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Effects of rotenone on inducible nitric oxide synthase and cyclooxygenase-2 mRNA levels detected by real-time PCR in a rat bladder ischemia/reperfusion model

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Abstract. We aimed to determine whether rotenone treatment prevents induced ischemia/reperfusion (I/R) damage in rat bladders by detecting inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) levels by real-time PCR (RT-PCR). A total of 18 Sprague-Dawley albino rats were used in this experiment. The experimental groups each consisted of 6 rats and were treated as follows: group I, control; group II, I/R; group III, rotenone + I/R. In the control group, the rat bladders were removed by lower abdominal incision without any procedure. In the I/R group, 1 h prior to the ischemia 1 cc physiological serum was administered and the abdominal aortas were clamped for 1 h to achieve bladder ischemia. Following the ischemia, reperfusion was induced for 1 h and the bladders were removed. In the rotenone + I/R group, the rats were treated with 25 mg/kg rotenone intraperitoneally. The iNOS and COX-2 mRNA levels in each group were detected using RT-PCR. In the I/R group, the COX-2 levels in the bladder tissue were higher compared with the control group ($P<0.05$). The COX-2 levels in the rotenone-treated group were statistically lower compared with the I/R group ($P<0.01$). Vascularization and edema were markedly increased in the I/R group. Following rotenone treatment these were abrogated inversely to inflammation. Although iNOS levels were slightly higher in the I/R group compared with the control group, iNOS levels did not decrease and no significant difference was observed between the groups with regard to rotenone treatment ($P>0.05$). We suggest that rotenone may be used clinically to treat I/R damage due to its diminishing effect on COX-2 levels.

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Abbreviations: iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; RT-PCR, real-time polymerase chain reaction

Key words: rotenone, bladder, rat, ischemia/reperfusion, inducible nitric oxide synthase, cyclooxygenase-2

Introduction

A lack of blood supply to the tissues is termed ischemia and leads to cell death and organ failure. The lesions become more severe with the supply of blood in the reperfusion phase (1). Ischemia/reperfusion (I/R) injury is a series of cellular events triggered by the inadequate supply of oxygen (O_2) to the tissues, and ultimately all organs and systems are affected. Reperfusion and reoxygenation of the ischemic tissue causes lipid peroxidation of cell membranes and produces free oxygen radicals (FORs) that lead to alterations in cellular functions (2,3). FORs, which are produced due to the reperfusion of ischemic tissue, have been found to be responsible for this damage (4). It has been shown that catheterization following the overdistension of the urinary bladder initiates reperfusion injury and that FORs are one of the main factors in this damage (5,6). Previous studies have shown that FORs and peroxynitrite, produced due to I/R injury, cause urinary bladder dysfunction by means of affecting bladder components, including smooth muscle, mucosa and peripheral nerves (5).

Bladder ischemia occurs as a result of a reduction in the blood supply caused by atherosclerosis or trauma. This leads to impairment in compliance during the contraction of the bladder and may consequently result in bladder dysfunction. It has been shown that an increase in bladder wall thickness mediates a cyclic I/R in each micturition (7). Bladder dysfunction observed following decompression or catheterization, which is performed following an acute overdistension or retention, is caused by I/R (5,8). Cyclooxygenase (COX) isoforms (COX-1 and COX-2) catalyze the initial step of arachidonic acid metabolism. COX-2 expression is increased in a number of organs during I/R injury. Previous studies have reported that COX-2 inhibitors may protect the tissue from I/R injury (9). Nitric oxide (NO) is a significant endogenous regulatory molecule that plays a role in various biological functions and is involved in the regulation of homeostasis. Previous studies

have highlighted that NO levels were increased in the damage which occurred after bladder I/R injury and that the therapies which aimed to reduce NO levels were successful in preventing this damage (10). Complex I is the first enzyme complex in the electron transport chain of mitochondria and is a significant source for the production of reactive oxygen species. Rotenone, a mitochondrial electron transport inhibitor, takes part in the anti-oxidative defense mechanism (11).

