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Additive Antinociception between Intrathecal Sildenafil and Morphine in the Rat Formalin Test

The possible characteristics of spinal interaction between sildenafil (phosphodiesterase 5 inhibitor) and morphine on formalin-induced nociception in rats was examined. Then the role of the opioid receptor in the effect of sildenafil was further investigated. Catheters were inserted into the intrathecal space of male Sprague-Dawley rats. For induction of pain, 50 μL of 5% formalin solution was applied to the hindpaw. Isobolographic analysis was used for the evaluation of drug interaction between sildenafil and morphine. Furthermore, naloxone was intrathecally given to verify the involvement of the opioid receptor in the antinociception of sildenafil. Both sildenafil and morphine produced an antinociceptive effect during phase 1 and phase 2 in the formalin test. The isobolographic analysis revealed an additive interaction after intrathecal delivery of the sildenafil-morphine mixture in both phases. Intrathecal naloxone reversed the antinociception of sildenafil in both phases. These results suggest that sildenafil, morphine, and the mixture of the two drugs are effective against acute pain and facilitated pain state at the spinal level. Thus, the spinal combination of sildenafil with morphine may be useful in the management of the same state. Furthermore, the opioid receptor is contributable to the antinocieptive mechanism of sildenafil at the spinal level.

Key Words : *Antinociception; Drug Interactions; Injections, Spinal; Phosphodiesterase 5 Inhibitor; Sildenafil; Morphine; Receptors, Opioid*

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INTRODUCTION

Experimental evidence indicates an important role of cyclic guanosine monophosphate (cGMP) in antinociceptive action (1, 2). This evidence is in line with the observation that intraplantar injection of dibutyryl-cGMP produced antinociception in inflammatory hyperalgesia rats (3). Moreover, intrathecal 8-bromo-cGMP reduced mechanical allodynia in neuropathic rats (4). Therefore, cGMP seems to be very critical for the regulation of the nociceptive transmission. Guanylyl cyclase catalyzes the formation of cGMP from GTP, leading to the synthesis of cGMP, whereas cGMP-specific phosphodiesterase catalyzes the hydrolysis of cGMP to GMP, thereby ending signal transduction (5). Accordingly, intracellular cGMP concentrations are regulated by the action of guanylyl cyclase and the rate of degradation by cGMP-specific phosphodiesterase (5, 6).

Sildenafil (Viagra®) is a novel inhibitor of cGMP-specific phosphodiesterase 5, which has been shown to be effective in the treatment of male erectile dysfunction (7, 8). Recently, it has been reported that intrathecal sildenafil produced an antinociception, which is mediated through the nitric oxide (NO)-cGMP pathway (9, 10). It is demonstrated that morphine reversed not only acute nociception but also tissue injury hyperalgesia through the action on spinal opioid receptor (11-14). Furthermore, several lines of evidence suggest that opioid-induced antinociception may be related to the activation of the NO-cGMP pathway (15-17). These observations, conversely, may lead to a hypothesis that the effect of the cGMP-specific phosphodiesterase inhibitor may be affected by the opioid system. Therefore, understanding the functional role of cGMP and opioid receptor in altered nociception may help provide novel targets for pain therapy.

The purpose of the present study was to evaluate the characteristics of pharmacological interaction between spinal sildenafil and morphine in the formalin test which shows tissue injury pain leading to the facilitated state as well as acute pain (11). We further clarified the possibility of contribution of spinal opioid receptor on the action of sildenafil.

MATERIALS AND METHODS

Animal handling and experimental procedures were approved by the Institutional Animal Care Committee of Research Institute of Medical Science in Chonnam National University. Adult male Sprague-Dawley rats weighing 250-300 g were used in all experiments. The animals were housed in

groups of four, with free access to standard rat diet and tap water in a room under 12:12 hr light/dark cycle. For the purpose of drug administration, an intrathecal catheter was implanted under enflurane anesthesia and aseptic surgical conditions as described previously (18). A polyethylene-10 tube was inserted into the subarachnoid space through a slit made in the atlantooccipital membrane. The catheter was advanced caudally 8.5 cm to reach the level of the lumbar enlargement. The external end of the catheter was tunneled subcutaneously, exiting at the top of the head and plugged with a piece of steel wire. The skin was closed using 3-0 silk sutures. After catheter implantation, rats were housed in individual cages. All animals with a neurological deficit postoperatively were rejected from further study and killed immediately with an overdose of volatile anesthetics. At least 5 days of postsurgical recovery were allowed before the behavioral study.

