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Independent Article

Methylene blue attenuates renal ischemia–reperfusion injury in rats

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ABSTRACT

Background and purpose: In our study, we investigated the effects of methylene blue (MB) on histopathological changes in renal ischemia/reperfusion (I/R) injury rat model.
Material and methods: Twenty-one Sprague–Dawley male rats were divided equally into three groups. Group 1 (control) was administered intraperitoneal saline solution. In Groups 2 (untreated group) and 3 (MB treatment), the renal arteries were clamped, and ischemia (for 1 hour) and then reperfusion (for 4 hours) were applied. Thirty minutes before ischemia, the untreated group received physiological saline, whereas the treatment group was administered 30 mg/kg MB through an intraperitoneal route. Blood samples were drawn, and renal specimens were harvested 5.5 hours after physiologic saline injection in the control and immediately after the reperfusion period in the other groups. The levels of tissue superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), total oxidant status (TOS), total antioxidant status (TAS), plasma urea, creatinine and ischemia modified albumin (IMA) were measured. Moreover, the histopathological damage score of the renal tissue was determined.
Results: MB significantly alleviated the severity of histopathological damage by increasing the levels of tissue SOD and TAS and decreasing TOS concentrations in the renal I/R model ($p < 0.05$).
Conclusion: Administration of MB in renal I/R damage may play a protective role.

Renal ischemia is characterized by transient decrease or cessation of renal blood flow. It can be observed in many conditions, including renal transplantation, partial nephrectomy, renal artery angioplasty, cardiopulmonary bypass surgery, trauma, sepsis, burns, hydronephrosis, and elective urological operations. As a result, acute renal failure can develop, resulting in tubular necrosis, decrease in glomerular filtration and increase in renal resistance [1,2].

Prolonged ischemia destroys cellular integrity or even leads to cell death as a result of the accumulation of toxic metabolites. Ischemic tissue should be reperfused to achieve the regeneration of tissue and elimination of toxic metabolites. The recovery of tissue perfusion by means of drugs or mechanical interventions is called reperfusion. Destructive changes during reperfusion are more severe than in paradoxical ischemic injury. In ischemia/reperfusion (I/R) injury, free oxygen radicals, especially those released by polymorphonuclear leukocytes, that accumulate within tissues exert important effects [3,4]. The free radicals interact with almost all biomolecules integrated into the structure of living organisms, and they can induce reversible or irreversible effects on these biomolecules [5]. Tissues possess enzymatic and non-enzymatic antioxidant mechanisms against oxidative damage. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are examples of antioxidant enzyme systems [6].

Cellular stress factors such as hypoxia, acidosis, free radical damage, and impairment of membrane integrity induce structural changes in albumin molecules, attenuating the binding capacity of albumin for transition metals such as copper, nickel, and cobalt. The resulting defective albumin is referred to as "ischemia modified albumin" (IMA). IMA is a known sensitive marker in pulmonary embolism and myocardial, muscular, mesenteric and cerebral ischemia [7–9].

As an agent with lower toxicity, methylene blue (MB) has been used in clinical practice [10]. It increases the level of SOD and inhibits the formation of oxygen radicals, with resulting antioxidant effects [11,12]. Experimental studies have demonstrated that it also corrects hemodynamic instability developed in hepatic I/R injury [13], protects kidneys from harmful effects of immune suppressive agents via its antioxidant effect [14], and decreases intraabdominal adhesion [15].

In our study, we investigated the effects of MB on histopathological changes in renal I/R injury; tissue oxidative stress parameters such as SOD, CAT, GPx, total oxidant status (TOS); total antioxidant status (TAS) and levels of serum IMA, which is a proper marker of ischemia.

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1. Materials and methods

The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine, Bezmialem Vakif University. A total of 21 male Sprague–Dawley rats weighing 265–320 g were used. The rats were divided into three equal groups:

Group 1 (control) was composed of normal healthy rats that were treated with intraperitoneal (i.p.) physiologic saline solution.

Group 2 (untreated): This group consisted of rats that were administered i.p. physiological saline 30 minutes before ischemia/reperfusion

Group 3 (MB treatment): This group consisted of rats that were administered i.p. 30 mg/kg MB (Merck, Darmstadt, Germany) in 1 ml of sterile water 30 minutes before ischemia/reperfusion.

Excluding rats in the control group, the rats in other groups were laid on the examination table under intramuscular (i.m.) 15 mg/kg xylazine (Rompun, Bayer, Turkey) and 50 mg/kg ketamine (Ketalar, Eczacıbaşı, Türkiye) anesthesia. The anterior abdominal wall was explored through a midline incision. Vessels were separated with blunt dissection, and the renal arteries were clamped with atraumatic vascular clamps. After a period of 60 minutes, the clamps were removed, and free blood flow was maintained. The incision was closed and disinfected with 10 % povidone iodine. In the control group, at the end of 5 hours following administration of physiological saline, blood samples were drawn from the rats; however, in groups 2, and 3, at the end of 4 hours after reperfusion, blood samples were drawn from rats, and their left kidneys were removed. After peeling of the renal capsule with a scalpel, the kidney was divided into two parts with a longitudinal section. One portion was placed in 10% formaldehyde solution for histopathological analysis, and the other portions together with plasma samples were stored at −70 °C until biochemical analysis was performed.

