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# Pre-emptive Use of Riboflavin in a Rat Model of Bilateral Cavernous Nerve Injury

## Bilateral Kavernozal Sinir Hasarı Sıçan Modelinde Preemptif Riboflavin Tedavisinin Etkileri

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**What's known on the subject? and What does the study add?**

Penile rehabilitation is an important approach for patients after nerve-sparing radical prostatectomy. Many treatment strategies are being applied aiming rapid healing for erectile function. Riboflavin has never been used for penile rehabilitation before. This study supports riboflavin use for penile rehabilitation, especially in preemptive approach.

## Abstract

**Objective:** Erectile dysfunction is commonly encountered after radical prostatectomy due to cavernous nerve injury (CNI). We investigated the effects of riboflavin (Rb) on bilateral CNI in a rat model.

**Materials and Methods:** Twenty-four male rats were divided into four groups: control (C), patients with bilateral CNI, those with CNI receiving postoperative Rb treatment (CNI+Rb), and those with CNI receiving pre- and post-operative Rb treatment (Rb+CNI+Rb). Bilateral CNI was performed in all groups except for C. The CNI+Rb group was treated with 30 mg/kg Rb daily after CNI for two weeks; the Rb+CNI+Rb group was treated with 30 mg/kg Rb daily one week before CNI and then for two weeks after injury. Mean arterial pressure (MAP) and intracavernosal pressure (ICP) were measured 14 days after CNI in all groups. Tissue malondialdehyde, cyclic guanosine monophosphate, nerve growth factor, superoxide dismutase and total nitric oxide synthase (NOS) activities, neuronal NOS (nNOS) and inducible NOS (iNOS) were analyzed.

**Results:** ICP/MAP ratio was significantly lower in the CNI ($p<0.01$) and CNI+Rb groups ($p<0.05$) compared to the control group, however, the Rb+CNI+Rb group had results comparable to the C group in terms of nNOS and iNOS expression in the Western Blot analysis.

**Conclusion:** Rb exerted anti-oxidative and anti-inflammatory effects on CNI in a CNI rat model. Rb can be a potential beneficial agent to improve erectile function in nerve-sparing radical prostatectomy patients as a preemptive penile rehabilitation strategy, although further clinical studies are needed.

**Keywords:** Cavernous nerve injury, Erectile dysfunction, Oxidative stress, Riboflavin

## Öz

**Amaç:** Çalışmamızın amacı radikal prostatektomi modelinde erektil disfonksiyon tedavisinde riboflavinin (Rb) olası etkilerinin araştırılmasıdır.

**Gereç ve Yöntem:** Yirmi dört erkek sıçan kontrol (K), bilateral kavernoz sinir hasarı (KSH), KSH+Rb ve Rb +KSH+ Rb olmak üzere 4 gruba ayrıldı. Ön-tedavili KSH grubunda cerrahiden 1 hafta önce başlayarak toplam 3 hafta ve KSH+Rb grubuna ise cerrahiden sonra 2 hafta süreyle 30 mg/kg dozunda Rb uygulandı. Deney sonunda intrakavernosal basınç (İKB) ve ortalama arteryel basınç (OAB) ölçümü yapıldı. Kavernoz doku örneklerinde malondialdehit (MDA), siklik guanozin monofosfat (c-GMP) ve sinir büyüme faktörü (NGF) düzeyleri ile süperoksid dismutaz (SOD) ve total nitrik oksit sentaz (NOS) aktiviteleri ölçüldü. Dokuların indüklenebilir NOS ve nöronal NOS protein ekspresyonları Western blot yöntemi ile tayin edildi.

