Effects of methylene blue in acute lung injury induced by blunt chest trauma


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Abstract

Background: We studied whether methylene blue (MB) treatment blunts chest trauma-induced lung injury in rats.

Material and Methods: Forty male Sprague-Dawley rats, 200-300g, were used. The rats were divided into five groups (n=8): control, early contusion (EC), early contusion + methylene blue (2 mg/kg, EC+MB), late contusion (LC), and late contusion + methylene blue (2 mg/kg, LC+MB).

Results: Histopathological analysis showed increased hemorrhage, alveolar wall thickness, edema, and inflammatory cell infiltrates in the EC and LC rats, which decreased upon MB treatment. Immunohistochemical studies revealed that MB reduced activation of inducible nitric oxide synthase (iNOS) and the number of active terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells. A significant increase was observed in the malondialdehyde (MDA) and nitric oxide (NO) levels in the EC group compared to the control group (p<0.05). In addition, a significant decrease was reported in the glutathione (GSH), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels (p<0.01), but no significant difference was observed in the catalase (CAT) levels among the groups. The MDA level was significantly higher in the LC group compared to the control group, whereas the GSH level was significantly lower compared to the control group. The NO level in the EC+MB group was significantly lower when compared to the NO level in the EC group (p<0.05).

Conclusion: The present study provides evidence that MB might serve as a therapeutic treatment for blunt chest trauma.


Keywords: Blunt chest trauma, contusion, methylene blue, oxidants, antioxidants

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Introduction

Pulmonary contusion (PC) is a frequent injury type depending on blunt chest contusion. PC is seen in 17-70% of major injuries. PC causes alveolar congestion and hemorrhage, corruption of alveolar integrity, edema, and leukocyte infiltration. The clinical reflection of this situation is the case defined as hypoxemia and enhanced bronchospasm changing with hypercarbia duration. The basic pathophysiological mechanism of contusion is ventilation/perfusion defects, enhanced intrapulmonary shunt, increases in lung liquid amount, lung tissue damage at least at the segmental level, and a decrease in compliance. PC leads to a progressive inflammatory response, which is controlled by local and systemic immunological changes, as has been proven in many studies.

As there is no specific treatment for PC, researchers have used the molecules thought to be useful for human beings in experimental models. Swennevig et al reported that methylprednisolone (30 mg/kg) causes a decrease in the mortality rate and some hemodynamic parameters of blunt chest trauma and patients with PC. In a model inspired by PC-induced tissue damage pathogenesis, use of molecules that block cytokine release, such as indomethacin, before contusion leads to healing in the clinical and histological condition. Zhang et al showed that N-acetylcysteine (NAC) treatment has a positive effect on edema, pulmonary vascular resistance, static compliance, and oxygenation. There are different routes of administration concerning the intravenous or intratracheal use of methylene blue (MB). MB has been used as an agent in clinics for years, and is less toxic and readily available. MB is competitive with molecular oxygen, which is used in electron transfer by xanthine oxidase, and inhibits the formation of oxygen radicals and superoxides. In addition, MB can prevent nitric oxide (NO) effects by inhibiting soluble guanylate cyclase, NO, endothelial NO synthase (eNOS), and inducible nitric oxide synthase (iNOS). MB plays an important role in repairing liver, kidney, and lung...
tissue ischemia reperfusion damage, refractory septic shock, methemoglobinemia, and in treating some neurological diseases. In recent years, studies have proven that MB prevents alveolar damage and increases arterial oxygenation in rats that have sepsis or intestinal ischemia/reperfusion damage by cecal ligation and puncture methods. These studies showing the MB antioxidant effect and its effectiveness on oxidative damage encouraged us to conduct this project. We investigated the literature, but found no study investigating the protective effect of MB in the case of pulmonary contusions. When we considered the antioxidant properties of MB and the role of oxidative stress, we hypothesized that MB would be useful in treating PC.

