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Ameliorative Effect of Combined Administration of Inducible Nitric Oxide Synthase Inhibitor with Cyclooxygenase-2 Inhibitors in Neuropathic Pain in Sprague-Dawley rats

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A B S T R A C T

This study aimed to evaluate the effects of the cyclooxygenase-2 (COX-2) inhibitors rofecoxib and meloxicam, the inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine hydrochloride, and their combinations on neuropathic pain in Sprague-Dawley rats. Neuropathic pain was produced by chronic constriction injury (CCI) of the right sciatic nerve in Sprague-Dawley rats under ketamine anesthesia. The analgesic effects of the ED_{50} doses of aminoguanidine hydrochloride, rofecoxib, and meloxicam administered orally were assessed using standard behavioural tests. In addition, combinations of aminoguanidine hydrochloride with rofecoxib or meloxicam were evaluated for their effects on neuropathic pain. Mechanical, thermal, and cold sensitivity tests confirmed the development of neuropathic pain following CCI. When administered individually, aminoguanidine hydrochloride, rofecoxib, and meloxicam significantly increased paw-withdrawal thresholds to mechanical stimuli at 6 hours in the ipsilateral hind paw. Combined treatment with aminoguanidine hydrochloride (30 mg/kg) and either rofecoxib (1.31 mg/kg) or meloxicam (1.34 mg/kg) produced a greater increase in mechanical pain thresholds at 6 hours. The combinations were more effective than individual treatments in reducing mechanical hyperalgesia. Combining aminoguanidine hydrochloride with meloxicam or rofecoxib may represent a promising therapeutic strategy for managing neuropathic pain.

Introduction

The efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) in managing neuropathic pain following nerve injury remains debatable. Previous studies have shown up-regulation of cyclooxygenase-2 (COX-2) in the injured sciatic nerve of Sprague-Dawley rats subjected to partial sciatic nerve ligation (PSNL), along with reversal of PSNL-induced tactile allodynia after local administration of the COX inhibitor ketorolac. These findings suggest that nerve injury leads to excessive prostaglandin production in peripheral tissues, which may sensitize nociceptors at the spinal level and contribute to central sensitization and persistence of allodynia. Increased COX-

2 expression has also been reported following spinal nerve ligation, chronic constriction injury (CCI), and complete sciatic nerve transection (Ma and Eisenach, 2003).

In contrast, other studies have demonstrated that rofecoxib and celecoxib failed to alleviate allodynia or hyperalgesia in certain neuropathic pain models, indicating that some forms of neuropathic pain may be COX-2 independent (Broom *et al*., 2004). However, early systemic administration of the selective COX-2 inhibitor meloxicam has been shown to attenuate the development of tactile allodynia, suggesting a possible role of COX-2 in neuropathic pain progression (Bingham *et al*., 2005).

Additionally, increased expression of inducible nitric oxide synthase (iNOS) has been observed in injured sciatic nerves, and iNOS inhibitors have consistently reduced neuropathic pain in experimental models (Takahashi *et al*., 2005).. Thus, both COX-2 and iNOS appear to be involved in neuropathic pain mechanisms. Despite evidence supporting their individual antihyperalgesic effects, the combined effects of selective COX-2 and iNOS inhibitors remain unexplored. Therefore, the present study evaluates the effects of rofecoxib, meloxicam, and aminoguanidine administered alone or in combination.

Materials and Methods

Experimental Animals

Adult male albino Sprague-Dawley rats weighing 200–220 g were obtained from the Laboratory Animal Resource Section of the Institute and used for the study. The animals were maintained in a temperature-controlled environment under a standard light–dark cycle. They were provided free access to food and water throughout the experimental period.

Drug Administration

Rofecoxib (Ranbaxy Laboratories Ltd., India), meloxicam (Intas Pharmaceutical Company, India), and aminoguanidine hydrochloride (Sigma Chemical Company, USA) were used. All drugs were administered orally as aqueous suspensions prepared in 1% Tween-80.

