

Effect of riboflavin on rat bladder contractility and oxidant damage following ischemia/reperfusion

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ABSTRACT: Ischemia/reperfusion (IR) encompasses the processes of organ function damage and oxidative injury depending on the successive blood flow obstruction and removal of the obstruction. Known as a vitamin, riboflavin (Rb) is known to be protective against tissue damage with its antioxidant and anti-inflammatory properties. The aim of this study is to investigate the effect of Rb treatment on bladder contraction dysfunction and tissue damage due to IR. The study was conducted with forty Sprague-Dawley rats. The abdominal aorta of anaesthetized rats was occluded to induce ischemia (60 min) and then allowed reperfusion (60 min). Rb (25 mg/kg), n-acetylcysteine NAC (100 mg/kg) or saline was administered orally 15 min before the IR model immediately. The bladder was assessed by biochemical and histological analysis, and the contractility of the I/R-related bladder was detected by organ bath. Compared to the control group, MDA, MPO and caspase-3 activities increased in the IR group, while GSH levels decreased. MDA, GSH and caspase-3 activities were reversed with Rb treatment, but there was no change in MPO level. Healing of IR-induced edema and oxidant damage with Rb and NAC treatment resulted in improvement of the thinning of the bladder wall. According to these results, it can be said that Rb therapy can regulate IR-induced bladder dysfunction by improving antioxidant properties and tissue damage.

KEYWORDS: Ischemia-reperfusion; riboflavin; bladder; contractility response

1. INTRODUCTION

Ischemia-reperfusion (I/R) injury impairs bladder contraction and relaxation functions [1]. Ischemia is a pathological condition that expresses the long-term cessation of blood flow associated with damage to the vessels due to surgery, another disease or accident [2]. It is well-known that long-term cessation of blood flow causes structural and functional modifications in the vessel. The reperfusion process is the restoration of blood flow in the vein without any obstruction. In this case, a structural dilemma arises: recirculation after a period of time in structures that have not been blooded for a long time causes an excessive presence of reactive oxygen species [3]. Although this may seem like a healing process, it causes it to be examined more carefully as the part that causes significant damage to the organs. Oxidative damage, inflammation, and cellular damage, processes that damage tissue and cause loss of function, occur following reperfusion. It is known that ischemia-reperfusion occurring in the abdominal aorta causes structural damage to the tissue and causes deterioration in retention and micturition. In patients in whom this situation is inevitable, the quality of daily life decreases [4].

Riboflavin (Rb; Vitamin B2) is an easily absorbed and heat-stable vitamin and it has antioxidant and anti-inflammatory effects. There are studies related to the fact that it causes growth-development retardation in case of deficiency. It plays a role in the metabolism of lipid, carbohydrate and protein metabolism and is necessary for energy metabolism [5]. It is the central component of the flavin adenine dinucleotide and flavin mononucleotide cofactors. Rb also scavenges free radicals and has anti-inflammatory properties. The therapeutic effect of Rb in diabetes-related liver and eye damage has been demonstrated in experimental models. Clinical reports have revealed that riboflavin-supplemented diets reduce the risk of colorectal cancer and breast cancer [3]. Its antioxidant properties are effective in alleviating hepatic damage caused by lipopolysaccharide. Experimental studies have shared data showing that carbon tetrachloride-induced liver fibrosis improves mitochondrial dysfunction. In our previous study, we examined the effects of Rb therapy in

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IR-related kidney injury. In this study, our aim is to evaluate the effects of Rb therapy on IR-induced bladder dysfunction and damage with organ bath contraction response, and biochemical and histological analyses.