Materials and methods

Chemicals

MB, hydrogen peroxide, reduced glutathione, thiobarbituric acid, phosphate buffer, butylated hydroxytoluene, trichloroacetic acid, EDTA, 5,5’-dithiobis(2-nitrobenzoic acid), disodium hydrogen phosphate, phenylenediamine, ethanol, hexane, sodium nitrite, sodium nitrate, and ethylenediamine dihydrochloride were purchased from Sigma Chemical Co. In addition, superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Randox, UK) and NO (Enzo, USA) commercial kits were used. All other chemicals and reagents used in this study were of analytical grade. Ultra-distilled water was used as the solvent.

Animals and experimental protocol

Male Sprague-Dawley rats, weighing 200-250 g, were housed in the Experimental Animals Research Laboratory, Trakya University, Turkey, under standard laboratory conditions (22 ± 1 °C, 12 h light/dark cycle). The Ethical Committee of Trakya University approved all procedures and the experimental protocol concerning the animals. Rats were fed with standard rat chow and tap water ad libitum.

Falling weight methods defined by Raghavedran et al. were applied to perform PC. Rats were anaesthetized with 50 mg/kg intramuscular ketamine hydrochloride and 15 mg/kg intramuscular xylazine. The rats’ right hemithorax was drawn, and a 500 g metal cylinder was dropped from a 50 cm height in the supine position. In this way, trauma was standardized by applying 2.45 j energy on the chest region according to the formula E=mgh [E: energy (joule), m: mass of cylinder (kg), g: gravity constant (9.8 m/s²), and h: height (meter)].

The rats were divided randomly into five groups (n=8): control, early contusion (EC), early contusion+methylene blue (EC+MB), late contusion (LC), and late contusion+methylene blue (LC+MB). In the EC+MB group, 2 mg/kg MB has given intraperitoneally once a day just after contusion and the day after contusion. In the LC+MB group, 2mg/kg MB was given intraperitoneally twice a day for six days; the first injection was performed just after contusion. After 48 h in control, the EC and EC+MB groups, and seven days later in the LC and LC-MB groups, thoracotomy was done after re-anasthetization. The right lung tissue of all rats was immediately biopsied. Half of the right lung was put into Bouin’s solution for histopathological examination. The other half of the right lung was preserved at -80 °C until the biochemical parameters were analyzed.

Histopathology evaluation

Lung tissue samples were fixed in Bouin’s solution and embedded in paraffin at two and seven days after contusion were examined with hematoxylin and eosin.

iNOS immunohistochemistry

At the end of the experiment, the animals were decapitated. Lung tissues were removed, fixed in Bouin’s solution, and, following routine laboratory methods, embedded in paraffin. Five-micrometer paraffin tissue sections were mounted on poly-L-lysine slides. The slides were air-dried and the tissue deparaffinized. The avidin-biotin complex method was used for immunohistochemical staining, as previously reported. The sections were incubated with rabbit polyclonal anti-iNOS (Abcam, dilution 1:100) (Abcam, USA). Diaminobenzidine (DAB) was used as a chromogen, and the sections were counterstained with hematoxylin. The specificity of the immunohistochemical staining was tested using phosphate buffered saline (PBS) in the same dilutions. Control tissue sections were used as positive controls.

TUNEL assay

Apoptotic cells were stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique using an apoptosis detection kit (Calbiochem, San Diego, CA, USA), as previously reported. The number of iNOS and TUNEL-positive cells in each specimen was also scored. Five to 10 randomly selected areas were scored for each specimen in every experiment as follows: 0 = no positive response; 1 = less than 10 % of cells; 2 = 11-20 % of cells; 3 = 21-40 % of cells; 4 = more than 40 % of cells. These analyses were performed in two sections from each animal at 400x magnification in at least 10 different regions for each section.