Dose Selection

Various workers have used drug doses based on inflammatory pain sensitivities in neuropathic pain, but with limited success (Broom *et al*., 2004; Bingham *et al*., 2005). Hence, we aimed to evaluate whether the anti-inflammatory ED_{50} could have any relevance in neuropathic pain, since prostaglandins (PGs) and inducible nitric oxide synthase (iNOS) are also involved in the pathogenesis of neuropathic pain. Therefore, the doses were selected from two separate experiments previously conducted in a carrageenan-induced hind paw edema model (Winter *et al*., 1962) in Sprague-Dawley rats (unpublished data).

In one experiment, individual dose–response curves of rofecoxib, meloxicam, and aminoguanidine hydrochloride were assessed for their anti-inflammatory effects in carrageenan-induced hind paw edema. From the dose–response curves, ED_{50} values (meloxicam 12.30 mg/kg; rofecoxib 9.77 mg/kg; and aminoguanidine hydrochloride 281.83 mg/kg), as well as effective and non-effective doses of all three drugs, were determined. A dose was considered effective or non-effective if it produced a significant or non-significant reduction in edema volume, respectively. In the second experiment, effective and non-effective doses of these drugs, as determined in the first experiment, were used in combination (rofecoxib + aminoguanidine hydrochloride and

meloxicam + aminoguanidine hydrochloride). From the dose–response curves of these drug combinations, the ED_{50} of the combinations (meloxicam 1.34 mg/kg + aminoguanidine hydrochloride 30 mg/kg and rofecoxib 1.31 mg/kg + aminoguanidine hydrochloride 30 mg/kg) and the combinations producing maximum percentage inhibition of the inflammatory response (meloxicam 30 mg/kg + aminoguanidine hydrochloride 30 mg/kg and rofecoxib 30 mg/kg + aminoguanidine hydrochloride 30 mg/kg) were determined.

Based on the above two experiments, different doses were selected for the present study. The Sprague-Dawley rats were divided into eight groups, each consisting of six animals (Table 1). Before and after drug administration, the animals were subjected to mechanical, thermal, and cold hyperalgesia tests.

Experimental Induction of Neuropathic Pain

The CCI model proposed by Bennett and Xie (1988) was used for the experimental induction of neuropathic pain. Male albino Sprague-Dawley rats were anaesthetized with ketamine hydrochloride (100 mg/kg, i.m.). After complete development of anaesthesia, the hair around the mid-thigh region of the right leg was clipped and shaved. The common sciatic nerve was exposed at the mid-thigh level by blunt dissection through the biceps femoris muscle. Four ligatures of chromic gut (4–0), presoaked in saline, were loosely placed at 0.5 to 1 mm intervals around the nerve just proximal to the trifurcation.

A sham operation was performed on the left sciatic nerve, which was exposed but not ligated. Both wounds were closed in layers using silk sutures. The ligated animals were housed individually and allowed to recover for ten days. Animals were inspected frequently after surgery to monitor pain-related behaviour; almost all animals exhibited guarding of the operated hind paw but not of the sham-operated hind paw. Evidence of autotomy was not observed.

Table.1 Dose Schedule of Different Drugs

Groups	Dose (mg/kg)
Vehicle-treated control	–
Aminoguanidine hydrochloride (AG)	281.73
Rofecoxib	9.72
Meloxicam	12.20
Rofecoxib + AG	1.30 + 30
Meloxicam + AG	1.31 + 30
Rofecoxib + AG	30 + 30
Meloxicam + AG	30 + 30

Abbreviation: AG – Aminoguanidine hydrochloride

Measurement of Mechanical Hyperalgesia

Paw withdrawal latencies to mechanical stimuli were measured using an analgesymeter (Ugo Basile, Italy) just prior to and at 0.5, 1, 2, 4, and 6 h after drug administration on the 11th day following surgery. This device is based on the Randall and Selitto (1957) test and was used to apply a linearly increasing pressure through a blunt perspex cone to the hind paw until the rat withdrew the paw; the applied pressure was recorded as the withdrawal threshold to a mechanical stimulus. The stimulus was applied between the 3rd and 4th metatarsals. Changes in pain threshold in the test groups were compared with those in the vehicle-treated control group. A cut-off pressure of 200 g was imposed to prevent tissue damage.

