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Beneficial Effect of Cyclosporine in Experimental Diabetes Induced Neuropathic Pain in Rats

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Abstract: The present study was designed to investigate the effect of cyclosporine on hyperglycemia induced decrease antinociceptive effect of morphine in rats. Streptozotocin (STZ) (50 mg kg^{-1}, i.p., once) was administered to induce experimental diabetes in the rats. Pain sensitivity was measured using tail-flick and paw withdrawal test. Urinary and serum nitrite concentration was estimated using Greiss reagent. Spleen Homogenate Supernatant (SHS) was prepared from spleen of 28th day diabetic rats and administered to normal rats (400 μL. i.v.) for 28 days. Experimental diabetes significantly decreased paw withdrawal latency to thermal stimuli on day 28 as compared to age matched control rats, indicating that diabetic rats exhibit thermal hyperalgesia. Moreover, analgesic effect of morphine (4 and 8 mg kg^{-1} s.c.), was progressively decreased in diabetic and SHS treated non diabetic rats. Further, the levels of nitric oxide were also elevated in 28th day diabetic and SHS treated non diabetic rats. However, administration of Cyclosporine (12.5 and 25 mg kg^{-1} i.p.), an IL-2 inhibitor and splenectomy attenuated diabetes and SHS induced decrease in nociceptive threshold and increase in serum and urinary nitrite levels. It is concluded that cyclosporine have beneficial effect in diabetic neuropathy and also improved the analgesic effect of morphine.

Key words: Spleen homogenate, streptozotocin, hyperalgesia, neuropathic pain, nitric oxide, hyperglycemia

INTRODUCTION

Neuropathy is the most common complication associated with diabetic patient and recognized as one of the most difficult types of pain to treat (Ziegler, 2008). It is well reported that hyperglycemia induces hyperalgesia to thermal (Courtex *et al*., 1993) and chemical noxious stimuli (Shingo *et al*., 2006), resulting due to hypersensitivity of neuron (Ibironke and Saba, 2006). Various class of drugs such as nonsteroidal anti-inflammatory drugs, antidepressants, anticonvulsants and opioids are currently under investigation in the management of diabetes induced neuropathic pain but the treatment with these drugs is limited because of their partial effectiveness and severe potential toxicity (Tavakoli and Malik, 2008). Opioids are effective antinociceptive drugs, however, their antinociceptive activity decreased in diabetes associated neuropathic pain (Boulton, 2005). Moreover, hyperglycemia is associated with decreased functional expression of opioid (μ) receptor which may contribute to the pathogenesis of diabetic neuropathy (Chen *et al*., 2002). The cause of diabetes induced neuropathic pain is still unclear (Calcutt and Backonja, 2007). However, various mechanisms have been proposed to be involved in the pathogenesis of diabetic neuropathy i.e., increased aldose reductase activity (Price *et al*., 2004), nonenzymatic glycation (Sugimoto *et al*., 2008), activation of protein kinase C (Shukla *et al*., 2006), increased oxidative stress (Ashok *et al*., 2010) and cytokines level (Yu *et al*., 2009) are the best studied. Among them, oxidative stress, resulting from increased production of Reactive Oxygen Species (ROS) and downregulation or insufficient upregulation of antioxidant defense is a well-recognized fundamental mechanism in diabetic complications (Obrosova *et al*., 2007). Superoxide, a primary free radical, produced in diabetic and hyperglycemic conditions rapidly combines with NO and the formed peroxynitrite causes protein nitration or nitrosylation, lipid peroxidation, DNA damage and cell death and has direct toxic effects on the nerve tissue leading to neuropathic pain (Drel *et al*., 2007; Obrosova *et al*., 2007).

