Intradermal methylene blue administration on the progression of burn injuries

Objective: A burn injury has two defined areas: central necrosis and an adjacent area of ischaemia, which may or may not progress to necrosis. The concentration of nitric oxide (NO) increases after burn injury and may originate from potent oxidising agents. Methylene blue (MB) may act as an antioxidant and is supposed to reduce burn progression. This investigation was carried out to evaluate the effects of intradermal MB on necrosis progression in burns.
Methods: Full-thickness burn injuries were performed by applying a heated metal comb on the shaved back of male Wistar rats. The animals were divided into three groups: Control (C, n=7); MB (2mg/kg) one hour after burn injury (MB1h, n=11); and MB (2mg/kg) six hours after burn injury (MB6h, n=8). After seven days the lesions were photographed for visual assessment of burn necrosis; full-thickness cuts of lesions were dyed with Masson and Giemsa for microscopic histopathology; and tissue fragments of unburned interspaces were processed for chemiluminescence with nitrite/nitrate (NOX) and malondialdehyde (MDA) as oxidative stress markers.
Results: No statistically significant differences between groups were observed during visual analysis and NOX dosage. However, in microscopic analysis, the MB1h and MB6h groups showed smaller areas of necrosis, less inflammatory infiltration, and a more significant extension of interspaces. Furthermore, the dosage of MDA revealed that the MB1h group showed lower values when compared with the control group (p=0.001).
Conclusions: The study provided good evidence that MB intradermal injection can reduce necrosis progression in ischaemic perilesional areas and suggests an alternative to treating burns.
Declaration of interest: The authors have no conflicts of interest to declare.

burn progression • burns • ischaemia • malondialdehyde • methylene blue • necrosis • nitric oxide • rat model • wound • wound healing

Burn injuries present two pathophysiologically defined areas: the first is the central area of cellular necrosis; the second is the adjacent area of ischaemia, which may or may not progress to necrosis. Many agents have been suggested to prevent the ischaemic area progressing to necrosis, thus, reducing the extent of the burn wound. Perfusion, alterations, inflammation and oxidative stress may play a role in the progression of necrosis in the burned area.[1]

Nitric oxide (NO) is synthesised by nitric oxide synthase (NOS) from L-arginine and oxygen, releasing L-citrulline and inducible NOS (iNOS), more specifically activated by cytokines released by macrophages. The NO released activates the enzyme soluble guanylate cyclase (sGC) which in turn produces cyclic guanosine monophosphate (cGMP). The cGMP subsequently relaxes the smooth muscle, causing vasodilation and bronchodilation. An increase in NO concentrations, as a result of the increase in inducible nitric oxide synthase (iNOS), is reported in burn models, and these concentrations are elevated locally and systemically.[2–6] NO, in vivo, is rapidly metabolised to NO_2^- and NO_3^- which can be measured in biological tissues, plasma, and urine.[7]

On the other hand, considering burn injury as a generating oxidative stress factor, the NO produced combines with free radicals, among them the superoxide anion (O_2^-); when this becomes a non-enzymatic reaction, peroxynitrite ($ONOO^-$), a potent oxidising agent,[8] is formed. As both a reactive oxygen species (ROS) and reactive nitrogen species (RNS), peroxynitrite is able to attack proteins, causing their nitration. In addition to reacting with nucleic acids, causing damage to deoxyribonucleic acid (DNA) and oxidising lipids, mainly from the cell membrane (lipid peroxidation).

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Lipid peroxidation results in the formation of lipidic peroxides or lipid hydroperoxides, both of which are cytotoxic substances.[9–11] The metabolism of the latter produces malondialdehyde (MDA), which can be used as a marker for lipoperoxidation.[12,13]

Some studies have shown an increase in the NO/NOS ratio in the plasma of burn patients.[14,15] At the same time, plasma concentrations of NO(NO_2^-/NO_3^-) metabolites were increased in this type of patient, unrelated to the total body surface area (TBSA) burned.[16] In one burn model,[17] the NO content measured in the burned region was significantly higher when compared with the intact region. In this same study, the NO concentration in plasma increased significantly in a biphasic pattern, reaching values of >1 at six hours after injury. However, in the second step of this experiment, NOS inhibitors were able to suppress vascular permeability at the same time point after burn injury.

Methylene blue (MB), a well-established chemical compound in medical practice, can act as an inhibitor of sGC, reducing vessel response to cGMP-dependent vasodilators such as NO,[18] and is therefore widely used in clinical practice for reversal or prevention of vasoplegic shock, by enabling the action of sympathomimetic substances.

We have found examples of this with positive results in burn models,[18] experimental or clinical models of cardiovascular surgeries,[8,19-24] anaphylaxis,[24,25] sepsis[26,27] or even ischaemia-reperfusion studies.[28,29] In addition, the antioxidant function of this substance has been studied.[30] MB has the property of inhibiting iNOS, reducing NO production and consequently the generation of oxidising agents, dependent on this metabolic step.[31] In addition, it inhibits the generation of ROS, particularly superoxides, competing with molecular oxygen for the electron transfer of xanthine oxidase (XO).[32,33]

In a previous study of this research line, from a burn model in rats,[1,34-37] MB was injected intraperitoneally after one hour and after six hours of burn injury. Although there were no alterations in the metabolic levels of NO (nitrite/nitrate; NOX) and in the measurement of MDA, there was a significant reduction in the progression of necrosis in the interspace areas between areas burned, as well as in areas of normal skin and areas with ischaemic and inflammatory alterations, corresponding to the re-epithelialisation process. At the same time, in photographic evaluation, there was a decrease in areas of necrosis, especially in the group whose MB injection was performed six hours after the burn.[38]

Continuing this line of research, this project was designed to study the same parameters but using the intradermal injection of MB. This investigation aimed to determine the effects of intradermally administered MB on the progression of the necrotic area to the interspaces between burned areas in an experimental burn model in rats.

