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Anti neuroinflammatory effect of Vildagliptin in ischaemia-reperfusion induced cerebral infarction in normal and STZ induced type-II diabetic rats

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Abstract

Diabetes is one of the major risk factor for cerebral ischemic stroke. Increased base line levels of oxidative stress in diabetes will lead to cerebral ischemic damage. In pathological conditions such as cerebral ischemia/reperfusion injury, free radicals cause oxidative stress and inflammation leading to increased injury of brain. Inflammation is one of the major pathological mechanisms involved in cerebral ischemia and reperfusion injury. Vildagliptin newer anti-diabetic drug of the class DPP-4 inhibitors is reported to have anti-inflammatory properties apart from its antihyperglycemic activity. Therefore the aim of the present study is to evaluate the anti-inflammatory effect of Vildagliptin against cerebral infarction induced ischemia reperfusion injury in normal and STZ induced diabetic wistar rats. Cerebral infarction was induced by bilateral common carotid artery occlusion followed by 4 hr reperfusion. Percent infarction, inflammatory markers such as MPO, TNF-α, IL-6 and IL-10 were analysed. Treatment with Vildagliptin for a period of four weeks produced significant reduction in percent cerebral infarct volume. Vildagliptin at 10 mg/kg dose showed significant reduction in markers like MPO, TNF-α, IL-6 and IL-1β in diabetic group when compared to normal group and in contrast significant increase in anti-inflammatory marker like IL-10 levels. Vildagliptin showed significant cerebroprotective effect by antiinflammatory mechanisms.

Keywords: cerebral ischemia reperfusion injury, diabetes, inflammation, vildagliptin.

1. Introduction

Cerebral ischemic stroke is the leading cause of morbidity and mortality worldwide [1][2]. Type 2 diabetes mellitus (T2DM) further aggravates this condition [3]. Occurrence of cerebrovascular stroke is more in diabetes condition than in normal individuals [4]. Rapid reperfusion is inevitable to prevent cerebral ischemic events, however reperfusion is associated with exacerbation of brain injury [5]. Cerebral I/R injury triggers profound oxidative stress and inflammatory response [6][7]. Cerebral I/R injury activates variety of inflammatory factors of which NF-κB is important and initiate the transcription of genes associated with inflammation including TNF-α, IL-1β, IL-6 and IL-10 [8][9].

GLP-1 has been found to play a significant role in the brain [10]. GLP-1 receptors are widely expressed throughout CNS [11]. GLP-1 was rapidly degraded by Dipeptidyl peptidase-4 (DPP-4). DPP-4 inhibitors are the newer class of antidiabetic drugs that increases the biological halflife of GLP-1by preventing its degradation [12]. Vildagliptin is one of the DPP-4 inhibitor currently in clinical use for the treatment of T2DM and reported to have the antioxidant and anti-inflammatory properties [13][14].

In the present study we investigated the anti-inflammatory effect of vildagliptin in cerebral ischemia following bilateral common carotid artery occlusion (BCCAO) in normal and diabetic rats.

2. Materials and Methods

2.1 Chemicals

Streptozocin (Sigma Aldrich, India), Nicotinamide (Sigma Aldrich, India), 2,3,5-triphenyl tetrazolium chloride (TTC), Thiopentone sodium (Neon laboratories, India) Vildagliptin (Novartis, Hyderabad), MPO, TNF-, IL-6, IL-1β and IL-10(BIOSPES, China) and all other chemicals used were of analytical grade and purchased locally.

2.2 Animals

Adult wistar rats weighing 230-270 g were purchased from NIN Hyderabad, Telangana, India. Animals were maintained under a 12/12 hr light/dark cycle in ambient room temperature i.e., 24±1^{0}C. Animals were taken care in

compliance with the CPCSEA New Delhi. Experimental protocols were conducted in accordance with the approval of the IAEC.

2.3 Experimental procedure

2.3.1 Induction of diabetes

Rats were rendered Type2 diabetes by single injection of STZ (30 mg/kg i.v.) and Nicotinamide (150 mg/kg i.p.). After 72 hr animals were confirmed diabetes and those blood glucose levels in the range of 200-300 mg/dl were used in the present study.

