

Effects of Neuronal NOS Selective Inhibitor, 7-nitroindazole on Inhibitory Effect of Calcium Channel Blockers on Development of Tolerance to Morphine Induced Analgesia

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ABSTRACT

The aim of this study was to investigate whether selective inhibition of neuronal nitric oxide synthase (nNOS) attenuated tolerance to morphine analgesia and whether does the dihydropyridines, the L-type Calcium Channel Blockers (CCB), have any effect on the inhibition of tolerance to morphine, when combined with 7-nitroindazole, a selective nNOS inhibitor. **Method:** It has been hypothesized that in central nervous system, the augmented level of Excitatory Amino Acids (EAA), during chronic morphine treatment might activate N-methyl-D-aspartate (NMDA) receptor resulting in increased intracellular calcium. This further activates nNOS due to increased calcium-calmodulin complex formation. **Results:** Thus, the nNOS level increased due to chronic morphine may increase the level of cyclic guanosine monophosphate (cGMP) leading to phosphorylation of some key proteins and finally inducing morphine tolerance. This study has demonstrated that the 7-nitroindazole and dihydropyridines can independently and dose-dependently was able to inhibit the development of morphine antinociceptive tolerance. Our results also suggested that dihydropyridine type calcium channel blockers such as nimodipine and lercanidipine, when administered prior to 7-nitroindazole, were able to produce an additive effect on the inhibition of tolerance to morphine-induced analgesia as compared to either of the drugs alone. **Conclusion:** These results suggested that increased intracellular calcium-induced activation of nNOS might be involved in the development of tolerance to morphine analgesia. Further we demonstrated for the first time that the dihydropyridines along with selective nNOS inhibitor produced an additive effect on attenuation of morphine antinociceptive tolerance.

Key words: Morphine, tolerance, neuronal nNOS inhibitor, 7-nitroindazole, dihydropyridines, nimodipine, lercanidipine

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INTRODUCTION

Morphine is an effective analgesic for the clinical treatment of severe pain. However its chronic use leads to the development of tolerance and dependence and hence its use is limited. The development of tolerance may be due to alteration in signaling cascade or change in endogenous opioid system (Chevlen, 2003) or change in the specific properties of the specific receptor or effector system (Ueda *et al.*, 2003). N-methyl-D-aspartate (NMDA) receptor activation, as well as Nitric Oxide Synthase (NOS) activity, has been widely shown to be dependent of opioid induced tolerance (Wong *et al.*, 2000). It has been reported that chronic use of morphine is associated with the release of Excitatory Amino Acids (EAA) such as glutamate, aspartate etc. from the central neurons. These EAA are responsible for the activation of

the central NMDA subtype of glutamate receptors, which results in the elevations in the levels of central NO, which has been implicated in the development of morphine tolerance and dependence (Kolesnikov *et al.*, 1998; Heinzen and Pollack, 2004). The NMDA receptor, an ionotropic glutamate receptor, is widely distributed in the mammalian central nervous system (Zhu *et al.*, 1998). Glutamate activation of these receptors stimulates Ca^{++} influx into cells, which binds to intracellular protein, calmodulin and thereby activates NOS resulting in increased NO formation (Mao *et al.*, 1995). There are three main isoforms of NOS: Neuronal NOS (nNOS), Endothelial NOS (eNOS) and Inducible NOS (iNOS). nNOS and eNOS are calcium-dependent whereas iNOS is calcium-independent (Alderton *et al.*, 2001). The neuronal NOS (nNOS), a Ca^{++} -dependent low-output NOS isoform, has a pivotal role in development of morphine tolerance and dependence (Heinzen and Pollack, 2004). The NO level enhanced by chronic morphine may increase the levels of cyclic guanosine

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monophosphate (cGMP) that causes phosphorylation of some key proteins and alters the physiological responses, finally inducing morphine tolerance (Noda and Nabeshima, 2004; Pagliaro, 2003). Earlier studies have reported the role of Calcium Channel Blockers (CCB) and nNOS inhibitors individually and independently in morphine-induced analgesic tolerance, but since calcium is also involved in the activation of nNOS, it was thought that the combination of both the drugs would have either additive or synergistic effect in the inhibition of tolerance to morphine. So, the main aim of the present study was to investigate the effect of neuronal NOS inhibitor and CCB combination therapy on the development of tolerance to morphine-induced analgesia. In the present study, low doses of nimodipine, a cerebroselective CCB that can cross blood brain barrier and another new highly lipophilic dihydropyridine, lercanidipine, were used to study their interaction with selective nNOS inhibitor, 7-nitroindazole (7-NI) on tolerance to morphine-induced analgesia.

