ISSN: 0975- 08232

IJPSR (2010), Vol. 1, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received 28 February, 2010; received in revised form 24 April 2010; accepted on 26 April 2010

POSSIBLE MECHANISM OF HYPERGLYCEMIA INDUCED DECREASE IN ANTINOCICEPTIVE EFFECT OF ANALGESICS IN RATS

Rajeev Taliyan [*], Manjeet Singh [#] and Pyarelal Sharma

I. S. F. College of Pharmacy, Moga (Punjab), India

Keywords:

Spleen homogenate,
Streptozotocin,
Hyperalgesia,
Diabetes,
Diabetic Neuropathy

***Correspondence for Author**

Rajeev Taliyan

I. S. F. College of Pharmacy, Moga (Punjab), India

E-mail- taliyanraja@gmail.com

Deceased on 30-03 2009

ABSTRACT

Diabetes induced neuropathic pain is recognized as one of the most difficult pain to treat and conventional analgesic are well known to be partially effective or ineffective. Non-steroidal anti-inflammatory drugs and opioids are effective antinociceptive drugs, however, their antinociceptive activity decreased in diabetic neuropathy. The study was designed to investigate the mechanism of diabetes induced decrease in the antinociceptive effect of analgesics in rats. Streptozotocin (STZ) (20 mg/kg, *i. p.* 4 days) was administered to induce experimental diabetes in the rats. One week after the administration of STZ, the tail-flick and paw withdrawal test was performed. 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. Thermal hyperalgesia was noted in both diabetic and SHS (400 µl, *i. v.*) treated non-diabetic rats. Moreover, analgesic effect of morphine (8mg/kg, *s.c.*), lysine acetylsalicylic acid (400mg/kg *i. v*) and indomethacin (10 mg/kg, *i. p*.) was progressively decrease in diabetic and SHS of 28 day diabetic treated non diabetic rats. However, analgesic effect of morphine (8mg/kg s. c.), lysine acetylsalicylic acid (400mg/kg *i.v*) and indomethacin (10 mg/kg, *i. p*.) were improved in splenctomised diabetic rat. Administration of Cyclosporine (25 mg/kg, *i.p*), an IL-2 inhibitor, attenuated diabetes and SHS induced decrease in nociceptive threshold. It is concluded that spleen derived factor (s) and cytokines may be responsible for the observed decrease in antinociceptive effect of analgesics in diabetic rats.

INTRODUCTION: Neuropathy is the most common complication associated with diabetic patient and recognized as one of the most difficult types of pain [1]. It is well reported that hyperglycemia induces hyperalgesia to thermal [2] and chemical noxious stimuli [3], due to hypersensitivity of neuron [4]. Moreover, experimental diabetes mellitus is reported to attenuate antinociceptive effect of morphine [5], acetyl salicylic acid and diyprone in [6].The cause of diabetes induced decreased antinociceptive effect of analgesics in diabetic neuropathy is remain poorly understood [7].Various mechanisms have been proposed to be involved in the pathogenesis of diabetic neuropathy i.e. increased aldose reductase activity [8], non-enzymatic glycation [9], activation of protein kinase C [10], increase oxidative stress and cytokines levels.

STZ induced hyperalgesia and allodynia was thought to result exclusively from altered neuronal activity in the primary sensory and spinal cord neurons, probably via the release of neuroactive factors such as prostanoids and cytokines [11]. Spleen mononuclear cells and microglia are regarded as a major source for the production of proinflammatory cytokines such tumor nacrosis alpha (TNF-α) interleukine-2 (IL-2), IL-6 and interferon gamma (IFN-γ) and are implicated in the genesis of neuropathic pain [12].Following nerve injury, activation of glial cell, specifically microglia and astrocytes has been reported to be involved in maintenance of neuropathic pain [13] Administration of minocycline, a microglia inhibitor, was shown to prevents hyperalgesia and allodynia [14]. Hyperglycaemia-induced pro-inflammatory cytokines such as TNF- α, IL-2, IL-6 and IFN-γ are well reported to be involved in development of analgesics tolerance. Previously, it has been reported that long-term treatment of diabetic rats with cyclosporine, an inhibitor of interleukin-2, restores decreased antinociceptive effect of morphine [16]. In addition, it has been found that splenectomy in diabetic rats restored the decrease analgesic effect of analgesics 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 possible mechanism of hyperglycaemia induced decrease in antinociceptive effect of analgesics in rats.