The following drugs were used in this study: sildenafil and morphine sulfate (Sigma Chemical Co., St. Louis, MO, U.S.A.), naloxone hydrochloride (Sigma). Sildenafil was kindly provided by Korea Pfizer. Sildenafil was dissolved in dimethylsulfoxide (DMSO), and morphine and naloxone were dissolved in normal saline. Intrathecal administration of these agents was performed using a hand-driven, gear-operated syringe pump. All drugs were delivered in a volume of 10 μL solution, followed by an additional 10 μL of normal saline to flush the catheter.

The formalin test was done as a nociceptive test. The animals were injected subcutaneously with 50 μL of 5% formalin solution into the plantar surface of the hind paw using a 30-gauge needle. The formalin injection produced specific pain behavior characterized as rapid and brief withdrawal or flexing of the injected paw. This behavior was called a "flinching response". Such pain behavior was therefore quantified by periodically counting the incident of flinching of the injected paw. The number of flinches was counted for 1-min period at 1 and 5 min and at 5-min intervals from 10 to 60 min. Formalin-induced flinches were observed in a characteristic biphasic response. The initial phase 1 (0-9 min) was followed by a relatively short quiescent period, which was then followed by a late phase 2 (10-60 min). At the end of the experiment, the rats were killed with volatile anesthetics.

On the day of experiments, the rats were placed in a restraint cylinder and allowed to adapt for 15-20 min. Rats were then placed into one of the experimental groups. The control study was done with intrathecal DMSO or saline. Animals were tested only once in the formalin test. The total number of rats used was 113 with 5-7 rats per group. The researcher was blind to the drug given to experimental animals.

For evaluation of the dose-response of the antinociceptive action of sildenafil (1, 3, 10, and 30 μg) and morphine (1, 3, 10, and 30 μg), experimental rats received two drugs intrathecally. Intrathecal drugs were injected 10 min before formalin injection. Each ED_{50} value (an effective dose producing a 50% reduction in control formalin response) for the agents was calculated separately in two phases.

The isobolographic analysis (19) was used to determine the nature of pharmacologic interaction between sildenafil and morphine in the formalin test. This method was based on the comparison of doses determined to be equieffective. At first, each ED_{50} value was determined from the dose-response curves of agents alone. Next, sildenafil and morphine were intrathecally coadministered at a dose calculated using the ED_{50} values and fractions (1/2, 1/4, and 1/8) of ED_{50} for each drug. The ED_{50} values of the mixture were calculated from the dose-response curves of the combined drugs, and the combinations were used to plot the isobologram. In this experiment, the isobolograms were used to express the effect of the sildenafil-morphine combinations. An isobologram was constructed by plotting the ED_{50} values of the single agents on the X and Y axes, respectively. The theoretical additive dose combination was then calculated. From the variance of the total dose, individual variances for the agents in the combination were obtained. Furthermore, to describe the magnitude of the interaction, a total fraction value was calculated.

$$\text{Total fraction value} = \frac{ED_{50} \text{ of drug 1 combined with drug 2}}{ED_{50} \text{ for drug 1 given alone}} + \frac{ED_{50} \text{ of drug 2 combined with drug 1}}{ED_{50} \text{ for drug 2 given alone}}$$

The fraction values indicate what portion of the single ED_{50} value was accounted for by the corresponding ED_{50} value for the combination. Values near 1 indicate an additive interaction, values greater than 1 imply an antagonistic interaction and values less than 1 indicate a synergistic interaction. The mixture was delivered intrathecally 10 min before the formalin test.

To determine whether the effect of intrathecal sildenafil was mediated through opioid receptor, opioid receptor antagonist (naloxone 0.3 μg) was intrathecally administered 10 min before the delivery of sildenafil (30 μg). The maximum dose of naloxone used was chosen based on pilot experiments and the previous study (20) and this dose was ineffective at ameliorating the control formalin response. The formalin test was done 10 min after administration of sildenafil. The experiment was conducted in phase 1 and phase 2, respectively.