Methods

Animals

Male Wistar rats, weighing around 250 grams, from the central vivarium of the campus of Ribeirão Preto, University of São Paulo were used in this study. The animals were kept in an environment with a temperature between 22–25 ºC and in a light/dark cycle (12:12 hours), with free access to water and feed. The project was approved (protocol no. 212/2014) by the Committee on Ethics in the Use of Animals of MSRP-USP (Medicine School of Ribeirão Preto–University of São Paulo).

Experimental design

The animals were randomly divided into three groups. Initially, we aimed to randomise eight animals per group but were actually able to allocate 12 animals to each group. However, due to the death of some animals throughout the experiment, the groups were formed as follows:

- Control (C group, n=7)
- Group treated with methylene blue one hour after thermal injury (MB1h group, n=11)
- Group treated six hours after thermal injury (MB6h group, n=8).

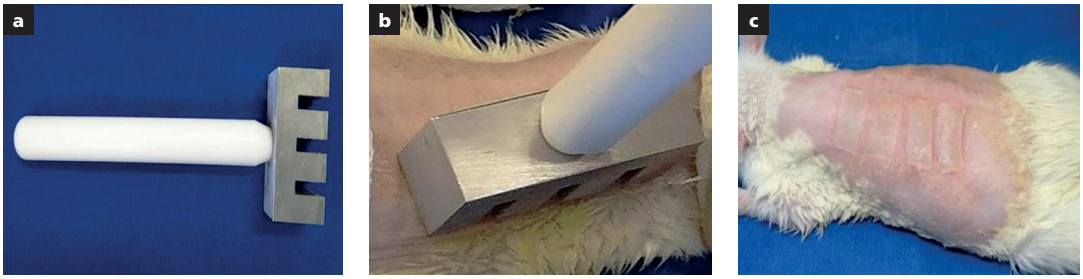
We attributed the death of the animals to an infection in our vivarium, apparently unrelated to burns. After taking appropriate measures, no more cases of infection were observed in other separate experiments.

Burn injury

The animals were anaesthetised with ketamine (50mg/kg) and xylazine (10mg/kg) intraperitoneally. Then the trichotomy of the dorsal region. The trichotomy of the dorsal region was carried out and the areas made sterile. Following an experimental model described in the literature for contact thermal burn injury,[1,34-37] a metal comb preheated for three minutes in boiling water (100 °C) was applied to the back of each animal for 30 seconds. The metal comb was attached perpendicular to the skin, without pressure, resulting in a full-thickness burn (Fig 1).

The comb presents four 10×20mm claws and three 5×20mm interspaces, resulting in four full-thickness

Fig 1. Creating the contact thermal burn injury. A metal comb **(a)** was preheated in 100°C water for three minutes before being placed on the shaved back of an anaesthetised rat **(b)**. Appearance of the animal immediately after the burn **(c)**



burns, separated by three interspaces of unburnt skin (Fig 1). After reheating the metal comb for one minute, it was applied to the other side of the rat's back, creating two burn areas, one on each side of each animal. Intraperitoneal application of buprenorphine (0.05mg/kg) was performed in all animals to reduce burn-associated pain when recovering from anaesthesia. This comb burn model was used to create the thermal injury and was adapted from Regas and Ehrlich.[21] [20]

Methylene blue

The MB was applied intradermally at a dose of 2mg/kg. The specific dose for each animal was diluted at 0.6ml in 0.9% physiological solution. After this dilution, 0.1ml was applied to each of the six burn interspaces.

In the MB1h group, MB was applied one hour after the burn was created. In the MB6h group, MB was applied six hours after the burn was created.

Group C received 0.1ml of physiological solution at each interspace one hour after the burn was created.

Measures

Photographic analysis

Wounds were observed and photographed for evidence of necrosis in the interspaces (unburnt spaces). When they became blackened, exceeding half the interspace area, they were considered to be necrotic. If they were less than half the interspace area, they were considered non-necrotic. The evaluation was conducted subjectively by three trained observers, blinded to the treatment employed.

Histopathologic analysis

Full thickness cuts of burn lesions were obtained after seven days in all animals. Interspaces were evaluated for histological evidence of necrosis and inflammatory infiltrate. For histopathological evaluation, the skin segments were fixed in 10% formaldehyde solution for 24 hours and afterwards, washed with 70% ethanol solution. The samples were then subjected to dehydration, xylene bleaching, imbibition and paraffin inclusion steps. After the inclusion of the material, 5µm thick histological sections were performed using a Reichert Jung 2040 rotary microtome (Reichert Microscope Services, US). The samples were stained by Masson's Trichrome technique (evaluation of the degree of necrosis) and Giemsa's technique (evaluation of inflammatory infiltrate). The slides were analysed using the Axioskop 2 Plus microscope ((Zeiss, Germany), increasing to 100 and 400 times magnification. The images obtained in the histopathological study were recorded by the camera (AxioCam Hrc, Zeiss, Germany) coupled to the microscope and later archived by the AxioVision 4.6 program (Zeiss, Germany).

Morphometric evaluation

In morphometric evaluation, the regions of the three interspaces of non-burned skin were measured (in millimetres). This evaluation was carried out on the right and left back areas of all animals in the studied groups.

