aJava

Asian Journal of Animal and Veterinary Advances

Effects of L-NAME on Antinociceptive Induced by Morphine in Formalin Test

M. Mokhtari, M. Shariati and L. Rezaeian
Department of Biology, Faculty of Sciences, Islamic Azad University,
Kazeroun Branch, Iran

Abstract: The present study intends to assess the effects of morphine, L-NAME and also the interaction of morphine and L-NAME together on the antinociceptive activity by using the formalin test as a test of nociception. The experiments were carried out on seventy adult male rats, weighting approximately 200-210 g. The animals were divided into seven groups, considered as control, sham and experimental groups, respectively. The experimental groups were divided into five groups which the first group that received 2 mg kg^{-1} of morphine, the other four groups received morphine in a dose of 2 mg kg^{-1} accompanied by L-NAME in the dose of 15, 20 and 40 mg kg^{-1} and the last group received only 40 mg kg^{-1} of L-NAME. The drugs were intraperitoneally injected 15 min before the formalin test. Minutes 0-5 and 15-60 were designated, respectively as the acute and chronic phases of pain. Statistical analysis showed that the groups which received morphine accompanied by different doses of L-NAME and those which received only morphine and L-NAME, showed more reduction of pain in comparison with the control group, specially in the second phase of the formalin test. The finding overall showing that two drugs act synergically. On the other hand, L-NAME decreases the intracellular signal activity of both cGMP and cAMP, which inhibiting of NO synthase. Consequently, the antinociceptive effect of morphine is reinforced.

Key words: L-NAME, morphine, antinociceptive, formalin test

Introduction

N^G-nitro-L-Arginine MethylEster (L-NAME) as been shown to cause antinociception by spinal, supra spinal, local or systemic administration. As L-NAME used extensively as a paradigmatic inhibitor of Nitric Oxide (NO) synthase and has been shown to cause antinociception in several experimental model.

There is much evidence showing that neurochemical systems such as opioid systems could control the pain (Khotib *et al.*, 2004). The opioid drugs, especially morphine, have a high efficiency in the mitigation of acute and chronic pains, but because they result in bearing and addiction following long or frequent usage, their usage in mitigating chronic pains has been restricted (Susanna, 1999).

The N-Methyl-D-Aspartate (NMDA) receptors have a role to play in the transmission of the pain induced by algestic stimuli created in the spinal cord and the increase of spinal sensitivity occurring under conditions of chronic pain. Following the activation of these receptors, calcium permeates the nervous cells, activating a number of events which are dependent on it. These events include the activation of the synthase and production of NO (Garthwaite, 1991; Meller and Gebhort, 1993).

Studies showed that NO is a well-known neurotransmitter and has a significant role in the vital functions of mammals, but its role in controling pain and the modulation of antinociceptive effects of opioid has been reported differently (Vaupel *et al.*, 1995). NO has been reported to activate soluble

---

Corresponding Author: Mokhtar Mokhtari, Department of Biology, Faculty of Sciences, Islamic Azad University,
Kazeroun Branch, Iran Tel: +98-7212239933 Fax: +98-7212230508

guanylate cyclase and to increase the intracellular 3':5':cyclic guanosine monophosphate (cGMP), which may then modulate a great number of physiological functions, such as circulatory vessel dilation (Stoclet *et al*., 1999) and immune system (Jungi *et al*., 1996). Furthermore, NO has also been reported to participate in both central and peripheral nociceptive processing by acting as a pronociceptive as well as an antinociceptive agent at supraspinal and peripheral sites (Machelska *et al*., 1997). However, precise mechanisms underlying the opposing effect of NO on nociception are yet unknown. A series of morphologic (Herdegen *et al*., 1994) physiologic and pharmacologic studies suggest that NO participates in some way in the process of nociception (Gartwaite and Boulton, 1995; Moncada *et al*., 1989).