In order to evaluate the behavioral changes of sildenafil and morphine, additional rats (n=10) received the highest doses of agents used, and were examined 5, 10, 20, 30, 40, 50, and 60 min after intrathecal administration. Motor functions were assessed by examining the righting and placing/stepping reflexes. The former was evaluated by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body

to an upright position. The latter was evoked by drawing the dorsum of either hind paw across the edge of the table. Normally, rats try to put their paws forward into a position for walking. Changes in motor functions were scored as: 0, normal; 1, slightly deficient; 2, moderately deficient; and 3, severely deficient.

Data were expressed as mean±SEM. In the formalin test, the time response data or the dose-response data were presented as the number of flinches or as percentage of control in each phase. To calculate the ED_{50} values for each drug, the number of flinches was converted to a percentage of control: % of control=([sum of phase 1or 2 flinching count with drug]/[sum of control phase 1or 2 flinching count])×100.

Dose-response data were analyzed by one-way analysis of variance with Bonferroni for *post hoc*. The dose-response lines were fitted using least-squares linear regression and ED_{50} and its 95% confidence intervals were calculated according to the method as described previously (21). The difference between theoretical ED_{50} and experimental ED_{50} was analyzed by t-test, with $p<0.05$ being considered statistically significant.

RESULTS

The pharmacological treatments employed with sildenafil and morphine did not produce any motor impairment in experimental rats as revealed by the righting and placing/stepping reflexes.

In control groups, the sum of the number of flinches did not differ from each other in both phases (saline:DMSO; 21±2:19±1 in phase 1, 159±10:159±13 in phase 2).

Fig. 1 displays the time course of intrathecal sildenafil and morphine, administered 10 min before formalin injection. Intrathecal sildenafil and morphine resulted in the dose-dependent inhibition of the flinching response during phase

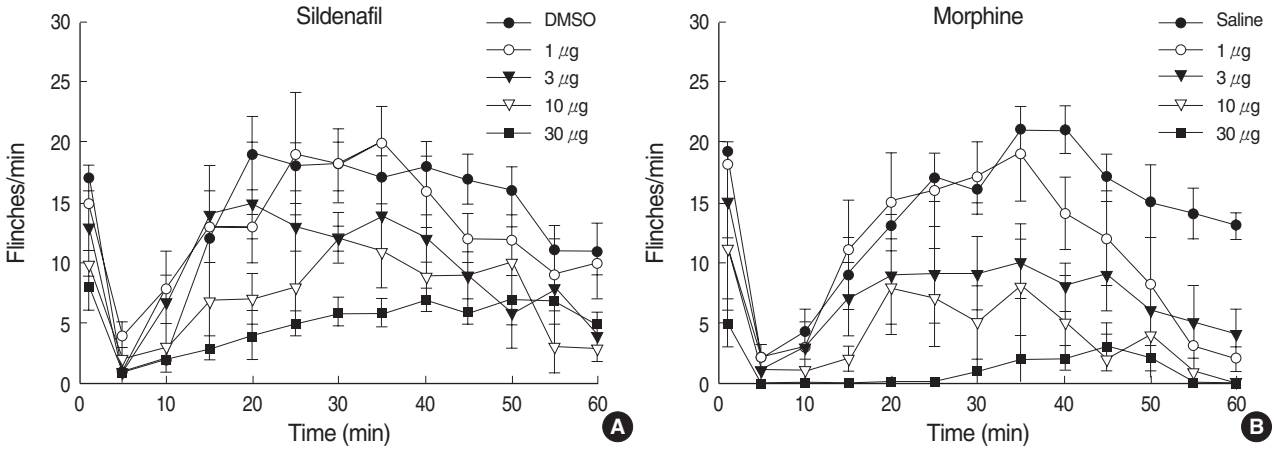

Fig. 1. Time effect curve of intrathecal sildenafil (A) and morphine (B) for flinching in the formalin test. Each drug was administered 10 min prior to the formalin injection. Formalin was injected subcutaneously at time 0. Data are presented as the number of flinches. Each line represents mean±SEM of 6-7 rats.

