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PROTECTIVE EFFECT OF ADENOSINE IN DIABETIC NEUROPATHIC PAIN IS MEDIATED THROUGH ADENOSINE A_1-RECEPTORS

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Abstract : Diabetic neuropathic pain is generally considered to be one of the most troublesome complications affecting diabetic patients and current therapy provides inadequate pain relief. In the present study, the effect of adenosine was investigated in a model of diabetic neuropathic pain. Diabetes was induced by streptozotocin (65 mg/kg, ip) in male Sprague Dawley rats and subjected to thermal (cold and hot) and chemical (formalin) stimuli. Diabetic rats developed hyperalgesia by the end of six weeks in thermal and chemical stimuli test. Adenosine (100, 200 and 500 mg/kg, ip) produced significant reversal of responses to thermal and chemical stimuli in diabetic rats. 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX 1 mg/kg, ip), an adenosine A_1-receptor antagonist, but not 3,7-dimethyl-l-propargylxanthine (DMPX 1 mg/kg, ip), an adenosine A_{2A}-receptor antagonist, reversed the protective effect of adenosine. These results indicate that adenosine is an effective analgesics in a model of diabetic neuropathy, and the protection produced by adenosine is via stimulation of adenosine A_1-receptors.

Key words : diabetic neuropathic pain, adenosine, thermal hyperalgesia, adenosine A_1–receptors, formalin, adenosine A_2-receptors

INTRODUCTION

Neuropathic pain is generally considered to be one of the most troublesome complications affecting diabetic patients (1, 2). Current therapy for painful neuropathy includes use of antidepressants, ion channel blockers, NMDA receptor antagonists, opioids, topical lidocaine and capsaicin (1, 3). Pain relief with current therapy is often inadequate and associated with side effects (1, 2, 4, 5).

Adenosine and adenosine receptor agonists have antinociceptive effect in animal models of acute (6–9), inflammatory (10–13) and nerve injury induced neuropathic pain (14–15). Systemic administration of adenosine and adenosine agonists produced analgesic action in patients with neuropathic pain (1, 16). It has been reported that there is a significant reduction in the circulating and levels of adenosine in the cerebrospinal fluid in neuropathic pain patients (17). Adenosine is involved in the antinociceptive effect of intrathecal morphine following nerve injury (18). Amitriptyline, a tricyclic antidepressant, is effective in alleviating neuropathic pain conditions and this action is believed to be due to interaction with endogenous adenosine systems (19–20). Adenosine kinase inhibitors, which enhance endogenous levels of adenosine, have shown protection in neuropathic pain states (21–22). Above evidence indicate antinociceptive

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potential of adenosine. However, effect of adenosine in diabetic neuropathic pain has not yet been evaluated. Therefore, in the present study, effect of adenosine was investigated in diabetic neuropathic pain model.

METHODS

Animals

Healthy male Sprague Dawley (140–160 g) rats were used for the study. They were procured from Central Animal Facility, NIPER. The animals were provided with the standard diet and water *ad libitum* and kept at standard conditions. All the animals were acclimatized for a minimum of one week prior to the study. The experimental protocol was approved by Institutional Animal Ethics Committee of NIPER.

Induction of diabetes

Diabetes was developed by administration of streptozotocin (65 mg/kg, ip). Before and after injection of streptozotocin, plasma glucose levels were analysed using the glucose GOD-PAP span diagnostic kit method (Qualigen, Glaxo, India). Only those animals showing blood glucose > 250 mg/dl were included in the drug treatment study.

Thermal stimuli: Cold and hot immersion tests

Cold and hot immersion tests were carried out according to the method described by Sharma et al., 2008 (23). In the cold immersion test, the tail of the rat was immersed in cold water maintained at 10°C, while in the hot immersion test, the tail was immersed in water maintained at 45°C. In both tests, basal tail flick latency (withdrawal response of tail) or signs of struggle were observed. The cut off time was 15 s. Cold and hot immersion tests were carried out each week for a period of six weeks in normal and streptozotocin diabetic rats and changes in tail flick latency in both groups were *compared.* Effect of adenosine was studied after six weeks of streptozotocin administration.