MATERIALS & METHODS: Wistar rats of either sex (250- 300g) were used. They were housed in animal house provided with 12-h light/dark cycle and free access to water and food. All experiments were conducted in accordance with National Institute of Health guidelines and Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals on the welfare of experimental animals and the study protocol was approved by Institutional Ethics Committee.

Preparation of Spleen Homogenate: After sacrificed 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 15 minutes. The supernatant of spleen homogenate (SHS) was used for the study. The supernatant of the spleen

homogenate (SSH) 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 [17]. 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 pre-treatment latency between 2 and 4 S in non diabetic control animals. The maximum cut-off latency time was fixed at 12 S in analgesic treated animals. Tail-flick latency was expressed as a percentage of the maximum possible effect (% MPE).

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

Pre-treatment 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: Heat hypersensitivity was tested according to the Hargreaves procedure [18] using the plantar test (Ugo Basile, Varese, Italy) before diabetes induction and subsequent to STZ injection for 28 days. 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 s to avoid tissue damage. In brief, each animal were placed in a clear Plexiglas box and hind paw was exposed to a constant beam of radiant heat through a Plexiglas surface. Time, in seconds, from initial heat source activation until paw withdrawal was recorded.

Experimental design: Rats in saline or vehicle treated group served as control. Tail flick and paw withdrawal latency was noted 30 min after administration of citrate buffer or drug vehicle on different days i.e. 0^{th}, 7^{th}, 14^{th},21^{h} and 28^{th} day. Fasting glucose levels were noted once weekly of citrate buffer or drug vehicle administration. In Group 2, rats were received intra-peritoneal injection of STZ at a dose of 20mg/kg, for 4 consecutive days to induce experimental diabetes, after verifying the blood glucose level more than 400 mg/dl considered as diabetic and used in the present study.

There were six groups of age-matched non-diabetic, diabetic and splenctomised diabetic rats. Non-diabetic, diabetic and splenctomised diabetic group were either given (8 mg/kg, *s. c*.) of morphine sulfate or lysine acetyl salicylic acid (400 mg/kg *i. v.)* and indomethacin (10 mg/kg *i. p.*) 30 min before the tail-flick and paw withdrawal test respectively on different days as described in group 1. Cyclosporine (25 mg/kg, *i. p*.) was administered, both in non-diabetic and diabetic rats, daily for 28 days, starting one day before the administration of STZ (20 mg/kg, *i. p*., 4days). After 12 h of the last dose of Cyclosporine, tail-flick latency was noted 30 min after administration of morphine (8mg/kg), Indomethacin (10

mg/kg) or lysine acetylsalicylic acid (400 mg/kg) on different days as described in group 1. In a separate group, non-diabetic rats were administered SHS (400 µl, *i. v.*), obtained from 28 days diabetic rats, for 28 days, and tail flick / paw withdrawal test were conducted respectively 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). Lysine acetylsalicylic acid and Indomethacin was purchased from Ciron Ltd, Mumbai, India. The solutions of these drugs were prepared freshly before use.

Data analysis: All the results are expressed as Mean ± S.D. One-way ANOVA followed by Tukey's test were employed to calculate the statistical significance for multiple comparisons between groups. P<0.05 was considered statistically significant.