In this present study, we aimed to ascertain whether rotenone treatment inhibits experimentally induced I/R injury in the rat urinary bladder, by means of measuring COX-2 and inducible nitric oxide synthase (iNOS) mRNA levels.

Materials and methods

Animals. This experimental study was conducted following approval of the ethics committee (date, 26.03.2008; number 44/2208). Sprague Dawley rats of both genders, aged 5-6 months and weighing between 250 and 300 g were used for the study. The rats were divided into 3 groups and 18 rats, 6 in each group, were used for real-time polymerase chain reaction (RT-PCR) assays. The rats were anesthetized by intraperitoneal (i.p.) injection of 50 mg/kg ketamine with 20 mg/kg rompun. Following the anesthesia, median abdominal laparotomy was performed.

Group I (control group, n=6). The urinary bladders of the rats were removed without any intervention immediately after performing abdominal laparotomy.

Group II (I/R group, n=6). The rats in this group were administered normal saline 1.0 cc i.p. 1 h before the ischemia, and bladder ischemia was induced by clamping the abdominal aorta for 1 h with a non-traumatic microvascular clamp (bulldog clamp). During this process, the laparotomy incision was sutured with 3/0 silk. After the ischemia period, the laparotomy incision was reopened, the abdominal aorta was unclamped and in order to provide reperfusion for 1 h, the laparotomy incision was re-sutured and closed. After 1-h reperfusion, the bladder tissues were removed.

*Group III (*rotenone + *IR group, n=6)*. The rats in this group were administered rotenone i.p. at a dose of 25 mg/kg 1 h prior to ischemia induction by clamping the abdominal aorta. Thereafter, laparotomy was performed through an abdominal incision and bladder ischemia was induced by clamping the abdominal aorta for 1 h with a non-traumatic microvascular clamp. The laparotomy incision was closed by suturing with 3/0 silk. After the ischemia period, the laparotomy incision was re-opened, the clamp was removed and reperfusion was provided for 1 h and then the laparotomy incision was re-sutured and closed. Following 1-h reperfusion, the bladder tissues were removed.

Histology. The specimens were fixed in 10% neutral formalin, dehydrated in increasing alcohol series, cleared in xylene and embedded in paraffin. Several 4-μm sections obtained from these specimens were mounted onto slides. All specimens were stained with hematoxylin and eosin (H&E) for histochemistry and evaluated under light microscopy.

RNA extraction and RT-PCR. The mRNA levels of iNOS and COX-2 in relation to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined using RT-PCR with SYBR-Green. Total RNA was extracted from urinary bladder tissue using an RNA stabilization reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm (Nanodrop 1000; Thermo, Wilmington, DE, USA). Aliquots of 20 μl of RNA from each group were used to produce complementary DNA (cDNA). The newly synthesized cDNA, stored at -20˚C, was used for the mRNA assay of iNOS and COX-2 with RT-PCR. cDNA (1 μl) from each group was amplified in 25 μl of reactive mixture with 0.25X SYBR-Green Supermix (Molecular Probes, Invitrogen, Carlsbad, CA, USA). RT-PCR was performed by monitoring the increase in the amount of SYBR-Green in real time using Rotor-Gene 6000 RT-PCR (Corbett Research, Sydney, Australia). The oligonucleotide sequences of the cDNA primers were designed at Gene Research Laboratories, UK. The forward primer for rat iNOS was 5'-CACCACCCTCCTTGTTCAAC-3' and the reverse primer was 5'-CAATCCACAACTCGCTCCAA-3'. The forward primer for rat COX-2 was 5'-TGCGATGCTCTT CCGAGCTGTGCT-3' and the reverse primer was 5'-TCA GGAAGTTCCTTATTTCCTTTC-3'. Sobajima *et al* also used GAPDH (housekeeping gene) to normalize iNOS (target gene) data using RT-PCR (12).