Chemical stimuli: formalin test

The formalin test was carried out according to the method of Courteix et al. (24). Formalin challenge was done once in all the groups. Formalin (0.1 ml 10%) was administered to the dorsal surface of the left hind paw in both normal and diabetic rats. Each animal was then placed in a plexiglass chamber and observed for 15 min for latency of licking, latency of paw elevation, duration of licking, duration of paw elevation. Changes in latency and duration of paw licking and paw elevation were observed after first, fourth and sixth week of streptozotocin administration. The effect of adenosine was studied out after six weeks of streptozotocin administration.

Drugs and treatment schedule

All drugs were procured from Sigma, U.S.A. They were prepared freshly and administered intraperitoneally by using a 26-gauge hypodermic needle. Streptozotocin was dissolved in citrate buffer (pH 4.4). Adenosine was suspended in 10% Tween 20. 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX) and 3,7-dimethyl-l-propargylxanthine (DMPX) were dissolved in dimethylsulphoxide (DMSO) and distilled water respectively.

Effects of adenosine on thermal (cold and hot immersion) and chemical (formalin) stimuli were carried out after six weeks of streptozotocin administration. Rats were pretreated (30 min) with different doses of adenosine (100, 200 and 500 mg/kg, ip). They were observed for a period of 2 h at the interval of 15, 30, 60 and 120 min in cold and hot immersion test. In case of chemical stimuli observations were made after 30 min of drug administration. To study the

involvement of adenosine A_1 or A_2-receptor in adenosine effect, DPCPX (1 mg/kg, ip), an adenosine A_1-receptor antagonist or DMPX (1 mg/kg, ip, an adenosine A_{2A}-receptor antagonist was administered 15 min prior to administration of adenosine (200 mg/kg). Control groups were also maintained with appropriate vehicles used for the different drugs.

Statistical analysis

Results were expressed as mean ± S.E.M. and analysed (SigmaStat) using student V test, repeated measures of analysis of variance (ANOVA) or ANOVA followed by post hoc comparison where appropriate. $P<0.05$ was considered statistical significance.

RESULTS

Streptozotocin (65 mg/kg, ip) induced diabetes in 80% of animals. Before administration of Streptozotocin plasma glucose levels were 82.16±1.10 mg/dl. Six weeks after Streptozotocin administration, blood glucose levels increased to 353.68±2.54 mg/dl. In normal rats, plasma glucose levels were 79.88±1.50 (0 weeks) and 83.18±1.51 mg/dl (after 6 weeks).

Streptozotocin treatment significantly decreased rat body weight 155.44±0.59 g (0 week) to 111.78±1.67 g (after 6 weeks). However, in normal rats body weight was significantly increased from 150.33±0.98 g (0 weeks) to 272.44±2.30 g (after 6 weeks).

Cold immersion test

Diabetic animals showed thermal hyperalgesia as evidenced by a significant reduction ($P<0.05$) in the tail flick latency in 83% of the diabetic animals by the end of six weeks in comparison to normal animals in cold immersion test. However, only 50% and 66% of the diabetic rats showed significant changes in tail flick latency by the fourth and fifth week respectively. Tail flick latency was 14.50±0.18 s before 'streptozotocin treatment and this was reduced to 9.67±0.66 s ($P<0.05$) and 7.83±0.61 s ($P<0.05$) after fourth and sixth week post treatment, respectively. However in normal rats tail flick latency was 14.12 ± 0.17 s, 13.67±0.45 s and 13.33±0.27 s after 0, 4th and 6th weeks, respectively (Fig. 1).

In diabetic animals, adenosine (200 and 500 mg/kg) produced significant reversal of thermal hyperalgesia while adenosine 100 mg/kg did not produce reversal (Fig. 2). This

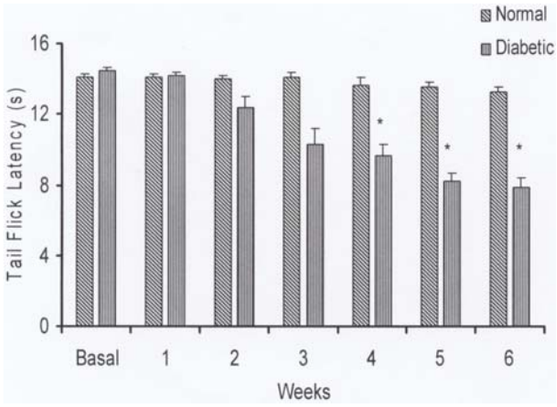

Fig. 1: Effect of duration of diabetes on tail flick latency (TFL) in cold immersion test. The values are expressed as Mean ± S.E.M. N= 10–12. *P<0.05 as compared to normal rats group.