1.1. Histopathologic evaluation

The renal tissues were individually immersed in Bouin's fixative, dehydrated in alcohol and embedded in paraffin. Sections of 5 μm were obtained, deparaffinized and stained with hematoxylin and eosin (H&E). The renal tissue was examined and evaluated in random order under blinded conditions with standard light microscopy. Tubulointerstitial injury was defined as tubular atrophy, dilatation, loss of brush border, cellular infiltration, and widening of the interstitium. The degree of tubulointerstitial damage in the cortex was determined using a semiquantitative graded scale [16], where 0 represents no abnormality, 1: minimal damage (involvement of 25% of the cortex), 2: mild damage (involvement of 25–50% of the cortex), 3: moderate damage involvement of 50–75% of the cortex), and 4: severe damage (involvement of 75% of the cortex). These analyses were performed with 2 tissue sections obtained from each animal examined less than ×400 magnification for at least 10 different regions for each section [1].

1.2. Biochemical tissue analyses

1.2.1. Preparation of tissue samples

The kidneys were cleaned, and each segment was homogenized in 10 volumes of 50 mM Tris–HCl at pH 7.4 using a rotor-stator homogenizer. The homogenate was centrifuged at 2000 ×*g* at 4 °C for 10 min, and a low-speed supernatant fraction was used for *ex vivo* assays.

1.2.2. SOD activity

The measurement of the tissue homogenate SOD enzyme activity was based on the generation of superoxide radicals by the action of xanthine and xanthine oxidase, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5 phenyltetrazolium chloride (INT) to form a red formazan dye [17]. One unit of SOD was defined as the amount of enzyme necessary to produce a 50% inhibition in the INT reduction rate. SOD activity is expressed as units per gram tissue protein.

1.2.3. CAT activity

The supernatants of the kidney were assayed by the method of Aebi et al. [18], which monitors the disappearance of H_2O_2 in the presence of the cell homogenate at 240 nm. The enzyme reaction was started by adding 0.1 ml of the sample (0.4–0.5 mg protein) to 2.9 ml of 50 mM phosphate buffer at pH 7.0 containing 12 mM H_2O_2. The absorbance was recorded immediately after addition of the sample at 240 nm at 15-second intervals for 2 minutes. A blank solution without sample was prepared. The absorbance (A) was read at 240 nm, and the A/min was calculated. The calculation was performed using the extinction coefficient of H_2O_2 i.e., 43.6 M^{-1} cm^{-1}. The activity is expressed as units per milligram tissue protein.

1.2.4. GPx activity

Glutathione peroxidase activity was estimated by measuring the changes in the absorbance at 340 nm owing to NADPH consumption in the presence of H_2O_2 [19]. The glutathione peroxidase activity is expressed as mmol per gram tissue protein (mmol/g protein).

1.2.5. Measurement of TOS and TAS

The TOS and TAS of the tissues were measured using automated colorimetric measurement methods [20]. The TOS assay was calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalents per liter (μ mol H_2O_2 Eq/L). The TAS assay method is based on the bleaching of the characteristic color of a more stable 2,20-azinobis [3 ethylbenzothiazoline-6-sulfonic acid] (ABST) radical cation by antioxidants. The assay has excellent precision values that are lower than 3%. The results are expressed as the mmol Trolox/mg protein.

1.2.6. Measurement of urea, creatinine and IMA

Using standard methods, the urea and creatinine values of venous blood samples were analyzed. In analysis of IMA, the decreased binding capacity of albumin to cobalt was evaluated using a rapid colorimetric assay developed by Bar-Or et al. [21,22]. The results are expressed in absorbance units (absu).

1.3. Statistical analyses

One-sample Kolmogorov Smirnov tests were used to determine that the quantitative data for every group were normally distributed. Therefore, intergroup comparisons were performed using one-way ANOVA and the post-hoc Tukey multiple comparison method. Histopathological scores were compared using Kruskal–Wallis and post-hoc Dunn tests. Statistical test results were considered significant at a $p < 0.05$.

2. Results

A total of 21 rats were included in the study. During the experiment, none of the rats died.

Histopathologic damage scores were compared between groups, and the severity of destructive changes in the untreated group were found to be significantly higher than that of the other groups ($p < 0.05$). In the MB treatment group, these histopathologic findings were statistically suppressed ($p < 0.05$). Histopathological sections are shown in Fig. 1, and comparisons of histopathological damage scores are shown in Fig. 2.

The SOD activity of the groups was compared. In the MB treatment group SOD activity was significantly increased when