Hemodynamic and Vascular Endothelium Function Studies in Healthy Pigs After Intravenous *Bolus* Infusion of Methylene Blue

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Objective: Clinical benefit of methylene blue (MB) treating NO-induced vasoplegia has been reported in sepsis, systemic inflammatory response syndrome (SIRS) in cardiac surgery and anaphylactic shock, but its safety is sometimes questioned, mainly regarding its hemodynamic effects and the possibility of causing endothelium dysfunction. To examine the nitric oxide plasma levels and cardiovascular effects of the infusion of MB *in vivo* and its effects on endothelium-dependent and endothelium-independent *in vitro* vascular relaxation.

Methods: The study protocol included two experimental groups of female pigs: Group I (Control) - the animals (n=6) did not receive MB; Group II (MB) – the animals received 3 mg/kg of MB intravenous bolus infusion. After fifteen minutes of hemodynamic parameter recording the animals were sacrificed by exsanguination, and *in vitro* studies were conducted using segments of coronary, hepatic, superior mesenteric and renal arteries, to determine the effect of MB on the arterial endothelium function with regard to NO release. Nitric oxide plasma levels (NOx) were measured in each of the experimental groups.

Results: The results obtained in the present investigation were: 1) intravenous infusion of MB (3.0 mg/kg) caused no hemodynamic changes; 2) absolute and percent plasma NOx values did not differ between the experimental groups; and 3) *in vitro* study of vascular relaxation showed no significant difference between groups. These results show that MB intravenous infusion seems to be safe. This finding agrees with data from clinical experiments where MB was used to treat vasoplegic syndrome after cardiopulmonary bypass, systemic inflammatory response syndrome (SIRS) and anaphylaxis. These results were not unexpected because, as in healthy subjects, hemodynamics is only fine tuned and not fully under NO control; therefore, MB inhibiting guanylyl cyclase is not expected to do anything.

Conclusion: Intravenous use of MB, at the investigated dose, did not cause any abnormal hemodynamic responses or impairment of endothelium-dependent relaxation.

Key words: Nitric oxide, methylene blue, distributive shock, vasoplegia.

Nitric oxide seems to play an important pathophysiological role in modulating the systemic changes associated with vasoplegia. Clinical benefit of methylene blue (MB) treatment of nitric oxide (NO) vasoplegia has been reported in sepsis[1-6], systemic inflammatory response syndrome (SIRS) in cardiac surgery[7-11], and anaphylactic shock[12-14].

Experimental studies demonstrate that NO synthesis inhibition by L-arginine analogs reverses hypotension and antagonizes the effects of vasoconstrictors released in consequence of sepsis and anaphylaxis. However, these inhibitors cause cardiac output decrease and pulmonary vascular resistance increase. Therefore, it is important to emphasize that L-arginine analogs inhibit both constitutional (ecNOS) and inducible (iNOS) nitric oxide synthase isoforms. The ideal would be the specific inhibition of the iNOS, responsible for vasoplegic reactions, preserving ecNOS activity, which has a vital role for microcirculation physiology. NO synthesis is still a matter of controversy and even a matter of bioethics. In view of these concerns about NO synthesis, it would be a good idea to block NO effects on the vascular smooth muscle by inhibiting the guanylate cyclase, which causes the increase in cGMP levels. Methylene blue is probably the safest drug for this, because it has been widely employed in clinics since the end of the nineteenth century.

The main objective of the present investigation was, basically, to answer the following question: should MB elicit some acute and undesirable effect if used independently of the vasoplegia situation with increased NO release? Over the last 12 years, our group has been studying MB to treat vasoplegia caused by adverse reaction to protamine, vasoplegic syndrome associated with cardiopulmonary bypass surgery, anaphylaxis, pancreatitis and ischemia/reperfusion injury. The fundamental idea is to show clearly that classic mean MB doses do not affect hemodynamic end vascular endothelium functions. Consequently, the aims of the present study were: 1) to assess experimentally MB *in vivo* action in pigs by using

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hemodynamic parameters; 2) to assess experimentally MB *in vitro* action on the endothelium-dependent vascular tone of coronary, hepatic, renal and superior mesenteric arteries of healthy pigs, and; 3) to determine alterations in plasma nitric oxide in experimental groups of pigs.

Methods

The study protocol included two experimental groups Group I (Control) - the animals were observed without MB infusion; Group II (MB) – the animals received MB 3 mg/kg intravenous infusion.

In vivo study- Female prepubescent Dalland pigs (22-26 kg) were anesthetized with a 15 mg/kg intramuscular injection of midazolan (Dormid®, manufactured by Cristalia, São Paulo, Brazil), a 10 mg/kg intramuscular injection of Tiletamine/ Zolazepam (Telazol®, Fort Dodge, IA, E.U.A.) followed by continuous intravenous infusion of Sulfentanyl 100 $\mu g.h^{-1}$ (Fastfan®, Cristália Produtos Químicos Ltda., Itapira, S.P., Brasil), and Propofol 10 mg.kg-1.h-1(Propovan®, Cristália Produtos Químicos Ltda., Itapira, S.P., Brasil), , using an infusion pump (Syringe Infusion Pump, Harvard Apparatus, South Natick, MA, E.U.A.). Pancuronium bromide, $6mg.h^{-1}$, (Pancuron®, Cristália Produtos Químicos Ltda., Itapira, S.P., Brazil) was used as the muscle relaxant.

