at the physiological level [33]. The estrous cycle of rats is characterized by four stages: proestrus, estrus, metestrus, and diestrus [34,35]. As in humans, SCI alters the estrous cycle of rats [36]. Hence, we used female rats to study SCI-induced changes in the ovary. Staging of the estrous cycle was achieved by characterizing cell types in the vaginal smear preparation [31]. In virgin SCI rats, the SCI-induced estrus arrest lasted for 21 ± 1.41 days in the vehicle and 12.83 ± 1.35 days in the GSNO treated group. The period of estrus arrest observed in the present study corroborates earlier reports of Hubscher et al. [36].

Mammalian ovaries are innervated by spinal nerves [7] and the abdominal branches of the vagus nerve [8]. SCI interrupts anterograde information transport from gonads to brain [9], which interrupts the release of gonadotropin from the hypothalamus, resulting in hypogonadism of SCI patients [37]. Even though the ovaries are supplied with nerve branches from both the vagus nerve and the spinal cord, the resumption of normal ovarian function after SCI could be mainly due to the preservation of neuronal tissue of the spinal cord. This hypothesis is based on the fact that both estrous and hind limb functional recovery occur around the same time as estrous resumption following SCI [38]. However, in the absence of influence from the spinal cord, the vagus nerve may play an important role in keeping ovarian structures responsive to the signals from the spinal nerves once they resume.

NFkB is a ubiquitous heterodimeric transcription factor that is sequestered in the cytoplasm by the inhibitor of kappa B (IkB) proteins. Degradation of IkB allows translocation of NFkB into the nucleus and subsequent activation of its target genes. Activated nuclear NFkB is often found at sites of inflammation, and NFkB pathways play important roles in the inflammatory response [10]. In the present study, the ovaries of SCI rats showed increased expression of NFkB when compared to the sham group of animals. GSNO treatment significantly reduced the expression of NFkB in the ovarian follicle.

As paracrine signaling is equally important to endocrine signaling, we next examined how NFkB-mediated signaling is conveyed among the cells of the ovary. In the ovary, GJIC is mediated by CX-43. In addition, the involvement of CX-43 in follicular maturation is well established [39–42]. Therefore, we hypothesized that SCI-induced ovarian pathology would be associated with an increase in CX-43 protein expression. In this study, we did observe this up-regulation of the CX-43 protein without any significant difference in phosphorylation level (p S-368; data not shown). This observation corroborates earlier reports showing up regulated CX-43 protein with no differences in phosphorylation levels [43]. Phosphorylation at serine residues of CX-43 results in the loss of gap junctional

communication [44]. The increased CX-43 protein with no concomitant phosphorylation observed in the present study denotes an increased level of communication among the ovarian cell types and a means to spread the apoptotic signal. We have also observed increased NFkB expression in the ovary of vehicle-treated SCI rats. On nuclear translocation, NFkB has been shown to bind to the promoter of the CX-43 gene and to activate its transcription. Hence, the increased CX-43 protein expression in the ovary of SCI rats may occur through the NFkB pathway [43]. The increased CX-43 level may reflect increased GJIC between the cells that could spread apoptotic signals to the neighboring cells of the ovary of SCI animals. In the present study, we found the inhibition NFkB expression in GSNO-treated animals. This inhibition may also occur due to the nitrosylation of NFkB by GSNO [45].

Estrogen influences NO, NOS, and NOS gene expression [46,47]. Variations in the level of NOS, and hence the level of NO, with stages of the rat estrous cycle [48] and the human menstrual cycle [49], have been reported. NO also directly inhibits apoptosis in estrogen-targeted cells [50]. Hence, the faster resumption of the estrous cycle in the GSNO-treated rats may occur through the modulation of NO levels. Moreover, the reduction in the level of apoptotic cells observed in the ovary of GSNO-treated SCI animals may occur through modulation of NO level.

The functional status of various constituent cell types of the ovary results in an altered HPO axis. SCI also alters the HPO axis [36]. Hence, the decrease in the expression of ER observed in the ovary of SCI animals in the present study may also be a consequence of altered levels of sex hormone. The cellular response of the ovary to estradiol depends on the concentration of estrogen receptors. In the present study, ER expression level was significantly decreased in the ovary of SCI animals. GSNO treatment significantly increased the expression of ER. However, the mechanism of the observed GSNO-mediated increase in ER expression level is yet to be determined.

Conclusion

In the present study, we have shown a faster recovery from SCI-induced transient arrest in the estrous cycle of GSNO-treated SCI rats. GSNO also blocks the nuclear translocation of NFkB in the ovarian cells, decreases the Cx-43 expression and increases the ER expression in the ovaries of SCI rats. The therapeutic potential of GSNO for stroke, traumatic brain injury, and SCI animal models has been shown already. Here, we have shown that GSNO is also effective in recuperating rats from SCI-induced transient arrest in estrous cycle. Our studies suggest that GSNO holds therapeutic value to SCI patients on multiple levels, offering neuroprotection as well as restoration from sexual dysfunction. Collectively, low-dose GSNO supplementation may provide an effective therapeutic approach to restore the normal ovulation process in SCI patients of reproductive age.

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Figure 1.