RESULTS:

Effect of STZ on serum glucose levels and body weight: Blood glucose levels in rats which had been rendered diabetic by streptozotocin were significantly elevated (Table-1, p<0.001) as compared to those in age-matched non-diabetic rats. Administration of morphine and SHS (28 day diabetic rats) in non diabetic recipient rats did not modulate serum glucose level. However, streptozotocin treatment significantly decreased rat body weight 258.4 ± 4.6 g (day 1) to 238.78 ±.5.67 g (day 28) (Table-1, p<0.05) as compared with age matched vehicle treated animals

TABLE 1: EFFECT OF STZ TREATMENT AND PHARMACOLOGICAL INTERVENTION ON SERUM GLUCOSE LEVELS AND BODY WEIGHT

S. No	Blood glucose (mg/dl)	Body weight (g)
Control (saline treated)	78.23 + 4.9	258.64 ± 4.6
Streptozotocin treated(DM)	428.42 a ± 14.76	218.28 a* ± 5.67
Morphine 8 mg/kg in DM	418.67 ± 7.78	220.62 ± 4.91
SHS treated Non DM	392.21 ± 9.21	252.82 ± 5.42

The mean of blood glucose levels and body weight in saline, STZ, morphine and spleen homogenate supernatant treated animals were measured before and after drug treatment once weekly and results are expressed as mean S.D.N=6; p<0.05; a=p<0.001 a*=p<0.05 vs saline treated (Body Weight).DM=Diabetic Rats

Effect of Streptozotocin (STZ) on pain perception: Before STZ administration, the rats paw withdrawal latency in both left and right hind paws from radiant heat was about 18 s. On day7, following STZ administration the pain sensitivity in diabetic rats (planter test) was similar to the control animals (18.01±0.92). However, STZ treated rats showed a progressive decrease in the paw withdrawal latency to thermal stimuli 18.21±0.92 (day7) to 8.45±0.72 (day 28, p<0.05 & p<0.001 on day 35), indicating that diabetic rats exhibit thermal hyperalgesia (Fig. 1).

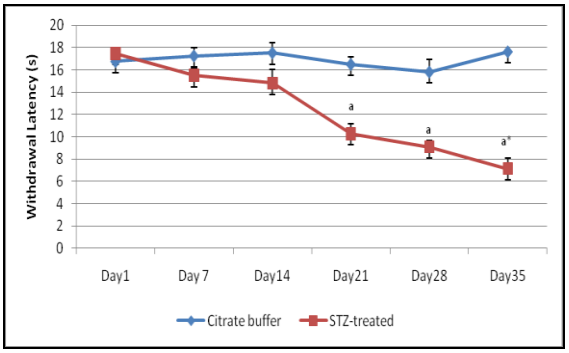

Fig 1: **Effect of STZ on Pain Perception**
Measurement of pain sensitivity on different days in both STZ treated and control rats subjected to thermal test (planter).Results are expressed as mean S.D: N=6 a=p<0.05 vs control: a*=p<0.01 Vs control

Effect of STZ treatment and pharmacological intervention on antinociceptive effect of morphine: Streptozotocin-induced hyperglycemia significantly attenuated the morphine-induced increase in %MPE as compared to that of non-diabetic rats ($p<0.001$) observed with tail flick test (Fig 2). Morphine analgesic effect, expressed as % MPE, was 67.70 ± 7.24 before streptozotocin treatment and this was reduced to 34.53± 4.41 (Fig. 2, $p<0.001$).However, administration of cyclosporine (25 mg/kg *i. p.*, once daily for 28 days) and splenectomy in diabetic rats was noted to improved diabetes induce decreased analgesic effect of morphine as compared with untreated diabetic rats (Fig. 2).

