

## **Methylene Blue does not Inhibit Capillary Blood Flow increase in Crush Injury**

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### **Abstract**

Background: Nitric oxide (NO) plays a central role in increasing capillary blood flow in muscles following crush injury. It activates soluble guanylate cyclase, which increases cyclic guanosine monophosphate (cGMP) production, thereby inducing smooth muscle relaxation and vasodilatation. The objective of this study was to evaluate the effect of methylene blue (MB), a guanylate cyclase inhibitor, on inhibition of nitric-oxide induced capillary blood flow increase in limbs injured by crush injury in a rat model. Materials and Methods: A standard crush injury was induced in rat hind limbs with and without intraperitoneal injection of MB. Capillary blood flow was measured in the crushed muscle. Results: Following crush, muscle capillary blood flow increased by 3-4 fold ( $P < 0.001$ ). This effect was observed only in limbs that underwent crush. Capillary blood flow in contralateral limbs was not affected. Intraperitoneal injection of MB did not affect either the enhanced capillary blood flow observed in crushed limbs or the capillary blood flow in the contralateral limb. Conclusion: Intraperitoneal injection of MB did not attenuate the increase in capillary blood flow observed in crushed limbs. The role of cGMP-dependent cGMP-independent mechanisms leading to vasodilatation and increased blood flow in muscles following crush injury should be further investigated.

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## 1 Introduction

Crush injury was first described in 1812 by Dominique Jean Larrey, who was a French surgeon in Napoleon's army [1]. Crush injury commonly affects survivors of structural collapse such as occurs in earthquakes, but may also affect victims of motor vehicle accidents and victims suffering from high velocity gun-shot wounds to their limbs. It results in severe electrolyte imbalance and acute renal failure as a consequence of rhabdomyolysis. The mechanism of crush injury was previously thought to be secondary to ischemia and reperfusion [2]. However, crush injury can also develop in limbs in which blood supply is maintained [3]. Furthermore, clinical experience shows that deprivation of limb's blood supply for several hours, a technique commonly used in orthopedic surgery, will not induce crush injury. Current thinking is that the main mechanism leading to crush injury and subsequent crush syndrome is destruction of muscle cells by direct pressure rather than ischemia [3].

Once muscle cells are crushed, increased membrane permeability allows sodium, calcium and excess water to enter the cells. This process culminates in muscle edema and cell death. A common feature described in crush injury models includes vasodilatation and increased blood flow to the muscle [4]. Both myocyte edema and increased blood flow may add to the increased tissue pressures observed in limbs following crush injury.

At the cellular level, nitric oxide (NO) is thought to be responsible for several of the untoward effects seen following crush [4]. NO, initially termed endothelium derived relaxing factor, is derived from L-Arginine via a chemical reaction mediated by NO synthase yielding NO and Citrulline [5,6]. Inducible nitric oxide synthase (iNOS) is one isoform that appears in pathologic states, such as crush and reperfusion injuries [7,8]. Compared to other isoforms, iNOS can produce large amounts of NO. NO activates soluble guanylate cyclase (sGC) resulting in increased production of the secondary messenger cyclic guanosine monophosphate (cGMP) [9]. cGMP induces smooth muscle relaxation with subsequent vasodilatation and increased blood flow.

Decreasing the vasodilatory effect of NO may be an important adjunct to treatment of muscles injured by crush. This could be achieved by one of two mechanisms: diminishing NO production by iNOS; or diminishing activation of sGC by NO. Methylene Blue (MB) is an antagonist of GC [10]. Unlike iNOS antagonists, there is considerable clinical experience with MB in humans. MB may have other positive effects in crush injury. It may induce noradrenaline secretion in blood vessel nerve endings and acts as a free radical scavenger in reperfusion injury [11,12].

The objective of this study was to evaluate the impact of MB on capillary blood flow in limbs injured by crush injury in a rat model that was previously described by our group [4]. Compartment syndrome does not develop after unilateral crush injury in rats because the limb muscles are of small volume relative to the limb. Therefore this model allows the effect of direct crush upon muscle to be examined without other effects from secondary increase in pressure induced by compartment syndrome.

## **2 Subjects and Methods**

### **2.1 Experimental Protocol**

This study was authorized and performed according to the guidelines of the Technion's committee for supervision of animal experiments. Forty four Sprague Dawley Rats, weighing 250-300 grams were divided into four treatment groups:

Group A (n=14) did not undergo direct crush and were not treated with MB.

Group B (n=14) underwent controlled crush only (to be explained below).

Group C (n=6) were treated with an intraperitoneal injection of 25mg/kg MB. These rats did not undergo controlled crush.

Group D (n=10) underwent controlled crush. Immediately following crush, 25mg/kg MB was injected intraperitoneally.

