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Methylene blue reduces pulmonary oedema and cyclo-oxygenase products in endotoxaemic sheep

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Methylene blue reduces pulmonary oedema and cyclo-oxygenase products in endotoxaemic sheep. O.V. Evgenov, N.V. Evgenov, T.E. Mollnes, L.J. Bjertnaes. ©ERS Journals Ltd 2002.

ABSTRACT: The authors recently demonstrated that methylene blue (MB), an inhibitor of the nitric oxide (NO) pathway, reduces the increments in pulmonary capillary pressure, lung lymph flow and protein clearance in endotoxaemic sheep. In the present study, the authors examined whether MB influences pulmonary haemodynamics and accumulation of extravascular lung water (EVLW) by mechanisms other than the NO pathway.

Sixteen awake, chronically-instrumented sheep randomly received either an intravenous injection of MB 10 mg·kg^{-1} or isotonic saline. Thirty minutes later, all sheep received an intravenous infusion of *Escherichia coli* endotoxin 1 μg·kg^{-1} for 20 min and either an intravenous infusion of MB 2.5 mg·kg^{-1}·h^{-1} or isotonic saline for 6 h.

MB markedly attenuated the endotoxin-induced pulmonary hypertension and right ventricular failure, and reduced the accumulation of EVLW. Moreover, MB reduced the increments in plasma thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$, and abolished the febrile response. However, MB had no effect on the changes in circulating neutrophils, serum hyaluronan, and total haemolytic activity of the alternative complement pathway.

The authors conclude that in sheep, methylene blue attenuates the endotoxin-induced pulmonary hypertension and oedema, at least in part, by inhibiting the cyclo-oxygenase products of arachidonic acid. This is a novel effect of methylene blue *in vivo*.

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Acute lung injury (ALI) represents the pulmonary manifestation of a global inflammatory process that is often associated with sepsis. Lung oedema, resulting from enhanced pulmonary microvascular pressure and permeability, is a pathophysiological hallmark of ALI [1]. Various mediators have been implicated in ALI, such as endotoxin, cytokines, eicosanoids, complement, degradation fragments of extracellular matrix, and oxygen free radicals [1–4]. A growing body of evidence also suggests that nitric oxide (NO) plays an important role in the pathogenesis [5, 6].

NO is a free radical that is synthesised from the amino acid L-arginine by a family of NO synthases (NOS). In normal lungs, NO is produced in minute amounts by two constitutive NOS isoforms, endothelial and neuronal NOS, that are localised at the pulmonary vascular endothelial cells, airway epithelial cells, and nonadrenergic, noncholinergic nerve fibres [5, 6]. Endothelial NO diffuses to adjacent smooth muscle cells and activates soluble guanylate cyclase, which generates cyclic guanosine 3′-5′ monophosphate (cGMP). In turn, cGMP causes relaxation of smooth muscle, hence regulating pulmonary vascular and bronchial tone [5–8]. Neuronal NO mediates neurotransmission and may also modulate bronchodilation [5, 6]. The expression and activity of a third isoform of NOS, inducible NOS, is upregulated in many cell types including macrophages, neutrophils, fibroblasts, vascular smooth muscle and airway epithelial cells in response to endotoxin and pro-inflammatory cytokines [5–7, 9]. Depending on the severity of the insult, this isoform may generate excessive amounts of NO, causing pulmonary microvascular damage and impairment of hypoxic pulmonary vasoconstriction along with peripheral circulatory failure. The deleterious effects of NO are, for the greater part, attributed to the enhanced production of cGMP, activation of cyclo-oxygenases (COX), alteration of the complement pathway, generation of reactive oxygen and nitrogen species, and induction of cell apoptosis [5–7, 10, 11].

Inhibition of inducible NOS activity has been found to attenuate morphological signs of experimental ALI and to improve arterial oxygenation [11]. However, a further increase in pulmonary vascular tone and even an aggravation of lung oedema have been reported following the administration of nonselective NOS inhibitors [12]. The current authors have recently demonstrated that in endotoxaemic sheep methylene blue (MB), a nonselective inhibitor of NOS and

soluble guanylate cyclase [8, 13], markedly attenuated the increment in lung fluid filtration, as assessed by lung lymph flow and protein clearance. The effect of MB was associated with reduced pulmonary capillary pressure and permeability-surface area product. Furthermore, MB improved gas exchange and precluded the increases in lung lymph and plasma cGMP, and in plasma nitrites and nitrates [14, 15]. The purpose of the present study was to investigate further whether MB influences pulmonary haemodynamics and protects against accumulation of extravascular lung water (EVLW) by other mechanisms in addition to inhibition of the NO pathway.