The jugular or femoral veins and the carotid or femoral arteries were isolated, respectively, to gain venous access and for arterial pressure monitoring. The nitric oxide blood samples were obtained in every step of each experiment in all of the groups.

In vivo studies were carried out by registering and measuring hemodynamic parameters through utilization of MP System 100 THE (BioPac System, Inc., Santa Barbara, CA, USA). The Vigilance System (Monitor and Swan-Ganz CCOmbo catheter CCO/SvO2 744HF75 - Edwards Lifesciences, Irvine, CA, U.S.A.) was used to measure continuous cardiac output, and the plasma nitrite/nitrate using chemiluminescence concentrations (Analyzer 280i NOA (Sievers, Boulder, CO, USA).

In vitro study - In vitro studies of the arterial segments were carried out in standard organ chambers. Upon conclusion of the *in vivo* observations, the animal was exsanguinated, the thorax and abdomen quickly opened, and the coronary arteries (left anterior descendent or circumflex artery), hepatic artery, superior mesenteric artery and renal artery were carefully dissected, freed of connective tissue, and immersed in cooled, oxygenated physiological pH 7.4 saline solution with the following millimolar composition: NaCl - 118.0; KCl - 4.7; $CaCl_2$ - 2.5; KH_2PO_4 -1.2; $MgSO_4$ -1.66; Glucose - 11.1; $NaHCO_3$ -25.0 (Krebs-Henseleit solution). The procedures and handling of the animals were reviewed and approved by the Institutional Animal Care review board.

Blood vessel segments (4 to 5 mm in width) were prepared from the aorta with great care in order not to touch the intimal surface. The artery segments were randomly selected for the experimental conditions so that, at most, four pairs of the segments per artery (with and without endothelium) were assigned to the same experimental condition. In some segments, vascular smooth muscle function was tested without the influence of the endothelium; in these segments, the endothelium was removed by gently rubbing the intimal surface of the blood vessel with a pair of watchmaker's forceps. This procedure removes endothelium but does not affect the ability of the vascular smooth muscles to contract or relax.

Arterial segments, with and without endothelium, were suspended in organ chambers (25 ml) and immersed in a control solution maintained at 37ºC, and bubbled with 95% O2 / 5% CO_2 (pH = 7.4). Each arterial ring was suspended by two stainless steel clips passed through the lumen. One clip was anchored to the bottom of the organ chamber and the other was connected to a strain gauge using Grass FT03 (Grass Instrument Company, Quincy, MA, USA) to measure isometric force. The rings were placed at optimal length-tension by progressively stretching them to maximal contraction in response to potassium chloride (20 mMol/l). In all the experiments, presence or absence of endothelium was determined by response to acetylcholine (Ach: 10^{-4}M) in rings contracted with potassium ions (20 mMol/l). The organ chambers were washed with control solution (Krebs-Henseleit solution), and the arterial segments were allowed to equilibrate before experimentation for 60 minutes in the presence of indomethacin (10^{-5} M) to prevent synthesis of endogenous prostaglandins. After that, prostaglandin $F_{2\alpha}$ was added to the organ bath and optimal tension was achieved. The rings were allowed to equilibrate for 30 minutes before the administration of drugs.

Drugs used included: Adenosine diphosphate (ADP: 10^{-9} to 10^{-4} M), sodium fluoride (0.5 to 9.5 mM), calcium ionophore (A_{23187} - 10^{-9} to 10^{-6} M), sodium nitroprusside (SNP: 10^{-9} to 10^{-4} M), prostaglandin $F_{2\alpha}$, and indomethacin (all manufactured by Sigma Chemical Company, St. Louis, MO, USA). All the drugs were diluted with distilled water, except indomethacin, which was dissolved in Na_2CO_3 (10^{-5} M). The concentrations were expressed as final concentrations in the organ chambers. Changes in wall tension were expressed as percent of the maximal tension achieved following exposure to prostaglandin $F_{2\alpha}$, a convention that corrects for inter-animal variability in tissue response to the drug. In all the experiments, (n) refers to the number of animals from which vascular segments were taken.

Statistical analysis - Results were expressed as means ± standard error and analyzed by ANOVA, paired T-test and, when necessary, Bonferroni post-test, using Prism 3.0 (GraphPad Software Incorporated, 1999). Values were considered to be statistically significant when the p value was lower than 0.05.

