

# Pharmacological Evidence for the Role of Nitric Oxide-cGMP in Antinociception

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## ABSTRACT

The aim of this study was to evaluate a possible role of nitric oxide (NO)-cyclic guanylate monophosphate (cGMP) in antinociception induced by sildenafil (phosphodiesterase-5 inhibitor), celecoxib (selective cyclooxygenase-2 inhibitor), and indomethacin (nonselective cyclooxygenase inhibitor). Each of these drugs was administered intraperitoneally into experimental mice at different doses either alone or combined with either N<sup>g</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME, NO synthase inhibitor) or methylene blue (guanylate cyclase inhibitor).

Antinociceptive activity was assessed by using a writhing test as the pain model.

The three drugs showed dose-related antinociceptive activity as defined by a reduction in writhing episodes in comparison with controls. Pretreatment of the mice with L-NAME or methylene blue led to inhibition of the antinociceptive effect of any of the three analgesic drugs tested. Ineffective doses of either celecoxib (0.5 mg/kg) or indomethacin

(2.5 mg/kg) achieved significant reduction of writhing episodes when concomitantly given with sildenafil at an ineffective dose (0.5 mg/kg). This possible synergistic effect of sildenafil was inhibited when animals were pretreated with either L-NAME or methylene blue. It is concluded that NO-cGMP may play an important role in induction of analgesia by sildenafil, celecoxib, and indomethacin.

## INTRODUCTION

The biochemical basis of inflammatory and nociceptive conditions has been primarily dependent on release of prostaglandins, which lower the pain threshold in A<sub>Δ</sub> and C nociceptive afferents to the non-noxious range.<sup>1</sup> Then, prostaglandins stimulate specific receptors located on A<sub>Δ</sub> and C fibers leading to accumulation of cAMP/Ca<sup>++</sup> and hyperalgesia.<sup>2,3</sup>

A new mechanism in nociception/antinociception has involved the nitric oxide (NO)-cyclic guanylate monophosphate (cGMP) pathway. L-arginine, which is a source of NO by the enzyme NO synthase, produced antinociception in rats with carrageenan-induced hyperalgesia. This effect was blocked by N<sup>g</sup>-nitro-L-arginine methyl ester

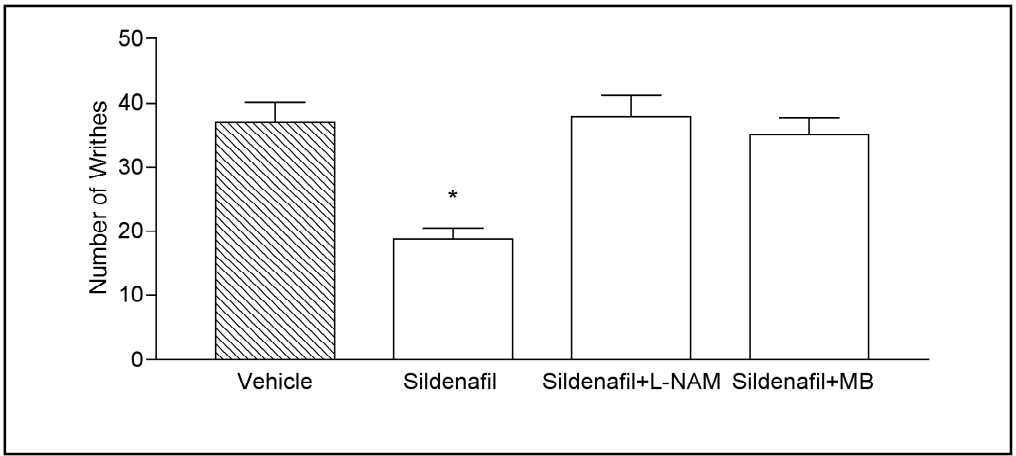
**Table 1.** Comparison of Antinociceptive Activity of Different Coses of Sildenafil, Celecoxib, and Indomethacin to their Corresponding Controls on Writhing Episodes in Mice

