THE EFFECTS OF RIBOFLAVIN AND METHYLENE BLUE ON NOCICEPTION AND VISCERAL PAIN

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THE EFFECTS OF RIBOFLAVIN AND METHYLENE BLUE ON NOCICEPTION AND VISCERAL PAIN (Abstract) Background: Methylene Blue (MB) can prevent electron leaking, increase mitochondrial oxidative phosphorylation, and reduce ROS overproduction under pathological conditions, currently being trace evidence that it can alter pain perception in humans by local administration during certain surgical procedures. Riboflavin or vitamin B2 (B2) constitutes a part of the vitamin B group, which in recent studies shows a growing implication in the treatment of some pathology that imply pain management. Aim: To investigate the effect of one dose of Riboflavin and Methylene Blue on nociception and visceral pain in mice. Methods: A total of 48 BALB/c male mice were divided into 3 groups: MB Group, B2 Group and C Group. MB (5 mg/kg b.w.), B2 (100 mg/kg b.w.) or an equivalent volume of saline was administered intraperitoneally. Mice were tested before (baseline) and after drugs administration over a 4h period. Nociception was evaluated by means of Hot Plate Test (HPT) and TFT (Tail Flick Test). Visceral pain was evaluated 2h after administration. Results: Four hours after MB administration we recorded an analgesic effect on the hot plate test (p<0.05 at 30, 60 and 240 min). No significant effect on the TFT was noticed. B2 vitamin had an antinociceptive effect as compared to control group only for HPT that persisted for 2h but had no effect on TFT. Both MB and B2 vitamin have shown an analgesic effect (p<0.01) on visceral pain when compared to the control group but the pain inhibition was more important after riboflavin administration. Conclusions: Even if the exact mechanisms are not clarified by our study, we demonstrated that both ATP modulators (MB & B2 vitamin) have analgesic effect on visceral pain and nociception. Keywords: METHYLENE BLUE, RIBOFLAVIN, PAIN

Methylene Blue (MB) is a synthetic redox compound containing a central thiazidic ring system (1) that had a wide usage from chemotherapy to dementia, antimalarial, and not least contributed to the discovery of the neuroleptic drug families due to its oxidation-reduction facilities (2). MB improves mitochondrial respiration and the metabolic rate stimulating the mitochondrial complex IV, enhancing it by 30% and increasing the oxygen consumption to 70% (3).

MB is inhibiting cGMP- mediated processes and neuronal NOS (4,5), and can
influence NO-synthases (ROS), mono- 
amino-oxidase A (MAO) and disulfide-reductases (6).

MB is able to interfere with peripheral and central nervous systems by modifying the glutamatergic, monoaminergic and cholinergic neuronal communication (7), and can also prevent leaking of electrons, increasing mitochondrial oxidative phos- 
phorylation and reduce ROS overprodu-
c tion (8).

In humans MB proved its analgesic ef-
tects after local administration either alone (intradermal anesthetic) in association with propofol injections (9) or in chronic low-
back pain (10). In animals models it being reported that it could have antinociceptive effects.

Riboflavin (RF) also known as vitamin B2 constitutes a part of the vitamin B group that is essential for cellular functions and development. It plays an important role in oxidation-reduction reactions, reducing oxidative stress and is involved in mediating the immune responses (11).

RF has a modulating role on mito-
chondrial oxidative stress in the brain (12), and is documented that although most of the conditions were associated with B2 defi-
ciency, it can be used to alleviate migraine as a prophylactic drug (13) or in diseases associated with metabolic disorders (14).

RF also demonstrated in recent studies that it can influence experimental nocice-
tion in mice by possibly influencing the inflammatory mediators (15) (TNF α, IL-1 or IL-6) and increase the effect of non-
steroidal drugs in rats (16).

The purpose of our study was to assess the effects of both MB and RF on nocicep-
tion and visceral pain in mice as long as both substances modulate serotonergic, noradrenergic, dopaminergic systems as well as NO and ROS all implicated in noc-
i ception modulation.

MATERIAL AND METHODS

Animals. The experiments were con-
ducted on 48 BALB/c male mice (28-34 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 laboratory for at least three days. The experimental protocols and pro-
c edures described in this article complied with the European Communities Council Directive 86/609/EEC and followed the International Association for the Study of Pain’s (17) and the University of Medicine and Pharmacy “Gr. T. Popa” ethical guide-
lines for investigations of experimental pain in conscious animals.