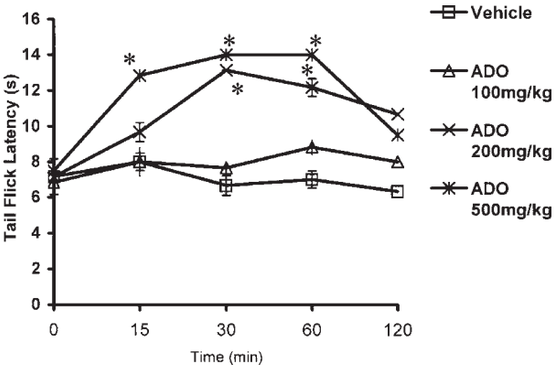

Fig. 2: Effect of adenosine (ADO) on tail flick latency in cold immersion test after six weeks of diabetes induction. All the values are expressed as mean ± S.E.M. N=6–8. *P<0.05 as compared to vehicle treated group.

reversal was dose dependent. Maximum protection was observed 30 min following drug administration. The adenosine A_1-receptor antagonist DPCPX, at 1 mg/kg, completely reversed the protection offered by adenosine 200 mg/kg in diabetic rats. However, DMPX, an adenosine A_2-receptor antagonist, failed to reverse the protection offered by adenosine (Fig. 3).

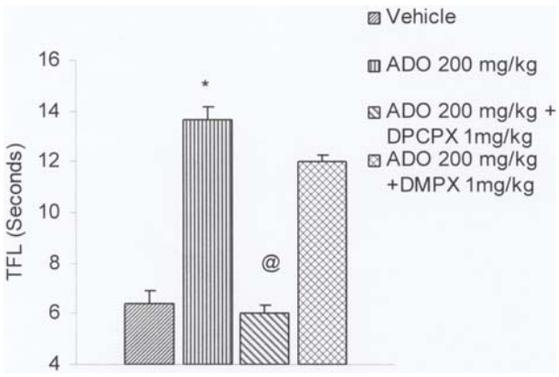

Fig. 3 : Effect of DPCPX, an adenosine A_1-receptor antagonist, and DMPX, an adenosine A_2-receptor antagonist, on adenosine (ADO) effects on the tail flick latency in cold immersion test after six weeks of diabetes induction. All the values are expressed as mean ± S.E.M. N=6–8. *P<0.05 as compared to vehicle and @P<0.05 as compared to adenosine group.

Hot immersion test

Diabetic animals showed thermal hyperalgesia as evidenced by a significant reduction in the tail flick latency in 91% of diabetic animals by the end of six weeks as compared to normal animals. Although the reduction was significant by fourth and fifth week, only 58% and 75% of the diabetic rats showed changes in tail flick latency by fourth and fifth week respectively. Tail flick latency was 14.40±0.24 s before streptozotocin treatment and this was reduced to 8.63±0.41 s (P<0.05) and 6.80±0.78 s (P<0.05} after fourth and sixth week post treatment, respectively. However in normal rats tail flick latency was 14.40±0.24 s, 13.60±0.31 s and 13.40±0.41 s after 0, 4th and 6th weeks, respectively (Fig. 4).

Adenosine (200 and 500 mg/kg) significantly reversed the thermal hyperalgesia in diabetic rats (Fig. 5). Reversal was dose dependent. The peak effect was at 30 min after 500 mg/kg and at 60 min after 200 mg/kg of adenosine administration. As with the cold immersion test, DPCPX at 1 mg/kg completely reversed the protection offered by adenosine 200 mg/kg, while DMPX at 1 mg/kg failed to reverse

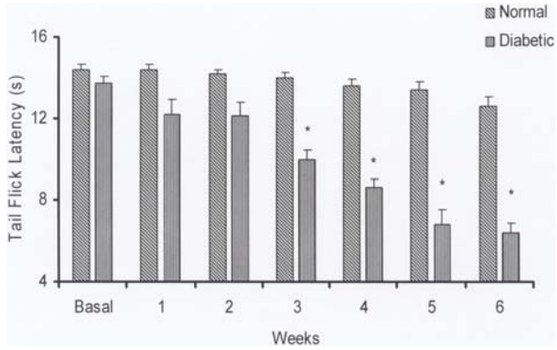

Fig. 4 : Effect of duration of diabetes on tail flick latency (TFL) in hot immersion test. The values are expressed as Mean ± S.E.M. N=10–12. *P<0.05 as compared to normal rats group.