Materials and methods

Animal preparation

The present study was approved by the Norwegian Experimental Animal Board. Sixteen yearling sheep of both sexes, weighing 37.6±1.6 kg (mean±SEM), were instrumented under endotracheal anaesthesia with halothane 0.8–1.25% (AstraZeneca, Macclesfield, Cheshire, UK). A medical-grade catheter (131162-24; Kebo Lab, Stockholm, Sweden) was implanted into the left atrium *via* a left thoracotomy in the 4th intercostal space. In addition, a 5-Fr introducer (CP-07511-P; Arrow International, Reading, PA, USA) was inserted into the left common carotid artery, and an 8.5-F introducer (CC-350B; Baxter Healthcare, Irvine, CA, USA) was placed in the left external jugular vein. After surgery, the animals were allowed to recover for 1 week.

Measurements and samples

On the day of the experiment, the awake sheep was placed in an experimental cage. A 4-Fr fibreoptic thermistor catheter (PV2024L; Pulsion Medical Systems, Munich, Germany) was advanced into the thoracic aorta. A 7-Fr flow-directed thermal dilution catheter (131HF7; Baxter Healthcare) was introduced into the pulmonary artery. The catheters were connected to pressure transducers (Transpac III; Abbott Critical Care Systems, North Chicago, IL, USA) and continuously flushed with a saline solution of heparin 10 $IU \cdot kg^{-1} \cdot h^{-1}$ (Nycomed Pharma, Oslo, Norway). Heart rate, mean arterial pressure (MAP), mean pulmonary arterial pressure (PAP), pulmonary arterial occlusion pressure (PAOP), and mean left atrial pressure (LAP) were displayed on a 565A Patient Data Monitor (Kone, Espoo, Finland). The pressures were recorded on a 79 Polygraph (Grass Instruments, Quincy, MA, USA) with the zero reference level at the shoulder of the front leg of the standing animal. Effective pulmonary capillary pressure (P_c) was derived from the PAOP tracing according to the technique of HOLLOWAY *et al.* [16].

Body surface area was calculated as $BW^{0.67} \times 0.084$, where BW is body weight in kg. Cardiac index (CI), EVLW, pulmonary blood volume index (PBVI), and right ventricular ejection fraction (RVEF) were determined by a thermal-dye dilution technique, as assessed by a Cold Z-021 (Pulsion Medical Systems). The dilution curves of 5-mL boluses of an ice-cold solution of indocyanine green (0.5 $mg \cdot mL^{-1}$ in 5% glucose) (Pulsion Medical Systems) injected into the right atrium were displayed and immediately inspected. Slow washout curves were rejected, and every variable was calculated as a mean of five successful measurements. In addition, body temperature was measured by means of the fibreoptic thermistor catheter. Systemic vascular resistance index (SVRI) was calculated as:

$$MAP/CI \times 80 \quad (1)$$

and pulmonary vascular resistance index (PVRI) as:

$$(PAP - LAP)/CI \times 80 \quad (2)$$

Venous blood leukocytes were counted using Evans blue and a haemocytometer, and leukocyte differential count was determined using slide smears stained with May-Grünwald-Giemsa. Samples of pulmonary arterial blood were collected into clot activator tubes (Vacutainer 367789; Becton Dickinson, Meylan Cedex, France) and tubes containing a solution of ethylene diamine tetra-acetic acid (EDTA; Sigma Chemical Co., St Louis, MO, USA) and 0.04 M indomethacin. Immediately after sampling, blood was centrifuged at 4°C (2,000×*g* for 10 min). The serum and plasma samples were stored at -70°C until analysis.

Protocol

After 2–2.5 h of stable baseline haemodynamic measurements, the values as of time -0.5 h were noted. Thereafter, the animals were randomly assigned either to receive intravenously an injection of MB 10 $mg \cdot kg^{-1}$ (Nycomed Pharma) (MB group), or a corresponding volume of isotonic saline (control group). Starting from time 0 h, *Escherichia coli* O26:B6 endotoxin 1 $\mu g \cdot kg^{-1}$ (Sigma Chemical Co.) dissolved in 30 mL of isotonic saline was infused intravenously for 20 min. The MB group additionally received an infusion of MB 2.5 $mg \cdot kg^{-1} \cdot h^{-1}$ for 6 h, whereas the control group received a corresponding volume of isotonic saline. After the final measurement, the animals were killed with thiopental sodium 100 $mg \cdot kg^{-1}$ (Abbott Laboratories, North Chicago, IL, USA).