Drugs. The drugs used in these experi-
ments were: methylene blue 1% (Tis Farm-
aceutic, Romania), acetic acid (Sigma, Germany) and riboflavin (≥98%, Sigma, Germany).

Both MB and RF were freshly diluted in saline and administered intraperitoneal (i.p.).

The Hot-Plate Test (HPT). The hot-
plate test was used to measure nociceptive response to heat according to the method described by Woolfe and MacDonald (18), with minor modifications. Briefly, the mice were placed into an open Plexiglas tube, on the hot-plate apparatus (Ugo Basile, model-
DS 37, Italy) 55 ± 0.1°C and the time be-
tween placing the animal on the hot-plate and the occurrence of licking, shaking of hind paws or jumping off the surface was recorded as response latency. An automatic 15 s cut-off was used to prevent tissue damage. Animals displaying baseline laten-
cies of more than 10 s were excluded from the study (20).
**The Tail Flick Test (TFT).** The tail flick test (19), was used to measure the latency for tail flick reflex following exposure to a heat stimulus. The heat source of the Tail-Flick unit (Ugo Basile, Italy) was focused on the distal portion of the tail at 4-5 cm from the tip and the reaction time necessary for the mouse to remove its tail was defined as the tail-flick latency. Animals displaying baseline latencies of more than 6 s were excluded from the study. The cut-off time was set at 12 s to avoid tissue damage (21).

Antinociception on the hot plate and tail flick tests was quantified as percentage of maximal possible effect (%MPE):

\[
\% \text{MPE} = \frac{(\text{post-treatment latency}-\text{baseline latency}) \times 100}{(\text{cut off latency}-\text{baseline latency})}
\]

**The Acetic Acid Induced Writhing Test (Visceral pain).** Was performed as previously described by us, after acclimatization in an acrylic observation chamber for at least 20 minutes, the mice received 0.1 ml /10 mg b.w i.p. injections with 1.0% (v/v) acetic acid (20).

The antinociceptive activity in the writhing test was expressed as the percentage of inhibition of nociceptive behavior using the ratio:

\[
\% \text{ pain score} = \frac{(\text{control mean} - \text{treated mean}) \times 100}{\text{control mean}}
\]

**Study design.** A total of 48 mice were divided into three groups as follows: Group MB, mice receiving a single intraperitoneal dose of methylene blue (5 mg/kg b.w); Group RF that received a single dose of 100 mg/kg b.w. riboflavin and Group C that received an equivalent volume of saline in the same manner.

The TFT, HPT tests were performed at baseline and thereafter 30, 60, 120, and respectively after 240 minutes after administration. The writhing test was performed 2h after administration.

The doses used in this study are in range with literature data and have been proved a maximum antinociceptive effect from the doses tested in our lab (data not shown).

**Statistical analysis.** The data obtained were expressed as the mean value ± SEM. Statistical evaluations were performed using SPSS v 20.0 software with one-way ANOVA. Post hoc comparisons were performed using Tukey’s Test. The significance level was set at p<0.05.

**RESULTS**

**The Tail Flick Test.** Administered i.p. neither MB 5mg/kg b.w. or B2 100 mg/kg b.w. did not influence the reaction of the mice to thermal stimuli over the time period allocated for the experiment, with no statistical significance as compared to the control group.

**The Hot Plate Test.** A single dose of riboflavin produced a significant analgesic effect (p<0.05) that started 30 minutes after i.p. administration (fig. 1) and maintained for 2h (MPE of 30.8% reaching a maximum of 56% at 60 minutes) showing a decrease in effect after 240 min (MPE= -51.7). MB demonstrated the same analgesic effect (p<0.05) that occurred at the same time as for B2, with a MPE between 29% and 38%, the effect lasting over 4h%. Although the MPE was higher in B2 Group, the analgesic effect lasted longer in the MB Group.

**The Writhing Test.** Intraperitoneal administration of vitamin B2 showed a significant analgesic effect on acetic acid induced pain (p<0.01) same as MB (p<0.05) when compared to control group (fig. 2). The pain inhibition score was higher for
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riboflavin (77%) as compared to the one MB demonstrated (23.2%) Tukey's mul-
tiple comparisons test showing strong signifi-
cance between the two.