Measurement of Thermal Hyperalgesia

The latency of foot withdrawal to noxious heat stimuli was measured using a previously described method (Bennett and Xie, 1988; Hargreaves *et al*., 1988) just prior to and at 0.5, 1, 2, 4, and 6 h after drug administration on the 11th day following surgery. A radiant

heat stimulus was applied by directing a beam of light through a hole (2 × 5 mm) in the light box to each hind paw through a glass window. The light beam was automatically turned off by an infrared sensor when the rat lifted the foot, allowing measurement of the time interval between the onset of the light beam and foot lift. This interval was defined as the foot withdrawal latency. The change in paw withdrawal latency was compared with that of the vehicle-treated control group. A cut-off latency of 20 s was used to prevent tissue damage.

Measurement of Cold Hyperalgesia

The paw withdrawal latency to cold stimuli was recorded in seconds (using a stopwatch) by immersing the hind paw of Sprague-Dawley rats in cold water (4 ± 1 °C) just prior to and at 0.5, 1, 2, 4, and 6 h after drug administration on the 11th day following surgery. The change in withdrawal latency in the treated groups was compared with that in the vehicle-treated control groups.

Statistical Analysis

One-way ANOVA was employed for data analysis, and the values were compared with those of the vehicle-treated control and individual drug-treated groups.

Results and Discussion

In the ipsilateral hind paw, significant ($P < 0.05$) changes in mechanical, thermal, and cold hyperalgesia were observed on the 11th day after surgery as compared with the sham-operated hind paw.

Effect of rofecoxib, meloxicam, and aminoguanidine hydrochloride alone and their combinations on mechanical, thermal, and cold hyperalgesia

Fig. 1 demonstrates a significant reduction in paw withdrawal latencies to mechanical stimuli in the right ipsilateral hind paw when compared with the sham-operated left hind paw just before drug administration, i.e., at 0 h. Following drug administration, no significant differences in paw withdrawal latencies to mechanical stimuli were noted at 0.5, 1, 2, and 4 h (data not shown). However, aminoguanidine hydrochloride, rofecoxib, and meloxicam, when given individually, produced a significant increase in paw withdrawal latencies to mechanical stimuli at 6 h in the right ipsilateral hind paw of CCI Sprague-Dawley rats (Fig. 2).

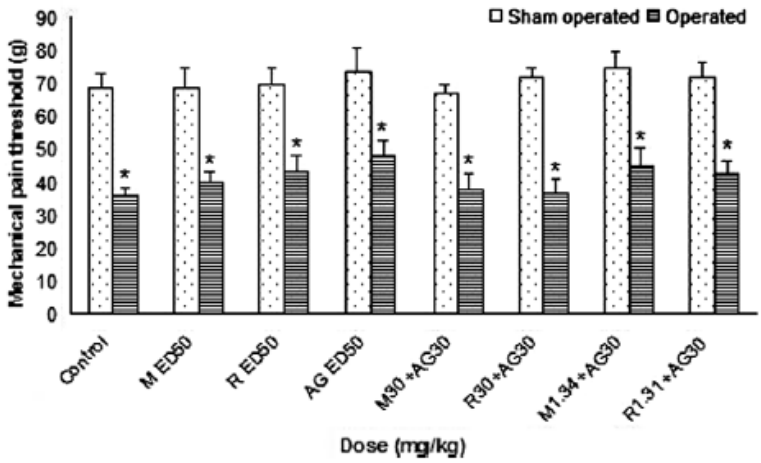

Fig. 1. Production of mechanical hyperalgesia at 0 h. Results are expressed as the mean pain threshold values. Each point represents mean ± SE (n = 6 animals). *P < 0.05 as compared with mechanical stimulation in the sham-operated hind paw. Abbreviations M, R, and AG denote meloxicam, rofecoxib, and aminoguanidine hydrochloride,

respectively.