GSNO augments the recovery from transient arrest in estrous cycle induced by SCI. Rats after SCI were divided into two groups. One received GSNO at a daily dose of 0.05 mg/kg body weight and the other saline. Estrous cycle was determined by vaginal smear method as described in material and methods. There was a significant difference in the recovery of estrus cycle between vehicle and GSNO group. Vehicle group acquired regular estrous cycle after 21 ± 1.41 days, while GSNO group in 12.83 ± 1.35 days (N: vehicle = 6; GSNO = 7; $**P < 0.001$ vs. Vehicle). Estrus cycle of sham operated animals (N = 4) was not affected and the animals showed regular 4 day cycle (data not shown).

Figure 2.

GSNO mediated structural changes in the ovary of SCI rats. Ovary of sham (A, D), vehicle (B, E) and GSNO (C, F) treated SCI animals were observed 14 days after injury following H&E staining. Sham animals showed degeneration of corpus luteum leading to the formation of corpus albicans (A). Vehicle treated ovary of SCI rats showed defective corpus albicans formation in addition to tissue sparing (B). Ovary of GSNO treated SCI rats show no tissue degeneration and the cellular architecture resembles that of sham animals (C). Sham animals also showed many ovarian follicles in the secondary follicle stage (D). Atrophying secondary follicles were evident with ovary of vehicle treated SCI animals (E). GSNO treated SCI animals showed normal healthy secondary follicles (F). Each photograph is a representative of N = at least 4 in each group.

Figure 3. GSNO is effective in inhibiting SCI induced cellular apoptosis in the ovary of rats. (A) TUNEL-positive cells in the ovarian follicle of sham operated and vehicle and GSNO treated SCI rats. Increased number of TUNEL-positive cells was evidenced in vehicle group. Sham and GSNO group showed no apoptotic follicular cells. Lower panel shows the DAPI stained respective sections of the ovary. (B) TUNEL-positive cells in the corpus luteum of sham, vehicle and GSNO group of rats. Corpus luteum of vehicle group showed higher incidence of apoptosis when compared to sham and GSNO group. Lower panel shows the DAPI stained respective sections of the ovary. (C) The number of TUNEL-positive in the corpus luteum were quantified blindly in at least 10 different microscopic fields ($N = 4$ in each group) and averaged. Data are represented as mean $\pm$ SD. $P < 0.001$ as compared with sham.

Figure 4.

Nuclear translocation of NFkB in the ovary of SCI rats. Ovary of sham and SCI rats treated with vehicle and GSNO for 14 days was immunolabelled with anti NFkB antibody. In sham operated animals, the NFkB was compartmentalized to cytoplasm (A&B). SCI and ensuing transient arrest in estrous cycle induced nuclear translocation of NFkB (C&D). Nuclear translocation of SCI induced NFkB in the ovary was inhibited by GSNO treatment (E&F). Each photograph is a representative of N = at least 4 in each group. (A, C, E; 200 $\times$; B, D, F; 400 $\times$).

Figure 5.

GSNO induced down regulation of CX-43 expression in ovary of SCI rats. SCI animals were divided into two groups. One group received GSNO (0.05 mg/kg body wt); the other received the same quantity of saline. Sham animals also received saline. (A) Ovary of sham, vehicle and GSNO group or SCI rats stained with CX-43. Ovary of different groups extracted 14 days after SCI was immunostained following the routine procedure. Bottom row shows the DAPI staining of the respective sections. Increased level of CX-43 expression was seen with the ovary of vehicle treated SCI rats. Administration of GSNO at a dose of 0.05 mg/kg greatly decreased in CX-43 expression. (B) Western blots showing the expression pattern of CX-43 in the ovary of SCI rats treated with vehicle and GSNO. Tissues extracted on day 7 and 14 after SCI were resolved on 4–20% SDS PAGE and probed with anti CX-43 antibody. Loading volume was determined by B-actin expression level. CX-43 protein expression was up regulated both on day 7 and 14 in vehicle group.

GSNO treatment reversed the increase in CX-43 up regulation seen with ovary of SCI rats (N: vehicle = 6; GSNO = 7). (C) Densitometry analysis of autoradiographs from at least 3 animals in each group. Data are represented as percentage over sham. *** $P < 0.001$.

Figure 6.

ER expression in ovaries of SCI rats. SCI animals were grouped into two. One group received GSNO (0.05 mg/kg body wt); the other and the sham animals received saline. (A) Ovary of sham, vehicle, and GSNO group or SCI rats stained with anti ER antibody. Ovary of different groups extracted 14 days after SCI was immunostained following the routine procedure. Decreased level of ER expression was seen with the ovary of vehicle treated SCI rats. Administration of GSNO at a dose of 0.05 mg/kg greatly increased in ER expression. (B) Western blots showing the expression pattern of ER in the ovary of SCI rats treated with vehicle and GSNO. Tissues extracted on day 7 and 14 after SCI were resolved on 4–20% SDS-PAGE and probed with anti ER antibody. Loading volume was determined by B-actin expression level. ER protein expression was down regulated both on day 7 and 14 in vehicle group. GSNO treatment reversed the decrease in ER down regulation seen with ovary of SCI rats (N: vehicle = 6; GSNO = 7). (C) Densitometry analysis of autoradiographs from at least three animals in each group. Data are represented as percentage over sham. * $P < 0.05$, *** $P < 0.001$.