Tissue determination of nitrate/nitrite

After each experiment, interspace skin fragments were frozen in liquid nitrogen, wrapped in foil and immediately stored in a freezer at –70 °C. The samples were homogenised at 10% mass/volume in 20mM Tris HCl ((hydroxymethyl) aminomethane hydrochloride) solution at pH7.4 using a Turrax tissue homogeniser (Rose Scientific Ltd., Canada) in three cycles with a one minute interval between them. After centrifugation at 5000rpm for 10 minutes at 4°C, the supernatant was used for NOX quantification. To avoid

possible interferences in the experiment, a homogenate deproteinisation process was carried out by precipitation in ethanol. In this technique, homogenate 300µL was added to 600µL of absolute ethanol at 4°C, followed by vigorous stirring, allowed to stand for 30 minutes at 0°C and then centrifuged at 10,000 rpm for five minutes. With the proteins deposited at the bottom of the tube, the supernatant was then withdrawn and stored at –20°C until used for analysis. The NOX dosage was performed by NO/ozone chemiluminescence by a Sievers 280 NO analyzer (GE Analytical Instruments, US). In the assay, 5µL of the sample was injected into a reaction vessel containing, as a reducing agent, a solution of 0.8% vanadium chloride at 1N HCl at 95°C. This agent converts NOX to NO in equimolar amounts. The NO produced was dredged with nitrogen gas to the chemiluminescence chamber of the Sievers 280 analyser. The NO reaction with ozone in the chamber emitted red light ($NO+O_3 \rightarrow NO_2+O_2+Light$), which was detected by the apparatus and converted into electrical signals. The generated current was then converted into digital signals analysed by the computer. The area under the generated curve allowed quantification of NOx in samples.

Tissue determination of MDA

Colorimetric determination of tissue levels of malondialdehyde (MDA), a product associated with oxidative stress, through its reaction with thiobarbituric acid was performed at 532nm in a Versamax microplate reader (Versamax Molecular Devices, US), using 1,1,3,3-Tetramethoxypropane (0–100µM) as standard and the results were expressed in µM/mg protein.[18]

Table 1. Isolated macroscopic evaluation of the examiners, comparing the control groups (C), methylene blue after one hour (MB1h), and methylene blue after six hours (MB6h). The Chi-squared test was used, and the result for each comparison and its respective level of significance (p) is shown below

Group	Chi-squared test	p-value
Examiner 1		
CxMB1h	1.286	0.25
CxMB6h	1.286	0.39
MB1hxMB6h	0.778	0.29
Examiner 2		
CxMB1h	0.143	0.26
CxMB6h	1.286	0.06
MB1hxMB6h	0.467	0.56
Examiner 3		
CxMB1h	1.286	0.26
CxMB6h	3.571	0.06
MB1hxMB6h	0.330	0.56

Statistical analysis

The Kappa statistic was used to analyse the agreement between observers regarding visual reading (macroscopic photographic analysis). To observe the differences between the proportions of results found between each of the examiners, considering the groups C, MB1h and MB6h, the McNemar test was used. For the analysis of NOX and MDA values, the Mann-Whitney test was used for independent samples in the comparison of medians. A significance level of 5% was considered in all analyses.

Computational performance: MedCalcStatistical software, version 16.4.1. (Medcalc Software Bvba, Belgium).

Results

Photographic analysis

Conceptually, the Kappa index is an interobserver agreement measure and measures the degree of agreement beyond what would be expected by chance alone. This measure of agreement has a maximum value of one representing total agreement, while values close to zero and negative indicate no agreement or agreement was exactly the one expected by chance. An eventual value of Kappa less than zero, i.e., a negative value, suggests that the agreement found was less than that which was expected by chance.

In this case, the hypothesis tested (H0; null hypothesis) is if Kappa is equal to zero, which would indicate zero agreement, or if it is greater than zero, concordance greater than chance.

Thus, the concordance of the MB effect among the experimental groups, in the view of three observers, generally showed good results when comparing sGC with MB1h; k=1 (CI: 0.9–1.0), when comparing guanylate cyclase (GC) with MB6h, and k=0.33 (CI: −0.40–1.0), when comparing MB1h and MB6h.

Proceeding with the macroscopic evaluation, we submitted the observers' data to the Chi-squared test in isolation, in order to understand the perception of each of the examiners on the treatment used. Table 1 shows, from the level of significance (p) obtained, that the observers were not able to perceive any difference between the groups in the macroscopic analysis.

After evaluation of the presence of inflammatory infiltrate in the dermis and hypodermis, a small presence of inflammatory infiltrate in the blood vessels and intercellular tissue was observed in the animals of

the three studied groups. The presence of inflammatory infiltrate was higher in the control group (mean: blood vessels=1.02 and intercellular tissue=0.26) when compared with the MB1h group (mean: blood vessels =0.93 and intercellular tissue=0.24) and the MB6h group (mean: blood vessels=0.8). Most animals in groups C, MB1h and MB6h showed a very mild degree of inflammatory infiltrate, but some animals in group C showed a mild degree of inflammatory infiltrate (Fig 2). For the three groups studied, the presence of inflammatory infiltrate in the blood vessels was greater when compared with the presence in the intercellular tissue (Fig 2).

Morphometric evaluation

After morphometric evaluation of measurements of the regions of the three interspaces (in millimetres) of unburned skin, a greater extension of interspaces was observed in the animals of the MB1h group (mean: 3.53mm) and mainly observed in the MB6h group (mean: 3.58mm) when compared with the animals in group C (mean: 3.45mm) (Fig 3).

Evaluation of the NOX concentration

Table 2 presents the NOX dosage means in the interspaces of the burned area samples. It can be observed that there is a tendency for NOx to be lower in groups MB1h (10.48±5.54) and MB6h (9.79±9.94) than in group C (12.38±10.97).