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## Öz

**Bulgular:** KSH, İKB/OAB değerlerinde anlamlı azalmaya (p<0.01) neden olurken preemptif Rb tedavili grupta bu değerler kontrol grubu değerlerine yaklaşmış olarak bulundu. KSH grubunda doku MDA düzeylerinin ve NOS aktivitesinin anlamlı olarak arttığı, c-GMP ve NGF düzeyleri ile SOD aktivitesinin anlamlı olarak azaldığı belirlendi. KSH sonrası uygulanan Rb tedavisi bu parametrelerdeki değişimleri geri çevirirken preemptif Rb tedavili gruplarda bu değerler kontrol gruba benzer düzeylere geldi.
**Sonuç:** Çalışmamızda Rb, hasarlı kavernoz dokuda antioksidan ve anti-enflamatuvar etki göstermiş ve böylece dokuyu koruyarak erektil fonksiyon kaybında iyileşme göstermiştir. Özellikle preemptif Rb kullanımının faydalı bir tedavi yaklaşımı sağlayabileceğini düşündürmekte olup insan çalışmalarıyla bu yaklaşım desteklenebilir.
**Anahtar kelimeler:** Kavernoz sinir hasarı, Erektil disfonksiyon, Oksidatif stres, Riboflavin

## Introduction

More than one million men are diagnosed with prostate cancer worldwide, leading to more than 300.000 deaths each year (1). Many patients who had radical prostatectomy (RP) for prostate cancer experience side effects, one of which is the post-operative erectile dysfunction (ED) (2). Cavernous nerve injury (CNI) is the reason behind ED after RP (3). Even though different surgical techniques and modifications of RP have been studied to maintain erectile function (EF), the cavernous nerve (CN) is often injured by manipulations such as traction, compression or thermal damage. Regeneration of neural tissues after neural injury following RP is limited and happens over a very long period of time. Moreover, only a very small proportion of men who were preoperatively potent have spontaneous erections after RP (4).

Nitric oxide synthase (NOS), nitric oxide (NO), soluble guanylyl cyclase (sGC) and cyclic guanosine monophosphate (cGMP) have major roles in penile erection physiology. NO is synthesized in the CN via neuronal NOS (nNOS) (5). NO, which is a ubiquitous neurotransmitter, consists of L-arginine and is released by nerve terminals. It then spreads into the neighboring smooth muscle cells and induces sGC, and this process increases the intracellular level of cGMP. Hence, the relaxation of smooth muscle in the corpus cavernosum as well as the penile arterioles is triggered by L-arginine. Meanwhile, phosphodiesterase type 5 inhibitors (PDE5i) prevent the catabolism of cGMP, which is the major supporting agent for penile erection (6). The pathophysiology behind CNI is not only nerve damage but also the accompanying oxidative stress (7), as the main cause of nerve damage is the increase in reactive oxygen species due to a decrease in NO. Superoxide radicals augment apoptosis, cause formation of peroxynitrite and can lead to dysfunction of the endothelium (5,8,9). Peroxynitrite, which stimulates superoxide dismutase (SOD), produces ineffective relaxation of smooth muscle and can stimulate adhesion of platelets to endothelium. Thus, endothelial dysfunction and increased NO destruction result in corporal veno-occlusive dysfunction and impairment of EF (5,6).

The nerve-sparing RP technique, which aims to protect the CNs and thereby provide penile innervation via regulation of the vascular homeostasis of the penis, maintains EF effectively (10). Other techniques or approaches include nerve grafting (11), nerve reconstruction (12), pharmacological neuromodulation using immunophilins (13), neuroprotection using erythropoietin (14), electro-stimulation of the CN or pelvic ganglion (15), regulation of FK506-binding protein (16) and transferring of the herpes simplex virus vector (17). Studies seeking to preserve EF peri-operatively have examined tissue healing procedures such as muscle-derived cell injection (18), neuronal embryonic stem cell injection (19), intracavernous injection of adipose-derived stem cells (20), inhibition of neuronal inflammation or neuronal cell death using neurotrophic and growth factors (21,22), and penile rehabilitation with PDE5i (4).