MATERIALS AND METHODS

Animals: The study was conducted on Swiss male mice of 20-25 g weight range; housed six animals per cage at Central Animal Facility (CAF), in a room with controlled temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$) and light (07.00-18.00 h). All the studies were performed in between 09.00-17.00 h to minimize diurnal variation. Food and water were made available *ad libitum*. In all the experiments, animals were used only once except for those used for tolerance study. The experimental protocol was duly approved by Institute animal ethics committee.

Drugs and chemicals: Morphine was obtained from Govt. opium alkaloid laboratories, Ghazipur, India. 7-NI was purchased from Sigma chemicals co., St. Louis, USA. Nimodipine was obtained as a gift sample from US Vitamins, Mumbai, India. Lercanidipine was obtained from Recordati Industria chimica, Milano, Italy.

Morphine was dissolved in 0.9% saline whereas, nimodipine as well as lercanidipine were solubilized in 20% DMSO (dimethyl sulfoxide) + 20% ethanol + 60% distilled water and 7-NI was dissolved in 100 mg mL⁻¹ DMSO and diluted with arachis oil prior to injection. Each drug was injected intraperitoneally (i.p.). CCB were administered twice-daily 30 min prior to morphine whereas 7-NI was administered once daily 15 min prior to morning dose of morphine.

Assessment of tolerance to tail flick analgesia: The modified tail flick technique of D'Amour and Smith (1941) was employed to measure analgesic response

(Hicon-medicraft tail flick analgesiometer, India). The source of radiant heat in the instrument was a nichrome wire-heating element. The intensity of the thermal stimulus was adjusted on a heat intensity of 4 on a scale 10 to give basal reaction latency between 4-6 sec. The cut-off time was fixed at 15 sec (three times of the basal) in order to avoid any damage to the tail of mice. Two basal (pre-drug) readings were taken at an interval of 30 min.

For acute experiments, either vehicle or morphine or respective drugs prior to morphine (10 mg kg⁻¹) were injected intraperitoneally and the reaction latency time was determined at regular interval for a period of 180 to 240 min (0, 30, 60, 90, 120, 180 and 240 min).

For chronic experiments, the animals were treated as per the treatment schedule for ten days and on 11th day, after 16 h of last injection of morphine, two basal readings were taken at an interval of 30 min and all the groups were challenged with 10 mg kg⁻¹ morphine and the reaction latency time was determined at regular interval for a period of 180 to 240 min (0, 30, 60, 90, 120, 180 and 240 min). The volumes of injection for the drugs or vehicle were 10 mL kg⁻¹ body weight of the mouse. The tail flick latencies were converted to percentage maximal possible analgesic effect (%MAPE) according to the following formula:

$$\text{MAPE (\%)} = \frac{\text{Reaction time of test} - \text{Basal reaction time}}{\text{Cut-off time} - \text{Basal reaction time}} \times 100$$

From MAPE% vs time plot, the area under curve (AUC_{0-180 or 240 min}) was calculated using trapezoidal method. The analgesia (AUC_{0-180 or 240 min}) was expressed as the Mean \pm SEM.

Acute effect of CCBs on morphine-induced analgesia: The effect of acute administration of nimodipine (0.1, 0.3 and 1.0 mg kg⁻¹) and lercanidipine (0.03, 0.09 and 0.3 mg kg⁻¹) on morphine (10 mg kg⁻¹)-induced analgesia was determined as described above in mice.

Chronic effect of 7-NI on tolerance to morphine-induced analgesia: Mice were rendered tolerant to the analgesic effect of morphine by twice daily administration of morphine (10 mg kg⁻¹) for ten days. 7-NI was administered once daily (OD) 15 min prior to morning dose of morphine (10 mg kg⁻¹). The control animals were administered similarly with vehicle following the same schedule. The development of tolerance to analgesia was measured after 16 h of the last injection of morphine to a challenge dose of morphine (10 mg kg⁻¹) on 11th day.