2. RESULTS

2.1. Contractility

Dose-dependent contraction responses with cumulative addition of carbachol (CCh; 10^{-9} to 10^{-4}) are as in Figure 1. Maximum contraction was observed at 10^{-5} M CCh dose in all groups and submaximal contraction was determined as 3×10^{-6} M CCh dose. The contractile response was generally seen to be significantly lower at each dose in the IR group compared to the control group. With the addition of 3×10^{-7} M CCh, the bladder tissue of the IR group contracted at a very low rate compared to the control group. In the submaximal dose, the contraction rate was 55.4% in the IR group, while the contraction response was 90.5% in the control group. In the Rb-treated IR group, the contractile responses of the bladder tissues were higher than the IR group, 10^{-6} M ($p < 0.05$) to 10^{-4} ($p < 0.05$) doses of CCh. On the other hand, as a positive control group, the NAC-treated IR group's contractility response gradual increase ($p < 0.001$) in contractile responses was evident compared to the IR group. In addition, the NAC-treated contractility response was 75% at the submaximal CCh dose.

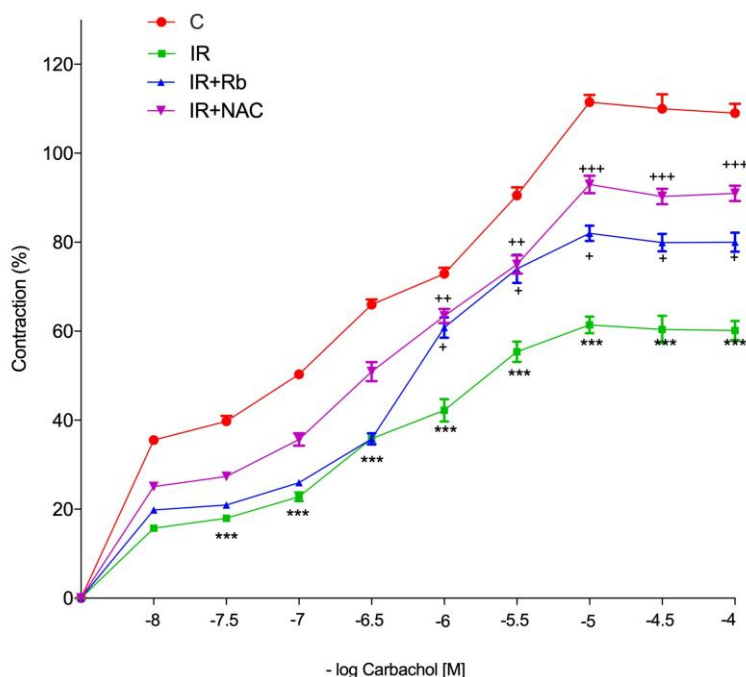


Figure 1. The concentration-response curve was obtained by the cumulative addition of carbachol to rat urinary bladder strips. Each point is expressed as a percentage of contraction induced by 124 mM KCl, and data are mean $\pm$ SEM. *** $p < 0.001$ versus control group; + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ versus IR group.

2.2. Biochemical Analyses

IR injury ensures oxidative damage to the urinary bladder. In this study, the MDA level of bladder tissues was remarkably ($p < 0.001$) elevated in the IR group, indicating ischemia and reperfusion period-induced injury (Figure 2a). IR-induced increase in MDA level was significantly reduced in the tissues of the Rb-treated IR and NAC-treated IR groups ($p < 0.01$ and $p < 0.001$, respectively). In parallel with these results, while the GSH level of the IR group was significantly ($p < 0.001$) lower compared to the control group, it relatively ($p < 0.05$) increased with Rb treatment. Bladder MPO activity is given in Figure 2c. There was a statistically significant increase in MPO activity in the IR ($p < 0.001$), IR+Rb, and IR+NAC groups compared

to the C group. Although Rb and NAC treatment caused a reduction in IR-induced MPO increase, it was not significant. According to Figure 2d, there is a dramatic ($p < 0.001$) increase in caspase-3 activity of the IR group compared to the C group. There was a significant ($p < 0.001$) decrease in caspase-3 activity in the Rb and NAC-treated-IR groups compared to the IR group.