Biochemical assay

The studied tissues were homogenized in tenfold volume of physiological saline solution by using a homogenizer (Ultra-Turrax T25, IKA; Werke 24,000 r.p.m.; Germany). The homogenate was centrifuged at 10,000×g for 1 h to remove debris. Clear upper supernatant was taken, and tissue analyses were carried out in this fraction. All procedures were performed at +4°C throughout the experiments. The tissue samples were stored in a polystyrene plastic tube at -80°C until the time of analysis. Malondialdehyde (MDA, as an important indicator of oxidative stress) levels were measured accord-
ing to Jain et al’s method. The principle of the method is based on the spectrophotometric measurement of the color that occurred during the reaction of thiobarbituric acid with MDA. Concentrations of thiobarbituric acid reactive substances (TBARS) were calculated with the absorbance coefficient of the malondialdehyde–thiobarbituric acid complex and expressed in nmol/mL. Glutathione (GSH) concentration was also measured with a spectrophotometric method. After whole blood was lysed and the precipitate was removed, disodium hydrogen phosphate and 5,5’-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) solution were added, and the color formed was read at 412 nm. SOD and GPx activities were studied on hemolysates by using commercial kits (Randox Laboratories, UK). Catalase (CAT) activity was measured according to Aebi’s method. The principle of the assay is based on determining the rate constant \( k (s^{-1}) \) of hydrogen peroxide decomposition by the catalase enzyme. The decomposition of the substrate hydrogen peroxide was monitored spectrophotometrically at 240 nm. The rate constant was calculated from the following formula: \( k = (2.3/\Delta t)(a/b) \log(A1/A2) \). NO was predicted as nitric oxide metabolites by Griess reaction after nitrate was transformed into nitrite by nitrate reductase. The nitrate present in the sample, which was derived from NO itself and peroxynitrite, was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase. NO products were presented as \( \mu \text{mol/g} \).

**Statistical analysis**

Statistical analysis of the control and the four experimental groups was compared using one-way analysis of variance (ANOVA) and Tukey’s posttest. For histological analyses, the non-parametric Bartlett test was used to determine whether the data was heterogeneous or homogeneous. The Bonferroni multiple comparison procedure with ANOVA was then applied to identify differences between means. A value of \( p < 0.05 \) was considered statistically significant. All values were expressed as mean ± standard deviation. Statistical tests were performed using SPSS version 12.0 PL for Windows (SPSS Inc., Chicago, IL, USA).

**Results**

**Histopathological findings**

To examine the effect of MB on blunt chest trauma-induced lung injury, we assessed the pulmonary morphological changes using light microscopy. The histology of the control group lung tissues showed normal morphology (Figure 1a). Two and seven days after blunt chest contusion in the EC and LC groups, markedly increased alveolar wall thickness, edema, bleeding, and inflammatory cell infiltrates were detected in rat lungs compared with the control (Figure 1b and Figure 1d). According to the experimental design, the rats were administered MB intraperitoneally after contusion. MB administered at a dose of 2 mg/kg after contusion significantly inhibited blunt chest contusion-induced histological changes in the EC+MB and LC+MB groups (Figure 1c) and (Figure 1e). These results indicated that MB was effective in relieving blunt chest contusion-induced inflammatory symptoms of rat lungs.

**Immunohistochemical and TUNEL findings**

In the control group, a few TUNEL-positive cells were observed in the lung tissue (Figure 2a). The TUNEL staining results showed that the number of TUNEL-positive cells in the EC group was remarkably increased compared with the control group (\( p < 0.001 \)) (Figure 2b). In addition, the number of TUNEL-positive cells in the EC group was higher than in the other groups (\( p < 0.05 \)). Treatment of MB significantly decreased the number of TUNEL-positive cells in the EC+MB group (Figure 2c). The number of TUNEL-positive cells did not differ between the LC (Figure 2d) and LC+MB (Figure 2e) groups (\( p > 0.05 \)). The number of iNOS-positive cells in the EC group (Figure 3b) was significantly higher than in the control (Figure 3a) and EC+MB (Figure 3c) groups (\( p < 0.001 \) and \( p < 0.05 \), respectively). Additionally, the numbers of iNOS-positive cells in the LC (Figure 3d) and the LC+MB (Figure 3e) groups were significantly lower compared to the control group (\( p < 0.05 \)). The number of iNOS-positive cells in the LC group was significantly higher than in the LC+MB group (\( p < 0.01 \)). Quantitative
evaluation of the iNOS and TUNEL staining results is presented in Figure 2f and Figure 3f.

