Effect of Methylene Blue on the Hemodynamic Instability Resulting From Liver Ischemia and Reperfusion in Rabbits


ABSTRACT

The experimental investigation was performed to study the effects of methylene blue (MB) on hemodynamic, biochemical, and tissue changes among rabbits undergoing liver ischemia and reperfusion (IR). Twenty-four rabbits were randomized into 5 groups: 1, SHAM, control; 2, MB infusion bolus (3 mg/kg); 3, IR, hepatic ischemia for 60 minutes followed by 120 minutes of reperfusion; 4, MB-R, undergoing ischemia that had received an MB bolus infusion (3 mg/kg) prior to reperfusion; 5, R-MB, undergoing ischemia and MB bolus infusion after hemodynamic instability caused by reperfusion. The analysis included continuous recording of vital signs. Blood samples were collected at 0, 60, and 180 minutes of IR to determine blood gases as well as biochemical markers of liver function, nitric oxide, lipid peroxidation, and neutrophil activity. At the end of each experiment, liver tissue samples were collected for histological evaluation of parenchymae markers. Statistical analysis used two-way analysis of variance (ANOVA) tests with significance set at \( P < .05 \). Vital signs significantly improved with MB infusion, irrespective of whether it was applied before or after reperfusion. Blood gas data revealed different patterns among the SHAM, MB, IR, MB-R, and R-MB groups, without statistical significance, except for favorable lactate results in the R-MB group \( (P < .01) \), which displayed greater survival. Biochemical tests did not show significant differences among the groups, whereas histological analysis revealed favorable appearances for the MB-R and R-MB groups. The MB effect lasted long after reperfusion, suggesting that improvement in the hemodynamic parameters was not based on liver integrity, but rather was possibly related to endothelial function.

PRIMARY graft dysfunction resulting from ischemia-reperfusion (IR) injury is still an important cause of morbidity and mortality among patients undergoing liver transplantation. Mortality can be higher when IR injury is associated with systemic repercussions such as cardiocirculatory collapse, culminating in the so-called Ischemia and Reperfusion Syndrome (IRS). The dye methylene blue (MB) has been used as a vasopressor in sepsis and acute liver failure. Sepsis and IRS share many hemodynamic and biochemical similarities, including the patterns of increased pro-inflammatory cytokines. Several modes of action have been postulated for MB, depending on the clinical situation. MB acts not only nitric oxide (NO) blocker by inhibiting guanylate cyclase (GC), but also as an inhibitor of nitric oxide synthase (NOS).

Koelzow et al. showed that MB attenuates the hemodynamic changes caused by IRS following transplantation via GC inhibition. However, no in vivo experimental investigations corroborated Koelzow’s clinical experience. The present work in rabbits sought to evaluate: (1) the effects of MB on cardiovascular repercussions due to liver IR and its hemodynamic implications; (2) the effects of MB on changes in arterial blood gases and markers of acute circulatory shock (differences in lactate and \( O_2 \) extraction); (3) the NO biochemical products and liver function plasma markers in blood collected during various steps; (4) the changes in liver tissue products derived from NO, lipid

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peroxidation, and protein oxidation at the end of each experiment; and (5) the histological changes in liver parenchyma in situations of IR.

MATERIALS AND METHODS

The experimental and animal procedures were approved by our Institutional Animal Care Review Board. This investigation was carried out according to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Before the experiments, the rabbits were housed under standard laboratory conditions (12-hour light/dark cycle at 21°C), with free access to water.

Characterization of Experimental Groups

Thirty white male New Zealand rabbits weighing between 2.5 and 3.5 kg were randomized into 5 groups: (1) SHAM controls; (2) MB bolus (3 mg/kg); (3) IR, animals undergoing 60 minutes of hepatic ischemia followed by 120 minutes of reperfusion; (4) MB-R, animals undergoing schema that received MB bolus (3 mg/kg) prior to reperfusion; and (5) R-MB, animals subjected to ischemia with MB infusion bolus after hemodynamic instability caused by reperfusion.