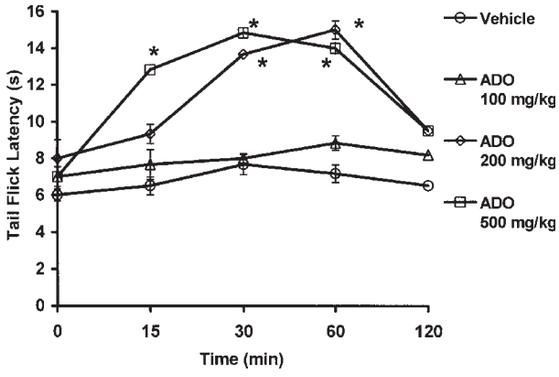

Fig. 5 : Effect of adenosine (ADO) on tail flick latency in the hot immersion test after six weeks of diabetes induction. All the values are expressed as mean ± S.E.M. N=6–8. *P<0.05 as compared to vehicle treated group.

the protection offered by adenosine 200 mg/kg (Fig. 6).

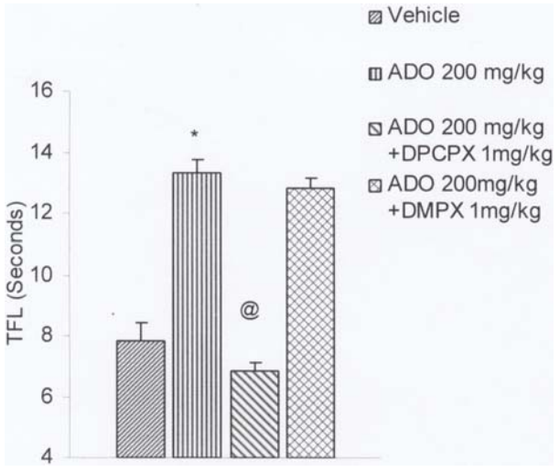

Fig. 6 : Effect of DPCPX, an adenosine A_1–receptor antagonist, and DMPX, an adenosine A_2-receptor antagonist, on adenosine (ADO) effects on the tail flick latency in hot immersion test after six weeks of diabetes induction. All the values are expressed as mean ± S.E.M. N=6–8. *P<0.05 as compared to vehicle and compared to adenosine group.

Chemical stimuli

Prior to streptozotocin treatment, there was no significant difference between controls and treated groups in latency of paw licking, latency of paw elevation, duration of paw elevation, and duration of paw licking. There was a significant difference in latency and duration of licking and paw elevation in diabetic rats by six weeks in comparison to normal rats. In diabetic rats, latency of paw licking and elevation after six weeks was 33.50±2.12 s (P<0.05) and 40.50±1.47 s (P<0.05), respectively as compared to normal rats 59.83±0.84 s (paw licking) and 60.83±0.80 s (paw elevation). In diabetic rats, duration of paw licking and elevation after six weeks was 108.33±1.41 s (P<0.05) and 221.67±1.44 s (P<0.05), respectively as compared to normal rats 45.17±0.81 s (paw licking) and 134.33±1.44 s (paw elevation). Significant decrease in latencies and increase in durations of licking and paw elevation indicates the development of hyperalgesia in diabetic rats.

Adenosine (50, 100 and 200 mg/kg) produced dose dependent reversal of hyperalgesia in diabetic rats (Fig. 7). There was significant improvement in the parameters of pain in comparison to vehicle treated diabetic rats. DPCPX at 1 mg/kg completely reversed the protection offered by adenosine 200 mg/kg, while DMPX failed to reverse the protection offered by adenosine (Fig. 8).

DISCUSSION

The present study confirms that streptozotocin-induced diabetes alters nociceptive thresholds and causes disturbances in responses to nociceptive and noxious stimuli. However, it requires several weeks to become evident. Alterations in pain thresholds were found to be progressive; the number of rats affected and the degree to which they were affected increased up to the sixth week of experimentation. These characteristics are close to the disturbances in pain sensation as observed in diabetic humans (24).