Riboflavin (Rb) is water-soluble B-complex vitamin that plays a role in various metabolic pathways and redox reactions via active coenzymes, flavin adenine dinucleotide and flavin mononucleotide (23). Rb protects tissues against neurotoxicity by alleviating oxidative stress, mitochondrial dysfunction, neurologic inflammation, glutamate and homocysteine toxicity (24).

The aim of this study was to investigate the essential role of Rb as an antioxidant and anti-inflammatory agent against ED in a bilateral CNI model in rats.

## Materials and Methods

### Animals and Experimental Design

Adult male Sprague-Dawley rats (250-300 g) were housed at a temperature-controlled room (22±2 °C) with a 12-hour light-dark cycle. Marmara University Animal Experiments Ethics Committee approved the study (number 78.2015.mar).

The rats were randomly divided into four groups with 6 rats in each: group 1, control (C) group, in which the rats underwent sham surgery and received carboxymethylcellulose (CMC) 0.5% as vehicle/solvent for intraperitoneal dosing; group 2, in which the rats underwent surgery to induce CNI and received CMC

0.5% only; group 3, CNI+Rb group, in which the rats underwent surgery for CNI induction and received Rb (30 mg/kg/day ip) for two weeks (25); and group 4, Rb+CNI+Rb group, in which the rats received Rb (30 mg/kg/day ip) before CNI induction then received Rb again (30 mg/kg/day ip) following the surgery. For the CNI and C groups, in which the sham operation was performed, CMC solvent was applied for 15 days following the operation. In the pre-treatment CNI group, Rb was applied at a dose of 30 mg/kg/day ip for a total of three weeks starting one week before surgery and continuing for two weeks after the surgery in the CNI+Rb group. Rb was dissolved in 0.5% CMC. Rb and CMC were purchased from Sigma Aldrich (St. Louis, MO, USA).

At the end of the experiment, under general anaesthesia, intracavernosal pressure (ICP) and mean arterial pressure (MAP) were measured and then cavernosal tissue samples were obtained for biochemical and histological analyses.

### Cavernous Nerve Stimulation and Intracavernosal Pressure/ Mean Arterial Pressure Measurement

To induce CNI, in all surgical procedures, the animals were anaesthetized with ketamine (100 mg/kg) and xylazine (6-9 mg/kg). After the animals were anaesthetized, following the shaving of the abdominal wall, a lower midline incision was made. The major pelvic ganglion and the CN, a distinct structure arising from the ganglion and running caudally along the prostate in a groove between the urethra and rectum, were detected as previously described (26). A vascular bulldog clamp was applied to each CN, 5 mm distal to the ganglion. It was applied for 30 seconds, removed for 30 seconds, and then reapplied for an additional 30 seconds. Two weeks after the induction of CNI and sham operation, all animals were anaesthetized as described previously, and they underwent an operation during which electro-physiological erection assessment was conducted. With a transverse neck incision, subcutaneous layers and the underlying muscles were separated. Dissection and subsequent cannulation of the left internal carotid artery with a heparinized polyethylene-50 tube, connected to a pressure transducer and an amplifier unit (COMMAT Pharmacology & Physiology Instruments, Ankara, Turkey) were performed. The amplifier which was connected to a module for data acquisition (MP 35 data acquisition system, Ankara, Turkey) allowed the MAP to be recorded using Biopac Student Lab PRO recording software (Biopac Systems Inc., Goleta, CA, USA). At the junction of the penis and pubic arch, dissection of the ischiocavernous muscle was performed and the penile tunica albuginea was visualized. ICP measurement was performed with a 24-gauge needle placed into the left penile crus which was connected to a transducer by a heparinized polyethylene-50 tube. The laparotomy incision was extended below until the penile root. The CN was located bilaterally which is situated on both sides of the prostatic tissue. Following CN dissection with a micromanipulator, a stainless steel bipolar electrode with 1 mm-apart parallel hooks was placed distally to the major pelvic ganglion. The electrode cable was connected to a STPTO2-A stimulator (COMMAT Pharmacology & Physiology Instruments, Ankara, Turkey). The stimulation parameters were 1.5 mA, 20 Hz, pulse width 5 milliseconds, 35 milliseconds delays and 7.5 volts for 60 seconds each. Stimulation of the CN was performed and the data were individually recorded. The maximum ICP/MAP ratio was calculated and reported as percentage (26,27).

