

**NEUROPROTECTIVE EFFECT OF EARLY TREATMENT WITH
PIOGLITASONE AND METHYLCOBALAMIN IN ALLOXAN INDUCED
DIABETES IN RATS.**

Aashish .S.Morani, Subhash.L.Bodhankar

*Department of Pharmacology, Bharati Vidyapeeth University, Poona College of
Pharmacy, Pune 411 038, Maharashtra, India*

Summary

Neuropathic pain in diabetics is characterized by both hyperalgesia and allodynia. This is attributed to both uncontrolled glycemia and the further complications which it leads to. We evaluated the effect of concurrent administration of Pioglitazone along with methylcobalamin in diabetic rats. The parameters used were hyperalgesia produced by tail immersion (hot water), hot plate method and allodynia produced by tail immersion in cold water. A gradual reduction in weight and locomotor activity along with a lowered pain latency was observed in alloxan treated animals. Whereas no change in pain latency was observed in drug treated animals indicating that the early treatment with pioglitazone and methylcobalamin could protect the animal from early hyperalgesia and allodynia produced by alloxan induced diabetes in rats.

Keywords: Pioglitazone, Methylcobalamin, Alloxan induced diabetic neuropathy, rats.

Address of Correspondance:

Aashish S Morani,
Department of Pharmacology,
Bharati Vidyapeeth University, Poona College of Pharmacy,
Erendwane, Pune 411 038,
Maharashtra, India.
Tel: 91 20 2543 72 37 (ext: 29).
Fax: 91 20 2435 93 86
E-mail: aashu110@gmail.com

Introduction

Diabetes mellitus is associated with several long term complications, prominent among them is peripheral neuropathy. Spontaneous pain, allodynia and hyperalgesia are frequently encountered in diabetic patients [1,2,3,4,5]. A review of published work on painful diabetic neuropathy suggests that a significant degree of neuropathic pain is more likely to occur in patients with uncontrolled diabetes and has been proposed that acute biochemical alterations in neural tissues might result from prolonged hyperglycemia and could contribute to the development of diabetic neuropathy [1,6]. Four main molecular mechanisms, namely increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms and increased hexosamine pathway flux have been implicated in glucose-mediated vascular damage and all seems to reflect a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain [7]. Clinical and experimental studies have revealed that reactive oxidant species (ROS) play a significant role in pathophysiology of neuropathic pain in diabetes [2]. Adequate metabolic control may reduce the symptoms of painful diabetic neuropathy [6]. Pioglitazone, a member of thiazolidinedione (TZD) family sensitizes peripheral tissues to insulin thereby ameliorating hyperglycemia and hyperinsulinaemia [8]. Ultra high doses of methylcobalamin (500µg/kg) was found to improve compound muscle action potential in acrylamide induced neuropathy. Also some reports suggest the probable role of methylcobalamin in nerve regeneration and its clinical use for the same in peripheral neuropathy [9]. Continuous treatment with methylcobalamin showed to ameliorate effects on peripheral lesions in experimental diabetic neuropathy [10]. The objective of this study was to evaluate the effect of early treatment with the combination of pioglitazone with methylcobalamin in prevention of neuropathic complications in alloxan induced diabetic rats.

Material and Methods

Animals: Adult male Wistar rats (200-250g) were obtained from National Toxicology Centre, Pune, India. On arrival, the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of $24\pm 2^{\circ}\text{C}$ and relative humidity of 30-70%. A 12:12 hr light : dark cycle was followed. All animals had free access to water and standard pellet laboratory animal diet. Animals were acclimatized to laboratory conditions before the experiments that were carried out between 0900 and 1700 hr. All the experimental procedure used in this study were reviewed and carried out in accordance with that described by Zimmerman et al [12]. The research protocol was approved by Institutional Animal Ethics Committee of Poona College of Pharmacy, Pune, India.

Preparation of the drug solution

Pioglitazone: Solution was prepared by dissolving pioglitazone (Alembic) in distilled water. The drug was prepared daily and was stored at room temperature away from sunlight and moisture. The volume of drug solution were calculated based upon the body weight of the animal.

Methylcobalamin:- Solution was prepared by dissolving methylcobalamin (Himedia) in distilled water. The drug solution was stored in air tight amber coloured bottles at room temperature in a cool and dry place away from moisture and sunlight.

Alloxan treatment: Following a 48 hr fast, the animals received intraperitoneal (i.p) injections of alloxan monohydrate (120mg/kg) dissolved in 0.9% sodium chloride. Control animals received i.p injections of 0.9% sodium chloride. Within 48 hrs following alloxan administration, blood glucose concentration were estimated by enzymatic GOD-POD (glucose oxidase-peroxidase) diagnostic kit method (Accurex) [13, 14]. The rats having serum glucose levels more than 250mg/dl were selected and used for the present study. After the induction of diabetes in the animals diabetic rats were randomly selected into three groups of 8 animals each. ie. control, diabetic untreated (DU) and diabetic animals treated with combination of pioglitazone (10mg/kg,O.D) with methylcobalamin (500mcg/kg, I.P) (DPM) treated. Treatment began from the day of BSL detection after

the alloxan treatment. Body weight was recorded daily and serum glucose level were measured on 15th and 30th day of study.