Biochemical analysis

The plasma concentrations of thromboxane (Tx) B_2 and 6-keto-prostaglandin (PG) $F_{1\alpha}$, the stable metabolites of thromboxane A_2 and prostacyclin (PGI_2), respectively, were determined by enzyme immunoassays (Biotrak RPN220 and RPN221; Amersham International, Buckinghamshire, UK). Serum hyaluronan concentrations were measured by a radiometric assay (10-9294-01; Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Total haemolytic activity of the alternative complement pathway was measured by incubating sheep serum diluted 1:15 in veronal buffered

saline, containing 0.1% gelatine (Sigma Chemical Co.) and 7 mM EDTA with 2% washed rabbit erythrocytes for 1 h at 37°C in 96-microtitre well plates. The plates were centrifuged and the supernatants were removed to another plate and optical density was read at 410 nm. The results were referred to a standard curve of normal human serum containing 100% lytic activity. The sheep alternative haemolytic activity was very close to human with values ~100%.

Statistical analysis

Data are expressed as mean±SEM. For each variable, normality was checked. Data were assessed by two-way analysis of variance. If the F-value was statistically significant, an unpaired, two-tailed t-test or paired t-test was used to evaluate differences between groups and within groups towards the baseline values, respectively. Probability values <0.05 were considered significant.

Results

Haemodynamics

MB markedly attenuated the haemodynamic responses to endotoxin (fig. 1 and table 1). In comparison to the controls, MB reduced the increments in PAP by ~40% from 0.5 to 2 h, and in PVRI and PAOP by ~60% from 0.5 to 1.5 h ($p<0.05$). In addition, MB reduced the increase in *P*c by ~50% throughout the experiment ($p<0.05$). In the control group, LAP increased slightly above baseline from 4 to 5 h ($p<0.05$). Moreover, PBVI rose from 0.5 to 1 h, at 3 h, and from 5 to 6 h ($p<0.05$). In the MB group, LAP and PBVI remained unchanged from baseline, the latter displaying an intergroup difference between 0.5 and 3 h ($p<0.05$). In parallel, MB attenuated the reduction in RVEF from 0.5 to 3 h and at 5 h ($p<0.05$). MB also counteracted the declines in MAP and CI ($p<0.05$), but SVRI displayed no intergroup difference.

Extravascular lung water content

EVLW increased by more than two-fold in the controls ($p<0.001$; fig. 2). In contrast, MB reduced the accumulation of EVLW by 30–50% throughout the study ($p<0.05$).

Cyclo-oxygenase products

In the control group, plasma TxB_2 peaked 85-fold above baseline at 0.5 h in parallel with the increment in plasma 6-keto-$PGF_{1\alpha}$, peaking 75-fold at 2 h ($p<0.01$; fig. 3). Thereafter, TxB_2 and 6-keto-$PGF_{1\alpha}$ gradually declined, albeit displaying intragroup differences throughout the experiment and at 4 h, respectively ($p<0.05$). As compared to the controls, MB reduced the peak increments in TxB_2 by 70% and 6-keto-$PGF_{1\alpha}$ by 50%, with intergroup differences still present at 4 and 3 h, respectively ($p<0.05$).