Group of Animals	Writhing Episodes (Mean ± SEM)	Post hoc*
<b>Sildenafil</b>		
Control (saline)	37 ± 3.2	
0.5 mg/kg	36 ± 3.7	
1.0 mg/kg	26.8 ± 3.7	P<0.05
1.5 mg/kg	21.5 ± 2.0	P<0.01
2.0 mg/kg	18.8 ± 1.7	P<0.001
3.0 mg/kg	15.7 ± 3.3	P<0.001
<i>ANOVA test</i>	P=0.001	
<b>Celecoxib</b>		
Control (Tween 80)	36.5 ± 3.4	
0.5 mg/kg	31.6 ± 4.7	
1.0 mg/kg	23.3 ± 1.7	P<0.01
1.5 mg/kg	17.2 ± 2.2	P<0.001
2.0 mg/kg	14.8 ± 1.7	P<0.001
<i>ANOVA test</i>	P=0.001	
<b>Indomethacin</b>		
Control (redistilled water)	40.8 ± 3.3	
2.5 mg/kg	38.7 ± 1.8	
5.0 mg/kg	29.5 ± 3.6	P<0.05
10.0 mg/kg	20.1 ± 1.8	P<0.01
12.5 mg/kg	16.2 ± 1.9	P<0.001
<i>ANOVA test</i>	P=0.003	
ANOVA=Analysis of variance (between all groups of each drug simultaneously).		
*Significant difference was assessed for each single dose group versus corresponding control group for the same drug. Significance at P<0.05.		

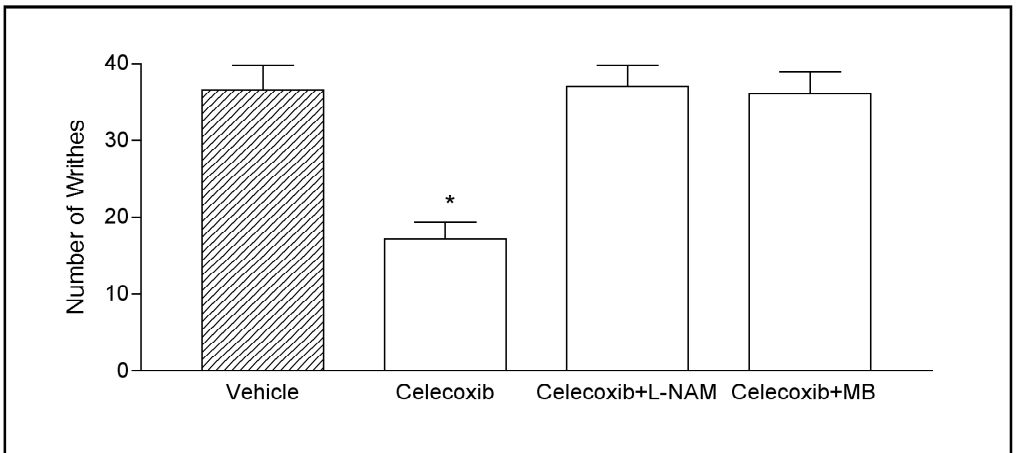
hydrochloride (L-NAME), which is an inhibitor of NO synthase enzyme. Furthermore, neuronal NO synthase may be involved in both peripheral and central analgesia since potentiation of antinociception produced by low doses of acetaminophen was observed after intraperitoneal (IP)<sup>4</sup> as well as intracerebroventricular or intrathecal administration of either nonselective NO synthase inhibitor (L<sup>G</sup>-nitro-L-arginine) or relatively selective inhibitor of neuronal NO synthases (7-nitroindazole).<sup>5</sup> Also, inhibition of the enzyme guanylate cyclase by methylene blue (MB, guanylate cyclase inhibitor) disabled it to catalyze

the formation of cGMP from guanosine triphosphate (GTP) with consequent blockade of cGMP-mediated antinociception.<sup>6</sup>

It is worth highlighting, however, that activation of the NO-cGMP pathway has been reported to result in hyperalgesia rather than antinociception.<sup>7</sup> On the contrary, some researchers considered cGMP and NO to work as mediators in pain perception. The cGMP seems to play an important role in the functional up- and down-regulation of nociception. Also, NO can participate in this neurophysiological process through its diffusion out of the neurons to acti-



**Figure 1.** Inhibition of antinociceptive effect of sildenafil 2 mg/kg by L-NAME (20 mg/kg) or methylene blue (MB, 1 mg/kg). Vehicle=saline. Bars are the mean  $\pm$  SEM for 12 animals. \*Significantly different ( $P<0.05$ ) from the other three groups as determined by analysis of variance followed by Tukey's test.