Results

Fourteen female animals were studied, and two of them died during the Swan-Ganz catheter manipulation, which induced ventricular fibrillation. Mean weight of the animals was 22.59 ± 1.18 kg. There was no difference ($p < 0.05$) in body weight among the studied groups.

Hemoynamic observations - Intravenous infusion of MB (2.0 mg/kg) caused no change in Mean Arterial Pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR), pulmonary artery pressure (PAP), pulmonary capillary "wedge" pressure (PCP wedge), pulmonary vascular resistance (PVR), and central venous pressure (CVP), compared to the control group (Figs. 1 and 2).

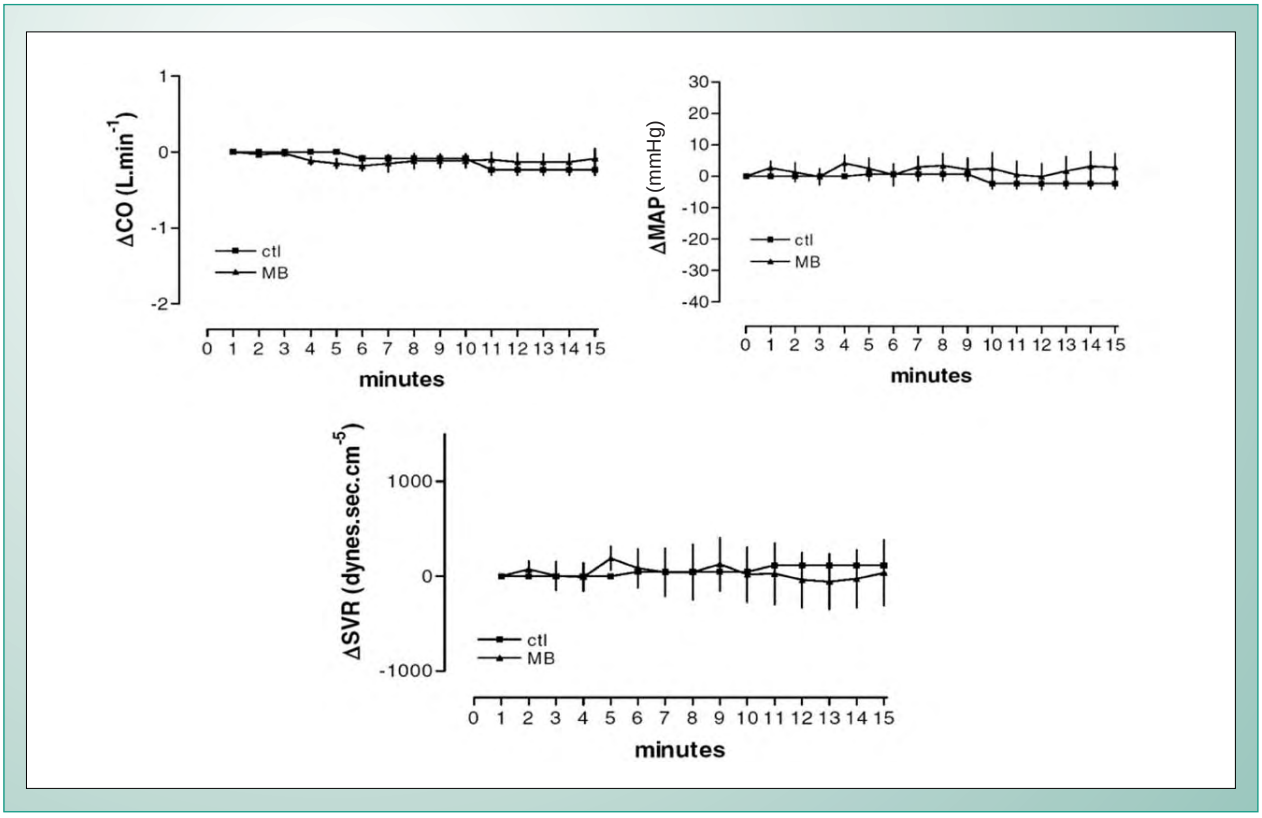

Fig. 1 - *Intravenous infusion of MB (3.0 mg/kg) caused no changes in Mean Arterial Pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR). Results are expressed as means ± SE;* n = 6; p<0.05.

Plasma nitrate/nitrite (NOx) - Absolute and percent plasma nitrate (NOx) values did not differ between the experimental groups (Fig. 3).

Endothelium-dependent vascular reactivity - After coronary, hepatic, superior mesenteric and renal artery rings $PGF_{2\alpha}$ contraction, progressive addition of adenosine diphosphate (ADP, 10^{-9} to 10^{-4}M) (Fig. 4), sodium fluoride (0.5 to 9.5 mM) (Fig. 5), A23187 calcium ionophore (10^{-9} to 10^{-6}M) (Fig. 6) induced endothelium-dependent vasodilatation in arteries with endothelium, which was significantly greater than in arteries without endothelium. But, significant differences between the Control and MB (n = 6, p > 0.05) groups were not observed.