Following nerve injury, neuropathic pain arises not only when these mechanisms are activated but also when peripheral and central mechanism sensitization is maintained (Troels and Nanna, 2009). There is accumulating evidence that indicates direct effects of hyperglycemia on the spinal cord, which modify sensory processing and contribute to behavioral indices of neuropathic pain (Calcutt and Backonja, 2007). Hyperglycemia-induced increase in cytokines such as tumor narcosis factor alpha (TNF-α), interleukin-2 (IL-2)

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and interferon gamma (IFNγ) is another important mechanism leading to both the development and progression of hyperalgesia in rats (Limiroli *et al*., 2002; Yu *et al*., 2009). Spleen mononuclear cells and microglia are regarded as a major source for the production of proinflammatory cytokines such as tumor necrosis alpha TNF-α, IL-2 and IFN-γ that are implicated in the genesis of neuropathic pain (Milligan *et al*., 2003). Minocycline, a microglial inhibitor, has been shown to prevent neuropathic pain and to attenuate heat hypersensitivity (Ledeboer *et al*., 2005). Pro-inflammatory cytokines are well reported to decrease the analgesic effect of morphine (Raghavendra *et al*., 2004). However, long-term treatment of diabetic rats with cyclosporine, an inhibitor of interleukin-2, is reported to restore the decreased antinociceptive effect of morphine (Kamei *et al*., 1993). Cytokines are known to induce the expression of inducible nitric oxide synthase (iNOS) (Deng *et al*., 1993) and iNOS inhibitors are reported to potentiate the analgesic effect of morphine (Ahmed *et al*., 2006). Moreover, we recently found that splenectomy in diabetic rats restored the decreased analgesic effect of opioids to the level seen in non-diabetic rats. Although, diabetic neuropathy is one of the most common etiologies of chronic pain in patients, the underlying mechanisms of analgesic resistance in diabetic patients remain poorly understood. Therefore, the present study was designed to investigate the effect of cyclosporine on hyperglycemia induced decrease antinociceptive effect of morphine in rats.

MATERIALS AND METHODS

Wistar rat (200-250 g) of either sex were used. They were housed in animal house provided with 12 h light/dark cycle and free access to water and food. The animal experiments were conducted in accordance with guidelines of Institutional Ethics Committee.

Preparation of spleen homogenate: After sacrificing the rats by cervical dislocation, spleen was removed and immersed in 1% Minimal Essential Media (MEM, pH = 7.8). The spleen was mashed, homogenized and centrifuged at 3000 rpm for 10 min. The supernatant of spleen homogenate (SHS) was used for the study. The supernatant of the spleen homogenate (SSCH) from each diabetic rat (28 day) (0.4 mL) was injected to each recipient rat (400 μL i.v.) through tail vein. Spleen Homogenate Supernatant (SHS) was used in place of mononuclear spleen cells to avoid any implication of immunogenic response.

Measurement of nociceptive threshold: The nociceptive latency was measured by tail-flick test (D'Amour and Smith, 1941). Tail-flick latency was considered as time between tail exposure to radiant heat and tail withdrawal. The intensity of radiant heat was selected so as to obtain a pretreatment latency between 2 and 4 S in non diabetic control animals. The maximum cut-off latency time was fixed at 10 S in analgesic treated animals. Tail-flick latency was expressed as a percentage of the maximum possible effect (% MPE):

$$\text{MPE} = \frac{\text{Post treatment latency time-Pretreatment latency time}}{\text{Cut off time-Pretreatment latency time}} \times 100$$

Pretreatment latency refers to the control latency before drug administration, while post-treatment latency refers to the latency after drug administration. Nociceptive latency was measured at 0, 15, 30, 60 and 180 min and expressed as mean latency.

Assessment of thermal hyperalgesia: The pain hypersensitivity to heat was tested according to the Hargreaves procedure (Hargreaves *et al*., 1988) using the Plantar test (Ugo Basile, Varese, Italy). The latency to the first sign of paw licking or withdrawal response to avoid heat pain was taken as an index of pain threshold.The withdrawal latency was averaged from at least three trials separated by a 10 min interval and the cut-off was set at 20 sec to avoid tissue damage. In brief, each animal were placed in a clear plexiglass box and hind paw was exposed to a constant beam of radiant heat through a plexiglass surface. The time, in seconds, from initial heat source activation until paw withdrawal was recorded.