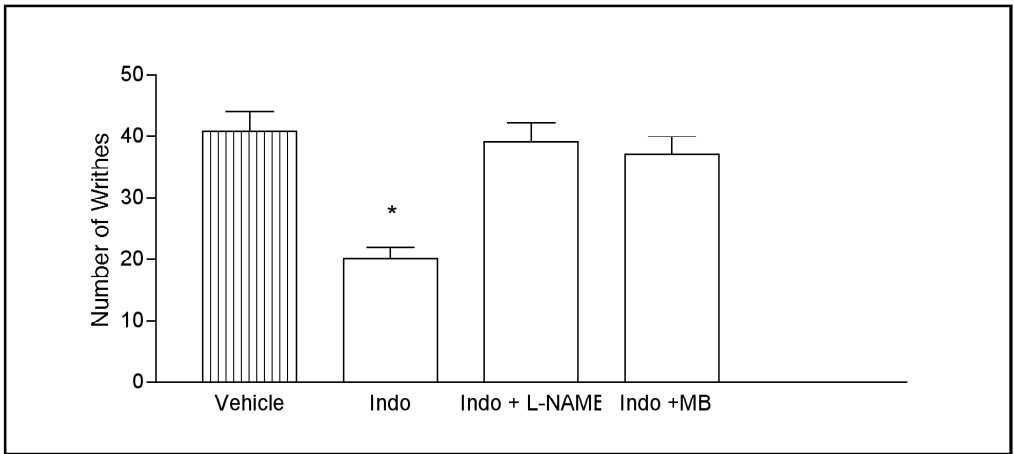
**Figure 2.** Inhibition of antinociceptive effect of celecoxib 1.5 mg/kg by L-NAME (20 mg/kg) or methylene blue (MB, 1 mg/kg). Vehicle=saline. Bars are the mean  $\pm$  SEM for 12 animals. \*Significantly different ( $P<0.05$ ) from the other three groups as determined by analysis of variance followed by Tukey's test.

vate guanylyl cyclase enzyme in neighboring cells.<sup>8,9</sup> Therefore, there is still a controversy about the role of NO/cGMP in nociception/antinociception.

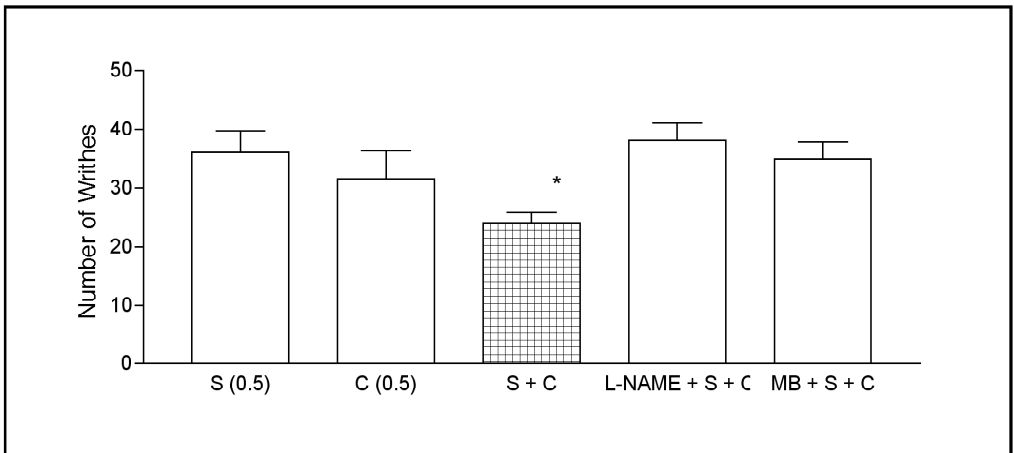
It has been proposed that the intracellular concentration and activity of cGMP is regulated through a symphony between NO, which activates guanylyl cyclase enzyme to form cGMP from GTP, and cGMP-specific phosphodiesterase-5 enzyme (PDE-5) that catalyzes the hydrolysis of cGMP.<sup>10</sup>

Sildenafil is reported to inhibit PDE-5. This inhibition reduces the degradation of cGMP and elevates its concentration in the cells.<sup>11</sup> It is believed that the recently reported antinociceptive effect of sildenafil in experimental animals is ascribed to peripheral activation of NO-cGMP pathway.<sup>6</sup>

The antinociceptive activity of nonsteroidal anti-inflammatory drugs (NSAIDs) has been established through inhibition of cyclooxygenase isozymes



**Figure 3.** Inhibition of antinociceptive effect of indomethacin 10 mg/kg by L-NAME (20 mg/kg) or methylene blue (MB, 1 mg/kg). Vehicle=saline. Bars are the mean  $\pm$  SEM for 12 animals. \*Significantly different ( $P<0.05$ ) from the other three groups as determined by analysis of variance followed by Tukey's test.