Group B and D rats underwent controlled crush injury of their hind left limbs. The rats were anaesthetized with intraperitoneal injection of sodium pentobarbital at a dose of 30mg/kg. Once anaesthetized, the rats were placed in the prone position. The left leg was immobilized by tape and the limb between the ankle and knee placed under a 4 cm<sup>2</sup> compression plate activated by a pneumatic piston. This allowed exposing these limbs to standardized external mechanical pressures of 4.25 kg/cm<sup>2</sup> for 120 minutes.

Once the pressure was released of their limbs, Group D rats were injected with 25mg/kg MB intraperitoneally. Group C rats that did not undergo controlled crush injury were also injected with MB using the same route and dosage. Group A rats did not undergo neither controlled crush injury nor intraperitoneal injection of MB.

The capillary blood flow to muscle in all the rats was evaluated 4 days following the crush injury and/or intraperitoneal injection of MB. This time span was chosen because it has been shown that capillary blood flow in rats peaks following four days from induced crush [4,13].

On the fourth day after the induction of crush, rats were anaesthetized with intraperitoneal injection of thiobutabarbital at a dose of 100mg/kg. At this dose, blood pressure is commonly maintained. Animals were then placed supine on a heated plate to preserve body heat at 38 degrees Celsius. The tibialis anterior muscle was exposed in both legs and paraffin oil heated to 38°C was applied to avoid tissue desiccation. Capillary blood flow was measured over 15 different areas in each limb using a dedicated laser Doppler monitor (Periflux4001 Master/4002 Satellite, Perimed AB, Sweden). Measurements were expressed as perfusion units (blood cell concentration multiplied by the mean cell velocity). Measurements were carried out over fifteen different areas since crush injury causes non-homogeneous damage to different areas within the muscle.

Capillary blood flow was the only endpoint used to measure the effect of MB in this study. In a previous experiment, in which L-NAME's effect on capillary blood flow was evaluated, pathologic assessment of the crushed muscle fibers revealed extensive damage [4]. Muscle fibers were edematous with areas of frank necrosis and abundance of polymorphonuclear cells and macrophages. Large lipid inclusions within myofibers were common. The nuclei of myofibers had very large and pronounced nucleoli.

These pathologic changes were observed in all crushed limbs, whether treated with L-NAME or not. The only distinctive change seen following crush, but attenuated by L-NAME, was capillary dilatation. Capillary dilatation correlated with increased capillary blood flow measured in the limbs by the laser Doppler monitor.

## **2.2 Statistical Analysis**

The average capillary blood flow was calculated for each limb of each of the rats. The average blood flow was then calculated for each group with its corresponding standard error, left and right limbs separately. Average blood flow in the left limb and right limb of each group were compared using one way ANOVA and by Tukey-Kramer Multiple Comparisons Test. P less than 0.05 was considered statistically significant. Data analysis was done using GraphPad InStat (GraphPad Software Inc., San Diego, CA).

## **3 Main Results**

The average capillary blood flow and corresponding standard errors as measured in each limb is presented in Figure I. Following crush, capillary blood flow in the crushed muscle increased by 3-4 fold ( $P < 0.001$ ). This effect was observed only in the limbs that underwent crush. Capillary blood flow in the contralateral limbs was not affected. Intraperitoneal injection of MB did not affect either the enhanced capillary blood flow observed in crushed limbs or the capillary blood flow in the contralateral limb. The standard error in each of the groups was small, revealing that variability between measurements was minimal.