Chronic effect of CCBs on tolerance to morphine-induced analgesia: The effect of twice daily administration of nimodipine (0.1, 0.3 and 1.0 mg kg⁻¹) and lercanidipine (0.03, 0.09 and 0.3 mg kg⁻¹) 30 min prior to morphine for ten days on tolerance to analgesic effect of morphine (10 mg kg⁻¹) was determined in both morphine naive and morphine tolerant mice by tail-flick analgesic response.

Chronic effect of 7-NI on inhibitory effect of CCBs on tolerance to morphine-induced analgesia: The effect of chronic administration of 7-NI (15 mg kg⁻¹) once daily, on inhibitory effect of either nimodipine (0.1 and 0.3 mg kg⁻¹) or lercanidipine (0.03 and 0.09 mg kg⁻¹) twice daily for ten days on tolerance to analgesic effect was determined in both morphine naive and morphine tolerant mice by tail flick analgesic response.

Statistical analysis: All the results were expressed as Mean±SEM. For multiple comparisons between groups, the results were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ has been considered as significant. All the data were analyzed by using statistical software sigma stat (version 2.03, USA) and sigma plot (version 8.0, USA).

RESULTS

Acute effect of CCBs on morphine-induced analgesia: The acute effect of pre-treatment of nimodipine (0.1-1.0 mg kg⁻¹) and lercanidipine (0.03-0.3 mg kg⁻¹) on the AUC_(0-240min) of morphine (10 mg kg⁻¹) induced analgesia has been shown in Table 1. Nimodipine (1 mg kg⁻¹) significantly affected acute morphine-induced analgesia. Similarly, lercanidipine *per se* didn't produce any analgesia but significantly affected acute morphine-induced analgesia in the doses of 0.09 and 0.3 mg kg⁻¹.

Chronic effect of CCBs on tolerance to morphine-induced analgesia: Nimodipine inhibited the development of tolerance to morphine-induced analgesia in a dose dependent manner. Nimodipine in the dose

of 1.0 mg kg⁻¹ has almost completely (99%) inhibited the development of tolerance to morphine-induced analgesia. The observed AUC_(0-180min) were 2621.06±231.32 (0.1 mg kg⁻¹), 4864.27±669.57 (0.3 mg kg⁻¹), 8539.76±885.17 (1.0 mg kg⁻¹) respectively as compared to 1524.80±173.30 for vehicle pre-treated morphine tolerant group (Fig. 1).

The inhibition of tolerance to morphine by nimodipine was further confirmed by a more lipophilic dihydropyridine, lercanidipine. The dose-dependent (0.03-0.3 mg kg⁻¹) effect of lercanidipine on the AUC_(0-180 min) is shown in Fig. 2. Lercanidipine (0.09 and 0.3 mg kg⁻¹) significantly inhibited the development of tolerance to morphine-induced analgesia. The AUC_(0-180min) values of morphine-induced analgesia at these doses were 2572.52±453.45 (0.03 mg kg⁻¹), 5162.49±571.83 (0.09 mg kg⁻¹), 8723.02±479.0 (0.3 mg kg⁻¹) respectively as compared to 1405.97±204.62 for vehicle pre-treated morphine tolerant group.

Chronic effect of different doses of 7-NI on tolerance to morphine-induced analgesia: 7-NI (5, 15 and 50 mg kg⁻¹) dose-dependently inhibited the

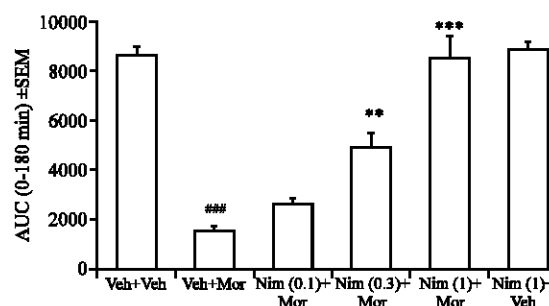


Fig. 1: Chronic effect of nimodipine (Nim) on tolerance to morphine (Mor)-induced analgesia (10 mg kg⁻¹) represented as AUC_(0-180 min). Values are expressed as Mean±SEM, (n = 6). ### $p < 0.001$ vs morphine-naive group; ** $p < 0.001$ and *** $p < 0.01$ vs morphine-tolerant group

Table 1: Acute effect of nimodipine (0.1-1.0 mg kg⁻¹) and lercanidipine (0.03-0.3 mg kg⁻¹) on morphine-induced analgesia represented as AUC_(0-240min)