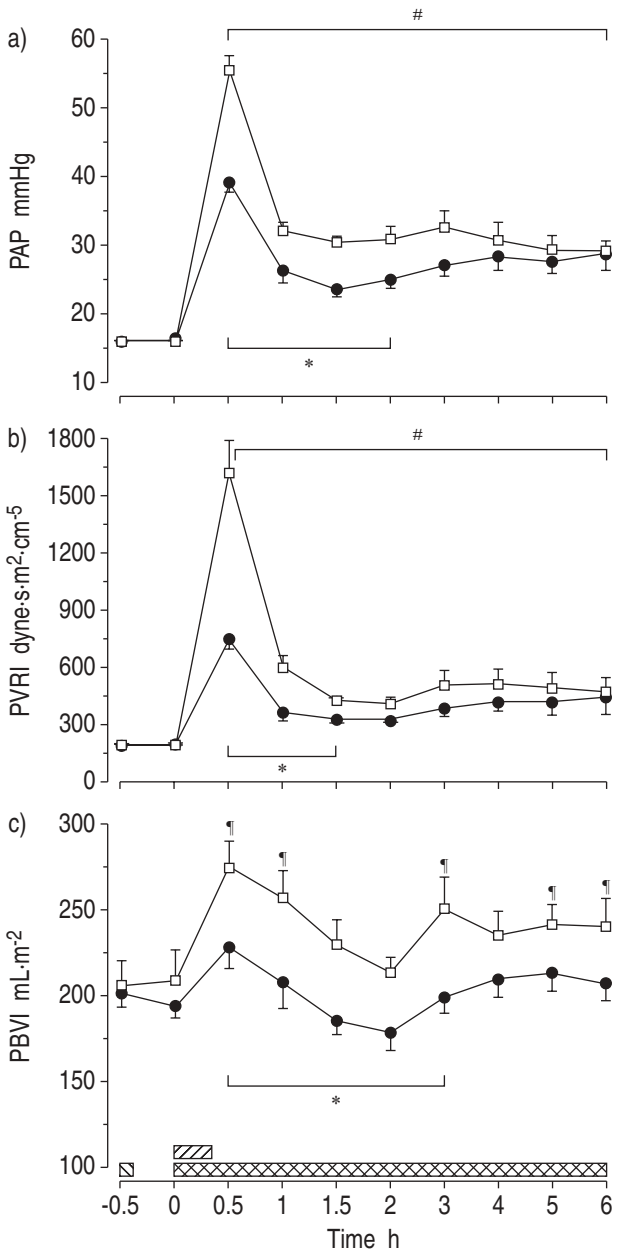

Fig. 1.–Effect of methylene blue (MB) on pulmonary haemodynamics in awake, endotoxaemic sheep (mean±SEM, n=8 animals/group). a) Mean pulmonary arterial pressure (PAP); b) pulmonary vascular resistance index (PVRI); c) pulmonary blood volume index (PBVI). □: control endotoxin (ET) group; ●: MB/ET group. In both groups, *Escherichia coli* ET 1 $\mu g \cdot kg^{-1}$ was injected at time 0 h (▨). In the MB group, sheep received MB 10 $mg \cdot kg^{-1}$ 0.5 h before ET (▩), followed by infusion of MB 2.5 $mg \cdot kg^{-1} \cdot h^{-1}$ from the onset of ET (■). *: $p<0.05$ between the groups for all individual time points within the bracket; #: $p<0.05$ from intragroup baseline in both groups for all individual time points within the bracket; ¶: $p<0.05$ from intragroup baseline in the control group.

Body temperature and laboratory variables

MB abolished the endotoxin-induced febrile response ($p<0.001$; fig. 4). In both groups, the circulating neutrophil count decreased rapidly by as much as 25-fold, whereas serum hyaluronan increased

Table 1. – Effect of methylene blue on haemodynamics in awake, endotoxaemic sheep

Measure	Time h									
	-0.5	0	0.5	1	1.5	2	3	4	5	6
PAOP mmHg										
Control	7.9±0.2	7.9±0.2	20.5±0.4#	14.9±0.7#	13.4±0.7#	13.8±0.9#	13.5±1.4#	13.1±1.2#	12.3±0.7#	11.9±0.2#
MB	8.4±0.3	8.5±0.3	14.0±0.7*#	12.0±1.0*#	10.8±0.6*#	11.8±0.7#	11.9±0.6#	12.1±1.1#	12.1±1.0#	11.5±1.0#
*P*c mmHg										
Control	8.3±0.3	8.3±0.6	28.5±0.7#	21.9±0.6#	18.7±0.9#	19.4±1.0#	17.4±1.0#	16.7±1.1#	16.4±1.0#	16.6±0.8#
MB	9.3±0.4	9.8±0.6	19.4±1.4*#	14.8±1.0*#	13.8±1.0*#	12.4±1.0*#	12.3±0.6*#	13.2±0.8*#	13.4±0.7*#	13.8±0.8*#
LAP mmHg										
Control	4.9±0.3	4.8±0.5	3.6±0.9	3.8±1.1	5.4±1.2	5.5±1.1	6.3±1.1	6.6±0.8#	7.0±0.8#	6.1±0.9
MB	5.3±0.2	5.0±0.5	5.1±0.7	5.1±1.2	4.7±1.3	4.6±1.1	5.0±1.2	5.4±0.9	5.7±1.0	6.1±1.0
RVEF %										
Control	38.0±1.9	38.4±2.0	18.1±1.9#	25.9±2.0#	25.9±2.0#	26.3±1.7#	26.4±2.0#	24.6±1.8#	23.6±2.0#	22.5±1.7#
MB	38.6±2.7	43.1±2.8	28.0±2.8*#	38.3±3.7*	34.0±2.1*	35.6±1.7*	36.0±1.6*	27.6±2.1#	28.8±1.1*#	25.6±1.9#
MAP mmHg										
Control	95±2	95±2	91±4	89±5	88±5#	86±4#	104±3#	107±3#	103±4#	91±4
MB	96±1	96±1	103±2*#	103±2*#	99±1*	97±1*	104±2#	105±3#	105±2#	105±4*#
CI $L \cdot min^{-1} \cdot m^{-2}$										
Control	5.5±0.4	5.5±0.4	2.8±0.3#	4.0±0.3#	5.1±0.3	5.5±0.3	4.7±0.4	4.3±0.3#	4.3±0.4#	4.7±0.5#
MB	5.1±0.2	5.3±0.1	3.7±0.2*#	4.9±0.2*	5.1±0.2	5.5±0.2	5.2±0.3	5.0±0.4	4.9±0.3	4.9±0.5
SVRI $dyne \cdot s \cdot m^2 \cdot cm^{-5}$										
Control	1407±88	1418±87	2733±260#	1754±129#	1378±44	1305±76	1839±170#	2111±196#	2075±256#	1719±238
MB	1520±59	1442±45	2234±111#	1693±80#	1575±91	1436±76	1631±87	1713±105#	1778±140#	1841±215

