Methylene blue attenuates lung injury after mesenteric artery clamping/unclamping

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Abstract

Background This controlled, experimental study was designed to assess the effects of intratracheal and intravenous methylene blue on reperfusion lung injury following superior mesenteric artery clamping/unclamping.

Materials and methods Superior mesenteric arteries of 144 anaesthetized adult male Wistar rats (n = 12/group) were clamped for 1 h. Ten minutes before unclamping, methylene blue or its vehicle was administered intratracheally or intravenously, followed by a 3 h-respiratory assessment and postexperimental assessment of survival.

Results Intravenous 3 and 9 mg kg⁻¹ but not higher methylene blue doses, and intratracheal 6-mg kg⁻¹ but not lower doses, significantly (P < 0.05) reduced the 100% increase in plateau pressure, 30% reduction in PO₂/FiO₂, fourfold augmented bronchoalveolar lavage-retrieved volume and the increased polymorphonuclear leukocytes/bronchoalveolar cells’ ratio associated with unclamping of the superior mesenteric artery. Lung tissue polymorphonuclear leukocytes count, total xanthine oxidase activity and wet-to-dry-weight ratio were also normal in these dose-treated groups. These effective regimens were also associated with longer animal survival.

Conclusions Intratracheal methylene blue mitigates lung reperfusion injury following superior mesenteric artery clamping/unclamping at a similar magnitude as an intravenous regimen. This finding is a novel potential use of methylene blue in vivo.

Keywords Injury, intestine, ischaemia-reperfusion, lung, methylene blue, trachea.

Introduction

The pathogenesis of acute lung reperfusion injury involves overproduction of radical oxygen species (ROS) [1] and activation of polymorphonuclear leukocytes (PMNL) [2]. Several compounds have been demonstrated as being capable of preventing or attenuating the xanthine oxidase (XO)-generated ROS-dependent injury in an isolated-perfused lung model, among them allopurinol, mannitol and N-acetyl-L-cysteine [3,4]; however, opinions are divided as to their efficacy in the intact animal.

Methylene blue (MB) blocks several ROS, particularly superoxides, by competing with the molecular oxygen for the transfer of electrons by XO [5]. Methylene blue was shown to prevent XO-induced aortic ring dysfunction upon exposure to postischaemic liver reperfusate [4,6]. Intraperitoneal (IP) MB was also used to reduce PMNL recruitment within the rat pulmonary vasculature [7]. More recently, the effects of IP MB were compared with the intratracheal (IT) potentials in attenuating haemodynamic and metabolic derangements that followed intestinal ischaemia-reperfusion (IR) [8]. Others have also administered antioxidants IT and demonstrated the efficacy of this route [9], as drugs are rapidly absorbed into the lung and do not undergo first pass metabolism [10]. Trans-tracheal drug administration is also used clinically in treating respiratory and cardiovascular emergencies [11].

In this study, an intestinal IR model which detrimentally affects murine lung function [7,8] was used in combination with a dose–response protocol of MB in order to test the hypothesis that MB given IT could attenuate lung injury following gut IR. This potential was compared with those obtainable with the clinically employed IV route.
Materials and methods

This study was performed in accordance with the Public Health Service policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act (7 US.C. et seq.). The protocol was approved by the Institutional Animal Care and Use Committee of Tel Aviv Sourasky Medical Center.

General protocol

Figure 1 displays the main experimental steps. Adult male Wistar rats ($n = 144$, 350–420 g) were anaesthetized with IP ketamine 7 mg kg$^{-1}$ plus diazepam 1 mg kg$^{-1}$; an additional dose (2 and 0.5 mg kg$^{-1}$, respectively) was given 2 h later. Animals were initially tracheostomized and mechanically ventilated with 95% air/5% CO$_2$ (the latter to prevent possible PCO$_2$ and pH changes during ventilation), using a piston-type rodent ventilator (10-mL kg$^{-1}$ of body weight tidal volume, 40 breaths min$^{-1}$, Rodent Respirator Model 683™, Harvard Apparatus®, South Natick, MA), aiming to maintain arterial PO$_2$ at $\geq$ 80 mmHg and PCO$_2$ between 32 and 40 mmHg. Positive end-expiratory pressure was maintained at 4 mmHg. The femoral artery was cannulated with an 18-gauge polyethylene catheter (Vigon 2™ Ohmeda®, Helsingborg, Sweden) for invasive blood pressure monitoring and blood sampling, while venous cannulas enabled fluid administration and drug injection where appropriate. Heparin 500 IU was administered IP 10 min after vascular cannulation was completed. Each animal was left to stabilize for 30 min before undergoing the following procedures.

A 25-mm-long midline laparotomy was performed and the superior mesenteric artery (SMA) was occluded with an atraumatic microvascular clip for 1 h. The most cephalad suture was reopened 1 h later to remove the clip; the absence of a pulsating SMA after the removal of the clip excluded the rat from subsequent experimentation. The suture was replaced and the animal was kept under observation for a 3-h reperfusion period. The acute experimental phase was then terminated: half of the group was euthanized with a high IV dose of potassium chloride while the rest were left to recover and their survival rate was recorded.