Fig. 1. The effects of B2 and MB on Hot Plate. The results are expressed as mean± S.E.M. * p < 0.05.

Fig. 2. The effects of B2 and MB on visceral pain in mice. The results are expressed as mean± S.E.M. * p < 0.05.

DISCUSSION
This study demonstrates, concordant with literature data, that methylene blue (22) and riboflavin (23) modify pain per-
ception. Thus, our study is one more proof that ATP modulators had an analgesic ef-
fekt on the hot plate test and visceral pain and a neglect effect of the thermo-
ociception evaluated by the tail flick test.

The substances used by us are mitochon-
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drial linked and have proved their ability to change the cellular ATP level and REDOX status of the cells. Thus, MB acts as an antioxidant and as an artificial electron donor to complex I-IV of the mitochondria, increasing ATP production and riboflavin has been shown to improve both complex I (NADH dehydrogenase) and complex IV (cytochrome c oxidase) activity. Riboflavin serves as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) coenzymes that participate in oxidative-phosphorylation reactions at the cellular level. A unique feature of FAD and FMN is their function as prosthetic groups in many enzyme systems and as catalysts of oxidation-reduction reactions.

There is a growing body of evidence that extracellular ATP has an important role in pain signaling (both the periphery and in the CNS). It is supposed that ATP is involved in acute pain; as long as ATP is released from damaged cells it excites directly primary sensory neurons by activating their receptors, by neither blocking nor suppressing the expression of P2X/Y receptors in sensory neurons or in the spinal cord, showing an effect on acute physiological pain. There is also evidence that endogenous ATP and its receptor system might be activated in pathological pain states, particularly in neuropathic pain. Thus, ATP is implicated in synaptic transmission of innocuous mechano-receptive and nociceptive input in the superficial dorsal horn; the purinergic ATP receptors being expressed in dorsal horn neurons and in DRG cells (24).

In our study we used two models of acute pain to evaluate the effects of methylene blue and riboflavin namely thermo-nociception models of pain (tail flick and hot plate tests) and visceral pain.

The hot plate and tail flick tests are commonly used for evaluating thermal pain sensitivity, heat selectively stimulating thermoreceptors and nociceptors. The tail-flick is considered mainly a spinal reflex, at least in its shorter latency form, persisting after section or cold block of upper parts of the spinal cord (25) but as with all reflexes, it is subject to supraspinal structures control (26).

By contrary, the hot plate test is supposed to have supraspinally integrated responses. The paw licking behavior is affected only by opioids but the jumping/shaking reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol (27). Thus we must keep in mind that mainly tail-flick and less the hot plate tests are predictive only for substances that are morphinomimetic in the strictest sense, with very few effects for partial agonist compounds and none at all for mild antalgics.

Visceral pain results from activation of sensory afferent nerves innervating internal organs (28). Viscero-sensory axons are almost exclusively thinly myelinated A-delta and unmyelinated C fibers while the receptors exhibit chemosensitivity, thermosensitivity and/or mechanosensitivity. The afferent fibres convey sensory information from the upper gastrointestinal tract to the CNS via vagal and splanchnic nerve pathways (29). A nociceptive stimulus evoked by i.p. injection of acetic acid or citric acid in mice (a model of inflammatory visceral pain) stimulates sensory afferents and the transmission of nociceptive information, centrally leads to the activation of descending antinociceptive mechanism to a noxious stimulus (30). It has been proposed that distension of visceral organs leads to re-
lease of ATP from the epithelium lining the organ; the released ATP then acts upon P2X receptors in subepithelial nociceptors to elicit visceral pain (31). However, the ATP inhibitors (32) were ineffective in reducing nociception in animal models of visceral pain.

Our behavioral data demonstrate that both substances known to stimulate cellular ATP level, decrease pain perception explored by hot plate test and visceral pain. As long as exogenous ATP proved its pronociceptive effect when administrated locally or in DRG, we suppose that the ATP modulator used act rather on the ATP-sensitive potassium channel (K_{ATP}) family imply in analgesia and/or on purinergic receptor imply in pain inhibition.

CONCLUSIONS

Even this study does not clarify the intimate mechanisms of action for methylene blue and riboflavin it is proving that ATP stimulators after one dose has analgesic effects. Long term administration effects and the analgesic neuromediation activated need further studies.

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