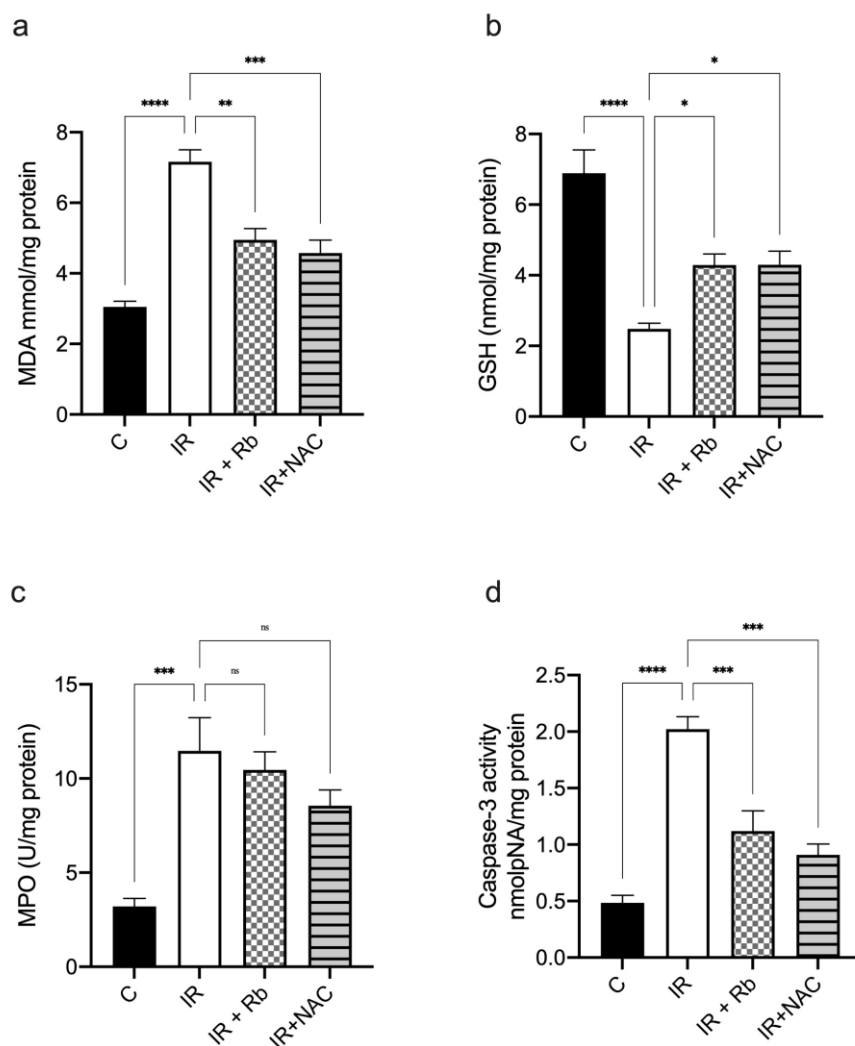


Figure 2. Effect of Riboflavin treatment on bladder after ischemia/reperfusion. A) Malondialdehyde (MDA), b) Glutathione (GSH), c) Myeloperoxidase (MPO), and d) Caspase-3 activity. ns: no significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

2.3. Histological Analyses

According to the macroscopic examinations (Figure 3), thinning of the bladder wall occurred in the samples belonging to the IR group compared to the C group. In addition, infiltration, edema and inflammation are observed in certain areas. After Rb and NAC treatment, there was a decrease in IR-induced infiltration, edema, and inflammation in the bladder tissue, and accordingly, the thinning of the bladder wall improved.