2.3.2 Induction of cerebral infarction

Rats were anaesthetized with thiopental sodium (30 mg/kg, i.p.). Cerebral infarction was induced by the BCCAO (Bilateral Common Carotid Artery Occlusion) method described by Iwasaki *et al*., 1989. Common carotid arteries were identified, exposed and isolated from vagus nerve. Carotid arteries were occluded for 30 min followed by reperfusion for 4 h. the rectal temperature was maintained at 37 ± 0.5^0C. Animals those do not lose righting reflex or which are convulsed during ischemia are excluded from the study.

2.3.3 Measurement of percentage cerebral infarction

Rats were divided into groups: Normal, sham, I/R (ischemia + reperfusion), Vildagliptin treated. Vildagliptin was suspended in 0.1% NaCMC and were administered orally at the dose of 10 mg/kg for a period of four weeks. After the treatment period rats were subjected to ischemia/reperfusion, the brains were removed quickly and washed with ice cold buffer and later sliced into coronal sections of 2 mm thickness [15]. The slices were immersed in 1% solution of TTC stain. Red formazan pigment was observed in viable cells, whereas dead cells appeared pale in colour and was unstained [16]. Necrotic infarcted tissue was separated and weighed. Percent cerebral infarction was calculated.

2.3.4 Estimation of inflammatory markers

Vildagliptin 10 mg/kg dose was used for the estimation of inflammatory markers in the selected group of animals. Brain tissues were separated immediately after reperfusion period and washed with ice cold buffer. Brain tissues were homogenized with ice cold tris buffer and the supernatant was used for the estimation of inflammatory markers like MPO, TNF-α, IL-6, IL-1β and IL-10.

Detailed assay procedure for inflammatory markers

Before adding to wells, ABC working solution and TMB substrate (Kit Component 8) was equilibrated for at least 30 min at room temperature (37^0C).

1) Standard, test sample and control (zero) wells were set on the pre-coated plate respectively, and then, recorded their positions. It was recommended to measure each standard and sample in duplicate.
2) 0.1ml of 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml standard solutions was transferred into the standard wells.
3) 0.1 ml of Sample / Standard diluent buffer (Kit Component 3) was added into the control (zero) well.
4) 0.1 ml of properly diluted sample rat serum was added into test sample wells.
5) Sealed the plate with a cover and incubate at 37^0C for 90 min.
6) The cover was removed and discarded the plate content, clapped the plate on the absorbent filter papers.
7) 0.1 ml of Biotin conjugated (anti-Rat IL-6, anti-Rat IL-10, anti-Rat TNF-α, anti-Rat IL-1β antibody) working solution was added into the above wells (standard, test sample & zero wells). The solution was added at the bottom of each well without touching the side wall.
8) Sealed the plate with a cover and incubate at 37^0C for 60 min.
9) Manual Washing: The solution in the plate was discarded without touching the side walls. The plate was clapped on absorbent filter papers or other absorbent material. Each well was filled completely with wash buffer (Kit Component 10) buffer and vortexes mildly on ELISA shaker for 2 min, then aspirated contents from the plate, and clapped the plate on absorbent filter papers or other absorbent material. This procedure was repeated two more times for a total of THREE washes.
10) 0.1 ml of ABC working solution was added into each well, and covered the plate and incubated at 37oC for 30 min.
11) The cover was removed and washed the plate 5 times with wash buffer (Kit Component 10), and each time let the wash buffer stay in the wells for 1-2 min.
12) 90 µl of TMB substrate (Kit Component 8) was added into each well covered the plate and incubated at 37oC in dark for 25-30 min. And the shades of blue can be seen in the first 3-4 wells (with most concentrated Rat IL-6 standard solutions) the other wells show no obvious color.
13) 0.1 ml of Stop solution (Kit Component 9) was added into each well and mixed thoroughly. The color changes into yellow immediately.
14) O.D. absorbance at 450 nm in a microplate reader was read within 30 min after adding the stop solution.