Experimental Procedure

Anesthesia consisted of intramuscular xylazine (10 mg/kg) associated with ketamine (50 mg/kg) once in the hind legs. This association was maintained during the experiment, repeating a third bolus every 90 minutes for analgesia and sedation. The animals received an intravenous infusion of 10 mL/kg/h sodium chloride (0.9%) to compensate for insensible loss during the procedure. They were maintained under spontaneous ventilation with have O2 catheter. After anesthesia, the right internal jugular vein and common carotid artery were cannulated with polyethylene catheters. Samples of arterial and venous blood obtained at the beginning of the experiment (time zero), the end of ischemia (60 minutes), and the end of reperfusion (180 minutes) were used to measure blood gases, lactate, nitrite/nitrate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). The surgical access was performed by median infraumbilical minilaparotomy. Except for the SHAM group, the hepatic artery of all the animals was clamped at the hilum for 60 minutes. Thereafter the clamp was removed for liver reperfusion. An MP 100 A system (Biopac Systems, Inc., Santa Barbara, Calif, United States) was used to monitor mean arterial pressure (MAP) and central venous pressure (CVP) and to record the electrocardiogram (ECG). At the end of the experiment, the animals were humanely killed by exsanguination and samples were taken from the liver parenchyma for histological examination under a light microscope.

Histological Analysis

Liver tissue samples were placed in 10% paraformaldehyde for 24 hours fixation before paraffin embedding, and 5-μm transverse sectioning using a Reichert Jung microtome model 2040. Selections were stained with hematoxylin and eosin (HE), Masson, Giemsa-Wolbach modified method to differentiate leukocyte nuclei followed by histological examination under a light microscope.

The histological study included evaluation of the hepatic parenchyma in all of the animals of each experimental group, taking the following characteristics into account: (1) interstitial hemorrhage, (2) parenchymal necrosis, (3) inflammatory reactions, and (4) interstitial edema. Each of these features was classified as mild, moderate, or severe.

The liver tissue stained with Giemsa was used for the neutrophil count among 5 animals in each study group. We chose locations with greater numbers of leukocytes: 2 fields near portal spaces and 2 in the liver lobe at 400 times magnification. The number of neutrophils present in the vessels was counted by a cytopathologist, blinded as to each rabbit's group. We did not count leukocytes in the process of diapedesis.

Blood Gases and Lactate

The arterial and venous blood gases as well as the lactate levels were measured using a conventional blood gas Gem Premier 3000 instrument (Instrumentation Laboratory Co., Bedford, Mass, United States) that had previously been calibrated using a cartridge-type IQM 150 (GEM Premier, Instrumentation Laboratory Co., Bedford, Mass, United States).

Nitrite/Nitrate (NO)

The NO contents in deproteinized liver tissue homogenates and plasma samples were measured using NO/Ozone chemiluminescence (Sievers 280 NO analyzer, GE Analytical Instruments, Boulder, Col, United States).

Lipid Peroxidation Assay in the Liver

Tissue samples from ischemic and nonischemic livers were homogenized with ice-cold Ringer’s solution to determine of MDA levels, using a commercial kit (Lipid Peroxidation Assay Kit, Calbiochem, Merck & Co. Inc., Whitehouse Station, NJ, United States), with readings of absorbance at 586 nm.

Statistical Analysis

The values corresponding to blood gas results, as well as hemodynamic and biochemical data are expressed as mean values with standard deviations. Two-way analysis of variance (ANOVA) was used for the statistical analysis, which was complemented with Bonferroni tests for paired observations. One-way ANOVA and
the Bonferroni post-test for multiple comparisons were used to compare groups of data obtained from tissue analyses of NO, MDA, and mean number of neutrophils. Calculations were accomplished using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, Calif, United States). A level of $P < .05$ was accepted to be indicative of statistical significance.

RESULTS
Hemodynamic Data, Mortality, and Time of Observation

MAP values for the SHAM and MB groups remained virtually stable during the 3-hour observation, with only slight reductions. All of the groups subjected to ischemia, namely IR, MB-R, and R-MB, displayed an initial increase in MAP, followed by a steady decrease during ischemia (60 minutes). During reperfusion, there was a sudden decrease in MAP among the group that did not receive MB (IR), with death of 4 of the 6 animals within the first 10 minutes. The group to which MB was administered prior to reperfusion (MB-R) showed no sudden decrease in MAP at the beginning of reperfusion. Their blood pressure levels were greater than the IR group up to 60 minutes of reperfusion ($P > .05$); there were 3 deaths at 20, 40, and 95 minutes reperfusion, respectively. In the R-MB group, the drug infusion produced an increase in MAP, yielding average levels greater than those obtained for the IR ($P < .01$) or MB-R ($P > .05$) groups up to 55 minutes of reperfusion; there were 2 deaths (65 and 100 minutes of reperfusion, respectively; Fig 1 and Fig 2).