The NOX dosage found in the interspaces of the groups did not present a normal distribution for the samples. Thus, we chose to apply non-parametric statistical tests. The NOX dosage found when comparing the interspaces of groups C (median: 6.83), MB1h (median: 9.69) did not show a statistically significant difference (p=0.58). The same was observed when comparing group C (median: 6.83) with MB6h (median 37.39) (p=0.36), and the BM1h group (median: 9.69) with MB6h (median: 37.39) (p=0.13) (Fig 4).

Evaluation of MDA concentration

Table 3 presents the MDA dosage means in the interspaces of the burned area samples. We can observe that there is a tendency for MDA to be lower in group MB1h (8.63±5.23) than in group C (18.75±9.84) and in group MB6h (16.58±15.67).

The dosage of MDA found in the interspaces of the groups did not present a normal distribution for the samples. Thus, we chose to apply non-parametric statistical tests. The MB1h group had a median of 7.40,

Fig 2. Photomicrography of the animal skin of group C showing large area of necrosis in the dermis (D) just below the epidermis (E) and delimited inferiorly by the arrows. Hair follicle (PF). Masson's trichrome, magnification100x

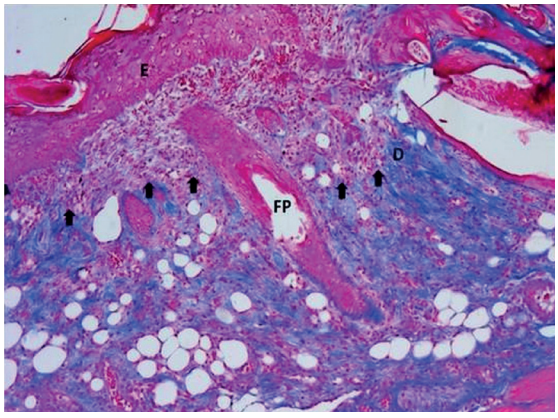


Fig 3. Photomicrography of the animal integument of the control group, showing mild inflammatory infiltrate in the tissue (arrows) and inside a blood vessel (star).Above and to the left, detail showing some neutrophils (arrow) in the lumen of a blood vessel.Giemsa: 100x; 400x.

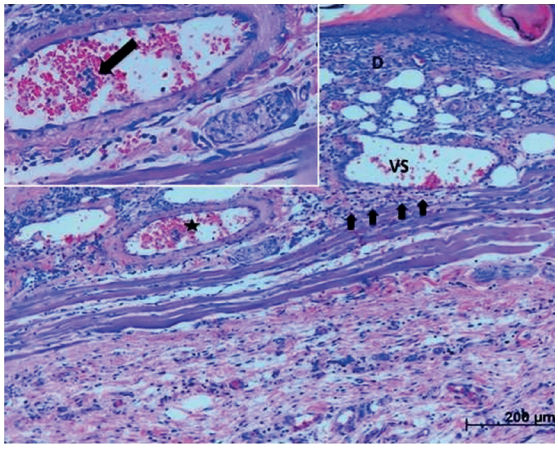


Table 2. Tissue nitrite / nitrate (NOX) titration in the interspace of the burned areas of the animals' backs. Group C (control), methylene blue after one hour (MB1h) and methylene blue after six hours (MB6h). These values represent the mean±standard deviation. (Group C: n=7, group MB1h: n=11, group MB6h: n=8)

NOX dosage in the interspaces of the burned area	
Control	(12.38±10.97)
MB1h	(10.48±54)
MB6h	(9.79±9.94)

Fig 4. NOX in µM in the interspaces between control (C) and methylene blue groups (MB1h) after 1 hour of thermal injury (p=0.58) **(a)**; NOX in µM in the interspaces between control (C) and methylene blue groups after 6 hours of thermal injury (MB6h) (p=0.36) **(b)**. NOX in µM in the interspaces between the methylene blue groups after 1 hour of the thermal injury (MB1h) and after 6 hours of the same (MB6h) (p=0.13) **(c)**

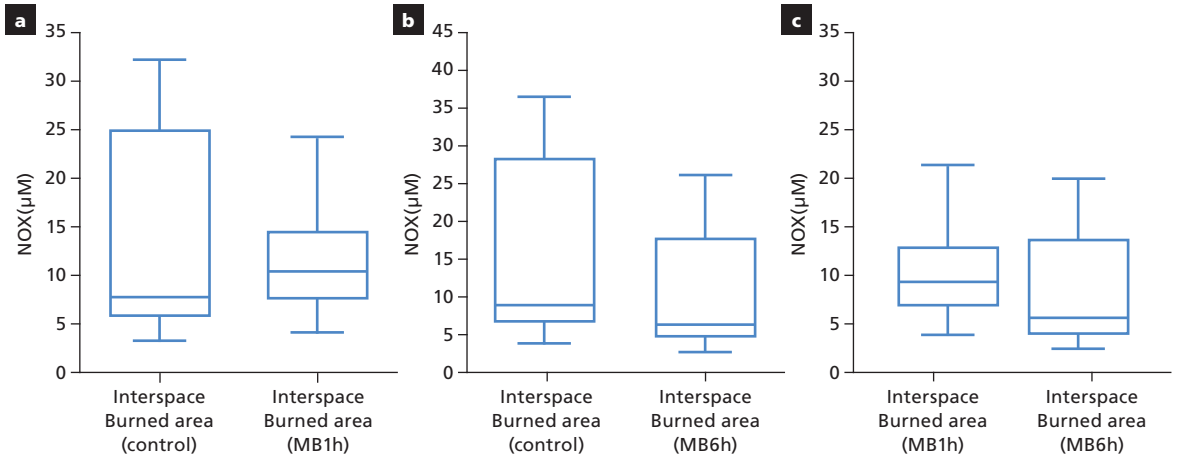


Table 3. Tissue dosage of malondialdehyde (MDA) in the interspaces of the burned areas of the animals' backs. Group C (control), methylene blue after one hour (MB1h) and methylene blue after six hours (MB6h)