2.3 Statistical analysis

All the values were expressed as mean ± SEM and analysed by one-way ANOVA followed by Tukey's *t* test ($p \leq 0.05$). The significance of differences was estimated by two-way analysis of variance followed by a post hoc test (Bonferroni's method) ($p \leq 0.05$). The statistical analysis was processed using Graph Pad Prism Version 5.0.

3. Results

3.1 Percentage Cerebral Infarction

Percentage cerebral infarction in normal rats following BCCAO was found to be 41.54±1.57. Significant reduction in infarction was observed at the dose level of 2.5, 5, 10 mg/kg of Vildagliptin (Table-1, Figure-1). Similarly, percentage cerebral infarction in diabetic rats following BCCAO was found to be 51.67±1.37. Significant reduction in infarction was observed at the dose levels of 2.5, 5, 10 mg/kg administration of Vildagliptin (Table-2, Figure-2). The effect of vildagliptin in diabetic groups was more pronounced when compared to normal groups. Hence the dose of Vildagliptin 10 mg/kg was selected for the study.

Table-1: Effect of Vildagliptin on Percentage Cerebral Infarction in normal rats. Vildagliptin (2.5, 5, 10 mg/kg,b.wt) was administered orally before BCCAO .

Groups (n=6)	Percentage Cerebral Infarction
Control	0.24±0.01
Sham	0.54±0.01
I/R	41.54±1.57*
Vildagliptin 2.5 mg/kg Treated	31.89±1.81*
Vildagliptin 5.0 mg/kg	19.17±2.33*
Vildagliptin 10 mg/kg	7.30±1.91*

Values were expressed as mean ± S.E.M (N=6 in each group) *P<0.05.

Figure 1: Effect of Vildagliptin on percentage cerebral infarction in normal rats

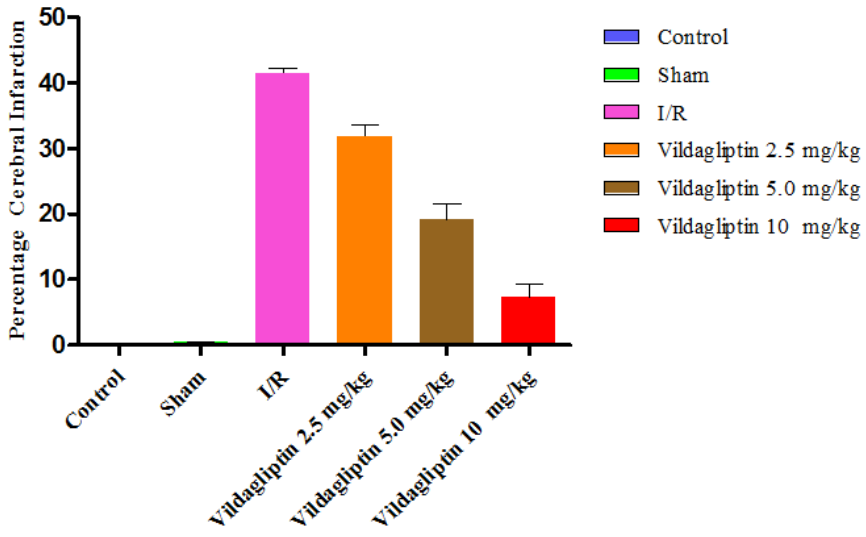
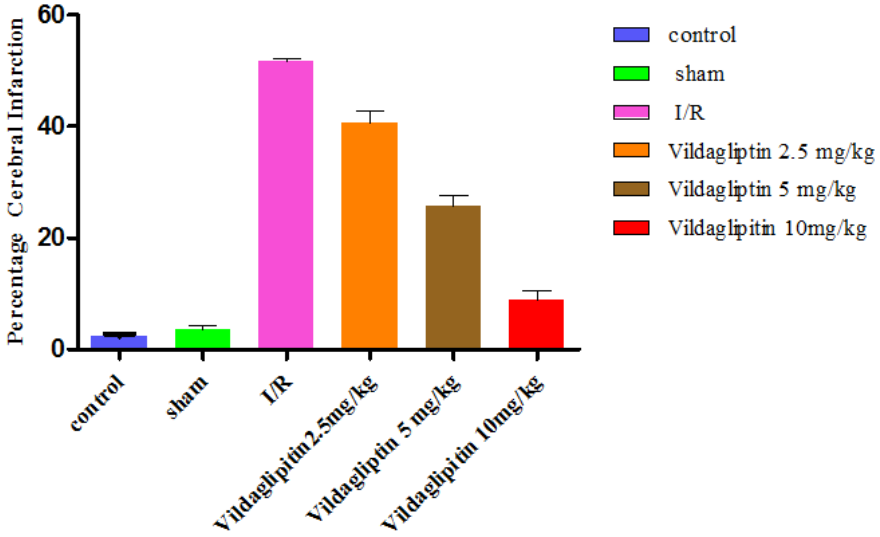