### Biochemical Analysis

### Measurement of Tissue Malondialdehyde Levels

Corpus cavernosal tissue samples were homogenized with ice-cold potassium chloride (150 mM) to determine MDA levels which shows the level of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as previously described. The results were expressed as mmol MDA/mg protein (24).

### Measurement of Superoxide Dismutase Activity

SOD activity was measured according to the method described by Tavukcu et al. (28). A standard curve was prepared with bovine SOD (3000 U; S-2515; Sigma, St. Louis, MO, USA) as a reference. Absorbance readings were taken at 0 and 8 minutes of illumination, and the net absorbance was calculated (27).

### Measurement of Cyclic Guanosine Monophosphate Levels

Amounts of cGMP in frozen tissue were determined in duplicate using an ELISA kit, according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale NY, USA). A total protein assay was performed using the Bradford method. cGMP values were given as pmol/mg protein (27).

### Measurement of Nerve Growth Factor Levels

Measurement of nerve growth factor (NGF) was detected by sandwich-ELISA according to the manufacturer's instructions (Chemicon International Inc., Temecula, CA, USA). A total protein assay was performed using the Bradford method (29). NGF values were given as pg/mg protein (27).

### Measurement of Tissue Nitric Oxide Synthase Activities

Tissue samples were homogenised with phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 10.000 x g at 40 $^{0}$C. Supernatant was used for the NOS activity assay to determine the levels of NOS activity (EnzyChrom, BioAssay Systems, Hayward, CA, USA), following the manufacturer's protocol. According to the assay, one unit of NOS catalyzes the production of 1 µmole of NO per minute under the assay conditions (pH 7.5 and 37 $^{0}$C). NOS activity was given as U/mg protein (27).

### Western Blot Analysis for Inducible Nitric Oxide Synthase and Neuronal Nitric Oxide Synthase

Western blot analysis and measurement was performed for inducible NO synthase (iNOS) and nNOS release as previously described (30). The Bradford assay was used to detect protein concentrations in homogenized samples (29). Afterwards, 25 µg of protein was resolved in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transferred to nitrocellulose membrane (sc-3718, Santa Cruz Biotechnology) which was blocked with 5% non-fat skim milk powder (Sigma, 70166) in Tris-buffered saline (TBS) and which was washed twice in (TBS+tween) TBST (TBS containing 0.1% Tween-20) and incubated overnight with primary antibody (1:500 monoclonal rat anti-iNOS, sc-651 anti-nNOS, sc-648, anti-b-actin, sc-47778, Santa Cruz Biotechnology). The membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 goat anti-mouse IgG1-HRP, sc-2060 and goat anti-rabbit IgG-HRP sc-2004, Santa Cruz Biotechnology) for 2 hours. Chemiluminescence reagents (sc-2048, Santa Cruz Biotechnology using a Chemiluminescent Imaging System, Syngene, USA) were used to detect the blot. Data were analyzed using the ImageJ OD analysis software. Signals were normalized with respect to β-actin.

### Statistical Analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego; CA; USA) was used for the statistical analyses. All data were expressed as means ± standard error of mean (SEM) Data groups were compared using an analysis of variance followed by Tukey's multiple comparison tests. A two-tailed p value of less than 0.05 was considered statistically significant.

## Results

ICP/MAP ratio was significantly lower in the CNI ($p<0.01$) and CNI+Rb groups ($p<0.05$) compared to that in the C group, however, the Rb+CNI+Rb treated group showed similar results to the C group (Figure 1).