The RT-PCR thermal cycling conditions were as follows: 5 min at 65˚C, 60 min at 37˚C for cDNA synthesis, 15 min at 95˚C, 15 sec at 95˚C, 1 min at 60˚C for 50 cycles and 1 min at 55˚C. RT-PCR data were collected using the Rotor-Gene 6000 detection system. Cycle threshold (CT) values were determined by automated threshold analysis. The primer quality (lack of primer-dimer amplification) was confirmed by melting curve analysis. The relative quantification of the gene expression was performed using the standard curve method, constructed with serial dilutions of control mRNA or RT-PCR amplicons. All experiments were carried out in triplicate. iNOS and COX-2 levels were standardized to GAPDH (ratio iNOS:GAPDH) to account for loading differences. Gene expression levels (mRNA) were reported using the median as a point estimator and the range of values.

Statistical analysis. SPSS (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) v. 10.0 package program was used for the statistical evaluation. Statistical significance between the groups was analyzed using the Tukey's HSD multiple comparison test. The data are presented as mean and standard deviation (mean ± SD). $P<0.05$ was considered to indicate a statistically significant result.

Results

Histological results. The specimens were evaluated under light microscopy and inflammation criteria, including edema, vascularization and infiltration of immune cells, were scored. Severe criteria were described as +++, mild criteria as ++ and slight criteria as +. During the evaluation of the slides it was noted that vascularization and edema were markedly increased in the I/R group. However, following rotenone treatment these were decreased to lower degrees. On the other

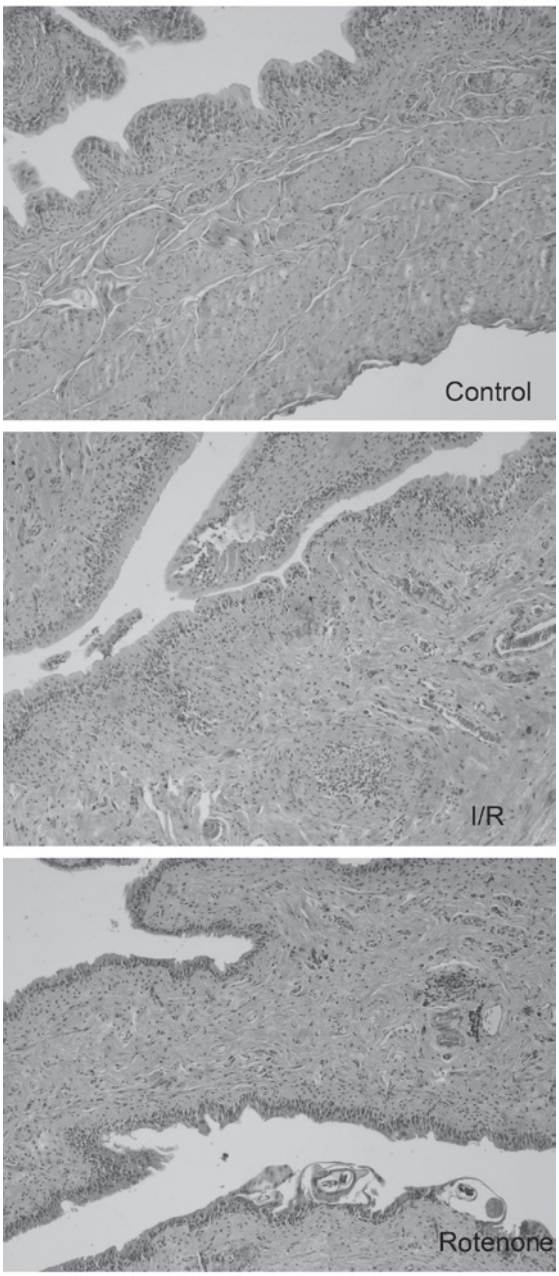

Figure 1. (A) Control. (B) Vascularization, inflammation and edema were markedly increased in the I/R group. (C) Following rotenone treatment, vascularization and edema were significantly reduced with a small decrease in inflammation. I/R, ischemia/reperfusion.