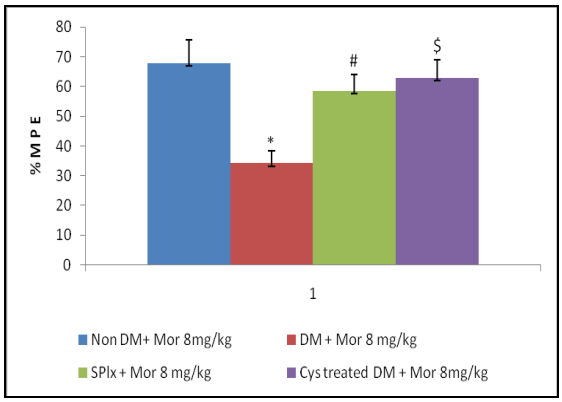

Fig-2: Effect of STZ treatment and pharmacological intervention on antinociceptive effect of morphine

Antinociceptive effect of morphine was measured using tail flick test and expressed as % maximum possible effect. * =p< 0.05 vs Morphine in non diabetic rats, # = p< 0.05 vs Morphine in diabetic rats;$= p< 0.05 vs morphine in diabetic rats.CYS=cyclosporine; DM=diabetic rats; SPLX=splenectomised diabetic rats. Mor =morphine

Effect of STZ and spleen homogenate supernatant on antinociceptive effect of morphine*:* STZ treatment progressively attenuated the morphine-induced increase in %MPE as compared to age matched citrate buffer treated control rats (Fig. 2, $p<0.001$). 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 STZ treated rats significantly decreased antinociceptive effect of morphine in non diabetic recipient rats ($p<0.05$)(Fig-3). On the other hand, administration of SHS obtained from CYS (25 mg/kg) treated diabetic rats did not modulate antinociceptive effect of morphine (Fig. 3, $p<0.05$) in recipient rats.

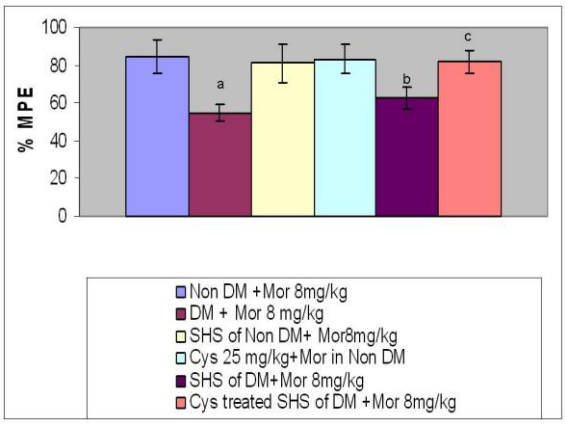

Fig-3: Effect of STZ and spleen homogenate supernatant on antinociceptive effect of morphine

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 = percentage maximum possible effect.

Effect of Splenectomy in diabetic rats and cyclosporine treatment on antinociceptive effect of lysine-acetylsalicylic acid and indomethacin: Streptozotocin induced diabetes significantly attenuated acetylsalicylic acid and indomethacin-induced increase in %MPE as compared to that of non-diabetic rats (Fig. 4, $p<0.05$). However, administration of cyclosporine (25 mg/kg) and Splenectomy in diabetic rats

significantly restored diabetes induced decrease antinociceptive effect of acetylsalicylic acid (400 mg/kg) and indomethacin (10 mg/kg) (Fig. 4,p<0.05).

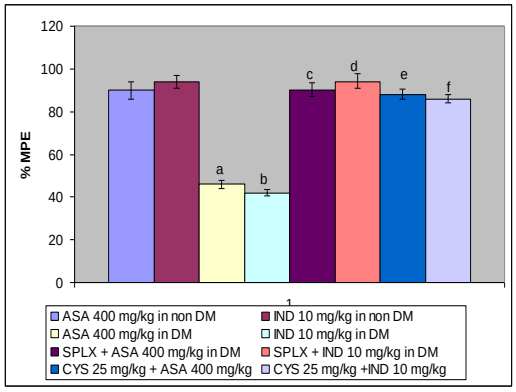

Fig 4: Effect of splenectomy in diabetic and cyclosporine treatment on antinociceptive effect of acetylsalicylic acid and indomethacin