Assessment of thermal hyperalgesia and cold allodynia [2]

Tail immersion (warm water) test: Tail of rat was immersed in a warm water ($47\pm 1^{\circ}\text{C}$) bath until tail withdrawal (flicking response) or signs of struggle were observed (cut-off 15 sec).

Tail-immersion (cold water) test: The procedure was same as warm water test but the temperature of water was set at $10\pm 0.5^{\circ}\text{C}$, a temperature that is normally innocuous. The cut-off time was 15 sec.

Hot plate test: In this test, animals were individually placed on a hot plate (Ugo Basile hot plate, Versace Italy) with the temperature adjusted to $55\pm 1^{\circ}\text{C}$. The latency to the first sign of paw licking or jump response to avoid heat pain was taken as an index of pain threshold. The cut-off time was kept 10 sec so as to avoid damage to the paw. Both hyperalgesia and allodynia were assessed weekly till the end of the study.

Statistical Analysis

Data was expressed as mean \pm SEM of animals in each group. To determine the statistical significance, ANOVA followed by Tukey-Kramer test (Instat/ Graphpad) was used. Differences between means were considered statistically significant if $p < 0.001$.

Results

Effect of DPM on blood sugar level (BSL) in alloxan induced diabetes in rats.

Blood sugar levels of control group of animals did not alter throughout the experiment. In DU group blood glucose levels increased steadily on day 15th (346.19±6.86 mg/dl) and day 30th (384.81±4.91 mg/dl) after alloxan treatment indicating the incidence of diabetes in the animals throughout the experiment. There was no significant difference in BSL of DPM treated animals (298.79±4.31 mg/dl) 48 hrs post alloxan treatment as compared to DU group of animals (269.4±8.16 mg/dl) indicating the incidence of diabetes. But a gradual reduction in the BSL of DPM group was observed on day 15th (119.16[#]±8.12 mg/dl) and day 30th (127.26[#]±5.62 mg/dl) indicating glycemic controls by drug treatment.

Table 1. Effect of DPM on blood sugar levels (BSL) in alloxan induced diabetes in rats.

	<i>Blood sugar level in mg/dl</i>			
	<i>Pre-Alloxan treated</i>	<i>48 hr post alloxan treated</i>	<i>Day 15</i>	<i>Day 30</i>
Control	97.62 ±5.35	106.64±6.16	102.59±4.8	104.23±3.84
DU	94.51±6.21	269.4±8.16	346.19±6.86	384.81±4.91
DPM	90.9±2.8	298.79±4.31	119.16*±8.12	127.26*±5.62

*Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test.. p <0.001 = *. Data of DPM was compared with that of DU. n=8.*

Effect of DPM on hyperalgesia produced by tail immersion (hot water) in alloxan induced diabetes in rats.

There was no change in tail flick latency (sec) observed in control group of animals throughout the experiment. A gradual decline in the latency was observed in DU group of animals from day 7th (8.1±0.26 s) onwards which was observed minimum on day 28th (4.1±0.76 s), indicating the presence of neuropathic pain due to diabetes. DPM group of animals recorded a reduction in latency on day 7th (9.9±0.49 s) which was followed by increase in pain threshold time on subsequent days, indicating absence of algesia produced by tail immersion in hot water.

Table 2. Effect of DPM on hyperalgesia produced by tail immersion (hot water) in alloxan induced diabetes in rats.

	<i>Tail Flick latency in seconds</i>				
	<i>Pre-Alloxan treated</i>	<i>Day 7</i>	<i>Day 14</i>	<i>Day 21</i>	<i>Day 28</i>
Normal	11.6 ±0.7	12.4 ±0.61	10.81±0.42	12.0 ±0.91	11.21±0.41
DU	10.9±0.51	8.1±0.26	6.1±0.82	5.3± 0.81	4.1±0.76
DPM	12.2±0.59	9.9.±0.49	11.2*±0.52	12.8*±0.42	12.9*±0.12

*Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test.. p <0.001 = *. Data of DPM was compared with that of DU. n=8.*

Effect of DPM on allodynia produced by tail immersion (cold water) in alloxan induced diabetes in rats.

No significant change in latency was observed in control group of animals throughout the study. For DU group of animals there was a gradual reduction in latency (sec) observed from day 7th (10.2±0.49 s) till day 28th (4.1±0.8 s) indicating the presence of allodynia. In the DPM group of animals, no significant lowering in pain latency was exhibited which implies the protective action of drug treatment on allodynia produced by cold water.

Table 3. Effect of DPM on allodynia produced by tail immersion (cold water) in alloxan induced diabetes in rats.