Table-2: Effect of Vildagliptin on Percentage Cerebral Infarction in Diabetic rats. Vildagliptin (2.5, 5, 10 mg/kg,b.wt) was administered orally before BCCAO .

Groups (n=6)	Percentage Cerebral Infarction
Control	2.32±1.10
Sham	3.53±1.54
I/R	51.67±1.37*
Vildagliptin 2.5 mg/kg	40.54 ± 2.34*
Vildagliptin 5.0 mg/kg	25.67 ± 1.91*
Vildagliptin 10 mg/kg	8.89 ± 1.57*

Values were expressed as mean ± S.E.M (N=6 in each group) *P<0.05.

Figure 2: Effect of Vidagliptin on percentage cerebral infarction in Diabetic rats



3.2 Effect on inflammatory markers

The effect of Vildagliptin following BCCAO on inflammatory parameters, such as MPO, levels in the brain were determined.

a) Effect on brain MPO levels

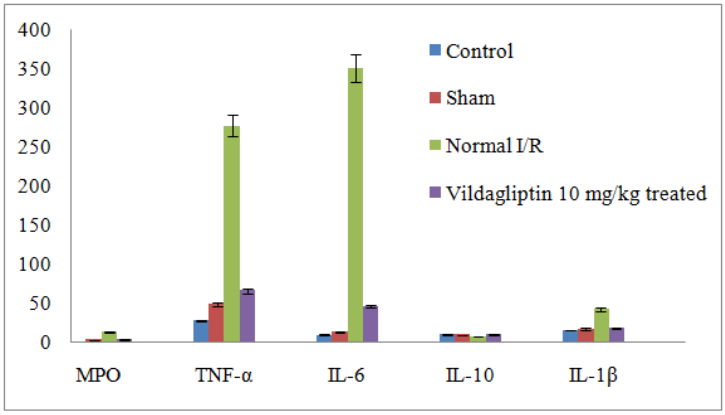
Myeloperoxidase levels in the brain were significantly higher in I/R group (12.92 ± 1.67) compared to sham operated rats (2.73 ± 2.22). In rats treated with vildagliptin 10 mg/kg b.wt, the elevated levels of MPO due to I/R injury were significantly attenuated in normal groups (3.06 ± 0.91) (Table-3). Similarly, MPO levels were higher in diabetic rats subjected to I/R injury (16.79 ± 1.65) compared to sham operated rats (3.45 ± 0.65). Vildagliptin treatment (4.23 ± 1.12) significantly attenuated the MPO levels when compared to diabetic I/R injury group (Table-4).

Table-3: Effect of Vildagliptin on Inflammatory Markers in Ischemia-Reperfusion Induced Cerebral Infarction in Normal Rats.

Parameter	Control	Sham	Normal I/R	Vildagliptin 10 mg/kg Treated
MPO (U/g tissue)	0.68 ± 1.45	2.73 ± 2.22	12.92 ± 1.67*	3.06 ± 0.91*
TNF-α (pg/mg of tissue)	27.34± 3.81	48.14 ± 4.43	276.42 ± 5.06*	65.33 ± 4.44*
IL-6 (pg/mg of tissue)	9.43± 1.34	12.32± 2.31	350.67 ± 24.34*	45.82 ± 3.57*
IL-10 (pg/mg of tissue)	10.02 ± 1.23	9.03 ± 1.42	6.86 ± 0.57*	9.39 ± 1.06*
IL-1β (pg/mg of tissue)	15.22 ±	16.87 ± 0.86	41.77 ± 1.77*	17.20 ± 1.33*

Vildagliptin (10 mg/kg,b.wt) was administered orally daily for a period of four weeks and were subjected to BCCAO . Values were expressed as mean ± S.E.M (N=6 in each group) *P<0.05.