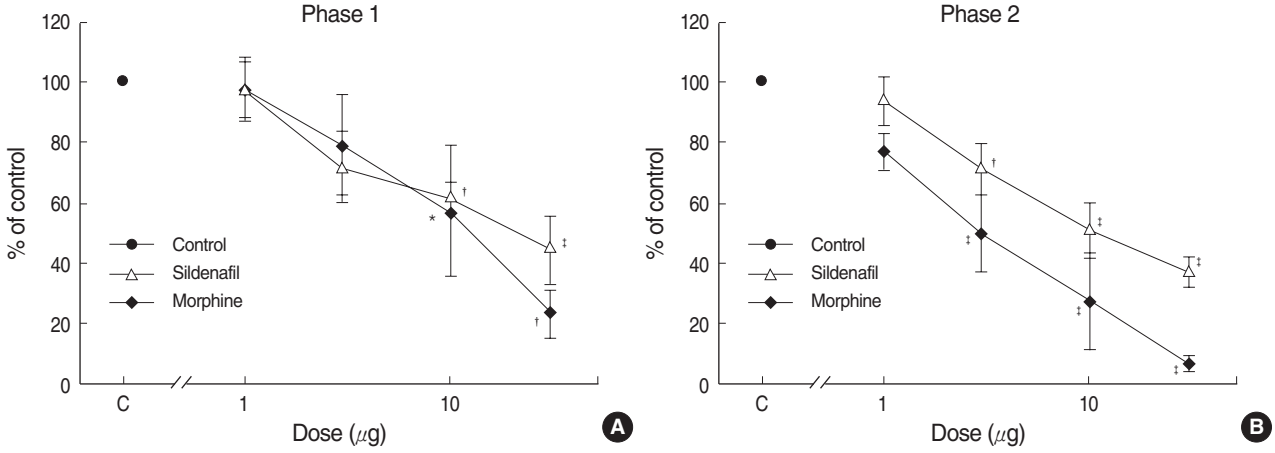

Fig. 2. Dose-response curve of intrathecal sildenafil and morphine for flinching in the formalin test. Data are presented as the percentage of control in phase 1 (A) and phase 2 (B). Sildenafil and morphine dose-dependently inhibited the flinches in both phases. Each line represents mean±SEM of 6-7 rats. C, control. *, $p<0.05$; †, $p<0.01$; ‡, $p<0.001$, compared with control.

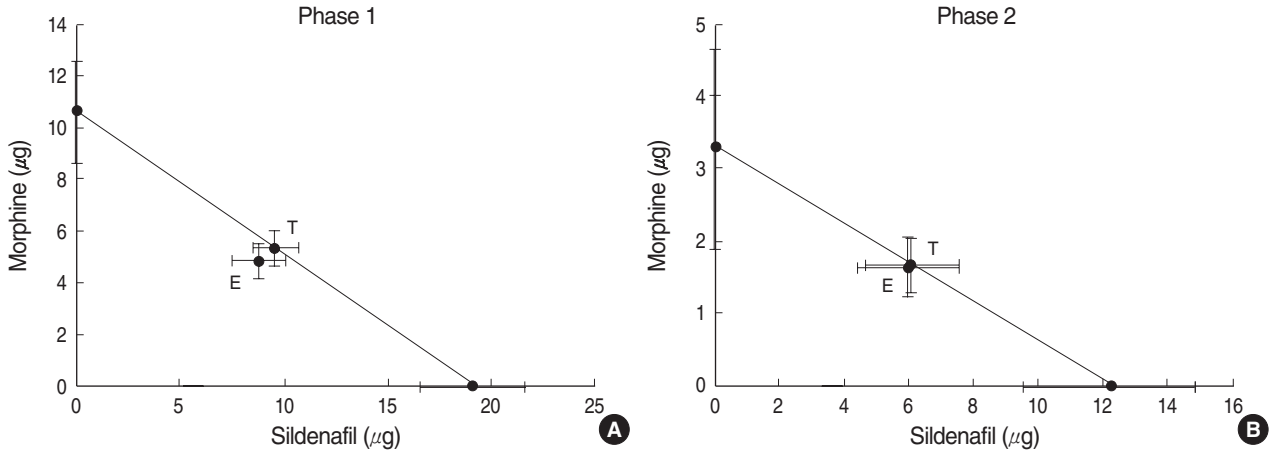

Fig. 3. Isobologram for the interaction between intrathecal sildenafil and morphine during phase 1 (A) and phase 2 (B) in the formalin test. The ED_{50} values for each agent are plotted on the x- and y- axes, respectively. Horizontal and vertical bars indicate confidence intervals. The straight line connecting each ED_{50} value is the theoretical additive line and the point on this line is the theoretical additive ED_{50} (T). The experimental ED_{50} (E) was not significantly different from T, indicating an additive interaction. Each point on the graph represents ED_{50} values from dose-response curves including 20-24 rats.