Decrease in pain threshold was observed with mildly noxious stimulus. Water at 10°C failed to induce tail withdrawal in normal rats before the cut off time (15 s) showing that this temperature is mildly noxious. However, the diabetic rats behaved in a way as if this temperature was painful which indicates the development of allodynia. By the fourth week, a considerable number of animals showed tail withdrawal latencies of less than 15 s, but after six weeks the percentage of responders was 83%. This data

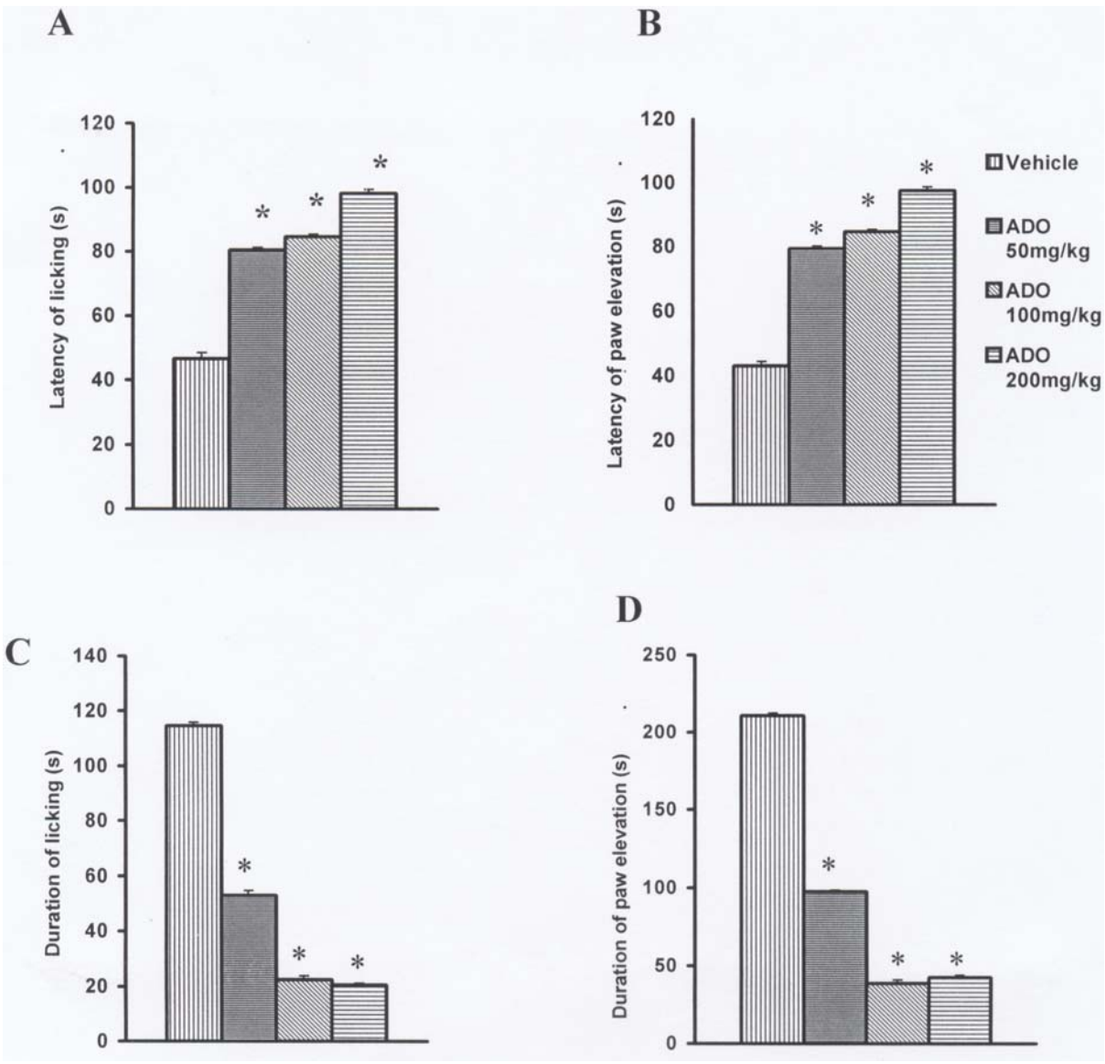

Fig. 7 : Effect of adenosine (ADO) on the (A) latency of licking (B) latency of elevation (C) duration of licking, and (D) duration of paw elevation in formalin test after six weeks of diabetes induction. The values are expressed as mean ± S.E.M. N=5–7. *P<0.05 as compared to vehicle treated group.