MDA tissue dosage (µM/mg)	
Control group, n=7	18.75±9.84
MB1h group, n=11	8.63±5.23
MB6h group, n=8	16.58±15.67
Values represent the mean±standard deviation	

lower than that for group C (median: 15.39), and this difference was statistically significant (p=0.001). The same did not occur when comparing group C (median: 15.39) with group MB6h (median: 11.06) (p = 0.24) and group MB1h (median: 7.40) with group MB6h (median: 11.06) (p=0.585), although these values show a p-value close to the limit proposed by this study (Fig 5).

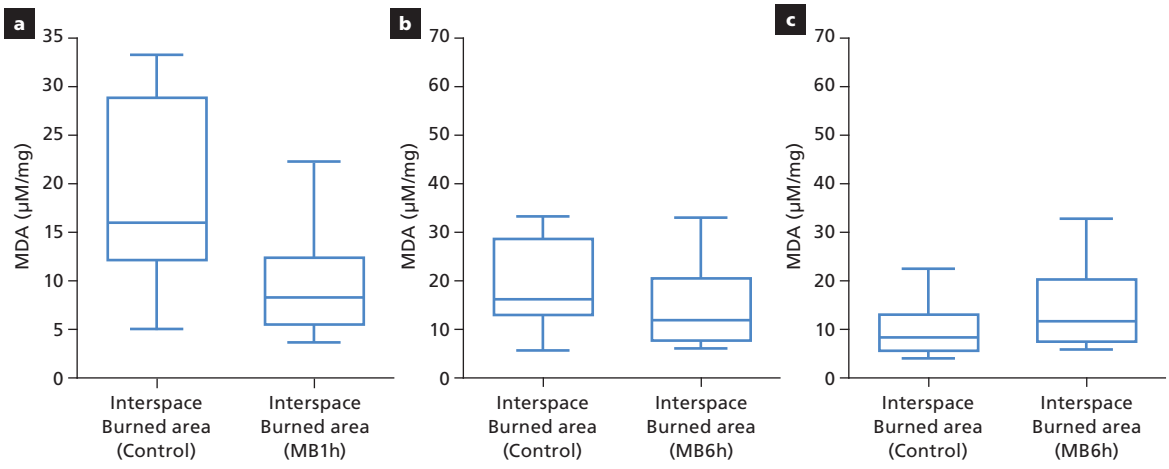
Discussion

The peripheral burn area, called the 'penumbra area', may or may not progress to necrosis. The phenomenon of ischaemia–reperfusion injury and oxidative stress play important roles in the process, although the exact mechanisms are not fully known. The reduced dermal circulation in the stasis zone surrounding the central necrosis area is probably due to obstruction of vessels with red, white, platelet and fibrin clot cells .[1]

Nitric oxide increases in situations that cause increased iNOS, such as burns.[39] During the first few hours after a traumatic injury, such as a burn, iNOS-mediated NO production is exacerbated, producing an increase in NO that greatly exceeds its basal levels. This increase in NO produces significant cell injury with several mechanisms. NO can directly promote peripheral vasodilation resulting in vascular decompensation, and can increase transcription of nuclear factor kappa-B (NF-kB), initiating an inflammatory signaling pathway that activates various inflammatory cytokines.[40,41] NO interacts with the superoxide anion (O_2–) to form peroxynitrite (ONOO–). This highly reactive compound can increase the damage produced by both superoxide and. NO alone.[42] In a burn model, an increase in NO was observed in the burned region with intact area.[17] Its plasma increase demonstrated a biphasic curve with a peak at one and six hours after the stressor event. From biphasic behaviour, since MB is able to block NO by inhibition of sGC and by inhibition of NOS,[28,31] its administration at one and six hours after thermal injury could reduce ONOO– and consequent cell damage by protein nitration, DNA damage and lipid oxidation.

In the clinical evaluation by photograph of the back of the animals seven days after the burn, observers were unable to detect the macroscopic difference between the control group and the groups using MB one hour and six hours after the burn event. We believe that the test was not objective enough to detect statistically significant differences and thus to demonstrate the positive effect of

Fig 5. Malondialdehyde (MDA), in µM/mg, measured in the interspaces between the control group (C) and the methylene blue group after one hour of thermal injury (MB1h) (p=0.01) **(a)**. MDA, in µM/mg, measured in the interspaces between the control group (C) and the blue methylene group after six hours of the same (MB6h) (p=0.24) **(b)**. MDA in µM/mg, measured in the interstices between blue methylene group after one hour of thermal injury (MB1h) and the blue methylene group after six hours of the same (MB6h) (p=0.058) **(c)**



the intervention.

In histopathological evaluation, intradermal injection of MB caused a significant reduction in the percentage of necrosis in the dorsum of animals in comparison with the control group. This reduction occurred in both the MB1h and MB6h groups. The presence of inflammatory infiltrate was also higher in the control group than in the MB1h and MB6h groups. These changes in necrosis percentages, extent, inflammatory changes, and normal skin in the burned and interspaces areas on animals' backs were similarly observed in both the MB1h and MB6h groups.

