The effects of intrathecal methylene blue and glyceryl trinitrate administration on orofacial pain in mice

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Background: Nitric oxide (NO) is involved in several types of painful conditions. In patients with chronic migraine, glyceryl trinitrate, a pro-drug for NO, can induce a delayed migraine episode. In this study, we decided to assess the effects of intrathecal administration of a NO pro-drug (glyceryl trinitrate-GTN) and a NO scavenger (methylene blue-MB) on orofacial pain (OFP).

Methods: 24 BALB/c mice were divided into three groups as follows: GTN group (0.1mg/kg, n=8), MB group (0.05mg/kg, n=8) and control group (NaCl, n=8). All groups received the substance by the intrathecal route. Two hours after drug/saline administration, formalin was injected into the upper lip and the time mice spent rubbing/liking the injected area was recorded.

Results: Intrathecal administration of MB and NTG had no effect on the acute phase of OFP when compared with control. In the second phase, however, both drugs had an analgesic tendency; for GTN, this was statistically significant (p=0.025), and for MB the effect was less important (p=0.083).

Conclusions: By centrally administering a NO pro-drug and a NO scavenger, we expected to modulate NO production in formalin-induced OFP. Our results demonstrated that the acute phase of OFP does not depend on NO (neither of the drugs had any effect) and that both substances diminish pain perception in the persistent/inflammatory phase but only the NO pro-drug has a clear-cut antinociceptive effect.

Keywords: Orofacial Pain, Nitric Oxide, Nitroglycerin, Methylene Blue, Analgesia, Intrathecal Injections

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Introduction

Nitric oxide is generated by the enzymatic cleavage of L-arginine by nitric oxide synthase (NOS) and exerts its function by converting GTP into cyclic guanine monophosphate (cGMP) [1]. In some cases, depending on concentration, NO can react with superoxide, producing the potent oxidant peroxynitrite, which can induce apoptosis or necrosis [2]. In mammalian cells, however, the main effector of nitric oxide (NO) is the afore-mentioned soluble guanylate cyclase (sGC), which catalyses conversion of GTP into the cyclic nucleotide cGMP.

Besides its well-known effects as a vasodilator, NO is one of the neurotransmitters involved in the nociceptive process. In nociception, NO seems to have a dual effect, depending on the NO quantity available in the synaptic cleft and on its site of action—peripheral vs. central [3].

Methylene blue (MB) has been investigated as a therapeutic agent in several neurological or painful conditions due to its beneficial effect on dysfunctional mitochondria as well as its ability to reverse the effects of NO donors [4]. Literature data indicate that MB can attenuate superoxide production because of its capacity to function as an alternative mitochondrial electron transfer carrier. In addition, MB can function as an anti-oxidant and can block the activity of monoamine oxidases (MAO).

Although it seems that MB could be an efficient drug in managing some disorders such as Alzheimer’s disease, Parkinson’s disease or Leber’s optic neuropathy, there is evidence that, in patients concurrently taking serotonergic reuptake inhibitors, MB administration may cause a condition called serotonin syndrome [5,6] secondary to MB’s ability to block central and peripheral MAO-A activity. In addition, MB acts as an artificial electron carrier, promoting mitochondrial respiration; the net outcome is that more energy is available, in the form of ATP, for cellular processes [7].

Glyceryl trinitrate (GTN, also referred to as nitroglycerin), is widely used as an exogenous NO donor whose bioactivation depends on the mitochondrion. Mitochondrial aldehyde dehydrogenase-2 (ALDH2) catalyses the bioactivation of GTN and generates 1,2-glyceryl dinitrate and nitrite, further metabolized to nitric oxide-based compounds (NO). Two main pathways have been proposed for GTN biotransformation, one based on GTN biotransformation, one based on GTN biotransformation that produces NO and contributes directly to vasodilation and the other based on the detoxification pathway that produces inorganic nitrite anions (NO₂⁻) [8].

In animals, GTN has proved its ability to sensitize trigeminal nucleus caudalis neurons to capsaicin [9], to induce hyperalgesia in rat hind limbs [10] and to activate leptomeningeal macrophages and their expression of iNOS [11]. The hyperalgesic effects of GTN have been linked to the drug’s NO production.