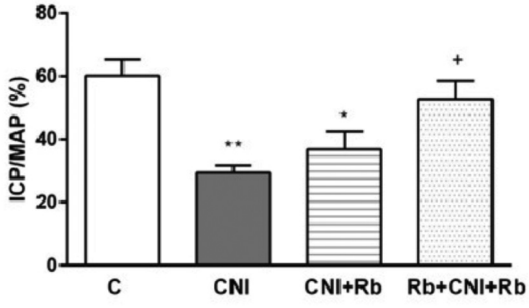


**Figure 1.** Intracavernosal pressure/mean arterial pressure results of all groups

*C: Control, CNI: Cavernosal nerve injury, Rb: Riboflavin. *p<0.05, **p<0.01: compared to group C, +p<0.05: compared to CNI*

MDA levels were significantly higher ($p<0.01$) in the CNI group, while treatment with Rb significantly reduced the MDA levels in the CNI+Rb and Rb+CNI+Rb groups, which achieved similar levels to that in the C group (Figure 2a).

SOD activity was significantly lower ($p<0.05$) in the CNI group when compared with the C group, while the Rb+CNI+Rb group showed no significant difference from the C group. However, SOD levels were significantly higher ($p<0.01$) in the Rb+CNI+Rb group than in the CNI group (Figure 2b). The total NOS activity was significantly higher ($p<0.001$) in the CNI group than in the C group, while NOS activity in the treatment groups was comparable to that in the C group and significantly lower than in the CNI group (Figure 3a). cGMP and NGF levels were significantly lower ($p<0.001$) in the CNI group when compared with the C group; moreover, the treatment groups had significantly higher levels ($p<0.001$) than the CNI group (Figure 3b, 4). Another important finding was that the Rb+CNI+Rb group demonstrated significantly higher NGF levels than the CNI+Rb group ($p<0.05$).

In the Western blot analysis for iNOS and nNOS, the Rb+CNI+Rb group demonstrated results similar to that in the C group, while the CNI group had significantly higher levels of iNOS and lower levels of nNOS (Figure 5).

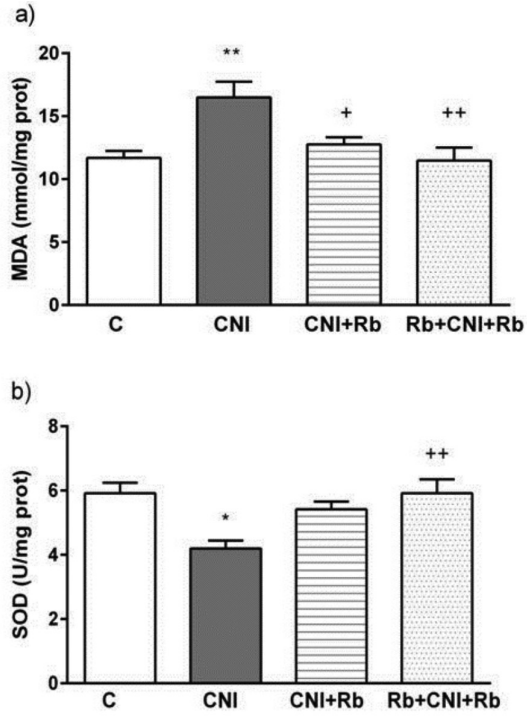


**Figure 2.** Cavernosal tissue analysis of; a) Malondialdehyde levels, b) Superoxide dismutase activity

*C: Control, CNI: Cavernosal nerve injury, Rb: Riboflavin. *p<0.05, **p<0.01: compared to group C, +p<0.05, ++p<0.01: compared to group CNI*

Histological analysis of cavernosal tissues revealed that the C group demonstrated good alignment of smooth muscle bundles and the endothelium (Figure 6a), whereas in the CNI group, endothelial deterioration was prominent in addition to mild accumulation of leukocytes (Figure 6b). In the CNI+Rb group, the endothelium showed moderate regression of degeneration (Figure 6c), whereas the Rb+CNI+Rb group demonstrated good regeneration of the endothelium (Figure 6d).