The results are expressed as % MPE; P<0.05,a = P < 0.05 Vs ASA in non diabetic , b = P < 0.05 vs IND in non diabetic ,c= P < 0.05 vs DM; d= P< 0.05 vs DM;e= P<0.05 vs DM;f= P<0.05 vs DM, % MPE = percentage máximum posible effect. CYs=cyclosporine (25 mg/kg.*i.p*).DM= diabetic rats,SPLX=splenectomy in diabetic rats

Effect of STZ and spleen homogenate Supernatant on antinociceptive effect of acetylsalicylic acid and indomethacin: Streptozotocin induced diabetes significantly attenuated acetylsalicylic acid and indomethacin-induced increase in %MPE as compared to those in non-diabetic rats (Fig. 5, p<0.05). Administration of cyclosporine (25 mg/kg) and SHS (400 µl) of non diabetic rats did not alter the antinociceptive effect of acetylsalicylic acid (400mg/kg) and indomethacin (10 mg/kg) in non-diabetic animals. However, SHS obtained from diabetic rats significantly decreased antinociceptive effect of acetylsalicylic acid and indomethacin in non diabetic rats. In contrast to this, SHS obtained from CYS (25 mg/kg *i. p.*) treated diabetic rat did not modulate antinociceptive effect of acetylsalicylic acid and indomethacin in recipients rats. (Fig. 5)

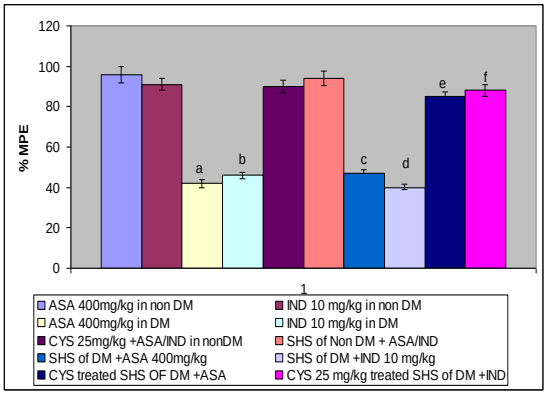

Fig 5: Effect of STZ and spleen homogenate supernatant (SHS) on antinociceptive effect of acetylsalicylic acid or indomethacin

The results were expressed as % MPE; P<0.05: a =P < 0.05 Vs ASA in non DM; b= P<0.05 vs IND in non DM; c= P < 0.05 vs SHS of non DM; d= P < 0.05 vs SHS of non DM; e=P< 0.05 vs SHS of DM; f=P< 0.05 vs SHS of DM; CYS=cyclosporine (25 mg/kg. *i .p*),

DM=diabetic Rats; % MPE = Percentage maximum possible effect

DISCUSSION: The results of the present study demonstrated a significant decrease in the antinociceptive effect of morphine, lysine acetylsalicylic acid and indomethacin in diabetic and spleen homogenate of diabetic recipient non diabetic rats. However, Cyclosporine treated diabetic and SHS rats was noted to attenuate hyperglycemia induced decrease in antinociceptive effect of analgesics. Accumulating evidence indicates that significant degree of thermal hyperalgesia and allodynia developed in rats after 3 weeks of STZ administration [19, 4]. Therefore, rats were kept for 4 weeks after STZ administration to provide sufficient time for hyperglycemia to affect pain perception. Administration of STZ was significantly increased blood glucose level (Table 1, p<0.001), however, body weight of STZ treated animals progressively deceased as compared to age matched control animals (Table 1, p<0.05).Moreover, nociceptive threshold was progressively decreased in STZ treated rats, and significant thermal

hyperalgesia was noted on day 28 (Fig. 1), which are fully consistent with previous report [19]. Hyperglycemia is well documented to decrease antinociceptive effect of opioids [20, 4], and non- steroid anti-inflammatory drugs [21-6].However, the mechanism of this decreased antinociceptive effect of analgesic as a consequence of diabetes is not known. Various mechanisms has been purposed to be involved in opioids tolerance such as activation of NMDA [22], PKA, PKC [23], receptor desensitization [24], increase oxido- nitrosative stress [25], cytokines 26 and NO level [27]. Spinal pro- inflammatory cytokines such as TNF-α, IFNγ and ILs are powerful pain-enhancing signals that contribute into neuropathic pain [28, 15].