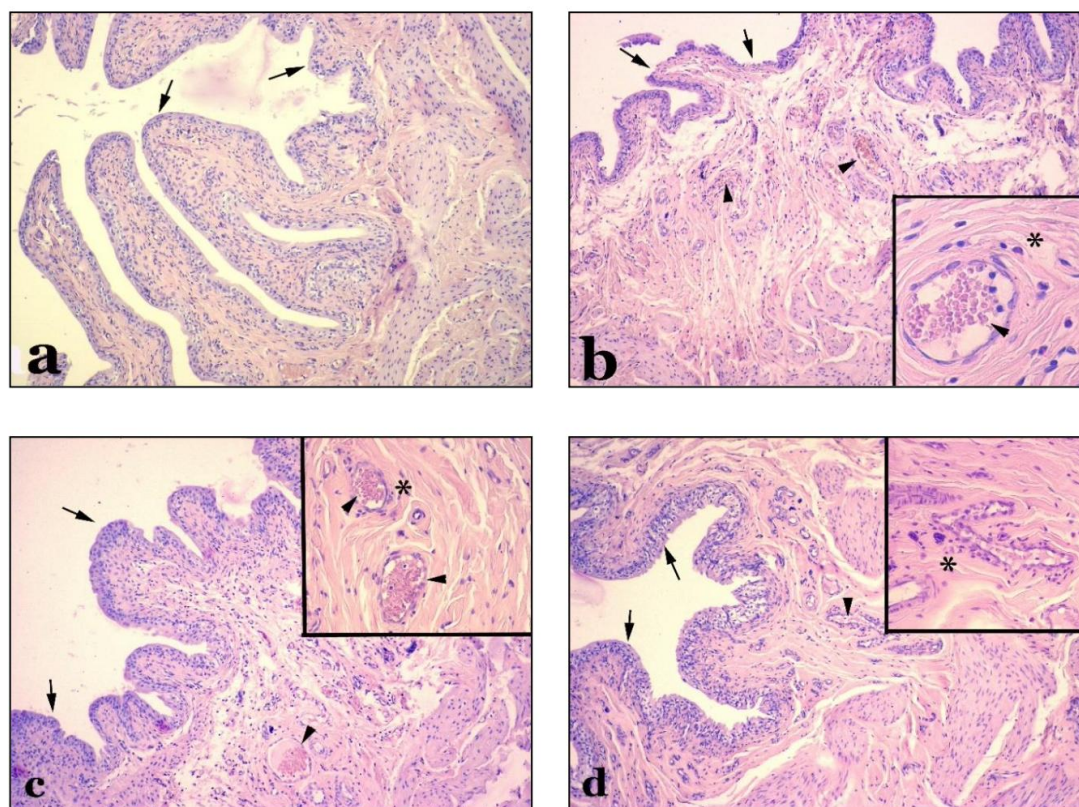


Figure 3. A histological examination of the H&E staining showed inflammatory cell infiltration, edema, and hemorrhage (b) in the lamina propria in the IR group, but no significant histological changes were found in the Control (a) group. We observed a substantial amelioration of the injury and inflammation in the IR+Rb group (c) and IR+NAC group (d).

3. DISCUSSION

The present study mainly demonstrated the effects of Rb treatment on IR-induced bladder contraction dysfunction and tissue oxidant damage. The results showed that IR decreased bladder contraction responses, increased MDA, MPO, caspase-3 activity levels and decreased GSH levels, and caused cellular damage in bladder tissue. It is seen that Rb treatment provides an improvement in IR-induced contraction responses and an improvement in tissue damage with an antioxidant effect.

Overall contractile responses (Figure 1) in rat bladder strips of 60 minutes of ischemia followed by 60 minutes of reperfusion were measured at a dose from 10^{-9} M CCh to 10^{-4} M. With increasing doses of carbachol, a normal contraction response curve was obtained in the control group. It is obvious that there is a decrease in the force of contraction in the IR group when compared to the control group. Increasing studies provide reports that bladder IR affects bladder contraction-relaxation function [6]. In fact, one study produced results showing that ischemia did not significantly impair the bladder contraction response, but reperfusion did cause damage to bladder smooth muscle. In age-related conditions such as atherosclerosis, urinary retention, embolization and thrombosis, ischemia and subsequent reperfusion of the bladder occur. IR-induced impairments in bladder function result in a decrease in the patient's quality of daily life [7]. Considering the effects of pretreatment with Rb, contraction responses starting with CCh at a dose of 10^{-6} M and up to a dose of 10^{-4} M show significant significance compared to the IR group. In order to better compare the effects of Rb pretreatment, we chose NAC treatment as the positive control group because there is no specific drug and the IR-induced contraction-relaxation responses of NAC have been extensively evaluated. Accordingly, we can also say that Rb pretreatment gave a similar contractile response to NAC pretreatment. In other words, 25 mg/kg Rb pretreatment can prevent IR damage in rat bladder domes.