As SMA clamping/unclamping can be associated with a low perfusion state [7,8], if the mean arterial pressure decreased to < 60 mmHg during the study, the animal received a 0.9% saline infusion (2 mL min$^{-1}$) until its blood pressure returned to the predetermined limit. If the arterial pH decreased to < 7.2, sodium bicarbonate 8.4% was administered (0.5 mL min$^{-1}$) to maintain it above this limit.

Study groups and drug protocol

Following the stabilization period, the animals were assigned to one of the 12 study groups ($n = 12$/group) as specified in Table 1. In addition to the four-dose groups/route, one group was also treated with MB’s vehicle (corresponding to the maximal dose used via that route) in order to determine whether the vehicle per se would have any effect on outcome.

Methylene blue (MB, 1%, Hope Pharmaceutics, Santa Ana, CA) was always diluted in 2.5 mL of 0.9% saline and administered 10 min before SMA unclamping. MB 3, 9, 15 and 27 mg kg$^{-1}$ was infused IV during a 5-min period; these doses were higher than the ones used in previous experiments [12–14] as, this time, I focused upon MB’s effects on the lungs. In view of its known liposolubility and direct uptake and minimal systemic metabolism when deposited within the alveolar space [10], taken together with recent findings [15], IT doses (at a ratio of 1 : 4 to the IVs) of 1, 2, 4 or 6 mg kg$^{-1}$ of body weight were administered in four different IR groups. Intratracheal MB was delivered by five puffs min$^{-1}$ for 5 min via a pump, which instilled MB into the distal airways. During these 5 min, oxygen mixed with air (1 : 1 ratio) was used in all animals.
Ventilatory and biochemical data collection

Inspiratory plateau pressure was used to assess ventilatory changes during the experiments. It was recorded directly and continually via a Statham Medical P132284™ pressure transducer (Mennen Medical®, Inc., Clarence, NY) positioned at the level of the main bronchi. The data were logged onto a physiological recorder (7D™ polygraph, Grass Instruments® Co., Quincy, MA) which was connected to a haemodynamic monitor (CS/3™, Datex-Ohmeda®, Helsinki, Finland).

Arterial blood (0·3 mL) was obtained and processed for oxygen and carbon dioxide partial pressures and for pH (AVL OMNI 8™, AVL, Graz, Austria); PO$_2$/FiO$_2$ ratio was later calculated and used to assess lung function. Saline was injected slowly IV at a 3:1-ratio to replace blood-sample volumes.

Pulse rate and mean blood pressure were also recorded directly from the arterial pressure waveform. Rectal temperature was constantly monitored and maintained at 37·5 °C.

Before sacrificing the animal, bronchoalveolar lavage (BAL) was performed using three 1-mL saline aliquots. The retrieved volumes were recorded and analyzed for PMNL, alveolar macrophages and bronchial cells.

At the end of this acute experiment, a thoracoabdominal incision was performed in six animals/group and 20 mL of saline was gently injected into the right ventricle to wash out the blood from the pulmonary circulation. Parts of the lungs were retrieved from each animal and kept at −70 °C to determine the total activity of xanthine oxidase (XO) plus its reduced form, xanthine dehydrogenase (XDH) (following Hashimoto’s method [16] with modifications). Wet-to-dry-weight ratios (WDRs) of lung tissues were determined by comparing the end-experimental wet weight with the dry weight of the same piece after being stored in an 80 °C oven for 5 days.

Statistical analyses

Data are presented as means ± SD. Analyses were performed at the Statistical Laboratory of the School of Mathematics, Tel Aviv University, using the SPSS Release for Windows, Version 11·01 (Chicago, IL, 2001). Normal distribution of the data was first ascertained by the Kolmogorov-Smirnov test. Comparisons of the normal values of each parameter between two groups at a time (e.g. control vs. IR-0) were then carried out by ANOVA with repeated measures, where time was the within-subjects’ effects and the drug variable was the between-subjects’ effects. Bronchoalveolar lavage volumes and rats’ survival periods did not distribute normally; their statistical analyses were carried out using the Mann–Whitney test. The ANOVA test with repeated measures was always followed by Tukey’s method for multiple comparisons test. Significance was set at $P \leq 0·05$.

Results

All IR groups treated with the vehicle solution yielded values that were similar to their corresponding IR-nontreated
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For clarity of data presentation, the IR-0-values include the vehicle-treated data as well.

Respiratory data

During stabilization, plateau pressure reached similar values among all groups; it did not change during the 1-h SMA occlusion (Fig. 2a,b). Superior mesenteric artery unclamping was associated with an immediate increase in plateau pressure which increased continuously during reperfusion. This, however, did not occur in the IR-IV 3- and 9-mg kg\(^{-1}\) treated groups. The IT-instilled MB also evoked dose-specific protective effects only in the IR-IT 6- and 4-mg kg\(^{-1}\) groups whose values were similar to those of the controls.