Endothelium-independent vascular reactivity - After $PGF_{2\alpha}$ contraction, progressive addition of the endothelium-independent agonist sodium nitroprusside (10^{-9} to 10^{-6} M) induced vasodilatation of all the studied artery (coronary, hepatic, superior mesenteric and renal) segments with and without endothelium. Maximal relaxations were not different for either experimental group. However, statistical analysis of relaxations showed differences in: 1) MB group (differences among coronary artery rings with and without endothelium in 3.8, 10^{-7} and 3.7M doses); 2) control group (differences among hepatic artery rings with and without endothelium, in 3.8, 10^{-7}, 37 and 10^{-6}M doses), and MB group (differences among hepatic coronary rings with and without endothelium in the 3.8; 10^{-7} and 3.7M doses); 3) control group (differences among superior mesenteric artery rings with and without endothelium in the 3.8; 10^{-7}, and 10^{-6}M doses), MB group (differences among superior mesenteric artery rings with and without endothelium in the 3.8, 10^{-7} and 3.7M doses); 4) control group (differences among renal artery rings with and without endothelium, in the 3.7 and 10^{-6}M doses) and MB (differences among renal artery rings with and without endothelium in the 3.7 and 10^{-6}M doses) (Fig. 7).

Among the rings with endothelium of the two experimental groups, there were no statistical differences, except for superior mesentery artery rings with endothelium, in which statistical differences were observed between control and MB groups at the dose of 10^{-7}M (n=6, p < 0.05).

Discussion

The present investigation found that 1) intravenous infusion of MB caused no relevant changes in the hemodynamic status; 2) absolute and percent plasma nitrate values were not affected by MB; and 3) methylene blue did not provoke endothelial dysfunction.

These results show that intravenous infusion of MB seems to be safe. This finding agrees with data from clinical experiments in which MB was used to treat vasoplegic syndrome after cardiopulmonary bypass[7], systemic inflammatory response syndrome- SIRS[7-11,15-20] and anaphylaxis[12-14]. These results are not unexpected since, as in healthy subjects, hemodynamics is only fine tuned and not fully under control of NO. MB inhibiting guanylyl cyclase is not expected to do anything.