IR damage causes a series of events such as protein, carbohydrate, nucleic acid and cell membrane lipid membrane destruction leading to cellular destruction with the formation of ROS it causes [8]. As an oxidative damage parameter, MDA is one of the end products of the decomposition of lipid peroxidation products [8]. An increase in MDA in tissue is associated with oxidative damage, and many studies point to an increase in

MDA in the bladder due to IR damage. Consistent with previous studies, our data indicate an increased level of MDA in the IR group compared to the control group. Available evidence suggests that riboflavin exerts pharmacological efficacy in preventing and alleviating various diseases. In a study we conducted in our laboratory, we showed [9] that Rb provides its therapeutic effect by correcting oxidant damage parameters in acetic acid-induced colitis model. Yu et al. [10] presented data that they can prevent abdominal aortic aneurysm by activating superoxide dismutase (SOD). It appears that Rb pretreatment provides a noticeable improvement in the level of GSH caused by IR. In addition, MPO, which is the most common oxidative stress marker, causes processes from initiation of inflammatory processes to apoptosis [11]. With this effect, it is one of the primary targets in mitigating oxidant damage. In our study, the increase in bladder MPO level caused by IR was prevented by Rb pretreatment. In addition, in parallel with these results, caspase-3 activity decreased in the Rb+IR group compared to IR and regressed to a level close to the control group.

Increased bladder wall thickness is indicated as the possible underlying cause of the IR-like pathology of the micturition and retention cycle. It has been reported that blood flow in reperfusion in the IR cycle paradoxically causes tissue damage with changes in cellular structures. Structural changes in the bladder tissue cause functional loss and disrupt the contraction-relaxation mechanism. However, compared to the control group, the IR group caused edema in the bladder tissues and thickened the inflamed bladder wall. Rb pretreatment prevented this IR-induced change and also provided similar improvement with the NAC pretreatment group.

The limitations of our study are the lack of different dose groups of riboflavin and the open-ended interpretation of organ bath data compared to techniques that yield better results for bladder dysfunction. In this respect, more comprehensive evaluations are needed.

4. CONCLUSION

In conclusion, this study shows that Rb therapy is an effective therapeutic in bladder dysfunction by reducing IR-induced oxidative stress responses and preventing tissue damage. More comprehensive studies are needed to see the underlying mechanisms.

5. MATERIALS AND METHODS

5.1. Animal model and Experimental group

All animal experiments followed a protocol that was approved by the ethics committee on animal research at Marmara University The Experimental Animal Implementation and Research Center (DEHAMER). The study was approved by the Local Institutional Animal Ethical Committee of Marmara University (with code 118.2016.mar). The Ethics Committee Guidelines were followed while working with the animals. Proper housing conditions (temperature (22 ± 2 °C), humidity (40-60%), and light (12 h/12 h light/dark)) for rats were provided and controlled until the end of the study. Forty Sprague-Dawley rats (250 $\pm$ 30 g) were divided into four groups (n=10): The sham-operated plus pretreated with saline group (Control group, C), the I/R plus pretreated with saline group (I/R group), the I/R plus pretreated with 25 mg/kg Riboflavin group (I/R + RB group), and I/R plus pretreated with 100 mg/kg N-acetylcysteine group (I/R + NAC group). NAC was included as a positive control since it was found to improve I/R-induced bladder contractility and prevent oxidant damage [12].