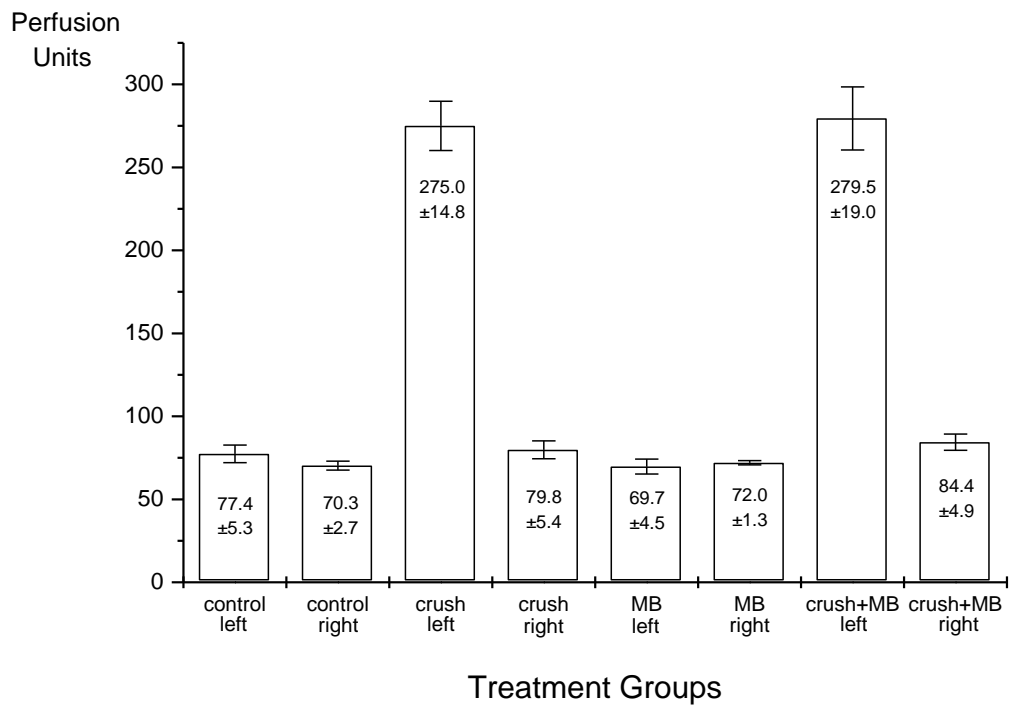


Figure 1: Perfusion measured in left and right limbs of different treatment groups (average and standard error)

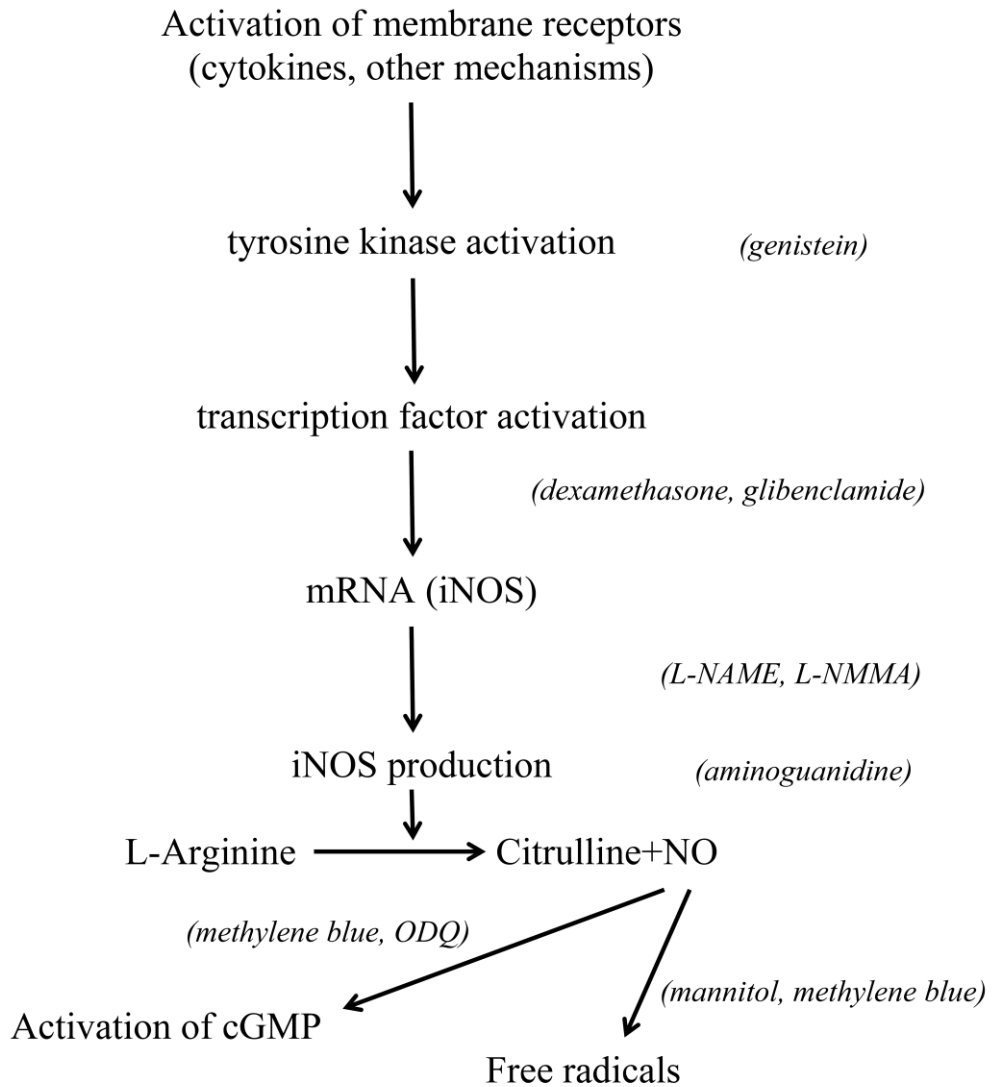


Figure 2: Possible interventions aimed at decreasing the untowards effects of crush injury (pharmacologic interventions in italics within parenthesis)

#### 4 Discussion

This study was based on the assumption that NO plays a significant role in the augmentation of capillary blood flow in crushed muscles and that targeting NO activation of cGMP may provide a target for intervention to attenuate the effects of crush injury.