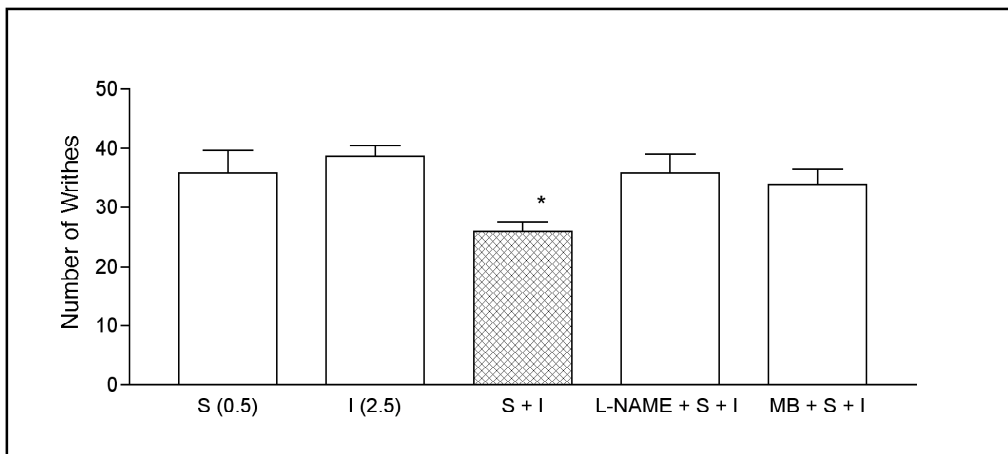


**Figure 4.** Antinociceptive activity of a combination of sildenafil (S, 0.5 mg/kg) and celecoxib (C, 0.5 mg/kg) together and with either L-NAME (20 mg/kg) or methylene blue (MB, 1 mg/kg). Bars are the mean  $\pm$  SEM for 12 animals. \*Significantly different ( $P<0.05$ ) from the other three groups as determined by analysis of variance followed by Tukey's test.

(COX) with consequent impairment of prostaglandin synthesis.<sup>12,13</sup> However, new mechanisms have been added, such as activation of NO-cGMP pathway as reported in previous studies on NSAIDs including nonselective COX inhibitor (eg, diclofenac) and selective COX-2 (eg, rofecoxib).<sup>14-17</sup> However, a controversy has been reported about the role of NO-cGMP pathway in antinociception induced by the NSAID

indomethacin, which is a nonselective COX inhibitor.<sup>16,18,19</sup>

In this study we have investigated the role of NO and cGMP in nociception because of contradictory results in the literature. Three drugs were examined: sildenafil (phosphodiesterase-5 inhibitor) as a new drug in the world of antinociception, celecoxib (selective cyclooxygenase-2 inhibitor), and indomethacin (nonselective cyclooxyge-



**Figure 5.** Antinociceptive activity of a combination of sildenafil (S, 0.5 mg/kg) and indomethacin (I, 2.5 mg/kg) together and with either L-NAME (20 mg/kg) or methylene blue (MB, 1 mg/kg). Bars are the mean  $\pm$  SEM for 12 animals. \*Significantly different ( $P < 0.05$ ) from the other three groups as determined by analysis of variance followed by Tukey's test.

nase inhibitor). Writhing test was the pain model used in the experimental animal study. The tested drugs were administered either alone or each was combined with L-NAME or MB (guanylate cyclase enzyme inhibitor) to assess the role of NO-cGMP in antinociception.

## MATERIAL AND METHODS

### Animals

Three hundred thirty six albino mice from our own breeding facilities were used in this study. They were of either sex with weight range of 30 to 35 g at the beginning of the experiment. The animals were housed in cages (4 per cage) in a conventional environment (temperature/humidity) and were subjected to natural daily dark-light cycles and had free access to food and water until 1 hour before the experiment. Each animal was used only once. The study was approved by Pharmacology Department Faculty of Medicine, Assiut University, and the experimental protocol followed the local guidelines of animal care in the animal house of the university.