Other studies showed the role of NO in causing sensitivity to pain and its interaction with morphine. Thus, nowadays, to recognize some physiological functions of nitric oxide, inhibitors of the biosynthesis of NO such as L-NAME are used (Hibbs *et al*., 1998). Studies show that the inflammations supposed to appear in response to formalin, are created following the production of nitric oxide and cause changes in the opioid receptors. This process can also be seen following chronic inflammations (Chizuko *et al*., 2003). Hence, the purpose of this research to assess the effect of different doses of L-NAME accompanied by morphine and the effect of morphine and L-NAME separately on the pains caused during the formalin test.

Prior studies showed that L-NAME antinociceptive effect of morphine increase in Tail-flick test, however this phenomen has not been obsereved in Hotplate test.

Therefore,the present study was conducted to evaluate different doses of L-NAME accompanied by morphine and L-NAME and morphine alone on antinociceptive effects in formalin test.

Materials and Methods

The experiments were performed on 70 male wistar rat (200-210 g).The animal were housed under natural light, with free access to food and water. All the experiments have been done in specified hours of day 9 and 12 to minimize the effects of environmental changes. To study the effect of morphine accompanied by L-NAME on the pain threshold induced by formalin, the animals were divided into seven groups, each group including ten members. The first group received nothing, the sham group an amount of the normal saline to that of morphine was intrapritoneally injected. The third received only morphine intrapritoneally of 2 mg kg^{-1}. For the fourth, fifth and sixth groups, L-NAME was injected at doses of 15, 20 and 40 mg kg^{-1}, followed by the intrapritoneally injection of morphine 15 min later, at the rate of 2 mg kg^{-1} and seventh group received only 40 mg kg^{-1} of L-NAME. The injection of L-NAME, morphine and the normal saline was carried out ten minutes before the injection of 2.5% formalin. Injections and behavioral responses of animals were carried out in a double blind way. In order to accustom the animals to the experimental conditions and eliminate their stress, they were put in a glass container. Ten minutes after the injection of the drugs or the normal saline, 50 µL of dilute solution of the 2.5% formalin was injected into the sole of the animal's right foot. The animals were immediately put into the experimental container which was made of pollexiglass, with dimensions of 30×30×30. The behavioral responses of pain was observed using a mirror which was set up beneath the container at an angle of 4 and was recorded every 15 sec using numbers 0, 1, 2 and 3 according to Dennis Dubiuison's method (Tjolsen *et al*., 1991). These quantitative data were enumerated in the form of twelve 5 min blocks and recorded according to the pain score formula. The recording of the data was continued till 60 min after the injection. The average pain score in each block was calculated using the followin formula (Tjolsen *et al*., 1991; Dubisson, 1977).

$$\text{Pain score} = \frac{0T_0 + 1T_1 + 2T_2 + 3T_3}{300 \text{ sec}}$$

In the average pain score of formula, T_0, T_1, T_2 and T_3 represent the number of the 15 sec intervals during which the animal shows each of the behaviors 0, 1, 2 and 3, respectively, within a five-minute period of time. In all groups, minutes between 0 and 5 were designated as the acute phase and minutes 16-60 were designated as the chronic phase of the experiment. The results are presented as Mean±Standard Errors of Mean $(\overline{X}\pm SEM)$.

The statistical analysis of the data obtained from the control, sham and experimental group was carried out using the Kruskal-Wallis test. The Tukey test was used to assess significant difference between the groups. Results with $p \leq 0.05$ were considered significant.

Results

The results obtained from this study indicated that L-NAME (40 mg kg^{-1}) and the different doses of L-NAME (15,20,40 mg kg^{-1}) accompanied with morphine (2 mg kg^{-1}) cause the reduction of pain score in acute and chronic phases in compared to control group significantly (Table 1 and Fig. 1).