Pharmacologic interventions to decrease NO production or decrease NO activation of cGMP could achieve this result (figure II). A similar model was used in experiments where the effects of mannitol 20%, hyperbaric oxygen and L-Nitro-Arginine-Methyl-Ester (L-NAME) upon capillary blood flow were examined (4,14). In these experiments, crush induced increase in capillary blood flow was attenuated by these interventions.

Methylene blue is a heterocyclic aromatic compound with many medical uses. It is used as a dye during endoscopy, anal procedures, photodynamic therapy and in sentinel lymphnode biopsy [15-18]. It has been shown to have efficacy in the treatment of different infections such as malaria [19]. It is an antidote for methemoglobinemia [20]. Extensive literature has documented its efficacy in treatment of vasoplegia seen following bypass surgery and reversal of sepsis induced hypotension [21,22]. These latter effects may be due to MB's effect upon NO.

Both the vast experience with MB use in clinical practice and its possible attenuating effects upon NO induced vasodilatation, make this drug an attractive option for treatment in crush injury immediately following victim extraction from under rubble. Results of this experiment, however, show that MB did not have any effect upon capillary blood flow increase observed following crush. Capillary blood flow increased 3-4 fold in both groups B and D. This effect was seen only in crushed limbs. Capillary blood flow in contralateral limbs in these groups remained similar to capillary blood flow measured in non-crushed limbs in groups A and C. The mechanisms underlying the increased capillary blood flow following crush remain largely unknown, but may depend on local regulatory mechanisms.

There are several possible explanations that may account for why MB did not prevent increases in capillary blood flow following crush:

1. NO may not be the main mediator leading to increase in capillary blood flow following crush. NO has been shown to cause vasodilatation [5]. It is extensively produced following crush injury [8]. However, the increased blood flow may be secondary to other processes as well, some of which have not been identified [23]. Nevertheless, previous experiments with L-NAME, a competitive inhibitor of all three isoforms of NOS, showed that increases in capillary blood flow were attenuated [4]. This latter finding indicates NO production plays a central role in increased capillary blood flow following crush.
2. MB may not have inhibited GC. The dosage of MB in this study was 25mg/kg. This dose is 12-25 times greater than doses used in different clinical scenarios in human beings [24]. This dosage was thought to be appropriate for rats, whose metabolic rate is increased compared to humans. Doses 3 to 4 fold higher were found to be extremely toxic and most rats so treated died following injection. Other, lower doses were not evaluated in this study, assuming that MB, if indeed effective, would exert its effect beyond a certain threshold. Nevertheless, MB may have dose dependent effects and we cannot rule out response at lower dosages.
3. Timing of MB administration may have been ineffective in inhibiting GC. Both L-NAME and MB are readily absorbed from the peritoneum [25]. Both have relatively similar half lives measured in hours (3-5 hours). Nevertheless, their biologic activity is different. L-NAME is an iNOS inhibitor while MB exerts its effect further downstream. One single dose of L-NAME proved to be sufficient in attenuating crush induced vasodilation [4]. One single dose of MB in this study did

not show a similar effect. Due to differences in biologic activity we cannot rule out that a different schedule, such as daily injections with intraperitoneal MB, could have achieved a different outcome.

4. NO activates other processes, apart from cGMP, that eventually lead to vascular smooth muscle relaxation and increased capillary blood flow [26,27]. These mechanisms bypass MB inhibition of GC. A possible alternate mechanism described is that of NO activation of sarco/endoplasmic reticulum calcium ATPase (SERCA). SERCA is important in regulating the amount of cytosolic free  $\text{Ca}^{2+}$  and subsequent muscle contraction. Activation of SERCA by NO will diminish cytosolic free  $\text{Ca}^{2+}$  leading to muscle vasodilatation. To exclude the involvement of a cGMP-dependent mechanism, sGC inhibitors other than MB, such as ODQ [1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one] should be employed [28]. Nevertheless, our choice to examine MB effect on crush induced vasodilatation relied on clinical applicability.

## 5 Conclusion

Although, NO has been shown to play a central role in increasing capillary blood flow in muscles following crush, MB in this study did not impede this increase in capillary blood flow. Further investigation is needed to evaluate the role of cGMP-dependent and cGMP-independent mechanisms leading to vasodilatation and increased blood flow in muscles following crush injury.

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