	<i>Tail Flick latency in seconds</i>				
	<i>Pre-Alloxan treated</i>	<i>Day 7</i>	<i>Day 14</i>	<i>Day 21</i>	<i>Day 28</i>
Normal	13.6 ±0.41	14.1±0.21	13.2±0.6	13.9±0.36	13.0±0.7
DU	14.0±0.6	10.2±0.49	7.4±0.51	5.5± 0.94	4.1±0.8
DPM	14.4±0.82	11.6±0.2	13.9*±0.26	13.1*±0.15	14.2*±0.63

*Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test.. p <0.001 = *. Data of DPM was compared with that of DU. n=8.*

Effect of DPM on thermal hyperalgesia (hot plate) in alloxan induced diabetes in rats.

No significant change in latency was observed in control group of animals throughout the study. For DU group of animals there was a gradual reduction in latency (sec) observed from day 7th (8.0 ± 0.44 s) till day 28th (3.8 ± 0.19 s) where the pain was observed to be maximum, indicating the presence of algesia by heat. In the drug treated group of animals, no significant lowering in pain latency was exhibited which implies the protective action of DPM on hyperalgesia produced in diabetic animals.

Table 4. Effect of DPM on thermal hyperalgesia (hot plate) in alloxan induced diabetes in rats.

	<i>latency in seconds</i>				
	<i>Pre-Alloxan treated</i>	<i>Day 7</i>	<i>Day 14</i>	<i>Day 21</i>	<i>Day 28</i>
Normal	9.8 ±0.4	9.4 ±0.16	9.7±0.34	9.7 ±0.1	9.1±0.84
DU	9.4±0.64	8.0±0.44	6.4±0.52	5.0± 0.32	3.8±0.19
DPM	8.9±0.14	8.3±0.93	9.6*±0.24	9.1*±0.54	9.3*±0.14

*Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test.. p <0.001 = *. Data of DPM was compared with that of DU. n=8.*

Discussion

A strong relationship exists between glycemia and diabetic microvascular complications in both type 1 and type 2 diabetes [7]. Generation of superoxide due to oxidative stress in diabetes may be responsible for vascular and neuronal complications of painful neuropathy [2]. Early in the course of diabetes, intracellular hyperglycemia causes abnormalities in blood flow and increased vascular permeability. Quantitative and qualitative abnormalities of extracellular matrix contribute to an irreversible increase in vascular permeability. With time microvascular cell loss occurs in part as a result of programmed cell death. Hyperglycemia may also decrease production of trophic factors for endothelial and neuronal cells. Together, these changes lead to edema, ischaemia and hypoxia induced neovascularization in the retina, proteinuria, mesangial matrix expansion, glomerulosclerosis in the kidney and multifocal axonal degeneration in peripheral nerves [7]. Impaired blood flow also seems to contribute to noxious stimulus hypersensitivity. Oxidative stress related reduction in perfusion is thought to play a part in cardiac autonomic dysfunction and also in small fiber sensory neuropathy [2].

Alloxan and the products of its reduction, dialuric acid, establish a redox cycle with formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxidant species (ROS) with a simultaneous massive increase in cytosolic calcium concentration cause rapid Beta cell destruction [15]. Early pharmaceutical intervention against the long-term consequences of hyperglycemia-induced cross-linking prevents the development of severe late complications of diabetes. Pioglitazone, a PPAR gamma receptor agonist has been approved by FDA for lowering blood sugar in type 2 diabetes. Also studies indicate that pioglitazone is a powerful inhibitor of glycation, AGE formation and cross-linking [8]. Intravenous methylcobalamin treatment is a safe and potentially beneficial therapy for neuropathy in chronic hemodialysis patients [15]. Methylcobalamin has established therapeutic uses in the treatment of diabetic neuropathy. Also methylcobalamin treated rats showed significantly faster compound muscle action potential recovery in acrylamide induced

neuropathy [9]. Continuous treatment with methylcobalamin had an ameliorative effect on the peripheral nerve lesions in STZ induced diabetic neuropathy in rats [10].

It was observed that alloxan treated rats (DU) exhibited a marked increase in glycemia [Table 1] and water intake and a clear cut reduction in the progressive gain in body weight as compared to control and drug treated group (DPM). In addition, polydipsia, polyphagia, polyurea and reduced motility was also observed in DU group of animals. These results were in accordance with Aibel et al (2004). Both hyperalgesia and allodynia were established after 14 days of alloxan treatment which was observed behaviorally [Table 2,3,4]. The hyperalgesic response in tail-withdrawal test is generally attributed to central mechanisms whereas the hyperalgesic response on hot plate is attributed to the combination of both central and peripheral mechanisms [2]

Our results indicate that in untreated group of animals (DU), there was no glycemic control, which led to hyperalgesia and allodynia in the models of tail immersion (hot and cold) and hot plate method. Whereas by a proper check on blood glucose levels by pioglitazone 10 mg/kg, O.D alongwith the supplementation of methylcobalamin 500 mcg/kg, I.P protected the diabetic animals from algesia produced by noxious and non-noxious stimuli.

Hence through our findings we conclude that the onset of neuropathic complications could be prevented by early glycemic controls by pioglitazone and concurrent administration of methylcobalamin in diabetic rats.

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