Data are presented as means±SEM. PAOP: pulmonary arterial occlusion pressure; *P*c: effective pulmonary capillary pressure; LAP: mean left atrial pressure; RVEF: right ventricular ejection fraction; MAP: mean arterial pressure; CI: cardiac index; SVRI: systemic vascular resistance index; Control: endotoxin group; MB: methylene blue/endotoxin group. n=8 animals/group. *: $p<0.05$ *versus* control group; #: $p<0.05$ *versus* intragroup baseline.

gradually 15-fold ($p<0.01$), without intergroup differences (table 2). The changes in total haemolytic activity of the alternative complement pathway were modest, although a few statistically significant differences were observed. In the controls, total haemolytic activity of the alternative complement pathway increased slightly at 0.5 h and then decreased below baseline from 1.5 to 3 h ($p<0.05$). In the MB group, a weak reduction occurred at 2 h and from 4 to 6 h, displaying an intergroup difference at 5 h ($p<0.05$; table 2).

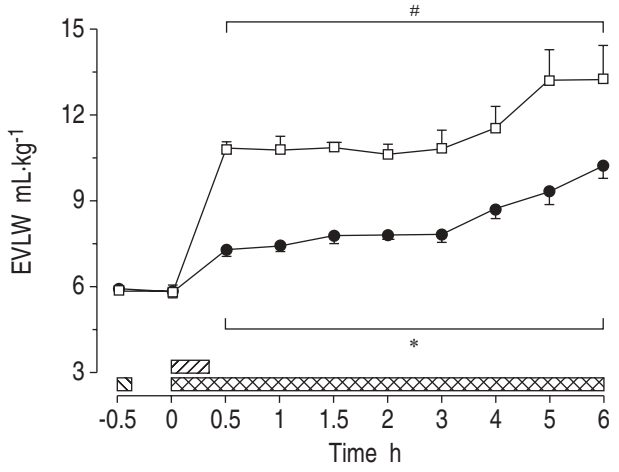

Fig. 2. – Effect of methylene blue (MB) on extravascular lung water (EVLW) content in awake, endotoxaemic sheep (mean±SEM, n=8 animals/group). □: control endotoxin (ET) group; ●: MB/ET group. In both groups, *Escherichia coli* ET 1 $\mu g \cdot kg^{-1}$ was injected at time 0 h (▨). In the MB group, sheep received MB 10 $mg \cdot kg^{-1}$ 0.5 h before ET (▧), followed by infusion of MB 2.5 $mg \cdot kg^{-1} \cdot h^{-1}$ from the onset of ET (■). *: $p<0.05$ between the groups for all individual time points within the bracket; #: $p<0.05$ from intragroup baseline in both groups for all individual time points within the bracket.

Discussion

The present study demonstrates that in endotoxaemic sheep, MB reduces the accumulation of EVLW in concert with attenuated pulmonary hypertension and increased RVEF. These effects of MB are associated with inhibition of the COX products TxB_2 and 6-keto-$PGF_{1\alpha}$, and of the febrile response to endotoxin.