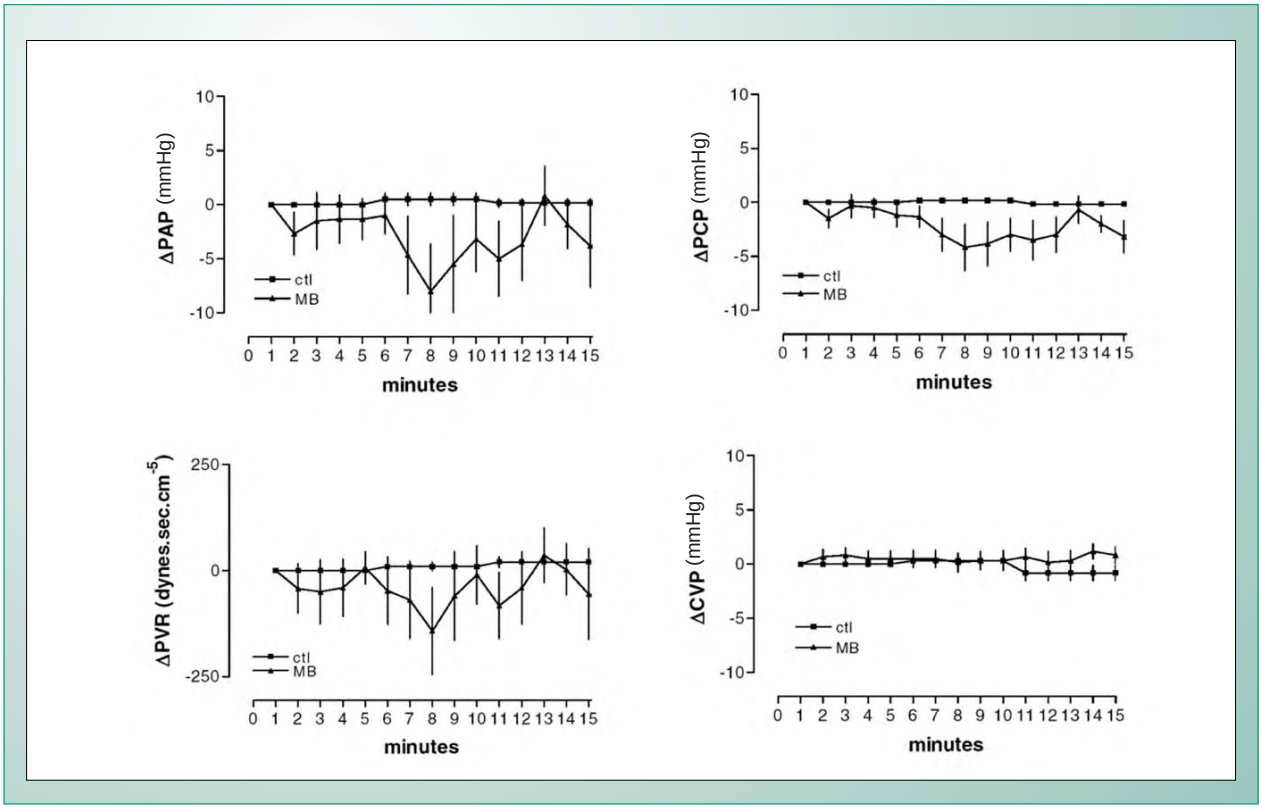

Fig. 2 - *Intravenous infusion of MB (3.0 mg/kg) caused no changes in pulmonary artery pressure (PAP), pulmonary capillary "wedge" pressure (PCP wedge), pulmonary vascular resistance (PVR), and central venous pressure (CVP) compared to the control group. Results are expressed as means ± SE;* n = 6; p<0.05.

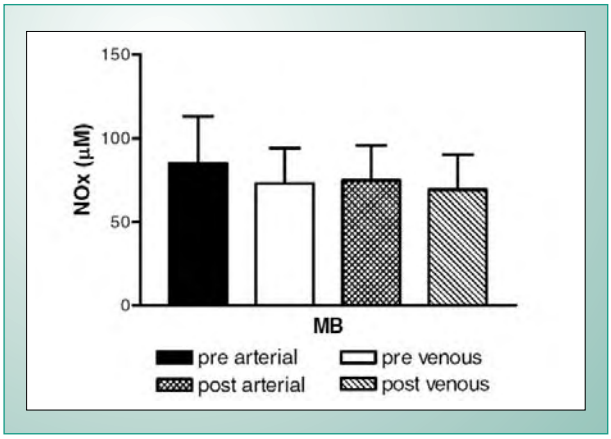

Fig. 3 - *Absolute and percent plasma nitrate (NOx) values did not differ between the experimental groups. Results are expressed as means ± SE;* n = 6; p<0.05.

Its elimination is rapid and may be observed by the quick change in urine color. In severe circulatory shock, it is convenient to use the bolus infusion followed by continuous infusion. The reason for observing the hemodynamic effects for only 15 minutes was to carry out the *in vitro* study in a time range in which plasma MB concentration is likely to be higher. Cumulated clinical and experimental evidence allows the inference that continuous infusion maintenance does not cause ischemic heart manifestations to the ECG, pulmonary hypertension and the fact that the tendency for arterial hypertension is caused by better response to amines allowing both MB suppression and the progressive decrease of vasopressor needs.

As expected, in the present study we did not observe alterations in the nitrite/nitrate (NOx) plasma levels, thus confirming that MB does not interfere with the nitric oxide pathway. However, during SIRS, plasma nitrate levels, proposed as an index for immune system activation, reaches its peak concentration levels only after 20 hours[21]. This observation could arouse some speculative considerations. But, summing up the results of the hemodynamic and vascular reactivity studies, it is possible to assume NOx normality as evidence of unaffected endothelium function in the case of *in vivo* MB intravenous infusion.

Moreover, MB safety from endothelial function capacity to release NO was proved, since the *in vitro* studies of the vascular reactivity did not show differences between control and MB groups. Statistically significant differences were not observed when the dose-response curves were compared for 1) adenosine difosphate (ADP), which is endothelial receptor-dependent; 2) sodium fluoride (NaF), which stimulates NO release acting G-proteins signal transduction; 3) calcium ionophore (A23187), which stimulates release of NO independent of receptors.

Differences were observed in the relaxations elicited by sodium nitroprusside (endothelium-independent relaxations) intermediary concentrations in artery rings without endothelium, but without differences in maximal relaxation. However, the same fact occurred with the control group, making it difficult to explain them. The most reasonable conjecture is to interpret this data in light of the