There was only a slight decrease in CVP during the experiment in the SHAM and MB groups. All the groups underwent steady CVP reduction during the 60 minutes of ischemia. During reperfusion, there was a sudden CVP decrease among the IR group. As for the MB-R and R-MB groups, the CVP increased upon reperfusion followed by a steady decrease after reperfusion. This difference was significant ($P < .05$) in the first 20 (for the IR and MB-R groups) and 60 minutes (for the R-MB group) of reperfusion ($P < .05$; Fig 3).

Compared with the SHAM group, there were no differences in the heart rate (HR) during the whole period of ischemia (60 minutes) among the IR, MB-R, and R-MB groups. During reperfusion, there was an abrupt decrease in HR in the IR group. In the case of the MB-R and R-MB groups, there was a slight enhancement in HR with reperfusion, followed by a progressive decrease. The difference in HR was significant ($P < .05$) in the first 15 (for the IR and MB-R groups) and 50 (for the R-MB group) minutes of reperfusion (Fig 4).

Fig 1. Survival curve (Kaplan-Meier analysis) of each group as a function of time (min).

Fig 2. MAP values (mm Hg) as a function of time (min). *In the R-MB group, drug infusion caused an increase in MAP and furnished average MAP levels higher than those obtained for the IR ($P < .01$) and MB-R ($P < .05$).
Serum Enzymes

Intergroup analysis of ALP revealed no differences among the groups. There was a significant increase in LDH among the last samples obtained from the SHAM and MB groups ($P < .001$) and the MB-R group ($P < .05$). The AST values in the last samples collected from both MB-R and R-MB groups were significantly higher compared with the SHAM and MB groups ($P < .001$ and $P < .05$, respectively), without a significant difference compared with the IR group. The ALT values measured in the last samples collected from the MB-R and R-MB groups were significantly increased compared with SHAM animals ($P < .001$ and $P < .05$, respectively), without a significant difference compared with the IR group (Table 1).

Plasma Nitrite/Nitrate (NO), Lactate, and Blood Oxygen Saturation Arterial-Venous Difference in Oxygen Saturation

Compared with the IR group, the MB-R and R-MB groups showed significant difference in lactate levels ($P < .001$), resembling the curves obtained for the SHAM and MB.
groups. There were no significant differences between the MB-R and R-MB groups \((P > .05; \text{Fig 5A})\). Intergroup NO statistical analysis revealed no difference. The MB group presented values quite similar to those recorded for the SHAM group, with no significant difference on comparison with the other groups \((P > .05; \text{Fig 5B})\). The MB-R and R-MB groups displayed significantly different in oxygen saturation levels \((P < .05\) compared with the IR group, resembling the curves obtained for the SHAM and MB groups. There were no significant differences between the MB-R and R-MB groups \((P > .05; \text{Fig 6})\).

Liver Tissue Nitrite/Nitrate (NO) and MDA

In the intergroup analysis, there were no significant differences in terms of tissue NO dosage. It is noteworthy that the curve obtained for the R-MB group was quite similar to that of the SHAM group \((P > .05; \text{Fig 7A})\). There were no significant differences among the groups with respect to tissue MDA measurements \((P > .05; \text{Fig 7B})\).

Histological Analysis

The histological analyses performed on the SHAM and MB groups were quite similar. The IR group revealed an inflammatory pattern with areas of congestion edema, and cellular necrosis (Fig 8A), as well as diffuse infiltrates with 1 animal showing a few hepatocytes containing cytoplasmic vacuoles (Fig 8B).