with cold immersion is similar to that of Courteix et al. (24), who reported that diabetes induced thermal allodynic responses were present as early as 2 weeks post streptozotocin treatment in a subpopulation of rats, and the number of responders increased with time. There was also a similar increase in hyperalgesic activity in diabetic rats in comparison to normal rats when subjected to thermal (hot immersion) stimuli. Our results are fully consistent with earlier reports in which streptozotocin diabetes produced a decrease in pain thresholds (24–25). Similar alterations in

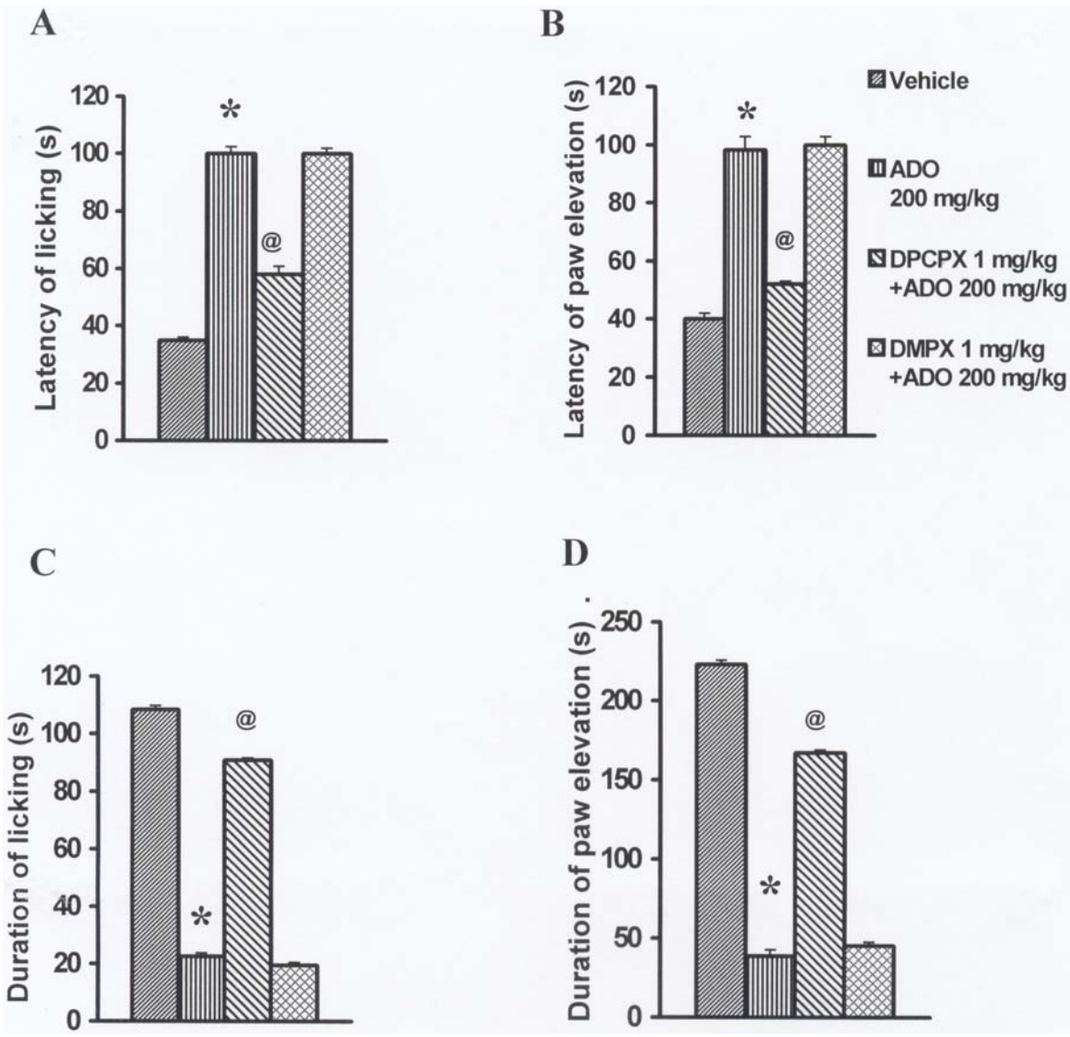

Fig. 8: Effect of DPCPX, an adenosine A_1-receptor antagonist, and DMPX, an adenosine A_2-receptor antagonist, on adenosine (ADO) response on (A) latency of licking (B) latency of elevation (C) duration of licking, and (D) duration of paw elevation in formalin test after six weeks of diabetes induction. The values are expressed as mean ± S.E.M. N=5–7. *P<0.05 as compared to control and @P<0.05 as compared to adenosine treated group.