Figure-3: Effect of Vildagliptin on Inflammatory Markers in Ischemia-Reperfusion Induced Cerebral Infarction in Normal Rats.



Vildagliptin (10 mg/kg,b.wt) was administered orally daily for a period of four weeks and were subjected to BCCAO . Values were expressed as mean ± S.E.M (N=6 in each group) *P<0.05.

b) Effect on brain* TNF-α *levels

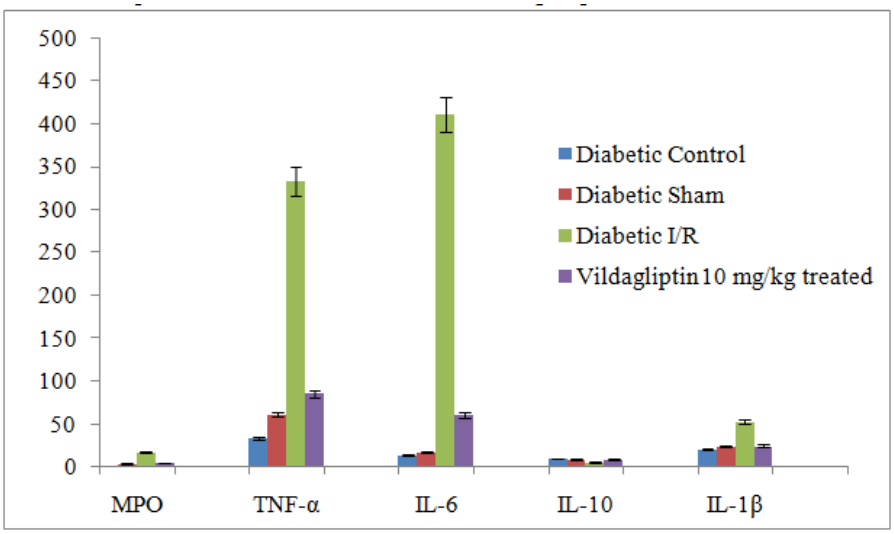
TNF-α levels in the brain of normal rats subjected to I/R injury (276.42 ± 5.06) were significantly increased compared to sham operated rats (48.14 ± 4.43). In Vildagliptin treated rats (65.33 ± 4.44), levels of TNF-α were significantly decreased when compared to I/R injury group (Table-3). Similarly in diabetic group subjected to I/R injury TNF-α levels (332.67 ± 7.54) were significantly increased when compared to diabetic sham group (61.22 ± 4.55). In Vildagliptin (85.44 ± 4.54) treated diabetic rats, levels of TNF-α were significantly decreased when compared to diabetic I/R injury group (Table-4).

Table 4: Effect of Vildagliptin on Inflammatory Markers in Ischemia-Reperfusion Induced Cerebral Infarction in Diabetic Rats.

Parameter	Diabetic Control	Diabetic Sham	Diabetic I/R	Vildagliptin 10 mg/kg treated
MPO (U/g tissue)	1.59 ± 0.44	3.45 ± 0.65	16.79 ± 1.65*	4.23 ± 1.12*
TNF-α (pg/ml)	33.94 ± 2.96	61.22 ± 4.55	332.67 ± 7.54*	85.44 ± 4.54*
IL-6 (pg/ml)	13.22 ± 2.22	16.33 ± 1.78	410.89 ± 8.22*	60.45 ± 2.92*
IL-10 (pg/ml)	9.52 ±1.22	8.93 ± 0.54	5.45 ± 0.55*	8.50 ± 0.43*
IL-1β (pg/ml)	20.23 ± 1.63	23.44 ± 2.10	52.23 ± 2.33*	24.44 ± 3.53*

Vildagliptin (10 mg/kg,b.wt) was administered orally daily for a period of four weeks and were subjected to BCCAO . Values were expressed as mean ± S.E.M (N=6 in each group) *P<0.05.