The majority of the pathological features of human ALI may be mimicked by systemic infusion of live bacteria or endotoxin. Sheep are particularly sensitive to endotoxin and respond with pulmonary hypertension, right ventricular failure, and increased lung fluid filtration [3, 17, 18]. In the control group of the present study, pulmonary oedema, assessed as EVLW, rose rapidly upon exposure to endotoxin, followed by a more gradual increase for the remainder of the experiment. In contrast, the accumulation of EVLW was markedly reduced in animals treated with MB. The single pathogenetic factor that differed most between the groups was pulmonary vasoconstriction. Although MB only reduced the early phase increments in PAP and PVRI, *P*c was halved throughout the whole study in comparison to the control group

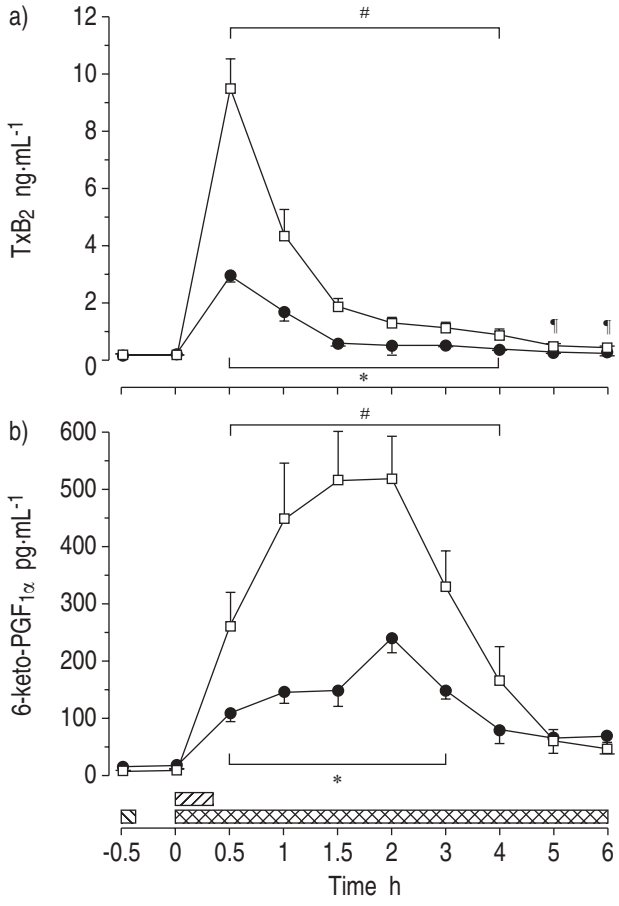

Fig. 3. – Effect of methylene blue (MB) on plasma concentrations of a) thromboxane B_2 (TxB_2) and b) 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in awake, endotoxaemic sheep (mean±SEM, n=8 animals/group). □: control endotoxin (ET) group; ●: MB/ET group. In both groups, *Escherichia coli* ET 1 μg·kg^{-1} was injected at time 0 h (▨). In the MB group, sheep received MB 10 mg·kg^{-1} 0.5 h before ET (▧), followed by infusion of MB 2.5 mg·kg^{-1}·h^{-1} from the onset of ET (■). *: $p<0.05$ between the groups for all individual time points within the bracket; #: $p<0.05$ from intragroup baseline in both groups for all individual time points within the bracket; ¶: $p<0.05$ from intragroup baseline in the control group.

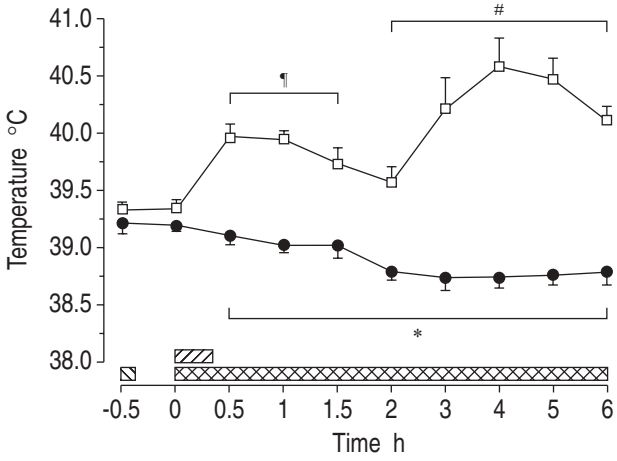

Fig. 4. – Effect of methylene blue (MB) on body temperature in awake, endotoxaemic sheep (mean±SEM, n=8 animals/group). □: control endotoxin (ET) group; ●: MB/ET group. In both groups, *Escherichia coli* ET 1 μg·kg^{-1} was injected at time 0 h (▨). In the MB group, sheep received MB 10 mg·kg^{-1} 0.5 h before ET (▧), followed by infusion of MB 2.5 mg·kg^{-1}·h^{-1} from the onset of ET (■). *: $p<0.05$ between the groups for all individual time points within the bracket; #: $p<0.05$ from intragroup baseline in both groups for all individual time points within the bracket; ¶: $p<0.05$ from intragroup baseline in the control group for all individual time points within the bracket.