Table 1: Pain score average of different animal groups obtained from formalin test

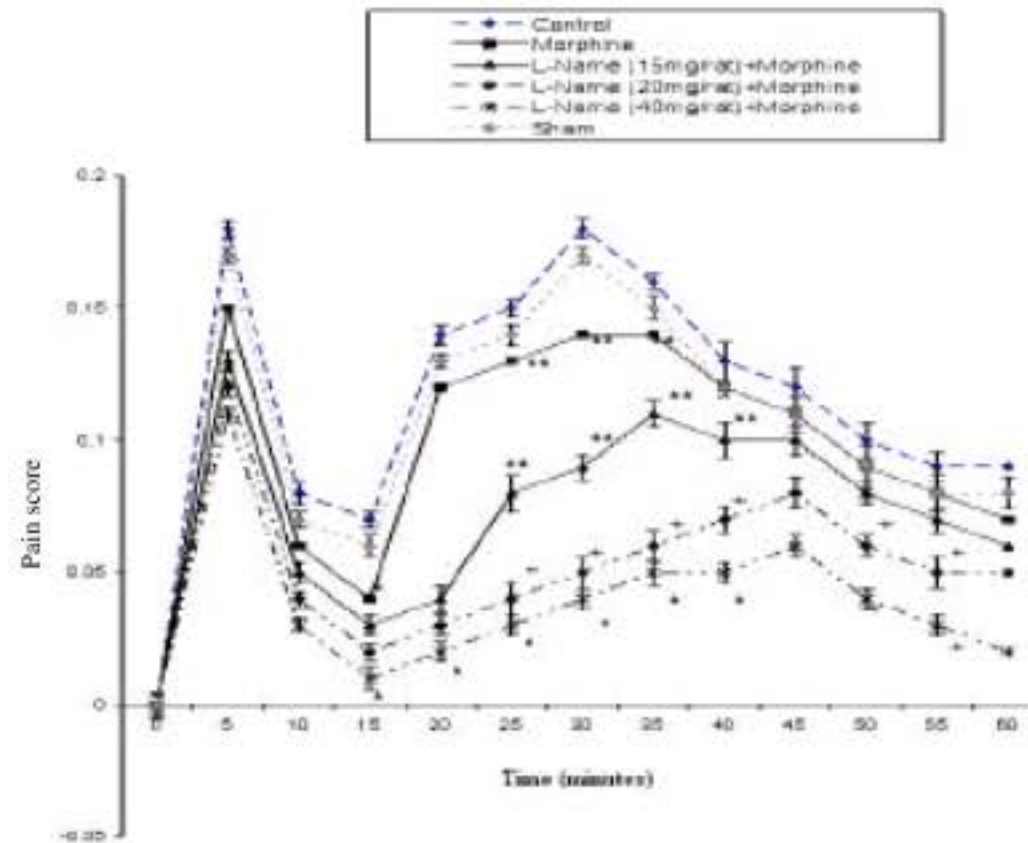| Groups	Pain score $(\overline{X}\pm SEM)$	
	Acute phase	Chronic phase
Control	0.18±0.003	0.12±0.002
Sham	0.17±0.003	0.11±0.002
Morphine (2 mg kg^{-1})	0.15±0.002*	0.10±0.002*
Morphine (2 mg kg^{-1})+L-NAME (15 mg kg^{-1})	0.13±0.003*	0.07±0.002*
Morphine (2 mg kg^{-1})+L-NAME (20 mg kg^{-1})	0.12±0.004*	0.05±0.001*
Morphine (2 mg kg^{-1})+L-NAME (40 mg kg^{-1})	0.11±0.005*	0.03±0.002*

* Values are significant ($p \leq 0.05$)

Fig. 1: Comparison of the average pain score in the acute and chronic phase for the groups receiving different doses of L-NAME accompanied by morphine, the group receiving only morphine, the control and sham groups. This figure has been drawn according to the negative and positive average of the standard deviation. The sign * indicates significant difference with the control group ($p \leq 0.05$). The symbol + indicates significant difference with the control group and two of the other experimental groups ($p \leq 0.05$). The symbol ** indicates significant difference with the control group and three of the other experimental groups ($p \leq 0.05$)

Table 2: Pain score average of different animal groups obtained from formalin test