Groups	AUC _(0-240min) ± SEM
Vehicle + Vehicle	794.02±216.23
Vehicle + Morphine (10 mg kg ⁻¹)	8062.69±256.40 ***
Nimodipine (0.1 mg kg ⁻¹) + Morphine (10 mg kg ⁻¹)	8104.96±637.64
Nimodipine (0.3 mg kg ⁻¹) + Morphine (10 mg kg ⁻¹)	8879.93±336.05
Nimodipine (1.0 mg kg ⁻¹) + Morphine (10 mg kg ⁻¹)	10624.27±544.77 **
Nimodipine (1.0 mg kg ⁻¹) + Vehicle	1121.27±256.00
Lercanidipine (0.03 mg kg ⁻¹) + Morphine (10 mg kg ⁻¹)	8723.50±163.83
Lercanidipine (0.09 mg kg ⁻¹) + Morphine (10 mg kg ⁻¹)	9560.16±406.55 **
Lercanidipine (0.3 mg kg ⁻¹) + Morphine (10 mg kg ⁻¹)	10630.75±192.16 ***
Lercanidipine (0.3 mg kg ⁻¹) + Vehicle	1332.50±267.21

Values are expressed as Mean±SEM, (n = 5), *** $p < 0.001$ vs morphine-naive groups; *** $p < 0.001$ and ** $p < 0.01$ vs morphine-treated group

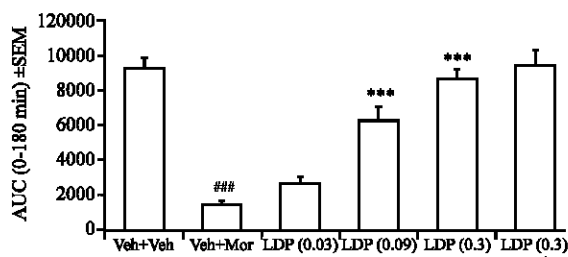


Fig. 2: Chronic effect of lercanidipine (LDP) on tolerance to morphine (Mor)-induced analgesia (10 mg kg^{-1}) represented as $\text{AUC}_{(0-180 \text{ min})}$. Values are expressed as $\text{Mean} \pm \text{SEM}$, ($n = 5-6$), ### $p < 0.001$ vs morphine-naive group; *** $p < 0.001$ vs morphine-tolerant group

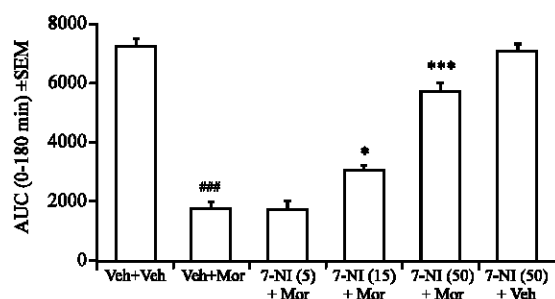


Fig. 3: Chronic effect of 7-nitroindazole (7-NI) on tolerance to morphine (Mor)-induced analgesia (10 mg kg^{-1}) represented as $\text{AUC}_{(0-180 \text{ min})}$. Values are expressed as $\text{Mean} \pm \text{SEM}$, ($n = 5-6$), ### $p < 0.001$ vs morphine-naive group; *** $p < 0.001$ and * $p < 0.05$ vs morphine-tolerant group

development of tolerance to morphine-induced analgesia as evident from the $\text{AUC}_{(0-180 \text{ min})}$ shown in Fig. 3. The $\text{AUC}_{(0-180 \text{ min})}$ values of morphine-induced analgesia at these doses were 1771.87 ± 271.53 (5 mg kg^{-1}), 3074.93 ± 150.76 (15 mg kg^{-1}), 5738.81 ± 289.17 (50 mg kg^{-1}) respectively as compared to 1792.40 ± 204.29 for vehicle pre-treated morphine tolerant group.