5.2. Surgery

In all protocols, a combination of 100 mg/kg ketamine and 10 mg/kg xylazine was used to induce anesthesia. The abdomen of the rats was incised approximately 3 cm. The abdominal aorta was fixed and separated from other vessels meticulously and gently. To induce ischemia abdominal aorta was occluded for 60 min and then allowed 60 min for reperfusion. In the sham group, the abdominal aorta was left intact. Following the I/R procedure, rats were sacrificed with cardiac puncture. The bladder dome was removed and separated from its base along the ureteral ophiries. Longitudinal strips (1.5 x 5 mm) were either immediately placed in organ baths or stored at -20 °C for biochemical analysis. Tissues were also taken for histological analysis [13].

5.3. In-vitro Organ Bath Experiments

Each strip preparation was placed in Tyrode's solution ((124.9 mM NaCl, 2.6 mM KCl, 23.8 mM NaHCO₃, 0.5 mM MgCl₂ 6H₂O, 0.4 mM NaH₂PO₄.H₂O, 1.8 mM CaCl₂, and 5.5 mM glucose) in a 20 mL organ bath. During the study, the system was ventilated with carbogen (5% CO₂ in O₂). Basal tension was determined

as 1 g for each tissue and cumulative contraction response was obtained after the tissues were in equilibrium for 1 hour. The contractile responses to carbachol (CCh) were expressed as force per 100 mg bladder muscle. The contractile responses to CCh (10^{-9} – 10^{-4} M) were cumulatively obtained [14].

5.4. Biochemical Analyses

5.4.1. Measurement of tissue malondialdehyde and glutathione activities

Bladder tissue samples were homogenized with ice-cold 150 mM KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 /M per cm and results are expressed as MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure. GSH levels were calculated using an extinction coefficient of 1.36×10^4 /M per cm. Results are expressed in μ M GSH/g tissue [15].

5.4.2. Measurement of tissue myeloperoxidase activities

Myeloperoxidase activity was measured in bladder tissues in a procedure similar to that documented by Hillegass et al. One unit of enzyme activity was defined as the amount of myeloperoxidase (MPO) present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue [15].

5.4.3. Caspase-3 activity

Caspase 3 activity assay was performed using the caspase 3 cellular activity assay kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. Tissue samples were treated for 10 min with iced lysis buffer supplied by the manufacturers. Then 40 μ L tissue samples and 50 μ L assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) were added to wells, and the microplate was equilibrated at 37 °C for 10 min. The reaction was initiated by adding 10 μ L DEVD-pNA substrate (200 mM final concentration). The colorimetric release of p-nitroaniline (pNA) from the Ac-DEVD-pNA substrate was recorded from 0 to 60 min at 405 nm using a specific activity of DEVD-pNA cleavage (pmol pNA/min) for each sample. The DEVD-pNA cleavage activity was calculated as pmol/min/mg protein [16].

5.5. Histological Analyses

Bladder specimens were collected and fixed in a 10 % neutral buffered formalin solution. After fixation, tissue samples were dehydrated in graded ethanol series and cleared in toluene. Paraffin-embedded samples were cut (5 μ m thick) by rotary microtome and sections were stained by hematoxylin and eosin stain. Sections were evaluated and photographed in all groups under a light microscope (Olympus BX51; Olympus, Tokyo, Japan). For each animal, five random tissue sections from the bladders were examined. The investigators were blinded regarding the treatment groups for microscopic examination.

5.6. Statistical Analyses

The data were expressed as mean $\pm$ standard error of the mean. The four groups were compared in terms of contractile responses by repeated measures ANOVA and in terms of other variables by the Mann-Whitney *U* test. GraphPad 8.0 (USA, San Diego) was used to evaluate the data. Differences were considered statistically significant if the null hypothesis could be rejected with >95% confidence ($p < 0.05$).

Author contributions: Concept; Design; Literature Search; Writing; Critical Reviews – B.E.

Conflict of interest statement: The author declared no conflict of interest in the manuscript.

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