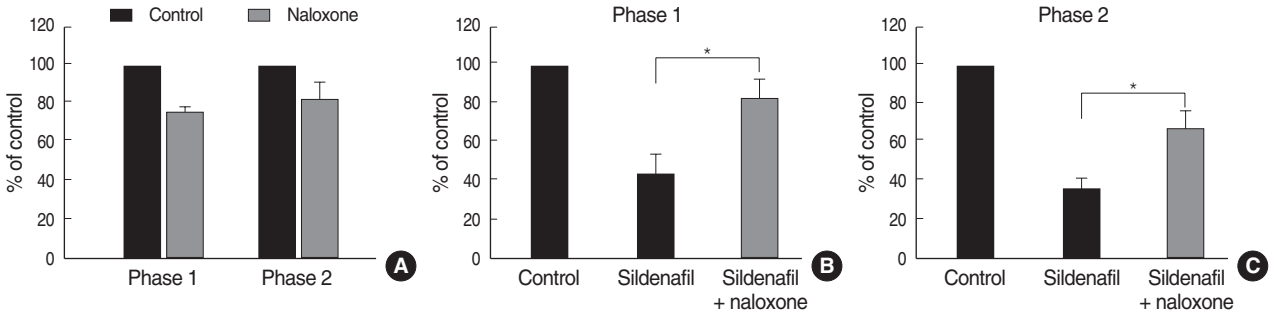

Fig. 4. The antagonistic effects of intrathecal naloxone (0.3 μg) for the antinociception of intrathecal sildenafil (30 μg) in the formalin test. Naloxone was administered 10 min prior to the injection of formalin. Naloxone alone had little effect on the control response with formalin (A). Naloxone reversed the effect of sildenafil during phase 1 (B) and phase 2 (C) of the formalin test. Data are presented as the percentage of control. Each bar represents mean ± SEM of 5 rats. *, $p<0.05$, compared with control.

1 and phase 2 in the formalin test (Fig. 2). The phase 1 ED_{50} values (95% confidence intervals) of sildenafil and morphine were 19.1 (6.3-58.2) and 10.6 μg (3.9-29.3 μg), respectively. The ED_{50} values (95% confidence intervals) of sildenafil and morphine for phase 2 were 12.3 (6.9-21.5) and 3.3 μg (1.8-6.1 μg), respectively.

Isobolographic analysis revealed an additive interaction between intrathecal sildenafil and morphine during phase 1 and 2 in the formalin test (Fig. 3). The experimental ED_{50} value was significantly lower than the theoretical ED_{50} value. Accordingly, the ED_{50} values (95% confidence intervals) of sildenafil in the mixture of sildenafil and morphine for phase 1 and phase 2 were 13.6 (4.7-38.9) and 7.6 μg (3.8-15.3 μg), respectively. Each total fraction value for the mixture of sildenafil and morphine in phase 1 and phase 2 were 0.91 and 0.98, indicating an additive interaction.

The antinociceptive effect of sildenafil was reversed by intrathecal naloxone (Fig. 4B, C).

DISCUSSION

Formalin-induced nociception consists of two different nociceptive states, acute nociception (phase 1) followed by the facilitated state (phase 2). The phase 1 response appears to result from the immediate and intense increase in the primary afferent activity. On the other hand, the phase 2 response mirrors the activation of a wide dynamic range of dorsal horn neurons with a very low level of ongoing activity in the primary afferent. Therefore, phase 2 reflects a facilitated state which appears to be a prominent and intensified pain state in spite of a reduced level of afferent input (22). This pain model may serve as a tool for observing the effects of various analgesic agents on these two pain types at once.

In the present study, intrathecal sildenafil suppressed the flinching response during phase 1 and phase 2 in the formalin test. These findings suggest that sildenafil may be active against acute pain and the facilitated state at the spinal level, which is consistent with the previous observation (9). Anoth-

er cGMP-specific phosphodiesterase inhibitor, zaprinast, was effective to attenuate the nociception induced by formalin (14). Therefore, it is conceivable that the increased cGMP level by inhibition of phosphodiesterase may contribute to the antinociception in the spinal cord.