Based on the dose-response curve for the respective drugs, the following doses were selected for combination therapy:

Nimodipine (0.1 and 0.3 mg kg^{-1}) + 7-NI (15 mg kg^{-1})

Lercanidipine (0.03 and 0.09 mg kg^{-1}) + 7-NI (15 mg kg^{-1})

Chronic effect of 7-NI on inhibitory effect of CCBs on tolerance to morphine-induced analgesia: The chronic administration of morphine for 10 days developed significant tolerance in mice. 7-NI

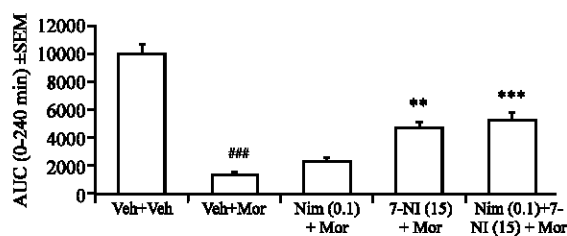


Fig. 4: Chronic effect of nimodipine (Nim, 0.1 mg kg^{-1}), 7-nitroindazole (7-NI, 15 mg kg^{-1}) and their combination on tolerance to morphine (Mor)-induced analgesia (10 mg kg^{-1}) represented as $\text{AUC}_{(0-240 \text{ min})}$. Values are expressed as $\text{Mean} \pm \text{SEM}$, ($n = 5-6$), ### $p < 0.001$ vs morphine-naive group; *** $p < 0.001$ and ** $p < 0.01$ vs morphine-tolerant group

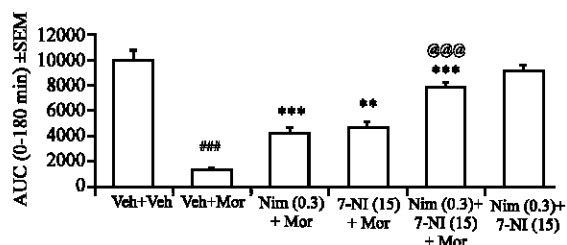


Fig. 5: Chronic effect of nimodipine (Nim, 0.3 mg kg^{-1}), 7-nitroindazole (7-NI, 15 mg kg^{-1}) and their combination on tolerance to morphine (Mor)-induced analgesia (10 mg kg^{-1}) represented as $\text{AUC}_{(0-240 \text{ min})}$. Values are expressed as $\text{Mean} \pm \text{SEM}$, ($n = 5-6$), ### $p < 0.001$ vs morphine-naive group; *** $p < 0.001$ and ** $p < 0.01$ vs morphine-tolerant group; @@@ $p < 0.001$ vs 7-NI and nimodipine pre-treated morphine-tolerant group

(15 mg kg^{-1}) as well as nimodipine (0.1 mg kg^{-1}) significantly inhibited the development of tolerance to morphine-induced analgesia but when both the drugs were combined, the effect of this combination was not found to be additive as represented by the $\text{AUC}_{(0-240 \text{ min})}$ (Fig. 4).

Further, 7-NI (15 mg kg^{-1}) in combination with nimodipine (0.3 mg kg^{-1}) significantly inhibited the development of tolerance to morphine-induced analgesia. However, it has been observed that when both the drugs were combined, the effect of this combination was found to be additive as represented by the $\text{AUC}_{(0-240 \text{ min})}$ (Fig. 5). The $\text{AUC}_{(0-240 \text{ min})}$ values for combination therapy were 7893.54 ± 377.21 as compared to 4688.91 ± 398.02 (7-NI 15 mg kg^{-1}) and 4175.21 ± 540.33 (nimodipine 0.3 mg kg^{-1}).

The effect of twice daily administration of lercanidipine (0.03 and 0.09 mg kg^{-1}) along with 7-NI

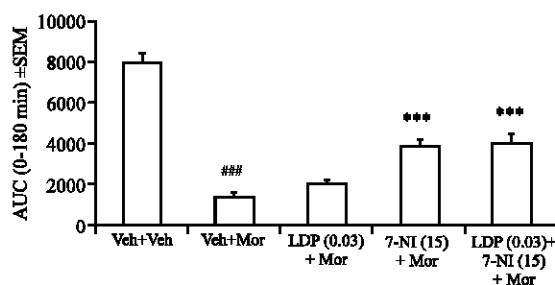


Fig. 6: Chronic effect of lercanidipine (LDP, 0.03 mg kg⁻¹), 7-nitroindazole (7-NI, 15 mg kg⁻¹) and their combination on tolerance to morphine (Mor)-induced analgesia (10 mg kg⁻¹) represented as AUC_(0-180 min). Values are expressed as Mean ± SEM, (n = 5-6), ###p < 0.001 vs morphine-naive group; ***p < 0.001 vs morphine-tolerant group