Compared with the controls' unaltered PO\(_2\)/FiO\(_2\), SMA unclamping was accompanied by a drop in the ratio, but less so for the IR-IV 3-, IR-IV 9-, and the IR-IT 6-mg kg\(^{-1}\)-treated animals (Table 2). The arterial methemoglobinaemia values are reported in Table 2.

Haemodynamics, fluids and metabolic data

Arterial blood pressure and heart rate decreased in all clamped/unclamped rats starting at SMA-reflow, but minimally in the IR-IV 9-MB-treated rats (Table 2). No IR-IT-MB regimen was capable of maintaining blood pressure or heart rate at baseline values.

The volumes infused in the IR-IT-treated rats in order to maintain the pre-established minimal blood pressure increased in parallel to the decrease in MB dosages, whereas the IV-treated rats exhibited a facedown bell-shaped trend (Table 2). IR-IT 6- and IR-IV 9 mg-kg-treated animals were the only groups in which fluid replacements were similar to those needed in the controls.

All IR groups required bicarbonate to maintain the desired pH. The amount of bicarbonate infused in the IR-0 rats was 10-fold higher than that required by the controls; the smallest amounts were administered in the IR-IV 9-, IR-IV 15- and the IR-IT 6-mg kg\(^{-1}\) groups.

BAL and PMNL data

Bronchoalveolar lavage-retrieved volumes in the IR-nontreated rats were significantly higher than in all IR-treated groups (Table 3). IR-IT 6-, IR-IV 9- and IR-IV 15-mg kg\(^{-1}\)-treated rats were the only groups that yielded volumes similar to those of the controls. The BAL-PMNL% values in the IR-IT groups were similar to those in the IR-IV groups but higher than those of the controls, with the exception of the low values of the IR-IV-9 and the IR-IT-6 groups. The rest of the cells in the BAL were alveolar macrophages and bronchial cells.

Total XO + XDH activity in lung tissues was higher in all IR rats compared with controls, except for the IR-IV-9 and the IR-IT-6 groups (Table 3). Here, too, activities increased as IT doses decreased and as IV doses increased.

Lung WDRs were higher in the IR-nontreated rats compared with the IR-treated or control groups. The ratios were relatively lower in the IR-IT 4-, 6- and IR-IV 3-mg kg\(^{-1}\)-treated lungs compared with their respective groups.

Fewer PMNLs were detected within the alveoli of the IR-MB-treated groups compared with the IR-nontreated group (Table 3). The inverse ratio between abnormal findings and IR-IT dosages and the inverse bell-shaped values vs. the IV doses applied for PMNL counts as well.

Survival data

No animal had difficulty in breathing and there were no iatrogenic injuries at the sites of acute interventions.
control rats survived the longest (> 3 days), followed by the IR-IV-27 and IR-IT -1 MB rats. The rest of the groups survived < 24 h (Table 3).

Discussion

The primary indices chosen to evaluate MB’s effectiveness were clinical, such as plateau pressure and PO\textsubscript{2}/FiO\textsubscript{2}; the secondary parameters were XO activity and PMNL lung recruitment. The main finding emerging from this study is that IT-MB dose-dependently contained the development of lung damage following 1 h of SMA clamping as effectively as the IV regimen, perhaps even better. Both strategies also resulted in sustained (3 h) effects instead of the short-lived beneficial effect of MB that was previously reported both in animals and in humans when it was administered after pathological conditions had developed [11,17–20].
It has been established that a variety of mediators associated with lung injury are released or activated in the circulation following SMA unclamping. The decisive role of XO and the generated ROS was demonstrated by some studies [3,4,7,17,21] while others focused on the role of neutrophils [2,7,22]. Xanthine oxidase was also reported to trigger PMNL lung sequestration [22], leading to lung ventilatory and vascular damage. Methylene blue is considered to be an antioxidant (e.g. superoxide [5] and peroxynitrite [23]) and was also used for the attenuation of XO-associated damage to organs other than the lung [4,6]. This is owing to MB’s potential of blocking ROS generation [5,23]. Methylene blue was also shown to inhibit NO synthase (NOS) [24], as well as the guanylate cyclase nitric oxide (NO)-binding site, besides causing the direct inhibition of NO synthase-dependent cGMP in the endothelial cells [25].

MB’s favourable effects were not seen in the 27-mg kg⁻¹ IV-treated lungs. This lack of effectiveness could be owing to an excessive MB-dependent blockade of guanylate cyclase, as had been reported previously [14]. Together with ROS’ endothelial-damaging potentials [22], which could ultimately lead to vascular tone impairment [6], an increase in metabolic acidosis could also down-regulate the beneficial effects of MB on the vascular bed [26] and generate a systemic vicious cycle of excessive need for fluid resuscitation and sodium bicarbonate. In addition, an excessive amount of MB-induced systemic and pulmonary vascular constriction could lead to changes in alveolo-capillary membrane integrity, thus enabling passage of fluid and large molecules into the alveolar space, resulting in the high WDR, XO activity and PMNL count, deterioration in PO₂/FiO₂ ratio and increase in plateau pressure. This line of reasoning is in agreement with the evidence of animals and humans.

References


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23 Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BE. Apparent hydroxyl radical production by peroxyxynitrate: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990;87:1620–4.