Estimation of nitrite/nitrate: Each rat was placed individually in metabolic cage and its urine was collected for 24 h. The animals were allowed to drink water ad libitum before the study but were denied water during 24 h study period. Urinary and serum nitrite was estimated using Greiss reagent (1% sulphanilamide and 0.1% naphth-ylethylene diamine in 5% phosphoric acid) (Green *et al*., 1982) and O.D. was measured at 540 nm (Spectrophotometer, Beckman DU 640B, Switzerland), which served as an indicator of NO. Nitrite concentration was calculated using standard curve for sodium nitrite. Nitrite levels in urine were expressed as amount excreted in 24 h.

Experimental design: Group 1 received saline or vehicle served as control and tail flick and paw withdrawal latency was noted 30 min after administration of citrate buffer on different day's i.e., 0th, 7th, 14th, 21th and 28th day. Serum/urinary nitrite and fasting glucose levels were noted once weekly of citrate buffer administration. Group II (n = 6): Rats in group 2 were received intra-peritoneal injection of streptozotocin (STZ) at a dose of 50 mg kg^{-1},

i.p., once to induce experimental diabetes, after verifying the blood glucose level more than 300 mg dL^{-1} considered as diabetic and used in the present study. In group III splenectomy was carried out under light anesthesia in diabetic rats. The age-matched non-diabetic, diabetic and splenctomised diabetic rat, were given either saline or morphine (4 and 8mg kg^{-1}, s.c.) and were treated once daily (12.5 and 25 mg kg^{-1}) with cyclosporine. Maximum effect of morphine and cyclosporine was observed at a dose of 8 and 25 mg kg^{-1}, respectively. Therefore, we had used only 8 mg kg^{-1} morphine and 25 mg kg^{-1} cyclosporine in the entire study. Non-diabetic rats were administered SHS (400 µL i.v.) obtained from 28 days diabetic and non-diabetic rats for 28 days. Tail flick and paw withdrawal latency was noted 30 min after administration of vehicle or drug treatment on different days as described in group 1.

Drugs: Streptozotocin was obtained from Sigma Aldrich, USA and was dissolved in 0.1 N citrate buffer. Morphine was supplied by Jackson Labs, Amritsar, India. Cyclosporine was obtained from Novartis (Mumbai, India). The solutions of these drugs were prepared freshly before use.

Data analysis: All the values are expressed as Mean±SD One-Way ANOVA followed by Tukey's test were employed to calculate the statistical significance for multiple comparisons between groups. The level of significance was fixed at $p<0.05$.

RESULTS

Effect of streptozotocin (STZ) on serum glucose levels and body weight: As shown in Table 1, blood glucose levels in rats which had been rendered diabetic by STZ were significantly elevated ($p<0.01$) as compared to those in age-matched non-diabetic rats. Administration of morphine, cyclosporine and SHS (28 day) in non diabetic recipient rats did not modulate serum glucose level. However, body weights are significantly reduced in STZ treated rats ($p<0.05$) as compared with age matched vehicle treated (Table 1).

Effect of experimental diabetes on pain perception: Before STZ administration, the rats paw withdrawal latency in both left and right hind paws from radiant heat was about 17 sec. On day 7, following STZ administration the pain sensitivity in diabetic rats (planter test) was similar to the control animals (15.21±0.82). However, diabetic rats showed significant decrease in the paw withdrawal latency to thermal stimuli on day 28 after STZ injection ($p<0.01$), indicating development of thermal hyperalgesia (Fig. 1).