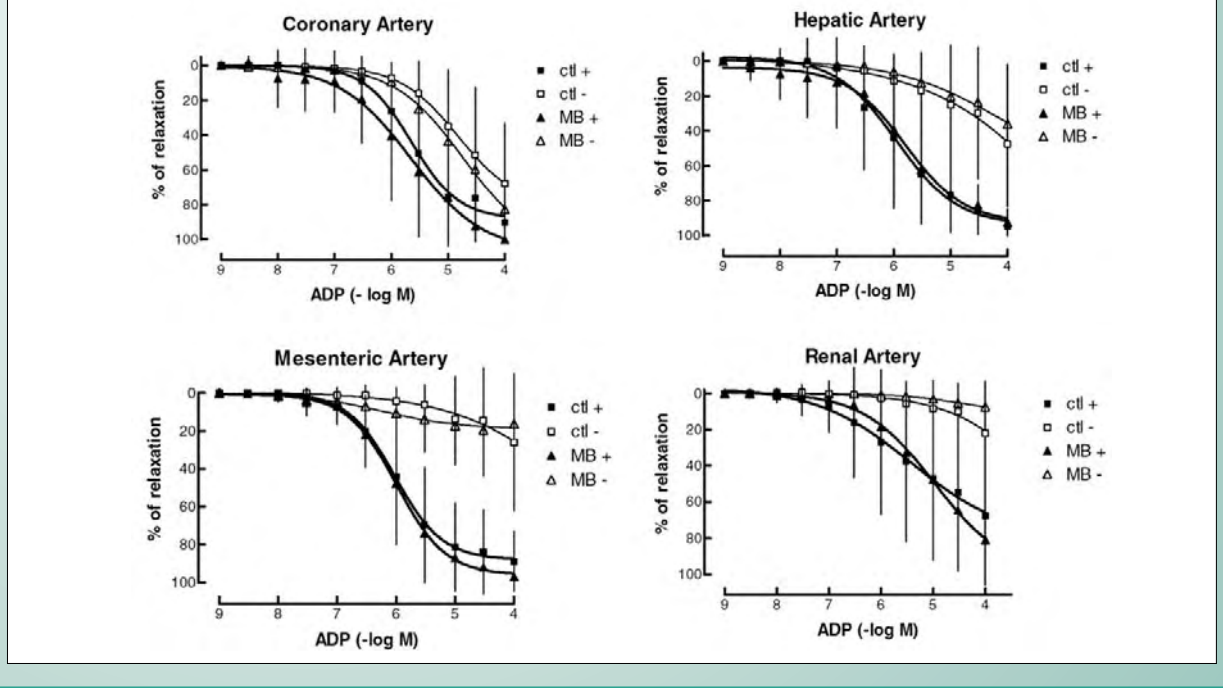

Fig. 4 - *Concentration-response curves to adenosine diphosphate (ADP). After coronary, hepatic, superior mesenteric and renal artery ring $PGF_{2\alpha}$ contraction, progressive addition of adenosine diphosphate (ADP, 10^{-9} to 10^{-4}M) induced endothelium-dependent vasodilatation in arteries with endothelium; this vasodilatation was significantly greater than in arteries without endothelium. However, significant differences were not observed between the control and MB groups. Results are expressed as means ± SE;* n = 6, *p<0.05.*