Moreover, pro-inflammatory cytokines are noted to interact with opioid [29] and cyclooxygenase (COX) receptors and modulate its action [30, 5]. Cyclo-oxgenase is an enzyme responsible for generating inflammatory mediators such as prostaglandin, leukotrienes and contribute significantly in pain hypersensitivity. Cox-1 was constitutive and that Cox-2 was exclusively a pro-inflammatory inducible enzyme was induced by proinflamatry cytokines.COX-2 inhibitor such as meloxicam has been reported to prevent diabetes induced neuropathy [31, 4]. It seems that nerve injury induced cytokines up- regulate the expression of COX enzyme and contributed in abnormal pain processing.

Following nerve injury, activation of immune-like glial cells such as astrocytes or microglia has been reported, and may contribute to hyperalgesia, mechanical allodynia or chronic inflammatory pain in animal models [12]. Microglia activation can be induced, following nerve injury and released diffusible substances from neurons such as PGs, nitric oxide, fractalkine, substance P, and excitatory amino acids that consequently increased release of cytokines and prostaglandins [13]. These inflammatory agents, commonly cytokines, have been shown to activate and/or enhance the sensitivity of primary afferents and spinal cord neurons, and thus cytokines play a key role in nociceptive processing [32]. Spleen mononuclear cells and microglia are regarded as a source of cytokines. Spleen derived factor is reported to modulate antinociceptive effect of morphine [33, 34]. Therefore, it is possible that the observed decrease in antinociceptive effect of opioids and NSAIDs analgesics in diabetic rats may be due to an increased formation and release of factor (s) from mononuclear cells of spleen.

In the present investigation, thermal hyperalgesia was observed in both diabetic (Fig. 1, $p<0.05$) and SHS, obtained from 28 day diabetic rats (400 µl, *I .v.*), treated non-diabetic rats (Fig. 3, $p<0.05$). In addition, on day 28 after STZ injection, significant analgesic tolerance to morphine (Fig. 2, $p<0.001$) and decreased efficacy of NSAIDs (Fig. 4, $p<0.05$).However, splenectomy was noted to improved diabetes induce decrease in antinociceptive effect of analgesics (Fig. 2 & Fig. 4). This indicates the involvement of spleen or spleen derived factor (s) in diabetes induce decreased analgesic effect of opioids and NSAIDs which was fully consistent with previous report [24]. Administration of cyclosporine, an interleukin-2 inhibitor, was observed to prevent SHS and diabetes induces

decrease in nociceptive threshold. It seems that the factor(s) synthesize and release from spleen may be cytokine or cytokines like substance. Pro-inflammatory cytokines are well documented to modulate nociceptive threshold and are thought to be involved in analgesic tolerance [26].

Further, to confirm the involvement of spleen derived factor (s), and its nature, we had prepared two fraction of diabetic spleen i. e. heated and non heated fraction and administered in non diabetic recipient rats for 28 days. We found that administration of heated SHS fraction of diabetic rats in non diabetic recipients rats did not produce any significant effect on nociceptive latency or nitric oxide level (data not shown). On the other hand, administration of non heated fraction of SHS significantly modulate pain perception (Fig. 3 & Fig. 5, $p<0.05$) supporting our speculation that spleen derived factor (s) may be involved in decreasing analgesic effect of morphine. Moreover, it indicates that released factor (s) of mononuclear cells of spleen is heat labile On the other hand, as depicted in Fig. 2 and Fig-4, administration of cyclosporine in diabetic rats and cyclosporine treated SHS (28 day diabetic) recipient rats significantly prevent decreased antinociceptive effect of morphine and acetylsalicylic acid/indomethacin respectively(Fig. 2 & 3). It seems that cyclosporine attenuated morphine and NSAIDs analgesic tolerance attributed to decrease cytokine or cytokines like substance that may be synthesized or released from spleen mononuclear cells. This is in accordance with previous reports which show enhancement of morphine antinociception following cyclosporine administration in rat [16].