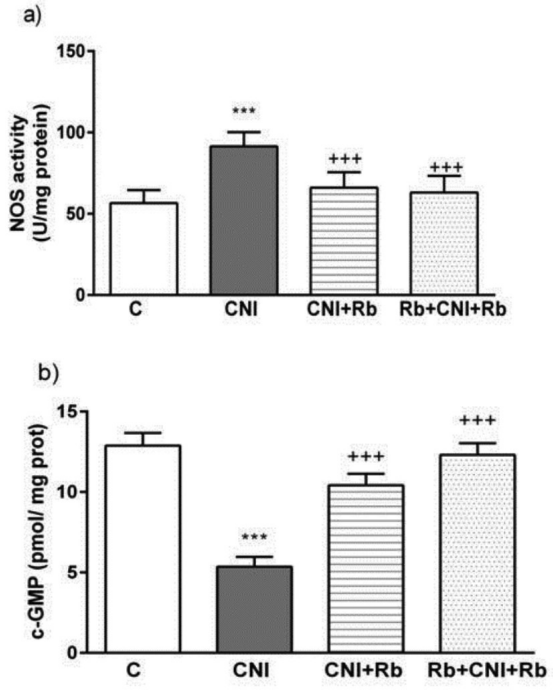


**Figure 3.** Cavernosal tissue analysis of; a) Nitric oxide synthase activity and, b) c-GMP levels

*C: Control, CNI: Cavernosal nerve injury, Rb: Riboflavin, cGMP: cyclic guanosine monophosphate, ***p<0.001: compared to group C, +++p<0.001: compared to group CNI*

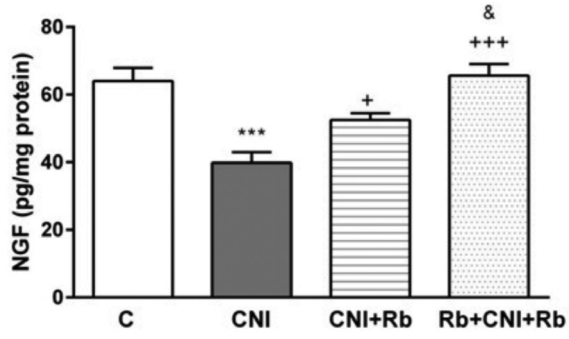


**Figure 4.** Nerve growth factor levels in cavernosal tissue

*C: Control, CNI: Cavernosal nerve injury, Rb: Riboflavin, NGF: Nerve growth factor, ***p<0.001: compared to group C, +p<0.05, +++p<0.001: compared to group CNI, &p<0.05: compared to group CNI+Rb*

## Discussion

The current study indicates that CNI leads to inflammatory and oxidative damage in erectile tissue, with increased MDA levels in the cavernous tissue and decreased SOD activity. The elevated oxidative injury results in a reduction of the tissue anti-oxidant enzymes (7). In addition, while cGMP levels decreased, NOS activity increased, and both parameters are important for EF. Furthermore, the findings of the study clearly demonstrate that Rb reverses these changes to the C level and protects cavernous tissues against CNI-mediated tissue damage.