MB acts as an antioxidant, pro-oxidant, inhibitor of prostacyclin synthesis and accelerator of the reducing processes in the cell.[30,43] The protective action of methylene blue against cell injury and evolution to necrosis could be observed in the groups at one and six hours after the burn injury. In vivo, NO is rapidly oxidised to nitrite and nitrate, which can be measured in biological tissues, plasma and urine.[7]

In the control group, without the presence of MB, the NO increased as a consequence of the burn and caused greater tissue damage, peroxynitrite formation, and cellular damage that evolved into necrosis. With the intradermal injection of MB, the NO (that would normally have increased production at the time of infusion at one and six hours after burn) would have its action blocked, reducing peroxynitrite formation and the resulting cellular injury due to oxidative stress. However, we did not find a statistically significant difference between the groups in NO measurements. Our sample may have been insufficient to generate this difference, as the graphs show a clear tendency to decrease NO metabolites in animals that used MB, at both one hour and six hours after thermal injury.

The trauma caused by the burn produces an inflammatory response that includes an increase in iNOS. Several studies have shown that increased iNOS expression contributes to post-burn cellular damage. In normovolaemic, low levels of NO produced by the constitutive pathway of endothelial NOS (eNOS) exert protective effects on cellular function. In addition, low NO levels play an important vasodilatory role in peripheral circulation, and prevent adhesion of platelets and neutrophils to microvasculature.[39]

MDA is a final product derived from the peroxidation of fatty acids. Its determination allows a convenient measure of lipid peroxidation. Increased plasma MDA levels were reported for burns.[39] In the evaluation of MDA tissue dosages in the burned areas, there was a reduction of MDA tissue in the interspaces of the burned areas in the MB1h group in comparison with the control group. Comparing the concentration of MDA in the burns, there was a statistically significant difference between groups C and MB1h. The reduction in tissue dosages of MDA allows us to infer that MB reduced lipid peroxidation and oxidative

stress in the interspaces in this group, which correlated with the findings of the histological evaluation. Thus, the reduction of the cellular lesion through the antioxidant action of MB was evidenced in less interspace tissue evolving to necrosis.

MB acts as an antioxidant by competitively inhibiting the reduction of molecular oxygen to a superoxide by acting as an alternative receptor for electrons during tissue oxidation.[32] Cellular oxidative stress is a critical step in burn-mediated injury, and antioxidant strategies to inhibit the formation of free radicals or to eliminate free radicals can provide tissue protection in patients with burns.[39] Oxidant-induced lipid peroxidation occurs in the lung and systemic tissues shortly after burn.[44] The oxidant changes are in part due to increased xanthine oxidase activity in the burned tissue. Both xanthine oxidase and neutrophil activation appear to be sources of burned oxidants.

Free radicals have beneficial effects on antimicrobial action and wound healing. However, following a burn, there is increased production of reactive oxygen species that are detrimental, and implicated in inflammation, systemic inflammatory response syndrome, immunosuppression, infection and sepsis, tissue damage, and multiple organ failure. Oxidative damage is a responsible mechanism in the events that occur in the local pathophysiology. The distance from the burn and antioxidant therapy can be beneficial in minimising the lesion in patients with burns. MB accepts xanthine oxidase electrons. Suppressing the uptake of superoxide (O_2) radicals.[32]

A major determinant of burn prognosis and outcome is lesion depth. While superficial burns heal from residual dermal-epidermal elements with minimal scarring, deep burns cause significant scarring and contractures.[1] Since MB has many biological effects, and the hypothesis of obtaining benefits in intraperitoneal use has already been tested, we decided to verify the intradermal route as an alternative mode of administration for this drug. The results of this study provide evidence that MB reduces the number of unburned ischaemic interspaces that evolve to necrosis when given one or six hours after burn in the experimental model used.

MB was the first drug used in humans; it was the precursor of antibiotics, chemotherapy, and antipsychotics. It is an extremely cheap drug, does not have a patent, and the drastic reduction in the consumption of vasoactive amines may already have had an economic impact.

Since 1994, the blockade of guanylate cyclase by MB in distributive shock has been the object of study in our Endothelial Function Laboratory, and has been used clinically by the Cardiovascular Surgery Group, both from the Ribeirão Preto Medical School of the Universidade de São Paulo (FMRP-USP). There is strong evidence that MB, a guanylate cyclase inhibitor, is a therapeutic option for the treatment of vasoplegic syndrome which can be catastrophic for patients with burns.

Evidence for the use of MB in burns is currently limited to that accumulated in regards to three cyclic adenosine monophosphate (cAMP)-independent vasoconstriction mechanisms:

- cGMP/NO-dependent vasoconstriction (the most important mechanism)
- Vasopressin administration
- Hyperpolarisation-dependent vasoconstriction.

Why do these therapeutic alternatives not always work? We believe that there are at least five aspects to this investigation:

- Lack of consideration of existing guidelines or evidence-based medicine about the accepted treatment options available
- The lack of more extensive knowledge of the different vasodilation mechanisms
- The possibility of interference between different vasodilation mechanisms
- The enzymatic activity of sGC
- The frequent use of MB as a therapeutic 'rescue' or 'final' attempt.

Concerning safety, MB can cause bluish stains that cause corneal and conjunctival injury in rabbits. Toxicological studies on the side-effects of intraperitoneal high doses of MB, ranging from 65–200mg/kg, were done and from this we inferred that MB is safe.[27]

Limitation.

Because it is an animal study, due to anatomical and physiological differences there are limitations in extrapolating to humans. Furthermore, the comb burn pattern may not adequately represent burn patterns in humans[1] where injury progression occurs both vertically and horizontally. There is also the limitation of sample number which may have been insufficient to demonstrate a marked difference between treatments.