CONCLUSION: In conclusion, we demonstrated that an increase in cytokines or cytokines like substances from spleen mononuclear cells was responsible for the observed decrease in antinociceptive effect of analgesics in diabetic rats.

ACKNOWLEDGEMENT: This work is dedicated to Prof Manjeet Singh, who expired, while this work was in progress. Sincere thanks to Mr Parveen Garg, Chairmen I.S.F. College of Pharmacy, Moga, Punjab for providing best research facilities.

REFERENCES:

1. Ziegler Den: Painful diabetic neuropathy treatment and future aspects. Diabetes Metab Res Rev.2008;1: S52-57

2. Courteix C, Eschalier A, and Lavarenne J; Streptozocin-induced diabetic rats: behavioural evidence for a model of chronic pain. Pain 1993; 53: 81-88.

3. Shingo Y, Yasuyuki S, Mitsutoshi Y, Yoshio K ,Shuichi T , Satoshi W , Masaki A and Ken-ichi M : Antinociceptive effect of methyleugenol on formalin-induced hyperalgesia in . Europ. J. of Pharmacol 2006; 1(3):99-103.

4. Ibironke GF and Saba OJ: Effect of hyperglycemia on the efficacy of morphine analgesia in rats. Afr J Med Med Sci.2006; 5(4):443-5.

5. Kamei J, Sodeyama M, Ohsawa M, Kimura M, Tanaka S. Modulation by serum glucose levels on morphine-induced antinociceptive effect in c57bl/ksj-dbidb mice. Life Sci 1998; 62(1)14

6. Tsiklauri N, Gurtskaia G, Tsagareli M**.** Is endogenous opioid system involved in non-opioid analgesics tolerance**?** Georgian Med News 2006; 137:121-125.

7. Calcutt NA and Backonja MM: Pathogenesis of pain in peripheral diabetic neuropathy. Cur Diab Reports 2007; 7: 429-434.

8. Price SA, Agthong S, Middlemas AB, and Tomlinson DR: Mitogen-activated protein kinase p38 mediates reduced nerve conduction velocity in experimental diabetic neuropathy: interactions with aldose reductase. Diabetes 2004; 53: 1851–1856.

9. Sugimoto K, Yasujima M, Yagihashi : Role of advanced glycation end products in diabetic neuropathy. Curr Pharm. 2008; 14(10):953-61.

10. Kasuya Y, Mizubayashi R, Miwa K: A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes.1999; 48: 2090–2095.

11. Elena L, Leda G, Alberto EP, and Paola S: Differential morphine tolerance development in the modulation of macrophage cytokine production in mice. Journ of Leuk Biol 2002; 72: 42-48.

12. Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, Tracey K, Martin D, Maier SF, and Watkins LR: Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J Neurosci 2003; 23: 1026–1040.

13. Inoue K, Tsuda M, Tozaki-Saitoh H: Modification of neuropathic pain sensation through microglial ATP receptors .Purinergic Signalling 2007; 3:311–316

14. Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, and Watkins LR: Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 2005; 115: 71-83.

15. Raghavendra V, Rutkowski MD, DeLeo JA: The role of neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham operated rats. J. Neurosci 2002; 22: 9980-9989.

16. Kamei J, Kawashima N, Suzuki T, Misawa M, and Kasuya Y: The effects of cyclosporine on morphine-induced antinociception in diabetic. Neurosci. Lett 1993; 158: 213–216.

17. D'Amour, WL, and Smith DL: A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther 1941; 72: 74–79.

18. Hargreaves KM, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1998; 32: 77–88.

19. Courteix C, Eschalier A, Lavarenne J; Streptozocin-induced diabetic rats: behavioural evidence for a model of chronic pain. Pain 1993; 53:81-88.