Table I. H&E-stained sections of the bladder samples.

Group	Inflammation	Edema	Vascularization
Control	+	-	+
I/R	+++	++	+++
Rotenone	++	+	++

H&E, hematoxylin and eosin; I/R, ischemia/reperfusion. +, slight; ++, mild; +++, severe.

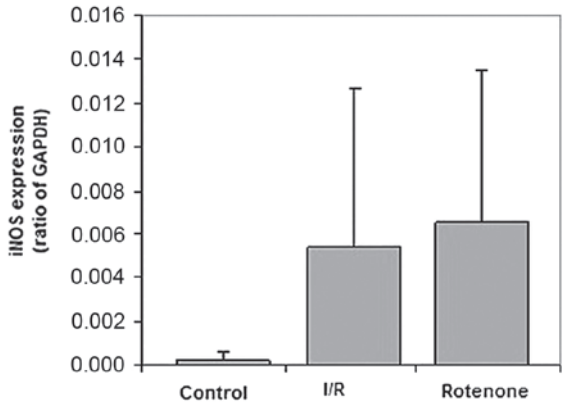

Figure 2. Effect of rotenone on bladder tissue iNOS levels. I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase.

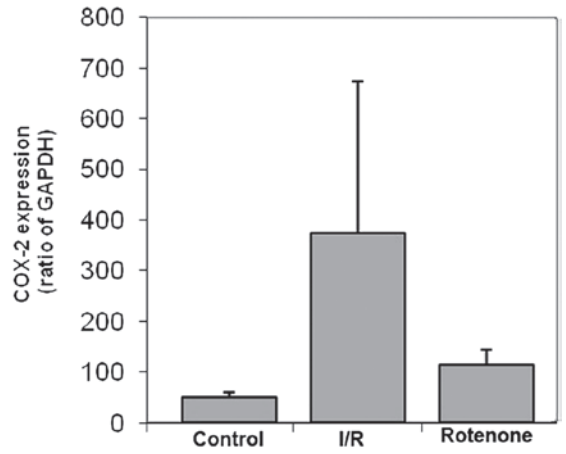

Figure 3. Effect of rotenone on bladder tissue COX-2 levels. I/R, ischemia/reperfusion; COX-2, cyclooxygenase-2.

hand, inflammation in the I/R group was markedly higher than in the control group. Following rotenone treatment, it was slightly decreased. All the scores are summarized in Table I. H&E staining of all the structures in the bladder are shown in Fig. 1.

Expression of iNOS in urinary bladder. iNOS levels were increased in the I/R group and iNOS values were not decreased with rotenone administration prior to I/R. According to one-way analysis of variance, the three groups were not significantly different (P=0.194). The three groups were also not different when the groups were compared with each other (all P>0.05 by the Tukey test; Fig. 2).

Expression of COX-2 in urinary bladder. I/R significantly increased the COX-2 values (P<0.05) and COX-2 values were not increased with rotenone administration prior to I/R, but were decreased to below the values noted in the control group (P<0.01; Fig. 3).

Discussion

An increase in FORs following I/R causes bladder tissue damage (5). In the present study, rotenone treatment prevented bladder damage via the COX-2 pathway. This hypothesis was verified in the histological examination which revealed that

inflammation, edema and vascularization were higher in the I/R group and that rotenone treatment reduced these damaging effects. In this present study, the analysis of the iNOS gene (target gene) expression using the RT-PCR method revealed that the extremely low, almost immeasurable iNOS gene expression was increased following stimulation, such as I/R injury. However, when the relative concentrations (the ratio to the reference GAPDH gene) were taken into consideration, we found that the increment in the I/R group was not statistically significant (P>0.05). We observed that iNOS expression was not decreased with rotenone treatment as compared with the I/R group (P>0.05).