The formalin test is a tonic model of continuous pain that results from assessing a rodent’s pain behavior after formalin-induced tissue injury [12]. The nociceptive behavior is assessed by measuring two commonly reported pain-like behaviors—flinching and licking/biting [13] in the first 40 minutes after formalin injection. It has been demonstrated that subcutaneous injection of formalin up-regulates the Homer1a protein in dorsal horn neurons; the factors responsible for Homer1a activation include N-methyl-D-aspartate receptor (NMDAR), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and proto-oncogene tyrosine-protein kinase (Src) [14]. In formalin-induced pain, the Homer proteins are, therefore, convergent factors that potentially link major glutamate receptors with Ca²⁺stores in neurons [15].

In this study we investigate the effect of intrathecal administration of two nitric oxide modulators, methylene blue and glyceryl trinitrate, on the orofacial pain induced by formalin injection.

Methods

The experiments were conducted on 24 male BALB/c mice (28±3 g), housed at 21±2°C under a 12-h light/dark cycle with access to food and water ad libitum. All animals were habituated to the testing room for at least five days. The experimental protocols and procedures described in this article comply with the European Communities Council Directive 86/609/EEC and follow the ethical guidelines for investigations of experimental pain in conscious animals of the International Association for the Study of Pain [16] and the University of Medicine and Pharmacy “Gr. T. Popa”. We used validated classical nociceptive models as described in the literature.

The drugs used in these experiments were: Nitrolingual Pumpspray (G. Pohl Boskamp GmbH & Co. KG), Methylene Blue 1% (Tis Farmaceutic, Romania), 37% formaldehyde (Fluka, Germany). MB and GTN were freshly diluted in normal saline and were injected intrathecally (i.t.).

The Orofacial Formalin Test (OFT) was performed as described by Luccarini, et al [17]. Briefly, animals were habituated for 20 minutes in individual Plexiglas observation chambers, after which they received an injection of 20 µL 5% formalin into the upper lip, right next to the nose with a 26-gauge needle. The nociceptive score was determined by recording the number of seconds the animals spent grooming the injected area with the ipsilateral hind paw or forepaw, often accompanied by the contralateral forepaw, for the first 6 min (first/neurogenic phase) and from 7 to 40 min (second/inflammatory phase).

During each experiment, the time (sec) spent in licking/biting or rubbing the injected area was recorded for 40 min. Results are presented either as the sum of seconds spent on licking or rubbing during each phase or percentage of inhibition of nociceptive behavior for phase I and for phase II.

We used lumbar intrathecal injections because they do not require surgery, are rapidly performed, do not produce
neurological deficits and hepatic clearance/metabolism is by-passed. Briefly, animals were firmly held by the pelvic girdle and a 37-gauge needle connected to a Hamilton syringe was inserted at an angle of approximately 15° relative to the horizontal plane. The needle was gently introduced along the groove between the spinous and transverse processes until slipping into the intervertebral space between L4 and L5. The L4–L5 position was selected for injection to minimize the risk of spinal cord injury. The flick of the tail was considered indicative of a successful i.t. injection [18]. Each mouse received 5 µL of saline, MB (0.05 mg/kg b.w.) or GTN (0.1 mg/kg b.w.).

The mice were divided into 3 groups: one group of mice received freshly diluted MB (n= 8, group MB), the second group received freshly diluted nitroglycerine (n= 8, group GTN) and the third group of mice served as control and received saline, the vehicle for drugs (n= 8, group NaCl). In each group, the inflammatory pain was induced two hours after substance administration. The antinociceptive activity was expressed as the percentage of inhibition of nociceptive behavior using the ratio: inhibition% = (control mean-treated mean) x 100 / (control mean).

Data are expressed as mean ± SEM. Statistical analysis was performed by means of SPSS-17.0 software. ANOVA was performed when appropriate. Post hoc comparisons of differences between treated groups and saline group were determined by the Dunnett coefficient. Significance was set at p=0.05.

Results

Intrathecal administration of MB and GTN was associated with changes in pain perception during the formalin test. However, these differences were significant only for the inflammatory phase (p=0.033 as assessed by ANOVA), with no statistically significant differences recorded in the first phase of the test (p=0.684).