We also determined NOS activities and cGMP levels, which are the main components of the NO/cGMP signaling pathway in

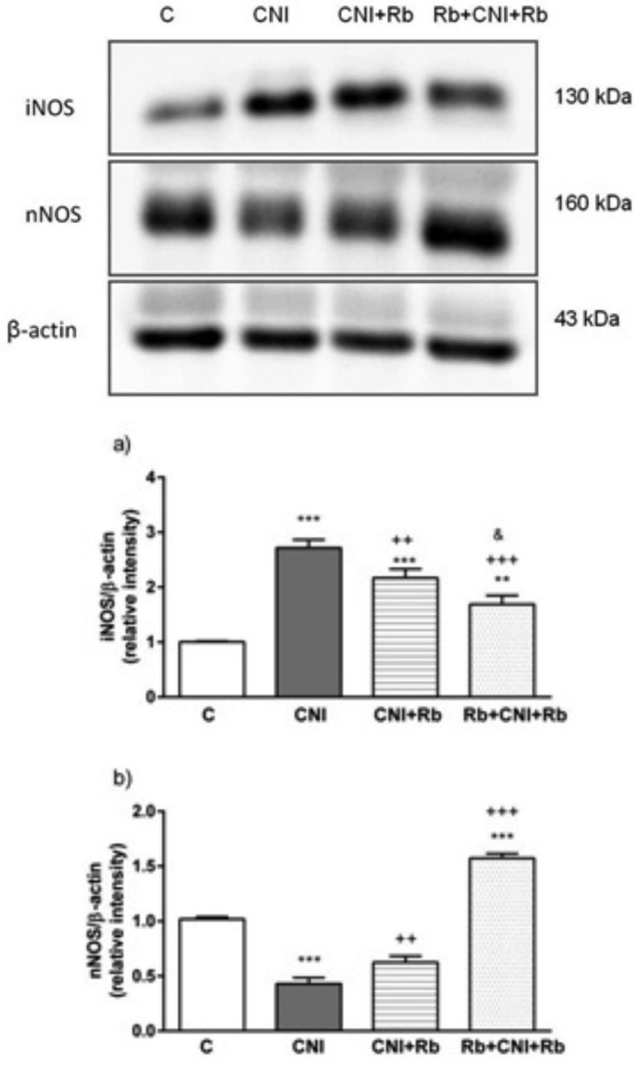


**Figure 5.** Western blot analysis in cavernosal tissue, a) inducible nitric oxide synthase ve b) neuronal nitric oxide synthase protein expressions

*C: Control, CNI: Cavernosal nerve injury, Rb: Riboflavin, nNOS: Neuronal nitric oxide synthase, iNOS: inducible nitric oxide synthase, **p<0.01, ***p<0.001: compared to group C, ++p<0.01, +++p<0.001: compared to group CNI, &p<0.05: compared to CNI+Rb group*

the cavernous tissue. We observed increased total NOS activity in CNI rats (Figure 3a). As shown by Western blot analysis, the drop in nNOS expression was associated with nerve fiber injury, compatible with previous studies (Figure 5) (16,19,31,32). Besides an elevation in iNOS expression, NGF levels also decreased, indicating that there was an apoptotic process in the CN fibers (Figure 4, 5) (12). With regard to a previous study, the increase in iNOS expression was normalized nearly to the C group by Rb, which was prominent in the Rb+CNI+Rb group (6). There was also a significant difference in iNOS expression between the Rb+CNI+Rb and CNI+Rb groups (Figure 5a; $p<0.05$). The increase in total NOS activity could be related to the elevated iNOS expression in the CNI group.

NGF, which is the first discovered neurotrophic factor and signaling molecule, has important neuroprotective effects against several diseases (12,33). We showed that NGF levels were significantly decreased after CNI, which could result in the development of ED. The current study showed that the Rb-treated pre-injury group (Rb+CNI+Rb) had significantly higher NGF levels than the CNI and CNI+Rb groups.

Similar to previous studies, the ICP/MAP values in CNI rats significantly decreased in our study (7,31). Furthermore, the Rb-treated groups had higher ICP/MAP values than the CNI group. The Rb+CNI+Rb group showed statistically significant differences from the CNI group and had values comparable to the C group (Figure 1). These data support the use of Rb for penile rehabilitation before CNI, as proven through biochemical analysis.