20. Raz I, Hasdai D, Seltzer Z, Melmed RN: Effect of hyperglycemia on pain perception and on efficacy of morphine analgesia in rats. Diabetes 1988; 37, 1253–1259.

21. Tortorici V, Nogueira L, Aponte Y, Vanegas H. Involvement of cholecystokinin in the opioid tolerance induced by dipyrone (metamizol) microinjections into the periaqueductal gray matter of rats. Pain 2006; 112 (1-2):113-120.

22. Chen SR, Samoriski G, and Pan.HL: Antinociceptive effects of chronic administration of uncompetitive NMDA receptor antagonists in a rat model of diabetic neuropathic pain. Neuropharmacol 2009; 257(2): 121-6.

23. Shukla PK, Tang L, Wang ZJ: Phosphorylation of neurogranin, protein kinase C,and Ca2+/calmodulin dependent protein kinase II in opioid tolerance and dependence. Neurosci Lett.2006; 404(3): 266-269.

24. Courteix C, Bourget P, Caussade F, Bardin M, Coudore F, Fialip F and Eschalier A: Is the Reduced Efficacy of Morphine in Diabetic Rats Caused by Alterations of Opiate Receptors or of Morphine Pharmacokinetics? J Pharmacol Exp Therap 1998; 285: 63–70

25. Irina G. Obrosova, Viktor R. Drel, Christine L. Oltman, Nazar Mashtalir, Jyoti Tibrewala, Groves J T, and Mark A Y: Role of nitrosative stress in early neuropathy and vascular dysfunction in streptozotocin-diabetic rats. Am J Physiol Endocrinol Metab 2007; 293: E1645–E1655

26. Johnston IN, Milligan ED, Wieseler-Frank J, Frank MG, Zapata V, Campisi J, Langer S, Martin D, Green P, Fleshner M, Leinwand L, Maier SF, and Watkins LR: A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J Neurosci 2004; 24(33): 7353-65

27. Grover V S, Sharma A, and Singh M; Role of nitric oxide in diabetes-induced attenuation of antinociceptive effect of morphine in mice. Eur Jour of Pharmacol 2000; 399:161-164

28. Doupis J, Thomas E L, Szuhuei Wu, Charalambos G, Thanh D, and Aristidis V: Microvascular Reactivity and Inflammatory Cytokines in Painful and Painless Peripheral Diabetic Neuropathy.J Clin Endocrinol Metab 2009; 94: 2157-2163.

29. Raghavendra V, Rutkowski MD, DeLeo JA. Attenuation of Morphine Tolerance, Withdrawal-Induced Hyperalgesia, and Associated Spinal Inflammatory Immune Responses by Propentofylline in Rats. Neuropsychopharmacol 2004; 29**:** 327–334.

30. Zhu X, Eisenach JM. Cyclooxygenase-1 in the spinal cord is altered after peripheral nerve injury. Anesthesiol, 2003; 99:1175–9

31. Satako K and Hitoshi K: Demonstration of Antiallodynic Effects of the Cyclooxygenase-2 Inhibitor Meloxicam on Established Diabetic Neuropathic Pain in Mice. Journal of Pharma Sci 2009; 110(2):213-217.

32. Yu LN, Yang XS, Hua Z, Xie W: Serum levels of pro-inflammatory cytokines in diabetic patients with peripheral neuropathic pain and the correlation among them. Zhongahua Yi Xue Za Zhi 2009; 89(7):469-71

33. Kamei J, Iwamoto Y, Misawa M, Nagase H, Kasuya Y: Evidence for differential modulation of mu-opioid receptor-mediated antinociceptive and antitussive activities by spleen-derived factor(s) from diabetic mice. Neuropharmacol 1994; 33(12):1553-8.

34. Nadeem K, Singh N and Jaggi A: Possible role of spleen derived factor, vanilloid receptor and calctonin gene related protein in diabetese induced hyperalgesia in mice. Yakkujakku jasshi 2008*; 12(*11): 1699-1705.