Effect of experimental diabetes and pharmacological intervention on antinociceptive effect of morphine: Administration of morphine (4 and 8 mg kg^{-1}, s.c.) was shown significant increase in %MPE as compared to that of age matched untreated rats ($p<0.05$). However, morphine induced increase in %MPE was significantly attenuated in diabetic rat ($p<0.05$) (Fig. 2) as compared with non diabetic rats. On the other hand, splenectomy in diabetic rat did not modulate analgesic effect of morphine ($p<0.05$) (Fig. 2). In addition, treatment of cyclosporine (CYS) prevent ($p<0.05$) diabetes induced decrease analgesic effect of morphine as compared with untreated diabetic rats (Fig. 2).

Table 1: Effect of STZ and pharmacological intervention on body weight and blood glucose levels

Treatments	Blood glucose (mg dL^{-1})	Body weight (g)
Control (saline treated)	84.23±6.89	32.54±2.43
Streptozotocin treated	488.42±14.76a	17.21±3.78a*
Morphine 8 mg kg^{-1} in DM	478.67±7.78	31.82±0.95
CYS 25 mg kg^{-1} in DM	392.21±9.21	30.22±2.52

Results are expressed as Mean±SDN: 6; a: $p<0.01$ vs. saline treated (mean blood glucose levels), a*: $p<0.05$ vs. control (mean body weight)

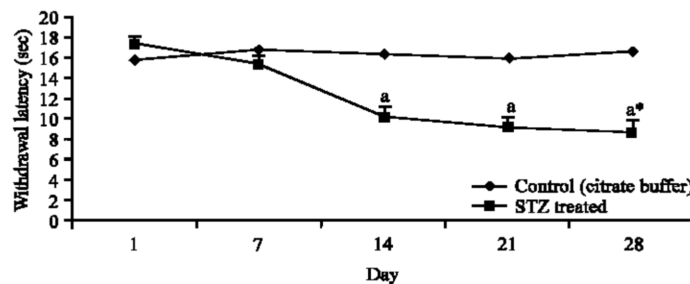

Fig. 1: Measurement of pain sensitivity on different days in both STZ and citrate buffer treated rats subjected to thermal test (planter test). Each value is expressed as Mean±SD: n = 6, a: $p<0.05$ vs. control: a*: $p<0.01$ vs. control

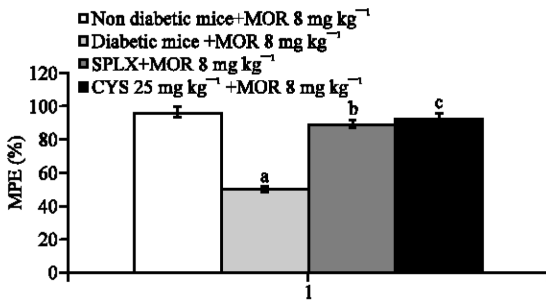

Fig. 2: Measurement of nociceptive latency by tail flick test (analgesiometer). The results are expressed as MPE%, $p<0.05$: a: $p< 0.05$ vs. Morphine in non diabetic rats, b: $p< 0.05$ vs. Morphine in diabetic rats; c: $p<0.05$ vs. morphine in diabetic rats. DM: diabetic rats, SPLX: Splenectomised diabetic rats. MPE% : Percentage maximum possible effect

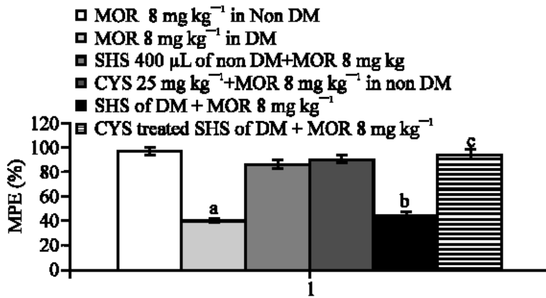

Fig. 3: The results are expressed as MPE%, $p<0.05$: a: $p<0.05$ vs. Morphine in non diabetic rats, b: $p<0.05$ vs. SHS of non diabetic rats; c: $p<0.05$ vs. SHS of diabetic rats. DM: Diabetic rats, CYS: Cyclosporine, MOR: Morphine, MPE % = Maximum possible effect%

