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Methylene blue reduces progression of burn and increases skin survival in an experimental rat model

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ABSTRACT

Following burn, increased nitric oxide (NO) combine with superoxide anion forming peroxynitrite. Methylene blue (MB) has NO blocking and antioxidant effects. Male Wistar rats (250 g) were burned bilaterally in dorsum with a comb metal plate heated inside boiling water and applied during 30 s, creating four rectangular 10 × 20 mm full-thickness burned areas separated by three 5 × 20 mm unburned interspaces (stasis zone). 30 rats were randomized into three groups (n = 10): treated groups received one dose of intraperitoneal (IP) MB injections (2 mg/kg), one or six hours after injury, and control group received saline. Seven days after injury, wounds were visually analyzed for interspaces necrosis; full-thickness sections were evaluated with Masson staining; tissue fragments were processed for nitrite/nitrate (NOx) and malondialdehyde (MDA) dosages. Photographic analysis: interspaces progression to necrosis were higher in control (64.8%) than in one (44.7%) and six (13.3%) hours MB groups (P = 0.0060). Histopathology showed lower necrosis percentage in one (34.85%) and six (41.62%) hours MB groups than control (77.03%) (P = 0.0034) and higher normal skin percentage in one (25.33%) and six (26.85%) hours MB groups than control (8.32%) (P = 0.0037). Re-epithelialization skin areas were higher in both MB groups (39.94% for one and 31.89% for six hours) than control (14.63%) (P = 0.0210). Interspace's NOx increased in both MB groups (P = 0.0130) with no difference in burned areas. No MDA difference was observed. IP MB injection one or six hours after injury reduced necrosis progression in stasis area in the rat comb burn model suggesting an antioxidant effect reducing oxidative stress.

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1. Background

Burn wound has a central necrotic area and the adjacent stasis area, which may or may not progress to necrosis [1]. The progression to necrosis extends the area requiring surgical treatment, translating into additional donor areas for skin graft and resulting in wound contracture.

Change in perfusion, inflammation and oxidative stress play a role in the necrosis progression in the burned area [2]. The release of cytotoxic substances such as cytokines, reactive oxygen, and nitrogen species (ROS and RNS respectively) contribute to cell damage and the extent of burn [3,4]. Oxidative stress occurs by the production of ROS such as hydroxyl radical (OH) and peroxide (OHH^-).

In a burn model, the nitric oxide (NO) content in the burned area was significantly higher than in the intact area. Also, the NO plasmatic content was significantly increased in a biphasic pattern one and six hours after injury. Finally, nitric oxide synthase (NOS) inhibitors suppressed vascular permeability one and six hours after burn [5].

The increased NO combines with superoxide anion (O_2^-) forming peroxynitrite ($ONOO^-$), a potent oxidizing agent [6] able to attack proteins, nucleic acids and oxidize cell membrane lipids (lipid peroxidation) [7–9].

NO is synthesized by nitric oxide synthase (NOS) from L-arginine and oxygen. An increase in inducible nitric oxide synthase (iNOS) with consequently increased local and systemic NO production, is reported in burn models [10–14]. Some studies have shown increased NO/NOS [15,16] and nitrite/nitrate (NOx) (NO metabolites) plasmatic concentrations in burned patients, unrelated to total burned body surface [17].

Many agents have been suggested to prevent the progression of stasis area to necrosis, reducing burn wound extension. Methylene blue (MB) has NO blocking agent properties [18]. It also acts as an antioxidant, pro-oxidant, prostacyclin synthesis inhibitor and accelerator of reducing processes inside cells [19,20].

Based on that, this study was designed to evaluate whether intraperitoneal injection of MB would reduce injury progression after burn.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing between 250 and 290 g were used in this study. Animals' housing and care were in accordance with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Local Animal Ethical Committee from Ribeirão Preto Medical School of the University of São Paulo. Animals were kept at room temperature of 22–25 °C and a 12 h day–night cycle. Water and standard diet were available ad libitum.

2.2. Experimental protocol

The rats were anesthetized using ketamine (50 mg/kg) (Cetamin, Syntec, Brazil) and xylazine (10 mg/kg) (Dopaser, Hertape Calier, Brazil) intra-peritoneally. The dorsum of each rat was shaved with electric clippers (Oster Golden A5, USA) and depilated with Veet (Reckitt Benckiser, Brazil).

The comb burn model [21] was used to create the thermal injury. A metal comb was heated inside boiling water for three minutes. Then, it was applied perpendicular to skin surface without pressure in animal's dorsum during 30 s resulting in four rectangular 10 × 20 mm full-thickness burned areas separated by three 5 × 20 mm unburned interspaces (zone of ischemia or stasis). Both sides of the dorsum were burned (Fig. 1A). No dressing was applied.

The animals were randomized into three groups: control group (n = 10): saline (0,3 ml) intraperitoneally; MB_1 group (n = 10): one dose of methylene blue (MB) (2 mg/kg) intraperitoneally one hour after thermal injury and MB_6 group (n = 10): one dose of MB (2 mg/kg) intraperitoneally six hours after thermal injury.

2.3. Measures and outcomes

Immediately and seven days after thermal injury, the wounds were photographed for unburned interspaces visual analysis of necrosis. Two independent observers, blind to applied treatment, considered as necrotic the interspaces that turned black.

All animals were sacrificed seven days after injury and lesions full-thickness sections from the interspaces and burned area were taken, fixated in 10% formalin and embedded in paraffin. 5 μm sections were stained with Masson's trichrome. A pathology specialist, blind to treatments, performed the histopathological analysis. Using a digital microscope (Nikon Eclipse 80i, Nikon, Japan) and a high-resolution camera (Nikon DS-Fi1, Nikon, Japan), images representing the section of each sample were digitalized and analyzed with NIS-Elements F3.2 software (Nikon, Japan). Considering in each group the presence of:

1) Normal skin: usual features without ischemic or inflammatory changes.
2) Skin with ischemic or inflammatory changes but without necrosis: crust on the epidermal surface, formed by leukocytes (predominantly neutrophils) and serous material or necrotic tissue, a possible re-epithelialization area.
3) Necrotic skin.

The three amendments were compared among the three groups separately.

Tissue fragments of burned areas and interspaces were separately wrapped and promptly stored at −70 °C. Tissue levels of nitrite/nitrate (NOx) were assessed by an ozone-based chemiluminescence assay. Briefly, samples were treated with ethanol (95%) at 4 °C for 30 min, followed by centrifugation at 10,000 × *g* for five minutes. NOx levels were measured by injecting 5 μL of the supernatant into a glass purge vessel containing 0.8% vanadium in 1N hydrochloric acid at 95 °C, which reduces NOx to NO gas. A nitrogen stream was bubbled through the purge vessel, then through 1N NaOH and then into a NO analyzer that detects NO released from NOx by chemiluminescence (Sievers, model 280 NO Analyzer; Boulder, CO, USA). NOx concentration was

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