The post-hoc tests showed that intrathecal MB administration had no effect on the nociceptive phase (Figure 1) of the orofacial pain test (phase I), but had a tendency towards an analgesic effect on inflammatory pain (phase II) (p=0.083). The inhibition percentage after MB intrathecal administration was 14.12 % for phase I and 39.40 % for phase II, respectively (Figure 2).

Intrathecal administration of GTN (Figure 1) had an effect that was similar to that of MB - it did not influence nociceptive pain (phase I) induced by formalin, but it significantly (p=0.025) reduced inflammatory pain (phase II). The inhibition percentage was 0.56 % for phase I and 50.05 % for phase II respectively (Figure 2).

There were no statistically significant differences between pain-related behaviors when comparing the GTN group with the MB group.

Discussion

In the present study, we have shown that intrathecal pre-treatment with low doses of methylene blue or glyc eril trinitrate 2h before formalin administration was associated with an analgesic effect on the inflammatory phase and had no effect on the nociceptive phase of the orofacial formalin test. These two nitric oxide-sGC modulators can have variable effects, depending on the administration pathway and on the selected dose.

Our study results concur with the results of Miclescu et al. [20], which have indicated that in patients with chronic, therapy-resistant neuropathic pain, methylene blue administration decreases pain intensity on the first 2 days after administration. Indeed, MB is a well-known inhibitor of the NO-stimulated sGC and has been widely used for inhibition of cGMP-mediated processes in order to reverse the effect of several NO donors, so its analgesic potential is not unexpected. Studies have shown that in the presence of ATP, NO binding to the sGC heme does not fully activate the enzyme [19]. Thus, the ATP which was found to be increased after MB administration could directly affect sGC activity. So MB might exert its analgesic effect through both NO inhibition and ATP production mechanisms.

GTN triggers headache in normal subjects, and migraine without an aura in migraine susceptible patients [21, 22]. In rodents, GTN-evoked hyperalgesia has been
developed as a model for sensory hypersensitivity associated with migraine [23, 24]; however, the doses used in rodents are 2000 times higher than those used in humans (10 mg/kg b.w. in rodents versus 5 microgram/kg b.w. in humans) and are administrated by systemic route. In our study, GTN, in a dose significantly smaller than that used in other animal studies, was administrated by the i.t. route. At this dose and administration pathway, GTN produced an analgesic effect in the formalin test 2 h after administration. These results are in line with other studies which have shown that intrathecal administration of low doses of L-arginine, a NO precursor, inhibit the nociceptive response evoked by the intraplantar injection of formalin in rats, whereas high doses of the NO precursor increase this response [25]. Moreover, in vitro studies on endothelial cells indicate that pre-treatment with 10–300 μM GTN results in time- and concentration-dependent loss of NO-stimulated sGC activity [26]. Thus, at small doses, GTN might decrease the sCG availability in neurons so that the painful effect of formalin injection might be limited by the decreased level of the neuronal sCG.

On the other hand, GTN might produce headache immediately after its administration, pain that activates the diffuse noxious inhibitory controls which decrease formalin pain experienced by rodents two hours later.

In our study, at a dose similar with that used in humans, GTN produced pronounced analgesia 2 hours after its administration when administrated via the intrathecal route, an effect opposite to the one obtained after large GTN doses administrated via the intraperitoneal route but in line with other studies that indicate the analgesic effect of NO [27].

Our study offers further evidence supporting the hypothesis that NO has a dual effect on pain. The dose, route of administration, the timing and the experimental pain model seems to be important when evaluating the effect of NO on pain.

**Abbreviations**

ALDH2: Aldehyde dehydrogenase-2; cGMP: cyclic guanine monophosphate; GTN: Glyceryl trinitrate; MAO: Monoamine oxidases; MB: Methylene blue; NMDAR: N-methyl-D-aspartate receptor; NO: Nitric oxide; NOS: Nitric oxide synthase; OFT: Orofacial Formalin Test; sGC: Guanylate cyclase

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**Competing interests**

The authors declare no conflict of interest.

**References**