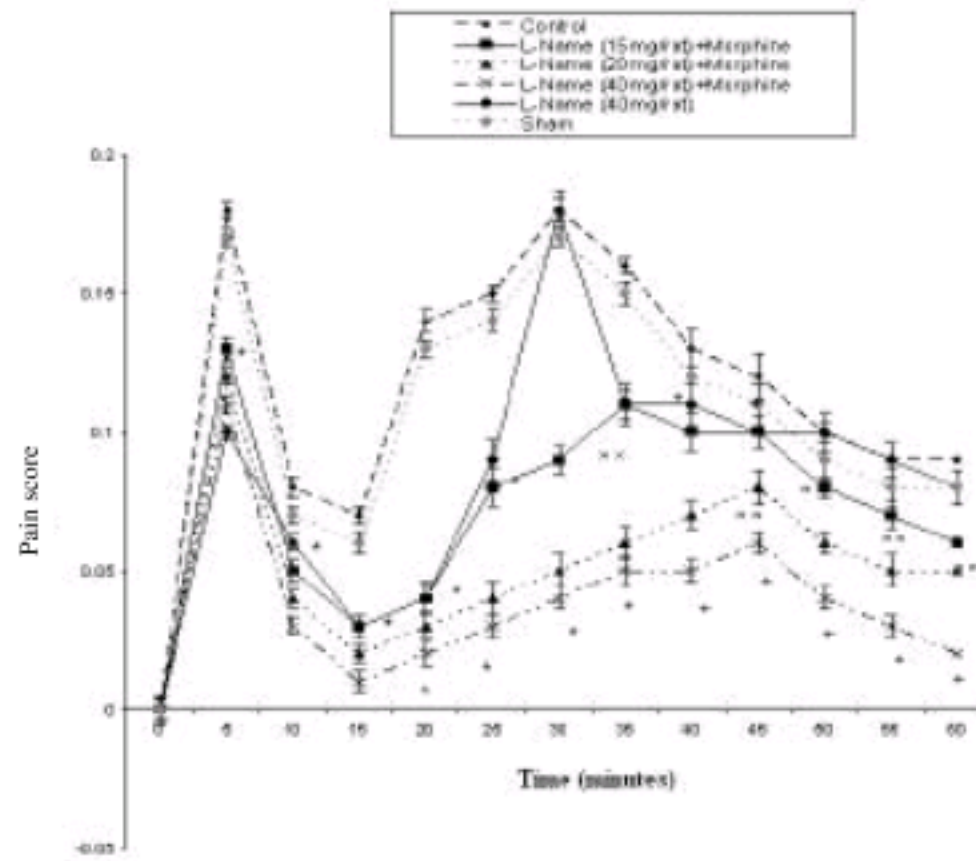| Groups	Pain score ($\bar{X} \pm$ SEM)	
	Acute phase	Chronic phase
Control	0.18±0.003	0.12±0.002
Sham	0.17±0.003	0.11±0.002
L-NAME (40 mg kg^{-1})	0.14±0.004*	0.09±0.007*
Morphine (2 mg kg^{-1})+L-NAME (15 mg kg^{-1})	0.13±0.003*	0.07±0.002*
Morphine (2 mg kg^{-1})+L-NAME (20 mg kg^{-1})	0.12±0.004*	0.05±0.001*
Morphine (2 mg kg^{-1})+L-NAME (40 mg kg^{-1})	0.11±0.005*	0.03±0.002*

Fig. 2: Comparison of the average pain score in the acute and chronic phase for the groups receiving different doses of L-NAME accompanied by morphine, the groups receiving only L-NAME, the control and sham groups. This figure has been drawn according to the negative and positive average of the standard deviation. The sign * indicates significant difference with the control group (p≤0.05). The symbol + indicates significant difference with the control group and two of the other experimental groups (p≤0.05). The symbol ** indicates significant difference with the control group and three of the other experimental groups (p≤0.05)

Besides, our results showed that the antinociceptive effect of L-NAME accompanied with morphine compared to L-NAME alone was high.