Phosphodiesterase enzymes occur widely in biological systems and are present in mammalian tissues (23). The cyclic nucleotide phosphodiesterase is responsible for degrading the second messenger nucleotides cAMP and cGMP. To date, eleven families of PDE isoenzymes can be distinguished on the basis of their functional characteristics, such as substrate specificity, cellular distribution and susceptibility to selective inhibitors (24). It has been reported that phosphodiesterases 5, 6, and 9 are specific for cGMP (5). cGMP-specific phosphodiesterase catalyzes the hydrolysis of cGMP to GMP. In particular, cGMP may play a critical role in the modulation of nociception. This proposal was based on the observation that local injection of dibutyryl-cGMP produced antinociception in a modification of the Randall-Selitto hyperalgesia (3). Furthermore, local sildenafil, a phosphodiesterase 5 inhibitor, caused antinociception in carrageenan-induced hyperalgesia, the writhing test and the second phase of the formalin test (25-29). Additionally, intrathecal 8-bromo-cGMP reduced mechanical allodynia in neuropathic rats (4). These findings suggest that inhibition of this enzyme, in turn, may increase the level of cGMP, thereby producing antinociception.

Intrathecal morphine reduced the flinching response in both phases of the formalin test in the present study, corroborating with previous results (11-14). Therefore, opioid receptors are involved in the modulation of acute pain as well as the facilitated state.

The isobolographic analysis of the current study showed an additive interaction between sildenafil and morphine in both phases of the formalin test. These results indicate that sildenafil cannot potentiate the antinociceptive action of morphine itself in acute pain and the facilitated state evoked by formalin and, *vice versa*. If fundamentally different mechanisms jointly contribute to the observed actions of two drugs on a given endpoint, such as antihyperalgesia, a synergistic interaction is considered likely. However, if mechanisms of action of one drug may be involved in those of another drug, a synergistic interaction may not be expected. In the current study, the antinociceptive effect of intrathecal sildenafil was reversed by intrathecal naloxone. Furthermore, sildenafil and morphine may have a common pharmacologic site of action, the NO-cGMP pathway. Thus, sildenafil may not interact with morphine in a synergistic fashion. On the other hand, zaprinast, another phosphodiesterase inhibitor, interacted synergistically with morphine in the spinal cord (14). Moreover, local sildenafil increased the antinociception produced by morphine (25). Although such a difference was not fully understood in this experiment, it could be caused by the species of animal used, the routes and doses of the drugs administered. In particular, sildenafil is different from zaprinast in terms of specificity for cGMP. Sildenafil is an inhibitor of cGMP-specific phosphodiesterase 5, but zaprinast is an inhibitor for phosphodiesterase 5, 6, and 9. Thus, further inhibition for phosphodiesterase 6 and 9 might enhance the effect of morphine. Another factor that might affect the drug interactions is the stimulus intensity of nociception. It was previously reported that morphine interacts synergistically with pentobarbital at a low intensity stimulus, while interacting additively with a higher intensity stimulus (30). The extent of antinociception produced was greater with the lower stimulus intensity (31). Therefore, a synergistic relationship might be observed with an injection of a lower formalin concentration which is believed to be a milder stimulus. An interesting part of this study was the pharmacologic antagonism of the effect of sildenafil by naloxone, which suggests that opioid receptor may be affordable to the antinociceptive action of sildenafil at the spinal level. However, it has not been known what increased cGMP by sildenafil could exert on opioid receptor, further research including receptor binding study will be necessary.

Morphine is the most common opioid for the treatment of severe pain. In particular, it can be used intrathecally for pain control. However, side effects, including nausea and respiratory depression, limit its use for pain control. Thus, a combination of drugs may be recommended. The advantage of such combination may decrease the necessary dose of each drug, thereby increasing a maximum achievable effect with a decreased incidence of side effects. Spinal sildenafil has not yet been available in clinics. However, in the future it may be used alone or in combination with morphine in the treatment of pain.

In conclusion, sildenafil attenuates acute pain and the facilitated state evoked by formalin injection. And sildenafil interacts additively with morphine. Also opioid receptor may be involved in the antinociception of sildenafil at the spinal level.

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