**Biochemical findings**

The oxidant (MDA and NO) and antioxidant (GSH, SOD, GPx, and CAT) levels of the experimental groups are shown in Figure 4. We determined that the lowest MDA level was in the control group and the highest in the EC group. Comparison among the groups revealed that MDA levels in the EC and LC groups were significantly higher than those in the control group (p<0.01 and p<0.05, respectively). However, the MDA levels in the MB administered (EC+MB and LC+MB) groups was not significantly different from the EC and LC groups (p>0.05) (Figure 4a). As for the GSH levels, there was a significant decrease in GSH levels in the EC and LC groups when compared to those in the control group (p<0.05) (Figure 4b). The SOD enzyme activity of the EC groups was lower than in the control group (p<0.01) (Figure 4c). In addition, GPx activity in the EC group decreased when compared to the control group (p<0.05) (Figure 4d). CAT enzyme activity showed no significant difference between the study groups (p>0.05) (Figure 4e).
The NO level of the EC groups was higher than in the control group (p<0.05). In addition, the NO level in the EC group was higher than in the EC+MB groups (p<0.05) (Figure 4f).

Discussion
People suffering from PC may need a wide variety of treatments, from simple oxygen supplement to serious mechanical ventilation. Many experimental and clinical studies have shown that these patients have oxidative stress and an inflammatory mechanism in tissue damage in their clinical story.

Potential inflammatory mediators, macrophages, and neutrophils are activated just after blunt chest trauma so that the progressive destruction cascade is initiated. Cytokines are released from activated neutrophils and macrophages and cause an increase in the number of reactive oxygen radicals and proteolytic enzymes and the amount of alveolocapillary membrane permeability, which in turn causes microvascular leakage. Alveolar macrophages can produce potential free oxygen radicals called superoxide radicals (SOR) and peroxynitrite. These released SOR may cause oxidative damage via lipid peroxidation. Finally, the direct damaging effect of PC and the primary and secondary inflammatory response combined with oxidative stress negatively affects the clinical period. Therefore, in this study, we examined the biochemical parameters (such as MDA, GSH, SOD, CAT, GPx, and NO) to observe the therapeutic effect of MB in the pathogenesis of pulmonary contusions.

With a pulmonary contusion, pathophysiological change is relative, depending on the time after the contusion occurred. In this study, we designed the experimental and therapeutic groups as two and seven days to observe the early and late onset activity of MB and its relationship with the physiopathology of pulmonary contusions. Raghavedran et al detected an increase in the erythrocyte number, albumin release in bronchoalveolar lavage, and arterial hypoxemia between the 8th minute and 4th, 12th, and 24th hours prior to contusion caused parenchyma in the rat model where pulmonary contusion was formed. In that same study, the concentration of all the inflammatory mediators returned to the basal values on the seventh day. In our study, early onset (day two) biochemical and histopathological findings were directly proportional with that study in terms of pulmonary parenchyma damage. However, histopathological findings and some biochemical parameters (such as an increase in MDA and a decrease in GSH) at the end of the seventh day were thought to indicate continuous parenchyma damage.

Salaris et al showed that MB has an effect as a parasitic electron collector by interrupting the electrons’ usual method of making leukomethylene. The production step of free oxygen radicals was skipped. MB, an alternative electron receiver for tissue oxidases, can be accepted as an antioxidant drug that competitively inhibits reduction of molecular oxygen to superoxides. In addition, MB competitively races against flavoenzymes such as xanthine oxidase to transfer oxygen and prevent oxygen radicals such as superoxides being produced.

An inflammatory reaction and oxidative stress occur after a pulmonary contusion. MDA is a reliable parameter, as it is the final product of lipid peroxidation caused by SOR. GSH is a very strong antioxidant used to measure oxidative stress, and its level decreases during oxidative stress. In our study, high MDA levels and low GSH levels at the early and late onsets were explained as a marker for lipid peroxidation and oxidative damage. Gokce et al reported decreased SOD activity, depending on the experimental pulmonary contusion. We also observed low SOD level at the early onset of the contusion. Türüt et al showed decreased CAT activity after the pulmonary contusion. Yet, in our study, CAT activity did not change significantly according to early or late onset of the contusion. Demirbilek et al reported that MB treatment decreases MDA levels (whereas the SOD, CAT, and GPx levels increase), prevents lipid peroxidation, and decreases lung damage. However, in this study, decreased MDA levels in the MB-treated groups and an increase in the SOD, CAT, and GPx levels were not observed.