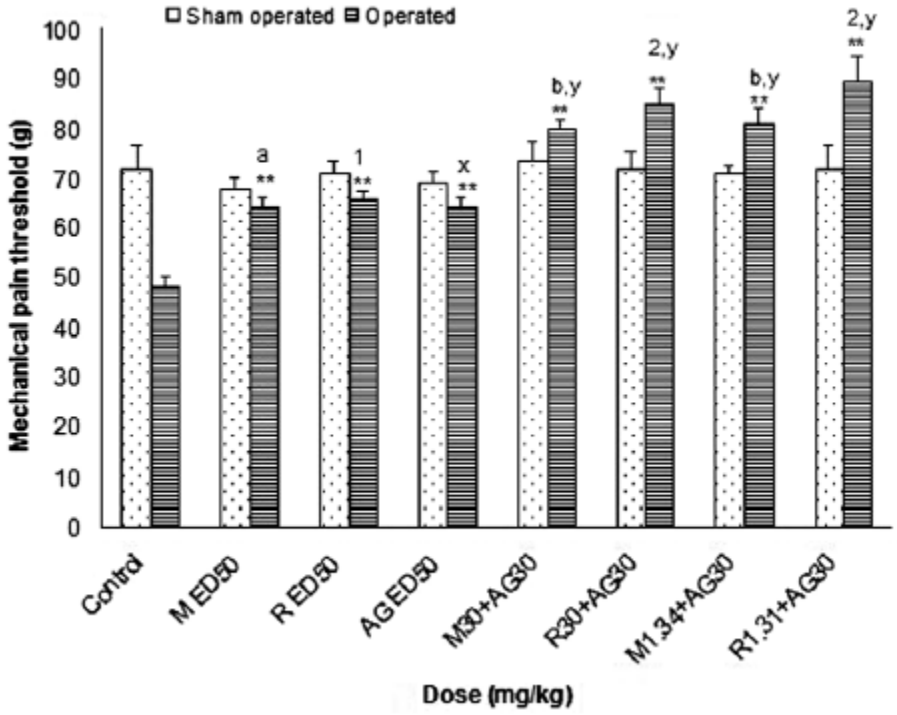

Fig. 2. Inhibition of mechanical hyperalgesia by rofecoxib, meloxicam, and aminoguanidine hydrochloride, alone or in combination, at 6 h. Each point represents mean ± SE (n = 6 animals). **P < 0.01 as compared with the vehicle-treated control. (a) and (b) values bearing different superscripts differ significantly with respect to meloxicam ED_{50} (P < 0.01); (x) and (y) values bearing different superscripts differ significantly with respect to AG ED_{50} (P < 0.01); (1) and (2) values bearing different superscripts differ significantly with respect to R ED_{50} (P < 0.01). Abbreviations M, R, and AG denote meloxicam, rofecoxib, and aminoguanidine hydrochloride, respectively.

Similarly, combined administration of aminoguanidine hydrochloride (30 mg/kg) with rofecoxib or meloxicam (30 mg/kg) produced a significant elevation in the pain threshold to mechanical hyperalgesia in the ipsilateral hind paw at 6 h (Fig. 2). Co-administration of aminoguanidine hydrochloride (30 mg/kg) with rofecoxib (1.31 mg/kg) or meloxicam (1.34 mg/kg) was also observed to significantly increase paw withdrawal latencies to mechanical stimuli at 6 h. Administration of the ED_{50} doses of the COX-2 inhibitors rofecoxib (9.77 mg/kg) and meloxicam (12.30 mg/kg), or aminoguanidine hydrochloride (281.83 mg/kg) alone, failed to produce any significant changes in paw withdrawal latency to thermal and cold stimuli (data not shown). Similarly, combined administration of aminoguanidine hydrochloride with rofecoxib or meloxicam did not result in any significant alteration in paw withdrawal latency to thermal and cold stimuli (data not shown).