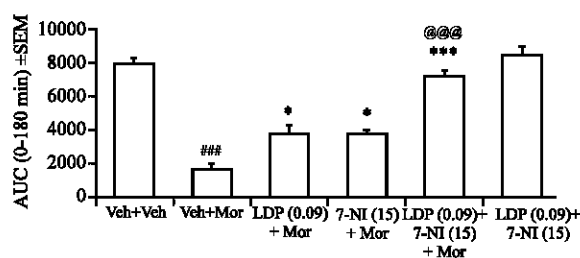


Fig. 7: Chronic effect of lercanidipine (LDP, 0.09 mg kg⁻¹), 7-nitroindazole (7-NI, 15 mg kg⁻¹) and their combination on tolerance to morphine (Mor)-induced analgesia (10 mg kg⁻¹) represented as AUC_(0-180 min). Values are expressed as Mean ± SEM, (n = 5-6), ###p < 0.001 vs morphine-naive group; ***p < 0.001 and *p < 0.05 vs morphine-tolerant group; @@@p < 0.001 vs 7-NI and LDP pre-treated morphine-tolerant group

(15 mg kg⁻¹) for 10 days on tolerance to morphine-induced analgesia was observed on 11th day by a challenge dose of morphine (10 mg kg⁻¹). The combination of lercanidipine (0.09 mg kg⁻¹) along with 7-NI (15 mg kg⁻¹) produced significant inhibition of tolerance and the effect was found to be additive (Fig. 7). The AUC_(0-180 min) value for the combination therapy was found to be 7208.89 ± 409.66 as compared to either of the drugs when used alone (3811.33 ± 514.52 and 3746.16 ± 251.05 for lercanidipine and 7-NI pre-treated group respectively) whereas lercanidipine (0.03 mg kg⁻¹) along with 7-NI (15 mg kg⁻¹) did not produced any significant additive effect as compared to either of the drugs alone (Fig. 6).

DISCUSSION

This study identified the L-type CCBs modulate the acute effect of morphine induced analgesia in mice. Acute

analgesic effects of morphine (10 mg kg⁻¹, i.p.) were potentiated by both nimodipine and lercanidipine in a dose-dependent manner, which was significant at 1.0 mg kg⁻¹ dose for nimodipine and 0.09 and 0.3 mg kg⁻¹ doses for lercanidipine. Nimodipine (1 mg kg⁻¹, i.p.) or lercanidipine (0.3 mg kg⁻¹, i.p.) per se did not produce any significant analgesia. The results of the chronic study demonstrates that both nimodipine (0.1-1.0 mg kg⁻¹, i.p.) as well lercanidipine (0.03-0.3 mg kg⁻¹, i.p.) dose-dependently inhibited the development of tolerance to morphine (10 mg kg⁻¹, i.p.) induced analgesia, however, the inhibition of tolerance to morphine-induced analgesia was found to be significant at 0.3 and 1.0 mg kg⁻¹ doses of nimodipine and 0.09 and 0.3 mg kg⁻¹ of lercanidipine. The highest dose of both the dihydropyridines used in this study were able to completely inhibit (restored 99% analgesia for 1 mg kg⁻¹ nimodipine and 94% for 0.3 mg kg⁻¹ lercanidipine) the development of tolerance to morphine-induced analgesia. However, nimodipine (1.0 mg kg⁻¹, i.p.) and lercanidipine (0.3 mg kg⁻¹, i.p.) administered alone twice daily for ten days didn't show any effect on morphine-induced analgesia. The effect of calcium channel blockers on tolerance to morphine induced analgesia was found in agreement with the previous reports (Gullapalli and Ramarao, 2002; Miranda *et al.*, 1992; Langley and Sorokin, 1989; Testa *et al.*, 1997; Michaluk *et al.*, 1998).

Further, the results of the chronic study indicated that, 7-NI, which is a selective nNOS inhibitor dose-dependently (5-50 mg kg⁻¹, i.p.) inhibited the development of tolerance to the antinociceptive action of selective μ -opioid receptor agonist, morphine (10 mg kg⁻¹, i.p.) in mice. Chronic administration of 7-NI prior to morphine significantly inhibited the development of tolerance to the antinociceptive activity of morphine.