The number of intravascular neutrophils was smaller among the MB-R revealing less inflammatory reaction and less perivascular leakage of blood vessels into the parenchyma (Fig 9A and 9B). The average number of neutrophils was greater among the IR, R-MB, and MB-R compared with the SHAM and MB groups \((P = .0016; \text{one-way ANOVA test})\): SHAM \(\times\) IR \((P < .01)\); SHAM \(\times\) R-MB and SHAM \(\times\) MB-R \((P < .05)\); MB \(\times\) IR \((P < .01)\); MB \(\times\) R-MB and MB \(\times\) MB-R \((P < .05)\); Bonferroni multiple comparison post-test; Fig 9C and 9D).

DISCUSSION

Apart from the setting of sepsis,\(^2\,^4\) MB has been used as a vasopressor in acute liver failure (1–3 mg/kg).\(^5\,^6\) When the MAP decreases, MB bolus causes it to increase by increasing peripheral vascular resistance and, in some cases, by enhancing the cardiac index. Sepsis syndrome and IRS share biochemical and hemodynamic variables, including higher levels of pro-inflammatory cytokines.\(^7\)

There are few literature reports on the use of MB during circulatory shock, more specifically when caused by liver graft reperfusion. Koelzow et al successfully used MB in humans.\(^1\) Since then, anesthesiologists who specialize in liver transplantation have described the use of MB to

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**Table 1. Liver Serum Enzymes**

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<th>SAMPLE-1</th>
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<tbody>
<tr>
<td>AST</td>
<td>ALT</td>
<td>AST</td>
</tr>
<tr>
<td>SHAM</td>
<td>42.83</td>
<td>57.33</td>
</tr>
<tr>
<td>MB</td>
<td>24.60</td>
<td>75.40</td>
</tr>
<tr>
<td>IR</td>
<td>35.66</td>
<td>57.00</td>
</tr>
<tr>
<td>MB-R</td>
<td>33</td>
<td>43.5</td>
</tr>
<tr>
<td>R-MB</td>
<td>29.83</td>
<td>48.50</td>
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Note: Blood samples were taken at the beginning of the experiment (time zero), at the end of ischemia (60 min), and at the end of reperfusion (180 min).
reverse situations of postoperative circulatory shock, despite the vasopressor properties of this compound.8,9

The experimental model used herein employed continuous registration of MAP, CVP, and HR values; biomarkers were determined at previously defined moments, namely baseline, end of ischemia, and end of the experiment. The histological samples were only collected at the end of the experiment. Male rabbits were used to avoid a possible influence of the menstrual cycle. In addition to the SHAM group, the investigated groups included animals undergoing IR either treated prophylactically prior to reperfusion (MB-R), as described by Koelzow et al.,1 or following reperfusion, according to Fischer et al.10 Subsequently, another SHAM group received MB but was not subjected to IR, seeking to assess whether the therapeutic response to MB depended on the ischemic injury.

The anesthetic technique was based on studies that used a similar methodology in rabbits.11 Moreover, anesthesia with ketamine/xylazine used herein provided good analgesia and spontaneous breathing without relevant hemodynamic repercussions. Supplementary oxygen was used; inhalation proceeded via a low-flow catheter (2 L/min) to prevent hypoxia manifested by clinical cyanosis. However, this maneuver invalidated the blood gas parameters because the absolute oxygen concentration could not be compared among animals, except for the relative concentration between arterial and central venous oxygen.

Concerning the IR model adopted in this investigation, it was necessary to establish a reasonable period of ischemia, so that the IRS would be triggered without a high animal mortality. Previous protocols have adopted 20,12 30,13 or 60 minutes of ischemia.11 Ischemic times shorter than 60 minutes were insufficient to trigger IRS in this pilot study, whereas longer ischemic times generated immediate circulatory shock and death. For this reason, we selected 60 minutes of ischemia. We used survival time after the onset of reperfusion as an indicator of MB effectiveness.

As for the reperfusion period, it is known that the expression of inducible NOS (iNOS) takes about 4–6 hours to be detectable, but in clinical practice the onset and verification of the effects IRS are almost immediate, allowing us to conclude that the observation period for MB activity could be shortened to 120 minutes.

Unfortunately, due to the size of the rabbits, a Swan-Ganz catheter was not feasible, so it was not possible to measure cardiac output, pulmonary capillary wedge pressure, or peripheral vascular resistance. Therefore, the criteria for cardiovascular collapse were based on MAP, CVP, and HR records. Plasma lactate levels and SpO2(a–v) determinations were performed to improve the sensitivity of the diagnosis of circulatory shock. In the specific case of lactate levels, we were concerned that MB would interfere with the measurement; however, the device (Gem Premier 3000) used herein employed an amperometric method, which was not susceptible to colorimetric interference.