Figure-4: Effect of Vildagliptin on Inflammatory Markers in Ischemia-Reperfusion Induced Cerebral Infarction in Diabetic Rats



Vildagliptin (10 mg/kg, b.wt) was administered orally daily for a period of four weeks and were subjected to BCCAO. Values were expressed as mean ± S.E.M (N=6 in each group) *P<0.05.

c) Effect on brain IL-6 levels.

IL-6 levels in the brain of normal rats subjected to I/R injury (350.67 ± 24.34) were significantly increased compared to sham operated rats (12.32± 2.31). In Vildagliptin (45.82 ± 3.57) treated rats, levels of IL-6 were significantly decreased when compared to I/R injury group (Table-3). Similarly in diabetic group (410.89 ± 8.22) subjected to I/R injury, IL-6 levels were significantly increased when compared to diabetic sham group (16.33 ± 1.78). In Vildagliptin (60.45 ± 2.92) treated rats, levels of IL-6 were significantly decreased when compared to diabetic I/R injury group (Table-4).

d) Effect on brain IL-1β levels.

IL-1β levels in the brain of normal rats subjected to I/R injury (41.77 ± 1.77) were significantly increased compared to sham operated rats (16.87 ± 0.86). In Vildagliptin (17.20 ± 1.33) treated rats, levels of IL-1β were

significantly decreased when compared to I/R injury group (Table-3). Similarly in diabetic group (52.23 ± 2.33) subjected to I/R injury, IL-1β levels were significantly increased when compared to diabetic sham group (23.44 ± 2.10). In Vildagliptin (24.44 ± 3.53) treated rats, levels of IL-1β were significantly decreased when compared to diabetic I/R injury group (Table-4).

e) Effect on brain IL-10 levels.

IL-10 levels in the brain of normal rats subjected to I/R injury (6.86 ± 0.57) were significantly decreased when compared to sham operated rats (9.03 ± 1.42). In Vildagliptin (9.39 ± 1.06) treated rats, levels of IL-10 were significantly increased when compared to I/R injury group (Table-3). Similarly in diabetic group (5.45 ± 0.55) subjected to I/R injury, IL-10 levels were significantly decreased when compared to diabetic sham group (8.93 ± 0.54). In Vildagliptin (8.50 ± 0.43) treated rats, levels of IL-10 were significantly increased when compared to diabetic I/R injury group (Table-4).

4. Discussion

Risk of cerebral stroke alone is 1.7-2.1 times higher in diabetic patients compared to non-diabetic patients [17]. Type 2 diabetes is associated with poor outcome of ischemic stroke [18]. Reperfusion is inevitable to prevent further damage caused by stroke, but reperfusion itself induces damage by production of free radicals and inflammation, and hence reperfusion injury termed as a double edged sword. Many studies have shown that inflammatory chemokines and cytokines play a central role in cerebral ischemia reperfusion injury [19][20].

Dpp-4 inhibitors such as vildagliptin extend the half-life of endogenous GLP-1 thereby increasing its effects on glucose control. GLP-1 have been shown to be neuroprotective in brain damage caused by ischemic insults [21]. It is therefore possible that diabetic patients being treated with Dpp-4 inhibitors might get an added benefit of protection from neuronal damage due to cerebral ischemia reperfusion injury. The purpose of present study was to investigate the effect of repeated administration of Vildagliptin on neurological outcome in normal and diabetic rats.

Vildagliptin significantly reduced the infarct volume in both normal and diabetic rats. Similarly, vildagliptin had significantly reduced the elevated levels of MPO, TNF-α, IL-6, IL-1β and increased the levels of IL-10 indicating the role of its anti-inflammatory. The results indicate that vildagliptin provided neuroprotection against cerebral ischemia reperfusion injury. The mechanism of neuroprotective effect of vildagliptin was mainly mediated through anti-inflammatory actions. There are few other possible mechanisms that may contribute to the neuroprotective effects like antioxidant and cAMP-mediated antiapoptotic actions of GLP-1 [11].

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