The statistical analysis of data illustrated that morphine (2 mg kg^{-1}) and morphine (2 mg kg^{-1}) with different doses of L-NAME (15, 20, 40 mg kg^{-1}) in compared to control group cause the reduction of pain score in acute and chronic phases significantly (Table 2 and Fig 2). Therefore, based on our finding it could be concluded that the antinociceptive effect of L-NAME with morphine compared to morphine alone was high.

Discussion

Nowaday, a great deal of efforts has been carried out to recognize the mechanisms of pain and the different approach to manage them. Currently, control of pain is mostly accomplished through anti-inflammatory non-steroidal and opioid antinociceptive drugs (Terayama *et al*., 2000). For many years,

morphine has been used as a antinociceptive drug. In fact, it is obvious that administration of morphine resulted in dependence and addiction. In this study, to assess the induced antinociceptive effects the formalin test was used, which is an authentic method for the measurement of chronic pain and specifies the degree of the induced sensitivity on the surface of the central spine following a topical inflammation. It should also be noted that some neurotransmitters such as substance P, bradykinin, glutamate and serotonin have a role in the induction of the pain resulting from the formalin test (Willis, 2001).

The results obtained from this study indicated that the injection of L-NAME accompanied by morphine results in a higher degree of decrease in pain score, compared with the injection of each of them separately. According to the results obtained in this study, during administration these drugs separately, the antinociception in the formalin test decreased. However, the degree of this decrease is lower compared to combination of these two drugs is injected. Secondly, L-NAME as an inhibitor of the synthesis of NO, decreases the pain of the chronic phase of the formalin test more than the acute one. In other words, L-NAME inhibits the responses caused by neurons and relayed by the (NMDA) receptors. If morphine is injected by itself, the pain receptors which are stimulated following the injection of formalin will inhibit the action of C fiber and consequently the responses of the initial phase will have more decrease than of the secondary one (Alvarez *et al.*, 2000).

With regard to our findings of this study, the simultaneous administration of these two drugs might cause an intensive inhibiting effect on the pain induced during both the acute and chronic phase of the formalin test. The pain induced in both phases; shows a higher degree of decrease in comparison with administration each drug alone. Other studies indicate that the action of the NMDA in hypothalamus increases the amount of the intracellular calcium, through binding to the calmodulin existing on the substrate of the enzyme, leads to increase the action of the enzyme of NO synthase and the production of NO from L-arginin. The intracellular cGMP is decreased following the activation of guanylate cyclase. Various behavioral studies indicate that the signal action of NOcGMP on the neurotransmitters of pain is the agent of the nociception effect of NO. Therefore, L-NAME may reinforce the antinociceptive effects induced through morphine by inhibiting the spinal system of NOcGMP (Andrew, 2004).

The inhibition of the synthase of NO may reduce the synaptic effect caused by the pain admission receptor of NMDA and in turn, increase the antinociceptive action of the opioids. In addition, the opioids could increase the antinociceptive effects due to the reduction of prostaglandin E_2 (PGE 2) caused by the action of NMDA. According to the studies carried out so far, the decrease of antinociceptive effect induced by the opioids, by inhibiting the NO synthase may be the result of the reduction of intracellular signal activity in both the cGMP and adenosine3': 5'-monophosphate (cAMP) routes. Opioids remove the depolarizer currents and neutralize the potential of the core by decreasing the intracellular cAMP. On the other hand; the decrease of the intracellular cAMP reduces the activity of the Protein Kinase A (PKA) which is dependent on cAMP, reduction of the discharge of neurotransmitters dependent on the action of the PKA. Thus, reinforces the L-NAME might increase antinociceptive effects induced by the opioids through stimulating antinociceptive actions caused by the opioid receptors (Brundege *et al.*, 1996).

In conclusion, the present study confirms that L-NAME and morphine causes analgesia and administration of L-NAME accompanied by morphine act synergically reduced the nervous activity induced by the formalin test to high degree and finally puts the animal in an analgesic conditions.

References

Alvarez, M., J. William and E. Bockstacle, 2000. Functional coupling between neurons and glia. J. Neuro. Sci., 20: 4091-4098.