The MB dose was injected as an endovenous bolus, according to literature reports.5,14 Generally, IRS is related to primary dysfunction of the tissue/organ subjected to ischemia. Therefore, in the present study we analyzed liver function to verify whether MB prevented IRS. Once MB was proven to be effective, we examined whether it depended on NO expression. A further question was whether MB action is systemic, or affect liver parenchyma only, or perceived systemically and in the liver. To address these questions, NO was measured in both liver tissue and plasma and indirect free radical activity (MDA) was also determined in the liver. At the end of the experiments, a
histological analysis verified whether the changes were irreversible.

Hemodynamic results confirmed the findings of Koelzow et al. The MAP of control animals remained stable, with a slight decrease in levels during the experimental period of 180 minutes. Animals belonging to the IR group underwent a sudden decrease in MAP within the first 5 minutes of reperfusion, with deaths of 4 of the 6 animals, thereby demonstrating the severity of IRS in terms of mortality. Two animals died among MB administrations (MB-R group), but their deaths occurred at 20 and 40 minutes of reperfusion. As for the group subjected to treatment with MB (R-MB group), only 1 animal died at 60 minutes of reperfusion. The CVP and HR profiles accompanied the MAP events, with minor variations, thus confirming the conclusions above.

Biochemical tests to assess liver function gave evidence of significant increases in ALT and AST levels among all groups, a finding that was consistent with the literature. The use of MB did not attenuate this increase. These results support the conclusion that MB does not confer protection to liver parenchyma with respect to the maintenance of normal physiological conditions.

Liver NO production showed no significant differences among the groups, thereby revealing that the establishment of IRS has no association with NO production in the liver parenchyma.

With respect to lipid peroxidation in the reperfused liver, no significant differences were detected in MDA concentrations among the investigated livers. This observation contrasts with suggestions by other authors that the extent of IR correlates with tissue MDA content. As well as our own rat studies.

All of the above-mentioned results indicated that the mechanism of IRS establishment is systemic, only being “triggered” by some factor driven from the parenchyma. Therefore, the systemic response is probably related to the NO/GMPc (cyclic guanosine monophosphate) pathway, as judged from the positive hemodynamic response to MB.

The ischemia period (60 minutes) used in the present study was sufficient to produce circulatory shock in animals, evidenced by the significant increase in lactate concentrations and the difference in arterial and venous blood oxygen saturations among the IR versus the control group. Treatment with MB (R-MB group) reversed the increase in circulatory shock parameters, which returned to values similar to those detected in controls. However, a different situation was observed when MB was used prophylactically.

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These data are consistent with other findings of the present investigation: they provide one more indication that MB becomes effective after some “triggering” factor is released into the bloodstream following systemic circulatory shock. This fact was not evaluated in the clinical study of Koelzow et al, who assessed only the prophylactic use of MB.

Our histological findings showed that MB attenuated the inflammatory response due to IR injury as measured by the number of neutrophils at the hepatic lobe and near the portal space. They were larger than those observed among animals from MB and SHAM groups, which was statistically significant (P = .0016; One-way ANOVA test) between three groups when compared with animals from MB and SHAM groups: SHAM × IR (P < .01); SHAM × R-MB and SHAM × MB-R (P < .05); MB × IR (P < .01); MB × R-MB and MB × MB-R (P < .05).

In conclusion, MB improved the vital signs and promoted lactate and oxygen extraction, leading to better survival during reperfusion. However, the duration of this effect was longer where we used MB treatment rather than prophylaxis. On the basis of the biochemical markers, we may hypothesize that GC blockade by MB does not protect the liver, strongly suggesting that the hemodynamic improvement in IRS was not based on liver integrity but possibly on a systemic vasoplastic endothelial dysfunction. In the present research ischemia was induced only by clamping the hepatic artery; it is well known that in this condition 75%–80% of the liver remains perfused through the portal vein. This explains the fact that normal values were obtained for the AST and ALT and reinforces the idea that hemodynamic instability occurs irrespective of liver injury.

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