Conclusions

In this study to evaluate the effects of intradermal MB on

necrosis progression in burns, it should be noted that:

- In the photographic evaluation, it was not possible to show a difference between the control group and the MB1h or MB6h groups
- In the histopathological and morphometric evaluation administration of MB appeared to reduce the necrosis percentage, ischaemic and inflammatory alterations, as well as the necrotic area of the interspaces
- MB was not able to reduce the tissue dosage of NOX in the interspace, either at one hour or six hours after thermal injury
- MB appeared to reduce the tissue dosage of MDA in the interspaces in the group given MB one hour after thermal injury.

However, based on the results presented in this paper, this study provides evidence that the intradermal injection of MB is able to reduce the progression of necrosis in the interspace, possibly by antioxidant action, reducing oxidative stress. Our work served to scientifically co-substantiate a new use, and possibly as an alternative treatment, for MB in burns.

Further studies will be carried out, aimed at the clinical application of this technique.

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References

1 Singer AJ, Taira BR, Lin F et al. Curcumin reduces injury progression in a rat comb burn model. J Burn Care Res 2011; 32(1):135–142. https://doi.org/10.1097/BCR.0b013e318203337b
2 Inoue H, Ando K, Wakisaka N et al. Effects of nitric oxide synthase inhibitors on vascular hyperpermeability with thermal injury in mice. Nitric Oxide 2001; 5(4):334–342. https://doi.org/10.1006/niox.2001.0350
3 Chen LW, Wang JS, Hwang B et al. Reversal of the effect of albumin on gut barrier function in burn by the inhibition of inducible isoform of nitric oxide synthase. Arch Surg 2003; 138(11):1219–1225. https://doi.org/10.1001/archsurg.138.11.1219
4 Chen LW, Hwang YC, Chen CJ et al. Burn-induced lung damage in rat is mediated by a nitric oxide/cGMP system. Shock 2003; 20(4):369–374. https://doi.org/10.1097/01.shk.0000086520.18735.df
5 Chen LW, Hwang YC, Wang JS et al. Inhibition of nitric oxide synthase reverses the effect of albumin on lung damage in burn. J Am Coll Surg 2005; 200(4):574–583. https://doi.org/10.1016/j.jamcollsurg.2004.11.010
6 Rawlingson A. Nitric oxide, inflammation and acute burn injury. Burns 2003; 29(7):631–640. https://doi.org/10.1016/S0305-4179(03)00079-2
7 Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol 1990; 30(1):535–560. https://doi.org/10.1146/annurev.pa.30.040190.002535
8 Binko J, Meachem S, Majewski H. Endothelium removal induces iNOS in rat aorta in organ culture, leading to tissue damage. Am J Physiol 1999 Jan;276(1):E125–E134 .
9 Del Maestro R, Thaw HH, Björk J et al. Free radicals as mediators of tissue injury. Acta Physiol Scand Suppl 1980; 492:43–57
10 Bruckdorfer R. The basics about nitric oxide. Mol Aspects Med 2005; 26(1–2):3–31. https://doi.org/10.1016/j.mam.2004.09.002
11 Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 1990; 9(6):515–540. https://doi.org/10.1016/0891-5849(90)90131-2
12 Slater TF. Free-radical mechanisms in tissue injury. Biochem J 1984; 222(1):1–15. https://doi.org/10.1042/bj2220001
13 Harris K, Walker PM, Mickle DA et al. Metabolic response of skeletal muscle to ischemia. Am J Physiol 1986; 250(2 Pt 2):H213–H220
14 Gamelli RL, George M, Sharp-Pucci M et al. Burn-induced nitric oxide release in humans. J Trauma Inj Infect Crit Care 1995; 39(5):869–878. https://doi.org/10.1097/00005373-199511000-00010
15 Preiser JC, Reper P, Vlasselaer D et al. Nitric oxide production is increased in patients after burn injury. J Trauma Inj Infect Crit Care 1996; 40(3):368–371. https://doi.org/10.1097/00005373-199603000-00007
16 Caneira da Silva MR, Filipe HM, Amaro Pinto RM et al. Nitric oxide and human thermal injury short term outcome. Burns 1998; 24(3):207–212. https://doi.org/10.1016/S0305-4179(98)00014-X
17 Sozumi T. The role of nitric oxide in vascular permeability after a thermal injury. Ann Plast Surg 1997; 39(3):272–277. https://doi.org/10.1097/00000637-199709000-00009
18 Farina Junior JA, Celotto AC, da Silva MF, Evora PR. Guanylate cyclase inhibition by methylene blue as an option in the treatment of vasoplegia after a severe burn. A medical hypothesis. Med Sci Monit 2012; 18(5):HY13–HY17. https://doi.org/10.12659/MSM.882718
19 Levin RL, Degrange MA, Bruno GF et al. Methylene blue reduces mortality and morbidity in vasoplegic patients after cardiac surgery. Ann Thorac Surg 2004; 77(2):496–499. https://doi.org/10.1016/S0003-4975(03)01510-8
20 Yiu P, Robin J, Pattison CW. Reversal of refractory hypotension with single-dose methylene blue after coronary artery bypass surgery. J Thorac Cardiovasc Surg 1999; 118(1):195–196. https://doi.org/10.1016/S0022-5223(99)70161-3
21 Ribeiro NA, Stolf NA, Silva Junior AF et al. Effect of methylene blue on the inflammatory and hemodynamic response in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. Rev Bras Cir Cardiovasc 2004; 19(1):17–23. https://doi.org/10.1590/S0102-76382004000100005
22 Santos MR, Silva LG, Evora PR et al. Use of methylene blue in reversing vasoplegia refractory to the use of catecholamines after aortobifemoral bypass. J Vasc Bras 2010; 9(1):74–77. https://doi.org/10.1590/S1677-54492010005000005
23 Evora PR, Ribeiro PJ, Vicente WV et al. Methylene blue for vasoplegic syndrome treatment in heart surgery: fifteen years of questions, answers, doubts and certainties. Rev Bras Cir Cardiovasc 2009; 24(3):279–288. https://doi.org/10.1590/S0102-76382009000400005
24 Evora PR. Should methylene blue be the drug of choice to treat vasoplegias caused by cardiopulmonary bypass and anaphylactic shock? J Thorac Cardiovasc Surg 2000; 119(3):632–633. https://doi.org/10.1016/S0022-5223(00)70152-8
25 Menardi AC, Capellini VK, Celotto AC et al. Methylene blue administration in the compound 48/80-induced anaphylactic shock: hemodynamic study in pigs. Acta Cir Bras 2011; 26(6):481–489. https://doi.org/10.1590/S0102-86502011000600013
26 Kirov MY, Evgenov OV, Evgenov NV et al. Infusion of methylene blue in human septic shock: a pilot, randomized, controlled study. Crit Care Med 2001; 29(10):1860–1867. https://doi.org/10.1097/00003246-200110000-00002
27 Ramamoorthy S, Patel S, Bradburn E, Kadry Z, Uemura T, K Janicki P,Shah RA, Bezinover D. Use of methylene blue for treatment of severe sepsis in imunossupressed patient after liver transplantation. Case Rep Transplant 2013; 2013.203791. https://doi.org/10.1155/2013/203791
28 Rosique RG, Rosique MJ, Rosique IA et al. Effect of methylene blue on the hemodynamic instability resulting from liver ischemia and reperfusion in rabbits. Transplant Proc 2011; 43(10):3643–3651. https://doi.org/10.1016/j.transproceed.2011.08.108
29 Greca FH, Gonçalves NM, Souza Filho ZA et al. The protective effect of methylene blue in lungs, small bowel and kidney after intestinal ischemia and reperfusion. Acta Cir Bras 2008; 23(2):149–156. https://doi.org/10.1590/S0102-86502008000200007
30 Riedel W, Lang U, Oetjen U et al. Inhibition of oxygen radical formation by methylene blue, aspirin, or alpha-lipoic acid, prevents bacterial-lipopolysaccharide-induced fever. Mol Cell Biochem 2003; 247(1/2):83–94. https://doi.org/10.1023/A:1024142400835
31 Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. Biochem Pharmacol 1993; 45(2):367–374. https://doi.org/10.1016/0006-2952(93)90072-5
32 Salaris SC, Babbs CF, Voorhees WD 3rd. Methylene blue as an inhibitor of superoxide generation by xanthine oxidase. Biochem Pharmacol 1991; 42(3):499–506. https://doi.org/10.1016/0006-2952(91)90311-R