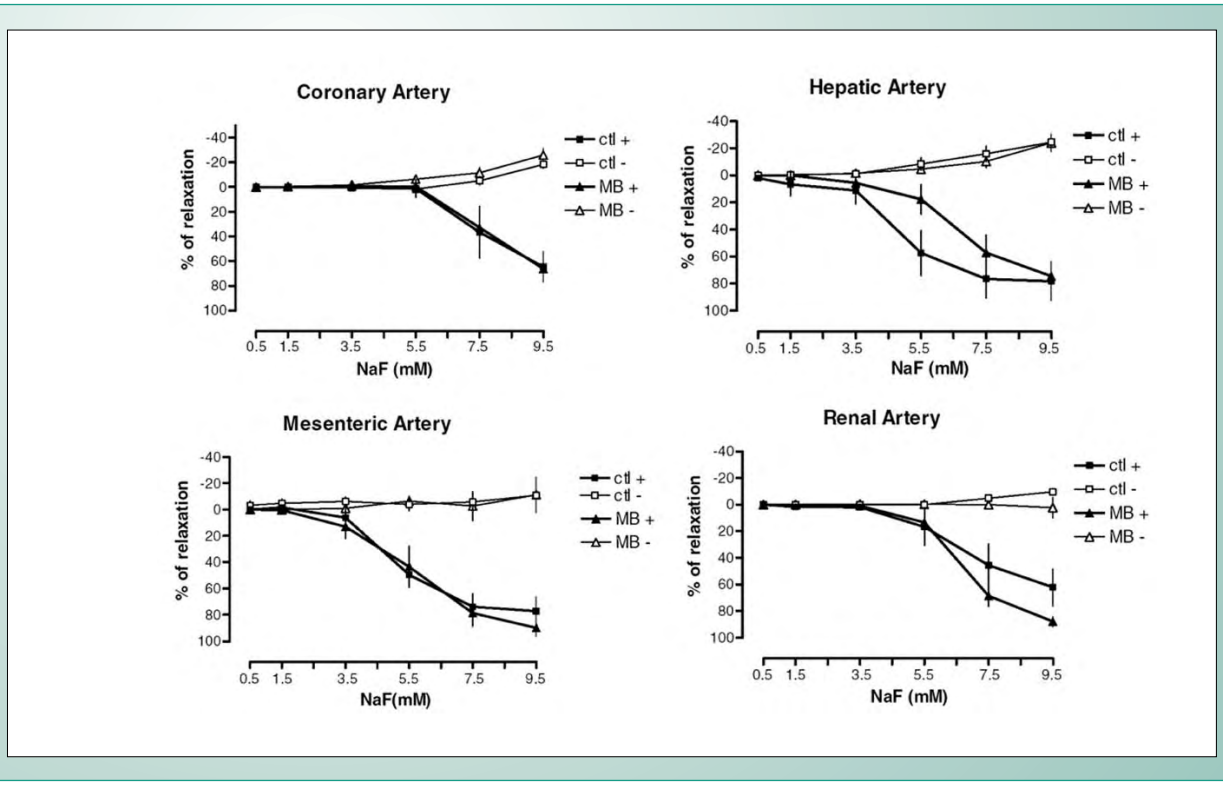

Fig. 5 - *Concentration-response curves to sodium fluoride (NaF). After the $PGF_{2\alpha}$ contraction the coronary, hepatic, superior mesenteric and renal arterial rings were exposed to increasing concentrations (0.5 to 9.5 mM) of NaF. Results were not statistically different and are expressed as means ± SE;* n = 6; *p<0.05.*

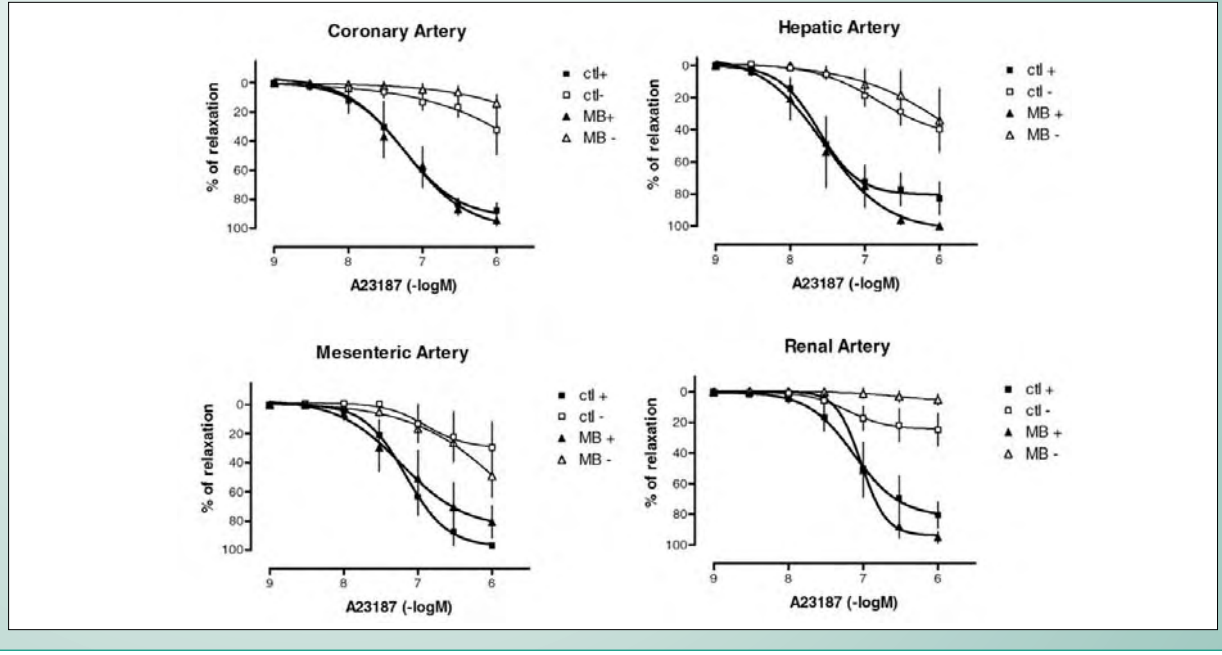

Fig. 6 - *Concentration-response curves to calcium ionophore (A23187). After $PGF_{2\alpha}$ contraction, the coronary, hepatic, superior mesenteric and renal arterial rings were exposed to higher concentrations (10^{-9} to 10^{-6}M) of calcium ionophore A23187. Results were not statistically different and are expressed as means ± SE;* n = 6; p<0.05.