pain threshold were observed in alloxan diabetes model (26). The mechanisms responsible for the decreases in pain threshold level in diabetic rats are not yet completely established. Alterations of receptor levels, hyperactivity of nociceptive fibres and injury to nerve fibres have been implicated (27–29).

An exaggerated response to formalin induced pain behaviours of licking and elevation of the injected paw was observed in diabetic rats as compared to normal rats. This indicates hyperalgesia to chemical

stimuli. The latencies to licking and elevation were reduced, whereas the duration of these behaviours were increased. About 90% of the animals kept their paw raised without ground contact for a much longer time than controls. The duration of the licking was also higher. Our results are in agreement with previous findings in which formalin evoked flinching are exaggerated in diabetic rats (30).

In the present study, we investigated for the first time, effect of adenosine on thermal and chemical hyperalgesia in diabetic rats. Adenosine (200 and 500 mg/kg) produced significant reversal of hyperalgesia in diabetic animals. With formalin, even the dose of 50 mg/kg of adenosine offered protection against flinching and licking behaviours. These results demonstrates the involvement of adenosinergic system in diabetic neuropathic pain. Results of the present study are further supported by a report in which adenosine administration showed improvement in motor nerve conduction and nerve blood flow in diabetic rats (31).

Adenosine and directly acting adenosine receptor agonists have been shown to reduce nerve injury (14, 32–33) and carrageenan (12) induced hyperalgesia in rats. Their administration also alleviates hyperalgesia and allodynia in human subjects (33–34); Eisenach et al., 2002). Adenosine kinase inhibitors also have shown to have antinociceptive effect in neuropathic animal models of nociception (21). Collectively, these reports suggest a significant role of adenosine in regulating neuropathic pain.

Adenosine stimulates two major receptor subtypes A_1, and A_2, which are linked to a number of effectors namely, adenylate cyclase, ionositol phosphate, K^+ channel, Ca^{2+} channel and neurotransmitters release. Activation of adenosine A_1-receptors results in a decrease in adenylate cyclase activity leading to decrease in intracellular level of cyclic adenosine monophosphate, while activation of adenosine A_2-receptors stimulates adenyl cyclase activity (35). In our study, the protective effect of adenosine (200 mg/kg) was reversed by DPCPX, an adenosine A_1-receptor antagonist but not by DMPX, an adenosine A_{2A}-receptor antagonist, indicating the involvement of adenosine A_1-receptors in alleviating diabetic neuropathic pain. Studies with adenosine receptor agonists and antagonists have demonstrated a pharmacological profile for spinal antinociception that is primarily adenosine A_1-receptor mediated (9). Specific activation of adenosine A_1-receptor reduced inflammation evoked responses of spinal cord dorsal horn neuron (36).

The exact mechanisms of adenosine protection in neuropathic pain is unclear given the multitude of effectors system linked to adenosine receptors. Pre or postsynaptic mechanisms such as inhibition of excitatory amino acids and control of calcitonin gene related peptides, neuropeptide and substance P release (37–39) may contributes to their effect. Supraspinal (40) and peripheral mechanisms (9) may also involve in antinociceptive and anti-inflammatory action of adenosine. In addition, there is also evidence that adenosine agonists produce analgesia by interactions with neurotransmitters especially dopamine, norepinephrine and serotonin (8, 41–42).

In summary, diabetes alters nociceptive thresholds indicating the development of diabetic neuropathic pain. Adenosine showed

significant effectiveness in a model of diabetic neuropathic pain and protection produced by adenosine was via stimulation of adenosine A_1-receptors. These findings suggest the potential of adenosinergic agents in diabetic neuropathic pain and may offer a therapeutic alternative to existing treatment.

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