33 Weinbroum AA, Goldin I, Kluger Y, Szold A. Methylene blue in preventing hemodynamic and metabolic derangement following superior mesenteric artery clamping/unclamping: an intratracheal vs. intraperitoneal dose-response study. Shock 2002; 17(5):372–376. https://doi.org/10.1097/00024382-200205000-00005
34 Choi M, Ehrlich HP. U75412E, a lazaroid, prevents progressive burn ischemia in a rat burn model. Am J Pathol 1993; 142(2):519–528
35 Regas FC, Ehrlich HP. Elucidating the vascular response to burns with a new rat model. J Trauma Inj Infect Crit Care 1992; 32(5):557–563. https://doi.org/10.1097/00005373-199205000-00004
36 Mahajan AL, Tenorio X, Pepper MS et al. Progressive tissue injury in burns is reduced by rNAPc2. Burns 2006; 32(8):957–963. https://doi.org/10.1016/j.burns.2006.02.010
37 Tobalem M, Harder Y, Tschanz E et al. First-aid with warm water delays burn progression and increases skin survival. J Plast Reconstr Aesthet Surg 2013; 66(2):260–266. https://doi.org/10.1016/j.bjps.2012.09.014
38 Rosique MJ, Rosique RG, Faria FM et al. Methylene blue reduces progression of burn and increases skin survival in an experimental rat model. Burns 2017; 43(8):1702–1708. https://doi.org/10.1016/j.burns.2017.04.021
39 Horton JW. Free radicals and lipid peroxidation mediated injury in burn trauma: the role of antioxidant therapy. Toxicology 2003; 189(1-2):75–88. https://doi.org/10.1016/S0300-483X(03)00154-9
40 Szabó C. The pathophysiological role of peroxynitrite in shock, inflammation, and ischemia-reperfusion injury. Shock 1996; 6(2):79–88. https://doi.org/10.1097/00024382-199608000-00001
41 Szabó C. Potential role of the peroxynitrite-poly(ADP-ribose) synthetase pathway in a rat model of severe hemorrhagic shock. Shock 1998; 9(5):341–344. https://doi.org/10.1097/00024382-199805000-00005
42 Szabó C, Billiar TR. Novel roles of nitric oxide in hemorrhagic shock. Shock 1999; 12(1):1–9. https://doi.org/10.1097/00024382-199907000-00001
43 May JM, Qu Z, Cobb CE. Reduction and uptake of methylene blue by human erythrocytes. Am J Physiol Cell Physiol 2004; 286(6):C1390–C1398. https://doi.org/10.1152/ajpcell.00512.2003
44 Demling RH, Lalonde C. Systemic lipid peroxidation and inflammation induced by thermal injury persists into the post-resuscitation period. J Trauma Inj Infect Crit Care 1990; 30(1):69–74. https://doi.org/10.1097/00005373-199001000-00010