level. These findings confirm the authors' recent observations in endotoxaemic sheep that pretreatment followed by continuous infusion of MB reduces the increments in PAP, PVRI, and P_c [14, 15]. Consequently, it is suggested that the decrease in EVLW after MB was a result, at least in part, of the reduced pulmonary microvascular pressure. However, MB could well reduce EVLW by means of a decrease in lung microvascular permeability and/or surface area [14]. The present observation that MB prevented the increases in PBVI and P_c after exposure to endotoxin supports the latter assumption. Furthermore, in the late phase of endotoxaemia, MB may enhance the pumping of the lung lymph, most likely by reducing excessive cGMP production in the lymph vessels

Table 2. – Effect of methylene blue on laboratory variables in awake, endotoxaemic sheep

Variable	Time h									
	-0.5	0	0.5	1	1.5	2	3	4	5	6
Neutrophils×10^9·L^{-1}										
Control	10.0±1.5	9.7±1.4	1.6±0.5#	0.8±0.1#	0.6±0.1#	0.4±0.1#	0.4±0.1#	0.6±0.1#	0.8±0.2#	1.1±0.2#
MB	10.2±1.1	10.4±1.3	1.1±0.4#	0.6±0.2#	0.4±0.1#	0.3±0.1#	0.4±0.1#	0.5±0.1#	0.5±0.1#	0.6±0.1#
Hyaluronan mg·L^{-1}										
Control	0.3±0.1	0.3±0.1	0.8±0.1#	1.3±0.2#	1.4±0.2#	1.6±0.3#	2.3±0.4#	3.0±0.4#	3.6±0.4#	4.5±0.6#
MB	0.3±0.1	0.4±0.1	1.0±0.2#	1.5±0.3#	1.5±0.3#	1.6±0.4#	2.5±0.4#	3.3±0.3#	3.7±0.3#	4.3±0.4#
THCA %										
Control	103±4	105±4	114±8#	101±3	94±4#	94±5#	96±4#	101±5	105±5	101±4
MB	100±2	100±3	104±4	103±3	97±3	93±4#	96±2	92±1#	91±3*#	92±3#

Data are presented as mean±SEM. THCA: total haemolytic activity of the alternative complement pathway. Control: endotoxin group; MB: methylene blue/endotoxin group. n=8 animals/group. *: $p<0.05$ *versus* control group; #: $p<0.05$ *versus* intragroup baseline.

[14]. This effect might also have contributed to lesser accumulation of EVLW in the present study.

In addition to the reduced pulmonary hypertension and oedema, MB attenuated the fall in RVEF after endotoxin exposure. The latter effect most likely resulted from decreased cardiac afterload. In turn, an improved right ventricle function might be responsible for better maintained CI [18] and, consequently, MAP since SVRI remained unchanged. Another possible mechanism for improved haemodynamics could be that MB counteracted endotoxin-induced reduction in myocardial contractility, which is mediated by cGMP [19].

Endotoxin and NO activate the COX pathway of arachidonic acid, resulting in synthesis of TxA_2, PGI_2, and other prostaglandins [2, 7, 10, 20]. TxA_2 is the primary mediator of early pulmonary vasoconstriction and bronchoconstriction. In addition, TxA_2 stimulates platelet aggregation and neutrophil adhesion, and may enhance pulmonary microvascular permeability. In contrast, PGI_2 exerts the opposite physiological effects. Because TxA_2 and PGI_2 are rapidly hydrolysed, their activity is measured by assaying their biologically-inactive metabolites, TxB_2 and 6-keto-$PGF_{1\alpha}$, respectively [2].

In the present experiments, endotoxin caused a rapid increase in plasma TxB_2 that was followed by a subsequent decline in parallel with a more gradual increase in plasma 6-keto-$PGF_{1\alpha}$. This pattern of prostanoid release is consistent with previous observations in endotoxaemic sheep [3, 18]. Interestingly, the authors found that MB markedly reduced plasma concentrations of TxB_2 and 6-keto-$PGF_{1\alpha}$. Earlier studies have also shown that MB may antagonise the arachidonic acid- and bradykinin-induced production of TxA_2 and PGI_2 in human platelets and porcine aortic endothelial cells by a mechanism independent of inhibition of soluble guanylate cyclase [21, 22]. However, the present investigation is the first to demonstrate that MB inhibits the endotoxin-induced systemic release of the COX products *in vivo*. To date, the exact mechanisms behind this effect remain unclear. The reduced systemic release of the prostanoids could result from either an interference with receptor coupling mechanisms that control the release of arachidonic acid from membrane phospholipids, a change in the amount and/or activity of COX and terminal PG synthases through oxidation of the ferrous haem moiety of the enzymes, or a decreased extracellular secretion [2, 22]. Moreover, some inhibitors of NOS, such as N^G-nitro-L-arginine methyl ester and aminoguanidine, have also been reported to reduce the formation of COX products, thus indicating the interactions between the NO and COX pathways [10, 11]. Consequently, MB inhibition of TxA_2 is most likely the explanation for the observed reductions in pulmonary hypertension and EVLW, and improved RVEF. This is in agreement with a number of studies showing that inhibitors of TxA_2 synthesis and antagonists of TxA_2 receptors attenuate the pulmonary vasoconstriction, oedema formation, and right ventricular failure in experimental ALI [17, 18, 23, 24]. In addition, other lipid mediators such as leukotrienes and platelet activating factor are also released in large quantities in ALI, contributing to pulmonary microvascular pressure and permeability changes [2, 25]. Therefore, a possible effect of MB on these mediators needs to be elucidated.