Urinary bladder ischemia followed by reperfusion may also be observed in age-related conditions, including urinary retention, atherosclerosis, vasospasms, embolism and thrombosis. The development of I/R secondary to these conditions may lead to urinary bladder dysfunction (13). It has been demonstrated that bladder wall blood flow (ischemia) and oxygen pressure (hypoxia) are decreased in partial bladder outlet obstruction and that the increase in the bladder wall thickness leads to cyclic I/R during each micturition (14). However, it has been reported that decompression following acute over-distension or retention and bladder dysfunction observed following catheterization were also associated with I/R (5,6). Saito and Miyagawa reported that histopathological changes, including severe erythrocyte extravasation and leukocyte infiltration, resulted from the rupture of microcirculation in the bladder. I/R injury occurred particularly in the smooth muscles and submucosa, but not in the mucosa, and these histopathological alterations associated with I/R injury were prevented by N(G)-nitro-L-arginine methyl ester (L-NAME) treatment (8).

Rotenone has been used as an antioxidant agent in the I/R injury of various organs. Ichikawa *et al* evaluated I/R in the intestinal mucosa and biochemical analyses revealed that the products that caused the damage were significantly decreased in the group receiving rotenone therapy compared with the ischemia group. The authors stated that rotenone induced this positive effect by inhibiting lipid peroxidation and decreasing mucosal inflammation (15). Vanden Hoek *et al* attempted to reduce the oxidative stress-induced damage on cardiomyocytes with complex 1 inhibitors and used rotenone, cyanide and antimycin for this purpose; consequently, the authors showed that rotenone was more effective than the other agents (16). Viñas *et al* reported that, according to quantitative analysis, I/R increased the renal expression of iNOS compared with the control and that no differences were found between I/R and the various selective NOS inhibitors (1). In the present study, quantitative iNOS expression increased in the I/R group and no significant difference was observed between the I/R and rotenone treatment groups, similar to the study performed by Viñas *et al*.

Similarly, we observed that urinary bladder I/R injury in rats was decreased with rotenone treatment. To the best of our knowledge, the expression levels of COX-2 and iNOS have been studied by RT-PCR to detect the I/R injury in various organs, but not in the urinary bladder. In the experimental unilateral lower extremity I/R injury model, Dupouy *et al* investigated the levels of COX-1 and COX-2 and reported that COX-2 mRNA levels in the skeletal muscle were significantly increased in the I/R group compared with the control group, and that COX-2 inhibitors may protect the tissue against this damage (9). In experimental gastric I/R injury, Hiratsuka *et al* reported that the COX-2 levels in mesenchymal cells was significantly increased in the I/R group compared with the control group (17). In their study on renal I/R injury, Matsuyama *et al* suggested that COX-2 played a significant role in organ and tissue homeostasis and that COX-2 inhibitor use may be beneficial in renal I/R injury (18). Tetsuka *et al* stated that rotenone did not inhibit IL-1β-induced Cox-2 mRNA and iNOS mRNA expression but inhibited IL-1β-induced Cox-2 and iNOS protein expression. These data suggest that rotenone inhibited the Cox-2 and iNOS expression at a post-transcriptional level (19). When the relative concentration of COX-2 mRNA (the ratio to the reference GAPDH gene) was evaluated, we also observed that COX-2 mRNA was increased in I/R injury, while significantly decreased following rotenone treatment (P<0.01).

In conclusion, we observed that rotenone therapy may be beneficial in decreasing the oxidative stress-induced COX-2 levels in urinary bladder I/R injury in rats. However, further studies are required for rotenone therapy to be used for therapeutic purposes in the I/R damage induced in the urinary bladder by various conditions.

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