Effect of SHS on antinociceptive effect of morphine: Experimental diabetes progressively decreases the analgesic effect of morphine as compared to that in non-diabetic rats ($p<0.01$) (Fig. 1). Administration of cyclosporine and SHS of Non diabetic rats did not alter the antinociceptive effect of morphine in non-diabetic rats (Fig. 3). However, administration of SHS of 28 day diabetic rats significantly decreased antinociceptive effect of morphine in non diabetic recipient rats ($p<0.05$) (Fig. 3). On the other hand, SHS obtained from CYS (25 mg kg^{-1}) treated diabetic rats did not modulate antinociceptive effect of morphine ($p<0.05$) (Fig. 3).

Effect of experimental diabetes and pharmacological intervention on urinary and serum nitrite levels: Experimental diabetes markedly increased urinary and serum nitrite concentration ($p<0.01$) (Table 2)

Table 2: Effect of experimental diabetes and pharmacological intervention on urinary and serum nitrite level

Treatment	Urinary nitrite concentration (µM/24 h)	Serum (µM L^{-1})
Non-diabetic ats	15.20±1.74	20.89±2.46
SHS of Non DM	16.01±2.41	20.14±3.01
Diabetic rats (DM)	30.40±3.90a	74.2±5, 45a
Splenctomised DM	17.05±1.76b	22.46±0.96 b
SHS (400 µL) of DM in Non DM	27.90±3.20a, c	62.87±6.09a, c
Cyclosporine treated, SHS of DM	16.40±2.5	21.15±2.87

Each value is the Mean±SD (n = 6). a: $p<0.01$ vs. non-diabetic rats, b: $p<0.05$ vs. diabetic rats, c: $p<0.05$ vs. non DM. DM diabetic rats, SHS: Spleen homogenate supernatant

Administration of SHS obtained from non-diabetic rats did not alter urine/serum nitrate level in non diabetic rats. However, administration of SHS obtained from 28th day diabetic rats significantly increased serum and urine nitrite levels in non diabetic rats (Table 2).On the other hand, SHS obtained from cyclosporine (25 mg kg^{-1} i.p.) treated diabetic rats and splenectomy in diabetic rats was noted to attenuate diabetes induced increase NO level in serum and urine ($p<0.05$) as compared with non treated diabetic animal.

DISCUSSION

The results of the present study demonstrated a significant decrease in the antinociceptive effect of morphine both in diabetic and spleen homogenate recipient non diabetic rats. However, Cyclosporine treated diabetic and SHS rats was noted to attenuate hyperglycemia induced decrease in analgesic effect of morphine. STZ administration was markedly increase blood glucose level (Table 1, $p<0.01$).However, body weight significantly reduced in STZ treated animals (Table 1, $p<0.05$) as compared to saline or vehicle treated. It is reported that significant degree of hyperalgesia and allodynia developed in rats after 3 weeks of STZ administration (Grover *et al*., 2000; Ibironke and Saba, 2006). Therefore, rats were kept for 4 weeks after STZ administration to provide sufficient time for hyperglycemia to affect pain perception. Following STZ administration, on day 28, paw withdrawal latency to thermal stimuli was markedly decreased (Fig. 1, $p<0.01$), indicating that diabetic rats exhibit thermal hyperalgesia.Similar models of thermal hyperalgesia and allodynia in streptozotocin-induced rats has been demonstrated previously (Courteix *et al*., 1993). Increase in blood glucose level is well documented to decrease antinociceptive effect of opioids (Courteix *et al*., 1998) and NSAIDs (Tsiklauri and Tsagareli, 2006). However, the mechanism of this decreased antinociceptive effect of analgesics as a consequence of diabetes is not known.