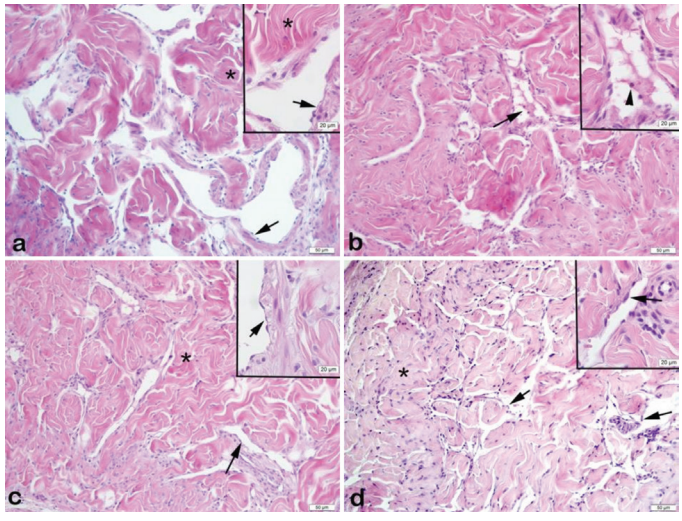


**Figure 6.** Histological analysis of cavernosal tissues, a) C group; smooth muscle bundles (*) surrounding vascular spaces, regular endothelial alignment (arrow). b) Cavernous nerve injury group; the prominent deterioration of vascular spaces and endothelium (arrow), sparsely observed leukocyte (arrowhead). c) Cavernous nerve injury + riboflavin group; the vascular endothelial cells have foamy cytoplasm (arrowhead) but vascular spaces were cleared away from deterioration (arrow), regular muscle bundles (*). d) Riboflavin + cavernous nerve injury + riboflavin group, clearly observed vascular spaces with regular endothelium cells (arrows), well-organized smooth muscle bundles (*)

Nevertheless, all penile rehabilitation strategies with PDE5i suggest the use of these agents after RP, following daily or on-demand protocols (34). No reported data was found except our previous study on the pre-injury treatment and pre-emptive penile rehabilitation, which is the first reported study on the data of pre-emptive penile rehabilitation before bilateral CNI in rats (35). According to these study results, pre-emptive penile rehabilitation with sildenafil (low- and high-dose) before bilateral CNI did not show a significant change in EF outcomes. Thus, our present study is the first one to demonstrate significant differences due to pre-emptive penile rehabilitation with Rb in CNI rats. Moreover, this is the first study in which Rb has been used for penile rehabilitation in a CNI rat model. The results of the current study show that Rb has protective effects on cavernous tissue after CNI.

### Study Limitations

We only evaluated the effects of Rb in a CNI model; one arm of the study might be ordered with PDE5i and alone.

## Conclusion

According to the results of this study, pathogenic CNI-induced ED causes oxidative stress to the cavernous tissue, and Rb can prevent injury to the erectile tissue. Furthermore, the results of this study suggest that preemptive use of Rb has a positive effect on oxidative parameters and improves all parameters towards the control levels. Rb can be considered a potential preventive agent in subjects undergoing RP, if supported with further studies, alone or with PDE5i.

### Ethics

**Ethics Committee Approval:** Marmara University Animal Experiments Ethical Committee approved the study (number 78.2015.mar).

**Informed Consent:** None (Animal experiment).

**Peer-review:** Externally and internally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: M.E., H.H.T., T.E.Ş., G.Ş., Concept: M.E., H.H.T., T.E.Ş., G.Ş., Design: M.E., H.H.T., T.E.Ş., G.Ş., Data Collection or Processing: Ö.Ç., N.B., Ö.A., Ş.Ç., G.Ş., Analysis or Interpretation: Ö.Ç., N.B., Ö.A., Ş.Ç., G.Ş., Literature Search: H.H.T., T.E.Ş., M.E., Writing: H.H.T., T.E.Ş., G.Ş.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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