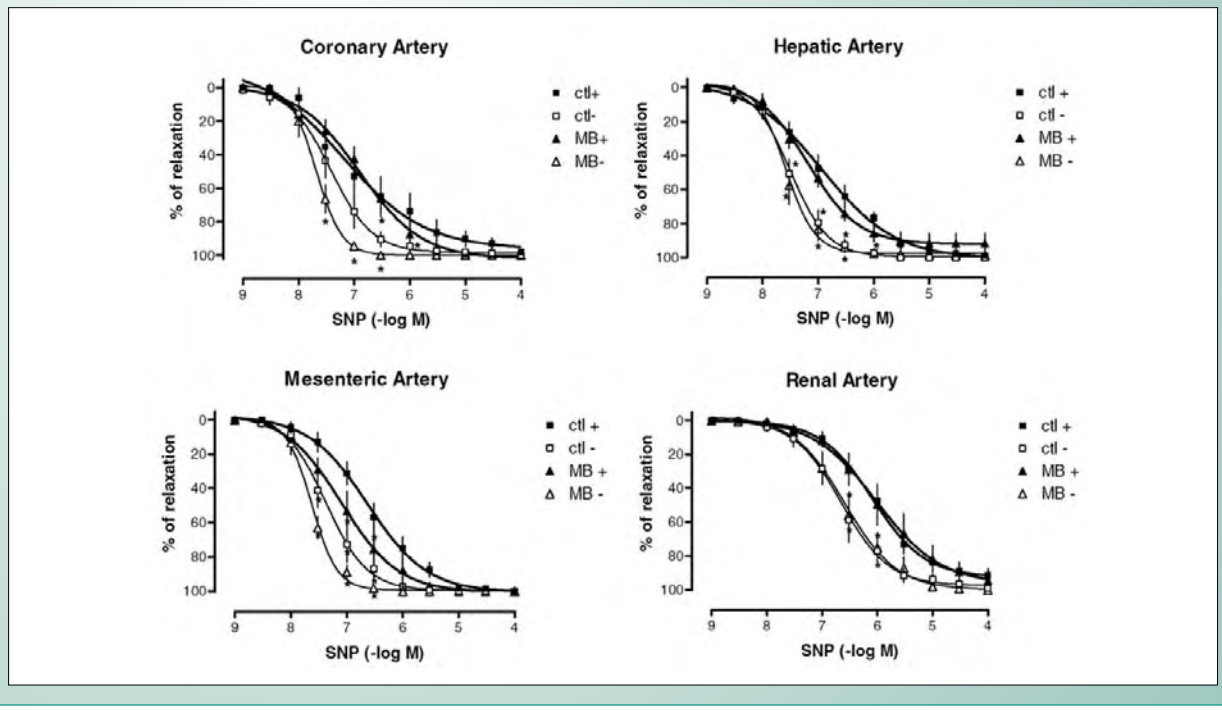

Fig. 7 - *Concentration-response curves to sodium nitroprusside (NPS). After $PGF2_{2\alpha}$ contraction, progressive addition of the endothelium-independent agonist sodium nitroprusside (10^{-9} to 10^{-6} M) induced vasodilatation of all the artery segments studied (coronary, hepatic, superior mesenteric and renal), with and without endothelia. Maximal relaxations were not different for either experimental group. However, statistical analysis of relaxations showed differences in: 1) MB group (differences among coronary artery rings with and without endothelium in doses 3.8, 10^{-7} and 3.7M); 2) control group (differences among hepatic artery rings with and without endothelium, in 3.8, 10^{-7}; 3,7 and 10^{-6}M doses) and the MB group (differences among hepatic coronary rings with and without endothelia in 3.8, 10^{-7} and 3.7M doses); 3) control group (differences among superior mesenteric artery rings with and without endothelia in 3.8, 10^{-7}, and 10^{-6}M doses), MB group (differences among superior mesenteric artery rings with and without endothelia in 3.8; 10^{-7} and 3.7M doses); 4) control group (differences among renal artery rings with and without endothelia, in 3.7 and 10^{-6}M doses) and MB (differences among renal artery rings with and without endothelia in 3.7 and 10^{-6}M doses). Among rings with endothelia in the two experimental groups, there were no statistical differences except for superior mesentery artery rings with endothelium, in which statistical differences were observed between the control and MB groups at the 10^{-7}M dose. Results were not statistically different and are expressed as means ± SE;* n = 6; p<0.05.

instrumentation for endothelium removal or sample size. Relating the finding to sex hormones[22,23] is very unlikely, because the studied female pig were prepubescent.

A word of caution is necessary in pointing out that conditions are totally different from the *in vivo* situation. The MB increment in the baths causes a decrease in endothelium-dependent relaxations, which is reverted by washing the preparation with physiologic solution. But, the idea here is to see if *in vivo* MB bolus infusion creates an endothelium dysfunction condition. The possibility that in vitro washing may be enough to normalize the arterial segments reactivity may reinforce the idea that possible endothelium dysfunction caused by MB, if any, is quickly reversible. Moreover, it must be emphasized that the MB pharmacokinetics is complex. The decrease of urinary excretion between 4 and 24 hours indicates that its half-life is around 5.25 hours, with very substantial drop in plasma concentration in the first hour.

Conclusions

In considering hemodynamic and endothelium functions, the present investigation augments the evidence of the safety of using MB in humans, at least acutely, since the experimental model used bolus infusion and observations lasting only 15 minutes. It is evident that later endothelium dysfunction cannot be discarded, considering the enzymatic characteristics of endothelial NO-synthase. However, this possibility is unlikely, because only a few authors refer to the possibility of MB NO synthesis inhibition[24-26]. As already mentioned, MB acts mainly as an inhibitor of guanilyl cyclase in the vascular smooth muscle. Moreover, clinical manifestations were not observed after the MB intravenous injections, except for the greenishness of the urine, confirming the favorable impressions in many clinical publications.

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Potencial Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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