Various mechanism has been proposed to be involved in opioids tolerance such as activation of NMDA (Chen *et al*., 2009), PKA (Smith *et al*., 1999), PKC (Shukla *et al*., 2006), receptor desensitization, increase oxidative stress (Ashok *et al*., 2010), cytokines (Johnston *et al*., 2004) and NO level (Ahmed *et al*., 2006). Spinal proinflammatory cytokines such as TNF-α, IL-2 and IFN-γ are powerful pain-enhancing signals that contribute into neuropathic pain (Doupis *et al*., 2009). Moreover, cytokines are noted to interact with opioid receptors and modulate its action (Raghavendra *et al*., 2002). Spleen is a rich source of cytokines. Spleen derived factor is reported to modulate antinociceptive effect of morphine (Kamei *et al*., 1994, 1998). Therefore, it is possible that the observed decrease in antinociceptive effect of various analgesics in diabetic rats was due to an increased formation and release of factor (s) from mononuclear cells of spleen.

In the present investigation, as depicted in Fig. 1 and 2, thermal hyperalgesia was observed in both diabetic ($p<0.01$) and SHS ($p<0.05$) obtained from 28 day diabetic rats (400 μL i.v.), treated non-diabetic rats. On day 28, after STZ injection, significant analgesic tolerance to morphine was observed as compared with control animals (Fig. 1). However, splenectomy was noted to improved diabetes induce decreased in antinociceptive effect of morphine (Fig. 2, $p<0.05$). The results indicate the involvement of spleen derived factor (s) in morphine tolerance, which was fully consistent with previous report by Kamaie *et al*. (1998). Moreover, administration of cyclosporine, an interleukin-2 inhibitor, was observed to prevent SHS and diabetes induces decrease in analgesic effect of morphine (Fig. 2, 3, $p<0.05$). It seems that the factor(s) synthesized and release from spleen mononuclear cells may be cytokines or cytokines like substance. Pro-inflammatory cytokines are reported to modulate nociceptive threshold and are thought to be involved in analgesic tolerance (Raghvandra *et al*., 2002). Cytokines are known to induce the expression of inducible nitric oxide synthase (iNOS) (Deng *et al*., 1993). Nitric oxide is reported to be involved in diabetes induced decreased antinociceptive effect of morphine (Yu *et al*., 2006). Therefore it is possible that cytokines unregulated the expression of iNOS and the formed nitric oxide interferes in pain perception. In the present study, as depicted in Table 2, both diabetic and SHS treated non-diabetic rats was noted to elevate NO concentration in both urine and serum test ($p<0.01$). However, heated SHS administration of diabetic rats did not produce any significant effect on nociceptive threshold or nitric oxide level (data not shown).On the other hand, non heated fraction of SHS significantly decrease nociceptive threshold and increase nitrite level, indicating the involvement of SHS of diabetic rats in decreasing analgesic effect of morphine mediated trough increase expression of iNOS. Furthermore, splenectomy was noted to attenuate diabetes induced increase nitric oxide levels in both urine and serum test (Table 2, $p<0.05$). We observed that administration of cyclosporine in diabetic rats and cyclosporine treated SHS (28 day) recipient rats did not modulate antinociceptive effect of morphine. These results support our speculation that cyclosporine attenuated morphine analgesic tolerance attributed to decrease cytokines and iNOS expression that consequently decrease nitric oxide level. This is in accordance with previous reports showing enhancement of morphine antinociception following cyclosporine administration in rat (Kamei *et al*., 1993; Banafshe *et al*., 2005).

CONCLUSION

On the basis of present data, it is conclude that an increase in cytokine (IL-2) release and factor (s) from spleen mononuclear cells was responsible for the observed decrease in antinociceptive effect of morphine in diabetic rats and cyclosporine can be used a novel therapeutic approach to manage painful diabetic neuropathy. Although further studies are necessary to reveal the exact mechanism involved in hyperglycemia induce decreased analgesic effect of morphine.

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