NO synthesis, as a free radical, induces iNOS production and increases pulmonary inflammation. The primary effect of MB inhibits guanylate cyclase by targeting NO. Moreover, MB has additional pharmacological effects, including production of oxygen radicals by inhibiting NO synthase. Gallili et al indicated that MB can decrease the alveolar injury by preventing the hazardous hemodynamic effects of NO. In another study, Gallili et al reported that in the intestinal ischemia/reperfusion-induced lung injury model, MB reduced interstitial and alveolar edema and neutrophil sequestration by inhibiting NO activity. In this study, we interpreted that MB could be effective against the damage caused by a pulmonary contusion, as MB application decreased NO level at early onset. Peroxynitrite formation significantly increases in increased NO and superoxide concentration and/or decreased SOD activity. Decreases in NO and superoxide may cause reductions in peroxynitrite. Thus, MB can be accepted as an antioxidant, as it decreases peroxynitrite formation. In addition, NO inhibits GPx activity. This condition could be the reason for the cytotoxic effect of NO and NO leading to apoptotic cell death. In our study, we considered damage in the EC group could be caused by increased peroxynitrite in addition to the decreased SOD and GPx levels. Starting from this point, we considered that MB decreases the level of NO and prevents peroxynitrite production, and thus has a protective effect against damage generation at early onset.

Our data also indicated that, in the immunohistochemical stains of the lung tissue, a marked increase in the iNOS-positive cells was evident at two days following blunt chest trauma. In addition, the iNOS-positive cell numbers decreased significantly in the lungs of traumatized animals at two days following treatment with MB. These histopathological findings supported the idea that MB application could effectively act on the damage...
caused by pulmonary contusions during early onset. Although MB treatment did not decrease the NO level during late onset, it is remarkable that MB application did decrease the number of iNOS positive cells during late onset. We did not encounter a study evaluating pulmonary contusions with iNOS staining.

In the last two decades, the importance of programmed cell death has been better understood in multiple organ failure and acute lung injury/adult respiratory distress syndrome pathogenesis8-10. Liener et al77 found the first proof that programmed cell death begins following PC. Apoptotic cell death is caused by massive oxygen radicals. This hypothesis is based on increasing myeloperoxidase levels of unfiltered PMNs, which are free radical sources. Seitz et al88 determined that type II cellular apoptosis increases in the 48 h after lung contusion. In their study, decreased type II cell number in the lung contusion model is correlated with the remaining population’s apoptosis markers, caspase-3, caspase-8, and Fas levels. In the present study, we demonstrated that MB inhibits apoptotic cell death in lung tissue alveolar cells and interstitial cells as determined with the TUNEL method. However, Seitz et al88 observed no changes in TUNEL staining. When lung sections were TUNEL stained, there was a significant increase in the number of positive cells in the EC rat group. Treatment with MB markedly reduced the reactivity and the number of TUNEL-positive cells. We considered that MB preventing apoptosis may be achieved by inhibiting the synthesis of NO.

In conclusion, we demonstrated that MB therapy could be used to treat the progression of experimental PC in accordance with our hypothesis. We posit that MB prevented PC by decreasing NO activity in the early period of contusion. Although we determined the beneficial effect of MB on oxidant and antioxidant parameters for treating contusion in the late period, we did not have statistically significant results. However, we demonstrated that MB treatment could be useful in the late period, as supported by histopathological findings. Moreover, the histopathological findings demonstrated that oxidative stress in PC was responsible for pulmonary parenchymal damage by inducing apoptosis, and MB prevented PC development by inhibiting apoptosis thanks to its anti-oxidant properties.

Conflict of interest
The authors declared no conflicts of interest.

References