7-NI, a selective nNOS inhibitor (Bhargava *et al.*, 1997) has attenuated the development tolerance to chronic morphine in mice. This suggests that chronic morphine treatment may enhance the level of neuronal NOS, which may be involved in the development of tolerance. In this study, selective neuronal NOS-inhibitor has been used; however, the role of non-selective NOS-inhibitors in the inhibition of tolerance and withdrawal to opioid-induced analgesia has been reported earlier in several studies (Bhargava, 1995, 1994; Bhargava and Cao, 1998; Bhargava and Kumar, 1997a, b; Bhargava and Throat, 1996; Bhargava and Zhao, 1996; Capasso *et al.*, 1998; Heinzen *et al.*, 2005; Vaupel *et al.*, 1997).

The doses of the drugs used in the combination therapy of CCB and selective nNOS-inhibitor on the development of tolerance to morphine-induced analgesia were selected based on the dose-response curves of the

respective drugs. The rationale for objective of studying the combination therapy was based on the hypothesis that nNOS has been thought to be Ca^{+2} -dependent, so it was expected that 7-NI along with CCBs, in the sub-maximal doses, will be able to completely restore the development of tolerance to morphine-induced analgesia (Alderton *et al.*, 2001; Watanabe *et al.*, 2003). It was found that 7-NI (15 mg kg^{-1} , i.p.), when used along with nimodipine, a cerebro-selective CCB (0.3 mg kg^{-1} , i.p.), in chronic morphine-induced analgesia, it shows an additive effect. Further, when 7-NI in the same dose was combined with another new, highly lipophilic ($\text{Log P} = 6.88$) dihydropyridine CCB, lercanidipine, which has highest Log P value among dihydropyridine type of CCBs (Sonkusare *et al.*, 2005), It has been found that 7-NI (15 mg kg^{-1}) along with lercanidipine (0.09 mg kg^{-1}) pre-treatment has similar additive effect as that observed in case of nimodipine in this study.

NOS-inhibitors as well as CCBs, both have been reported to have effect on blood pressure. However in the present study, lower doses of CCBs were used which were reported to have negligible effect on blood pressure. Moreover, 7-NI used in the study, is a highly selective neuronal NOS-inhibitor in vivo and has been reported to have a negligible effect on blood pressure. On the other hand, the arginine derivatives, which are not selective for the three isoforms of NOS like L-NNA or L-NMMA, increase the systolic blood pressure (Bhargava *et al.*, 1997; Vaupel *et al.*, 1997; Watanabe *et al.*, 2003).

7-NI, a selective neuronal NOS (nNOS) inhibitor significantly inhibited the development of tolerance to morphine-induced analgesia. Moreover, along with CCBs (nimodipine and lercanidipine) it has been found to have an additive effect on the inhibition of tolerance to morphine-induced analgesia. This may be due to the reason that the activation of nNOS has been found to be calcium dependent. Since nNOS activates formation of NO and NO has been thought to play a key role in the development of tolerance to morphine. So the combination therapy of neuronal NOS-inhibitor along with CCBs has shown an additive effect on the development of tolerance to morphine suggesting the involvement of a common pathway including both calcium and NOS together in the phenomena of tolerance development. But when higher doses of 7-NI were combined with CCBs, it did not show any potentiation over 7-NI alone (data not shown). This may be because of the reason that the dose of 7-NI used in combination with CCBs, itself might have completely inhibited nNOS and hence there was no potentiation over 7-NI alone on tolerance to analgesic effects of morphine.

There are also reports about two different nNOS isoforms (nNOS-1 and nNOS-2) having opposite effects on morphine analgesia both spinally and supra-spinally. (Kolesnikov *et al.*, 1997) had reported that nNOS-1 diminishes the antinociceptive action of the morphine and causes development of tolerance, whereas nNOS-2 enhances them and causes antinociception without tolerance (Pagliaro, 2003; Hayashida *et al.*, 2004). However, it seems to be more important to elucidate the mechanisms, which are involved in the induction and expression phases of tolerance and dependence, as well as the role of specific nNOS isoforms in these mechanisms (Ozek *et al.*, 2003). Moreover, the role of calcium on these two isoforms needs to be addressed. Therefore, to further explore the NOS inhibitors that could inhibit NOS isoforms, which are specifically involved in pain perception and have a role in opioid analgesia, tolerance and dependence, may very much contribute to the achieve better results in the treatment of pain and morphine tolerance.

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