Andrew, M., 2004. Modulation of dural nociceptor mechanosensitivity by the nitric oxide cyclic GMP signaling cascade. J. Neurophysiol., 30: 722-766.

Brundege, J., L. Dlao, W. Proctor and T. Dunwiddie, 1996. The role of cyclic AMP as a precursor of extracellular adenosine in the rat hippocampus. Neuropharmacolosy, 36: 1201-1210.

Chizuko, W., S. Tsukasa, O. Kazuhiro and S. Chikai, 2003. The role of spinal nitric oxide and glutamate in nociceptive behavior evoked by high-dose intrathecol morphine in rats. Pain, 106: 269-283.

Dubisson, D., 1977. The formalin test a quantitative study of analgesic effects of morphine, meperidine and brain stem stimulation in rats and cats. Pain, 4: 161-176.

Garthwaite, J., 1991. Glutamate, nitric oxide and cell-cell signaling in the nervous system. Trends. Neurosci., 14: 60-67.

Garthwaite, J. and C. Boulton, 1995. Nitric oxide signaling in the central nervous system. Ann. Rev. Physiol., 57: 683-706.

Herdegen, T., S. Rudiger, B. Mayer, R. Bravo and M. Zimmermann, 1994. Expression of nitric oxide synthase and colocalisation with Jun, Fos and krox transcription factors in spinal cord neurons following noxious stimulation of the rat hindpa. Mol. Brain Res., 22: 245-258.

Hibbs, J., R. Tainjor, Z. Varin and E. Rachlin, 1998. Nitric oxide synthase. In methods in nitric oxide: A cytotoxic activated macrophage effector molecule. Biophys. Res. Commun., 157: 87-94.

Jungi, T., H. Adler, M. Thöny, M. Krampe and E. Peterhans, 1996. Inducible nitric oxide synthase of macrophages: Present knowledge and evidence for species-specific regulation. Vet. Immunol. Immunopathol., 54: 323-330.

Khotib, J., M. Narita, M. Suzuki, Y. Yajima and T. Suzuki, 2004. Functional interaction among opioid receptor types: Up-regulate of mu-and delta-opioid receptor functions after repeated stimulation of kappa-opioid receptors. Neuro Pharmacol., 46: 531-540.

Machelska, H., D. Labuz, R. Przewlocki and B. Przewlocka, 1997. Inhibition of nitric nxide synthase enhances antinociception mediated by mu, delta and kappa Opioid receptors in acute and prolonged pain in the rat spinal cord. J. Pharmacol. Exp. Therap., 282: 977-984.

Meller, S.T. and G.F. Gebhort, 1993. Nitric Oxide (NO) and nociceptive processing in the spinal cord. Pain, 53: 127-136.

Moncada, S., R.M.J. Palmer and E.A. Higgs, 1989. Biosynthesis of nitric oxide from L-arginine: A pathway for the regulation of cell function and communication. Biochem. Pharmacol., 38: 1709- 1715.

Susanna, F., 1999. Transmitters involved in antinociception in the spinal cord. Brain. Research. Bulletin, 48: 129-141.

Stoclet, J., B. Muller, K. Gyorgy, R. Andriantsiothaina and A. Kleschyov, 1999. The inducible nitric oxide synthase In vascular and cardiac tissue. Eur. J. Pharmacol., 375: 139-155.

Tjolsen, A., O. Berge, S. Hunskaar, J. Rosland and K. Hole, 1991. The formalin test: An evaluation of the method. Pain, 51: 5-17.

Terayama, R., Y. Guan, R. Dubner and K. Ren, 2000. Activity-induced plasticity in brain stem pain modulatory circuitry after inflammation. Neuroreport, 11: 1915-1919.

Vaupel, O., A. Kimes and E. London, 1995. Nitric oxide synthase inhibitors, preclinical studies of potential use for treatment of opioid widraual. Neuropsychopharmacology, 13: 315-322.

Willis, W., 2001. Role of neurotransmitters in sensitization of pain responses. Ann. N.Y. Acad. Sci., 933: 142-156.