### Drugs

Sildenafil citrate (Viagra tablets, 50 mg) and celecoxib (Celebrex tablets, 100 mg) were given as a gift from Pfizer Company, Cairo, Egypt. The active substance sildenafil was extracted from the Viagra tablets according to the protocol suggested by Tseng and Lin.<sup>20</sup> Then, it was dissolved in 0.9% saline as the proper vehicle. Celecoxib was extracted from the Celebrex tablets according to Guirguis et al,<sup>21</sup> and the extracted product was dissolved in polysorbate 80 (Tween 80 1% in saline, purchased from Merck Company, Germany) as the proper vehicle. Indomethacin was purchased as Liometacin ampoules, from Nile Company, Cairo, Egypt. Liometacin is a water-soluble salt of indomethacin (N-methyl-D-glucamine salt of indomethacin, ie, meglumine indomethacinate). Each lyophilized ampoule contains 77.2 mg of indomethacinate salt equivalent to 50 mg indomethacin. L-NAME and MB were purchased from Sigma Company, Germany. They were both dissolved in distilled water before use.

## Measuring of antinociceptive activity (writhing test)

In this study, we have used a writhing test as a peripheral model to test the antinociceptive activity of different drugs. In this test, 1% acetic acid solution (10 mL/kg) was injected intraperitoneally into the mouse and the number of writhing episodes induced was counted. Writhing reflex was defined as constriction of the abdomen, twisting of the trunk, and extension of the hind legs. Observation of writhes was performed during 20-minute periods starting 3 minutes after injection of acetic acid.<sup>22</sup> Selection of the writhing test as the model of pain in this experiment was based on its high sensitivity to both central and peripheral analgesics, especially NSAIDs.<sup>23,24</sup>

## Experimental procedure

Mice received IP injection of vehicle or increasing doses of sildenafil (0.5 to 3 mg/kg), celecoxib (0.5 to 2 mg/kg), or indomethacin (2.5 to 12.5 mg/kg) 20 minutes before the noxious stimulus (acetic acid 1%) to assess the antinociceptive effect of each agent in the writhing test. The control group (n=36) was divided into three equal subgroups (n=12 each) according to the vehicle injected (0.9% saline, Tween 80 1% in saline, or redistilled water). The number of mice exposed to different doses of sildenafil, celecoxib, or indomethacin was 12 per individual dose of any of the drugs tested for antinociception. To determine the participation of NO-cGMP pathway in the peripheral antinociceptive effect of the tested drugs, mice were pretreated with L-NAME, a NO synthase inhibitor or with MB, an inhibitor of guanylyl cyclase enzyme. These inhibitors were administered intraperitoneally throughout the study at doses of 20 mg/kg for L-NAME and 1.0 mg/kg for MB 30 minutes before the onset of writhing test; that is 10 min-

utes before the antinociceptive drug. Tested drugs and other inhibitors were injected in a vehicle volume of 50 mL/animal.

## Statistical analysis

Results were expressed as mean  $\pm$  standard error of the mean (SEM) and data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey or Student's t-test (post-hoc test) wherever suitable to determine the statistical difference between groups. To test for equality of variance, Levene's test was applied. The criterion for statistical significance was  $P < 0.05$ . Statistical program SPSS version 8 (SPSS Inc, Chicago, IL) was used for statistical analysis.

## RESULTS

### Antinociceptive activity

Sildenafil (1.0, 1.5, 2.0, and 3.0 mg/kg), celecoxib (1.0, 1.5, and 2.0 mg/kg), and indomethacin (5.0, 10.0, and 12.5 mg/kg) produced significant, dose-dependent decreases in the number of writhing episodes in mice (Table 1). As estimated by log-dose-response curve, the experimental doses of antinociceptive drugs that result in a 50% analgesia ( $ED_{50}$ ) relative to their corresponding control responses were 1.8 mg/kg, 1.4 mg/kg, and 8.5 mg/kg for sildenafil, celecoxib, and indomethacin, respectively.

Effect of inhibition of NO-synthase or guanylyl cyclase inhibitor (MB, 1 mg/kg) on the antinociceptive effects of sildenafil, celecoxib, and indomethacin. Pretreatment of mice with L-NAME at a fixed dose of 20 mg/kg or MB at a fixed dose of 1 mg/kg led to inhibition of the antinociceptive activity of sildenafil, celecoxib, or indomethacin given at doses that achieved significant antinociception as mentioned in Table 1 (Figures 1-3).