CCI-induced neuropathic pain serves as a valuable experimental model for assessing the role of inflammatory mediators in neuropathic pain. The postoperative behaviour of the Sprague-Dawley rats revealed hyperalgesia in the ipsilateral hind paw, but not in the contralateral hind paw, which may be attributed to the participation of various inflammatory mediators (Levine *et al*., 1990; Clatworthy *et al*., 1995; Tracey and Walker, 1995). Peripheral nerves contain resident macrophages that account for nearly 1–4% of

the total cell population in the rat sciatic nerve (Oldfors, 1980). Following peripheral nerve injury, macrophages are recruited to the site of nerve damage (Perry *et al*., 1987; Frisen *et al*., 1993), where they assist in the clearance of degenerating axons and myelin sheaths (Perry, 1994). Macrophages release a broad spectrum of substances (Nathan, 1987), some of which can activate nociceptors either directly or indirectly. These include PGE_2 and PGI_2, which directly sensitize primary afferent neurons (Schaible and Schmidt, 1988). In addition, macrophages secrete cytokines such as IL-1β and IL-6 that induce hyperalgesic responses mediated through prostaglandin synthesis (Cunha *et al*., 1992). Macrophages also produce nitric oxide, which may contribute to peripheral hyperalgesia (Kawabata *et al*., 1994). Furthermore, CCI has been reported to induce local expression of iNOS in both macrophages and Schwann cells at and distal to the site of injury (Levy *et al*., 1999). The increase in iNOS mRNA expression was found to parallel both temporal and spatial protein expression. Thus, prostaglandins (Syriatowicz *et al*., 1999) and the iNOS–NO pathway (Levy *et al*., 1999) are regarded as major mediators of neuropathic pain.

The present study demonstrated a decrease in pain threshold in the ipsilateral hind paw of Sprague-Dawley rats, which was significantly elevated following oral administration of rofecoxib, meloxicam, and aminoguanidine hydrochloride, indicating the involvement of COX-2 and iNOS in neuropathic pain. In a previous investigation, subcutaneous administration of meloxicam or SC5812 (COX-2 inhibitors) into the sciatic nerve–transected hind paw of Sprague-Dawley rats produced marked attenuation of mechanical hyperalgesia with a shorter duration of action (Syriatowicz *et al*., 1999). Similar injections into the contralateral hind paw and abdomen did not alter hyperalgesia, suggesting that the observed effects were localized rather than systemic. In contrast, the present study demonstrated a systemic effect of meloxicam of shorter duration. Another COX-2 inhibitor, etodolac, has also been shown to reduce heat-evoked hyperalgesia in Sprague-Dawley rats, emphasizing the significance of COX-2 in neuropathic pain (Suyama *et al*., 2004). Comparable effects were observed with rofecoxib and meloxicam in the present investigation. Collectively, these findings further support the role of COX-2-derived products in neuropathic pain.

Conflicting evidence exists regarding the role of iNOS in neuropathic pain, particularly in studies involving aminoguanidine. Earlier reports indicated that aminoguanidine (30 mg/kg, i.p.) failed to reduce mechanical sensitivity in spinal nerve-ligated Sprague-Dawley rats (Lee *et al*., 2005). In contrast, the present study demonstrated a reduction in pain sensitivity, consistent with our earlier findings (Naik *et al*., 2006). These observations are further corroborated by studies showing that several iNOS inhibitors with varying sensitivities can attenuate neuropathic pain responses to different stimuli (Levy and Zochodne, 1998; Sung *et al*., 2004; Labuda *et al*., 2006; De Alba *et al*., 2006). Taken together, these findings suggest that COX-2 and iNOS represent important therapeutic targets in neuropathic pain.

The efficacy of COX-2 inhibitors in neuropathic pain associated with nerve injury remains controversial. Up-regulation of COX-2 has been demonstrated in spared nerve injury, CCI, and complete sciatic nerve transection models in Sprague-Dawley rats (Ma and Eisenach, 2003). However, rofecoxib, a COX-2 inhibitor, administered at doses of 1 and 3.2 mg/kg for 5 and 3 days, respectively, did not alter the development of allodynia and hyperalgesia in the spared nerve injury model, suggesting that hyperalgesia