The authors observed that the favourable effects of MB on haemodynamics and EVLW occurred in parallel with a preclusion of fever. The latter effect could be due to inhibition of the synthesis of PGE_2, which mediates the febrile reaction. There is supporting evidence that MB counteracts the interleukin (IL)-1β-induced production of PGE_2 in cultured human airway epithelial cells [10]. Another explanation could be that the beneficial effects of MB resulted, at least in part, from reduced formation of reactive oxygen species that contribute both to the febrile response and the endothelial injury [26]. However, the latter hypothesis has not been specifically addressed in the present study.

Hyaluronan, a glycosaminoglycan constituent of the pulmonary extracellular matrix, is an important regulator of normal interstitial architecture and tissue hydration [27]. Increased concentrations of hyaluronan are present in bronchoalveolar lavage fluid and serum from patients with sepsis-induced ALI [4]. In turn, degradation fragments of hyaluronan may upregulate inducible NOS expression in inflammatory cells, contributing to propagation of pulmonary inflammation [28]. In the present investigation, the authors found that endotoxin increased the serum hyaluronan concentration. The latter possibly reflects an excessive hyaluronan outflow from the lungs, in agreement with a previous study in septic sheep [29]. However, MB had no effect on the hyaluronan changes, indicating that lung fluid filtration was not influenced by this mechanism.

The lung haemodynamic response to endotoxin is apparently more prominent in ruminants than in other species. This is thought to be mainly due to the presence of resident pulmonary intravascular macrophages. Endotoxin primes pulmonary macrophages for increased production of NO and pro-inflammatory cytokines such as tumour necrosis factor-α and IL-1β. In turn, the latter cytokines stimulate neutrophil recruitment and retention within the lung capillaries with subsequent lung damage [1, 5, 9]. In ovine endotoxaemia, a low peripheral leukocyte count has been shown to correlate with increased lung fluid filtration and hypoxaemia [17, 18]. In the present study, the authors found that MB did not modify the endotoxin-induced reduction in the circulating neutrophil count. This could imply that MB had no influence on neutrophil trapping in the lungs. Nevertheless, MB administered intraperitoneally to rats has been shown to reduce pulmonary neutrophil sequestration and alveolar damage after bowel perforation [30]. The differences between the species and the models used could explain discrepancies between the studies.

Activation of complement stimulates polymorphonuclear leukocytes and may induce transient lung injury in its own right [1, 4]. Since assays for detection of sheep complement activation products are not available, total haemolytic activity of the alternative complement pathway, as an indicator of complement

activation, was measured. The authors found only minor changes in complement haemolytic activity, which were not affected by MB. Thus, complement activation does not contribute to the pathophysiological changes observed in this model. This is in accordance with the authors′ recent *in vitro* observation that endotoxin, in doses corresponding to those used in the present study, hardly activates complement, in contrast to whole bacteria (T.E. Mollnes, personal communication).

To conclude, methylene blue protects against endotoxin-induced acute lung injury in sheep, as indicated by attenuated pulmonary hypertension and oedema, and improved right ventricular function. The present authors suggest that these effects are, most likely, caused by inhibition of the cyclo-oxygenase products of arachidonic acid that occurs in addition to inhibition of the nitric oxide pathway. The finding that methylene blue impairs the release of prostanoids *in vivo* refutes the contention that this dye acts only as a blocker of nitric oxide synthases and soluble guanylate cyclase. Further experiments are warranted to elucidate whether combined inhibitors of the nitric oxide pathway and the cyclo-oxygenase products, such as methylene blue, are beneficial in the treatment of sepsis-associated acute lung injury.

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