Administration of sildenafil at a noneffective dose of 0.5 mg/kg concomitantly with either celecoxib or

indomethacin at their noneffective doses of 0.5 and 2.5 mg/kg, respectively, achieved significant reduction in writhing episodes ( $P<0.05$ ). This effect was blocked when the administration of any of the two drug combinations was preceded by L-NAME or MB (Figures 4 and 5).

## DISCUSSION

In this study, sildenafil showed a dose-dependent antinociceptive activity as demonstrated by reduction of writhing episodes following the IP injection of acetic acid 1% in the animal model. This result supports the proposed analgesic activity of sildenafil.<sup>6</sup>

It is suggested that inflammatory pain involves nociceptor sensitization that may be a result of neuronal biochemical imbalance between cAMP and cGMP concentrations with predominance of cAMP. Sildenafil by its potent and selective blocking activity on PDE-5 could inhibit cGMP degradation with consequent biochemical imbalance between cAMP and cGMP that leads to predominance of cGMP and development of antinociception.<sup>25</sup> The doses of sildenafil that achieved analgesia in our study were within the range of doses used for the same purpose in other studies.<sup>6,25</sup> Furthermore, blockade of the analgesic activity of that drug by concomitant administration of either L-NAME (NO synthase inhibitor) or MB (guanylate cyclase inhibitor) indicated a possible involvement of NO-cGMP-PDEs-5 pathway in sildenafil-induced antinociception.<sup>6</sup>

Similarly, the NO-cGMP pathway may be also involved in the antinociception induced by celecoxib and indomethacin, especially in the pretreatment of animals with L-NAME or MB that led to inhibition of analgesia obtained by either of the two drugs.

A controversy has been reported about the role of NO-cGMP pathway in

indomethacin-induced antinociception. The results of our study (Figure 3) support the opinion of a possible role of NO-cGMP in the analgesic effect of indomethacin. In a previous study on pain-induced functional impairment model in the rat (PIFIR model), indomethacin administration reduced significantly the nociceptive response elicited by uric acid injected into the knee joint of the animal.<sup>19</sup> This analgesic effect was reduced by local pretreatment with L-NAME while local administration of L-arginine (NO synthase substrate) increased the antinociceptive activity of indomethacin.<sup>15</sup> In contrast, previous studies reported no influence of L-NAME or MB on indomethacin-induced antinociception.<sup>6,17</sup> The discrepancy in results between the different studies may be explained on the basis of difference in pain models selected for analysis, the intensity and type of nociceptive stimulus employed, as well as the animal species used.<sup>16</sup> An example of the influence of the selected pain model on antinociceptive activity of different NSAIDs was the observation that in formalin-induced inflammatory pain, peripheral administration of celecoxib could not produce antinociception at two formalin concentrations (1% and 5%), while diclofenac produced a dose-dependent antinociception in the second phase of both concentrations of formalin.<sup>26</sup> Furthermore, the peripheral pronociceptive/antinociceptive activity of NO-cGMP pathway may be affected by tissue levels of NO and/or the intracellular content of cGMP. So, changes in concentration of these mediators, eg, by L-NAME and/or MB, may lead to contrasting effects.<sup>27,28</sup>

Sildenafil administration at a noneffective dose (0.5 mg/kg) with celecoxib (0.5 mg/kg) or indomethacin (2.5 mg/kg) increased the antinociceptive activity, a finding that may reflect a possible

potentiation in the analgesic effects between sildenafil and either of the other two drugs. Sildenafil was reported to increase the antinociceptive activity of centrally acting analgesics such as morphine.<sup>29,30</sup> Also, it potentiated the antinociceptive activity of peripherally acting analgesics such as nimesulide or diclofenac.<sup>6,31</sup> The observed blockade of possible synergistic activity between sildenafil and both celecoxib and indomethacin at noneffective doses by L-NAME or MB supports the possible role of NO-cGMP in antinociception induced by any of the three drugs.

In conclusion, NO-cGMP pathway plays a role in antinociception induced by the NSAIDs celecoxib and indomethacin. This mechanism could be added to their known inhibitory action on prostaglandin synthesis for induction of analgesia. Sildenafil demonstrated an antinociceptive activity in experimental animals, an effect that needs further investigation especially because the drug may show other effects when it is used as antinociceptive agent.

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