may not be dependent on COX-2 activity (Broom *et al*., 2004). Similar results were reported with rofecoxib and celecoxib in models of mechanical and thermal hyperalgesia (Bingham *et al*., 2005). In contrast, the present study demonstrated an increase in paw withdrawal latencies to mechanical stimuli at 6 h in the ipsilateral hind paw of CCI Sprague-Dawley rats following treatment with rofecoxib and meloxicam, indicating an antihyperalgesic effect attributable to COX-2 inhibition. The higher doses employed in this study may explain the antihyperalgesic effects of rofecoxib that were not reported previously (Broom *et al*., 2004; Bingham *et al*., 2005). Variations in experimental outcomes may also reflect differences in the neuropathic pain models used by different investigators. Meloxicam, another COX-2 inhibitor, administered orally at 12.30 mg/kg, produced a significant increase in paw withdrawal latencies in the ipsilateral hind paw of CCI Sprague-Dawley rats. Repeated systemic administration of meloxicam (2 and 4 mg/kg, i.p.) at 0, 12, 24, and 36 h following nerve injury attenuated the development of tactile allodynia, indicating partial involvement of COX-2 in the development of tactile allodynia in the L-5 single spinal nerve injury model (Takahashi *et al*., 2005). In contrast, the present study observed an antihyperalgesic effect of meloxicam at a much higher dose (12.34 mg/kg) several days after nerve injury, suggesting a role for COX-2 in the progression of CCI-induced neuropathy.

Immunoreactivity resembling iNOS has been detected at 7 and 14 days at both constriction and distal regions of the sciatic nerve in CCI Sprague-Dawley rats (Levy and Zochodne, 1998). It has also been reported that CCI is associated with a localized inflammatory response mediated, at least in part, by iNOS. Activation of the local iNOS–NO system plays a critical role in the pathogenesis of peripheral nerve injury and neuropathic pain (Levy *et al*., 1999). Earlier studies from this laboratory further demonstrated significantly elevated nitrate and nitrite levels in the sciatic nerve of CCI Sprague-Dawley rats (Naik *et al*., 2006), indicating local up-regulation of NOS, particularly iNOS. Aminoguanidine, a NOS inhibitor with greater selectivity toward iNOS, significantly increased paw withdrawal latencies to mechanical stimuli in the present study, highlighting the therapeutic relevance of iNOS inhibitors in neuropathic pain. It is likely that aminoguanidine reduced nitric oxide-mediated microvascular vasodilatation, thereby decreasing pain sensitivity, as previously reported (Levy and Zochodne, 1998).

The precise mechanism underlying the interaction between aminoguanidine hydrochloride and rofecoxib remains unclear and cannot be conclusively determined from the present data. Previous studies suggest a close interaction between the iNOS and COX-2 pathways, with iNOS enhancing COX-2 activity, possibly through interaction with the heme moiety that binds to the active site of the COX-2 enzyme (Salvemini *et al*., 1993; Kim *et al*., 2005). Inhibition of iNOS by aminoguanidine hydrochloride reduces nitric oxide production, which in turn fails to stimulate COX-2 activity and leads to decreased synthesis of both nitric oxide and prostaglandins, resulting in a potentiated or synergistic effect. This action is in addition to the direct inhibition of COX-2 by rofecoxib and meloxicam and iNOS by aminoguanidine hydrochloride.

In the present investigation, however, treatment with aminoguanidine hydrochloride, rofecoxib, and meloxicam, either individually or in combination, did not produce significant alterations in paw withdrawal latencies to thermal and cold

stimuli. This may be attributed to differences in the underlying mechanisms governing thermal and cold hyperalgesia. In addition, distinct nerve fibre populations mediate mechanical, thermal, and cold hyperalgesia. Responses to mechanical stimuli are reduced in C and A-delta fibres in neuropathic pain (Shir and Seltzer, 1990; Shim *et al*., 2005). Afferent responses to thermal stimuli primarily involve C fibres rather than A-delta fibres (Shir and Seltzer, 1990; Shim *et al*., 2005), whereas cold-sensitive C fibres play a key role in cold hyperalgesia (Craig *et al*., 1994). It is therefore likely that A-delta fibres predominantly mediate mechanical hyperalgesia in the present study.

In conclusion, in Sprague-Dawley rats with CCI-induced neuropathic pain, co-administration of aminoguanidine hydrochloride with rofecoxib or meloxicam provides an additional benefit by producing a potentiated effect compared with administration of either drug alone.

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