



Modulatory effect of sildenafil in diabetes and electroconvulsive shock-induced cognitive dysfunction in rats

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Abstract:

The nitric oxide/guanylyl cyclase, cyclic guanosine monophosphate/phosphodiesterase 5 (NO/cGMP/PDE5) pathways play a key role in physiological and pathological situations, such as synaptic plasticity, learning and memory formation, diabetic gastropathy and neuropathy, long-term potentiation (LTP), epilepsy, cerebral ischemia, and neurodegenerative diseases. Several studies have demonstrated the alteration of NO-cGMP pathway in cognitive impairment. The present study was aimed to study the effect of sildenafil, a PDE5 inhibitor on diabetes and electroconvulsive shock (ECS)-induced cognitive dysfunction in rat using one-trial step-through type of passive avoidance and elevated plus maze task. Diabetic and ECS-treated rats showed poor learning performance in step-through passive avoidance and plus-maze task. Acute administration of sildenafil significantly reversed the diabetes and ECS-induced retention deficits in both the test paradigms. Sildenafil also significantly improved the cognitive performance in young rats in both the paradigms. Furthermore, L-NAME, a non-selective NOS inhibitor and methylene blue, a guanylate cyclase inhibitor blocked the effect of sildenafil. The results thus suggest that cognitive impairment might be due to the modulatory effect of nNOS or PDE5 enzyme on cGMP levels. Moreover, sildenafil-induced reversal of cognitive impairment suggests the protective role of PDE5 inhibitors in neurodegenerative disorders.

Key words:

sildenafil, cognition, diabetes, electroconvulsive shock

Introduction

Nitric oxide (NO) acts in the central nervous system (CNS) as the intercellular messenger mediating the increase in cyclic guanylyl mono phosphate (cGMP) levels that follows activation of glutamate receptors [10]. NO, a putative neurotransmitter, plays a key role in the central synaptic transmission efficiency, and is formed through nitric oxide synthase (NOS)-cata-

lyzed conversion of L-arginine to L-citrulline. Of all the forms of NO, the neuronal nitric oxide synthase (nNOS) has been vastly studied. Major brain areas including cerebral vessels, glial cells, and neurons in abundance contain nNOS [3, 22]. nNOS located pre- or post-synaptically is particularly implicated in neural signaling, neurotoxicity, synaptic plasticity and modulation of behavioral pathways (learning or expression of pain). On the other hand, endothelial nitric

oxide synthase (eNOS) present in fewer neurons is mainly involved in the regulation of vascular function, whereas inducible nitric oxide synthase (iNOS) is implicated in pathological conditions and unspecific immune response including that of the brain [16].

Diabetes mellitus can lead to functional and structural deficits in both the peripheral and CNS. The pathogenesis of these deficits is multifactorial and may involve microvascular dysfunction and oxidative stress. Cognitive deficits are also reported to occur in animal models of diabetes (streptozotocin, STZ-induced) which can be prevented, but not fully reversed by insulin treatment. STZ-induced cognitive impairment affects long-term potentiation (LTP) in the hippocampus. Recent studies have shown that the expression of nNOS mRNA and protein is decreased in the hippocampus of STZ-treated rats which may contribute to the cognitive deficits. Additionally, the phosphodiesterases regulate the activity of second messengers (cAMP and cGMP) that play a crucial role in synaptic plasticity. Studies using reverse transcription polymerase chain reaction have shown that chronic exposure to the electroconvulsive shock produces differential alteration in expression of phosphodiesterase (PDE) isoforms 1, 2, 3, 4, 5 and 7 in the rat hippocampus and striatum [6].

Based on these findings of altered nNOS expression in diabetes and differential expression of PDE activity upon exposure to electroconvulsive shock (ECS), the present study was aimed to evaluate the effect of sildenafil, a PDE5 inhibitor on diabetes and ECS-induced cognitive dysfunction in the rat. One-trial step-through type passive avoidance and elevated plus maze task were used to evaluate cognitive function of rats.

Materials and Methods

Animals

Wistar rats, weighing 200–250 g, were housed under standard laboratory conditions and kept under natural 12 h light : 12 h dark cycle. The animals procured from Central Animal House, Panacea Biotec Ltd., Lalru, Punjab, were housed six per cage with free access to standard food and water. Rats were acclima-

tized to laboratory conditions before testing. Experiments were carried out between 9:00 and 18:00. All the experimental protocols were approved by the Institutional Animal Ethics Committee. Animals were divided into different groups for behavioral tests, i.e. passive avoidance test, elevated plus maze test and locomotion.

Drugs

Sildenafil (Panacea Biotec Ltd., Lalru, India), N^G-nitro-L-arginine methyl ester (L-NAME) and carrageenan type IV (Sigma, USA), methylene blue (MB), streptozotocin (STZ) (Sigma, USA) were used in the study.

Doses of 0.25, 0.5 and 1.0 mg/kg of sildenafil were used. The dose selection was based on the preliminary studies, which showed no effect on the blood pressure.

Electroconvulsive shock (ECS) and drug treatment

Animals received ECS (2 mA, 0.2 s) once daily for 15 days with (*via* corneal electrodes). The drug treatment was given to the animals immediately after the ECS and only those animals were selected that did not develop any tonic-clonic seizures.

Sildenafil citrate dissolved in artificial cerebrospinal fluid (CSF in mM; NaCl-118, NaHCO₃-21, KCl-2.6, CaCl₂-1.3, MgCl₂-0.9) was directly injected intracerebroventricularly (*icv*) to the rat at doses of 50, 100 and 200 µg/rat in the volume of 10 µl over a period of 1 min using a Hamilton microsyringe. *Icv* administration was carried out as described earlier by Singh et al., [26]. Briefly, the rats were anesthetized with a combination of thiopental sodium (25 mg/kg, *ip*) and light ether anesthesia, and a polyethylene cannula was implanted into the lateral ventricle (1.5 mm lateral to sagittal suture and 1.5–2 mm anterior to corneal suture) and anchored to the skull with stainless steel screw and dental cement under aseptic conditions. The animals were housed individually in the cages for at least 3 days to recover from surgical trauma. Age matched control animals received only artificial cerebrospinal fluid (CSF).

ECS-treated animals were administered the test drug immediately after the learning trials (day 0) in both the paradigms and transfer latency (TL) was observed on day 1.

Induction of diabetes and drug treatment

Diabetes was induced in rats by a single injection of STZ (60 mg/kg, *ip*) freshly dissolved in citrate buffer (pH 4.5). Age-matched control animals were injected with citrate buffer. Diabetes was confirmed after 8 weeks; only the animals with blood glucose level above 400 mg/dL were included in the study.

Sildenafil citrate was dissolved in 0.9% normal saline. Doses of 0.25, 0.5 and 1.0 mg/kg, *ip* of sildenafil were used. Control (age matched) animals received saline (0.9% NaCl, 10 ml/kg, *ip*) treatment.

Diabetic animals were treated with test drug immediately after the learning trials (day 0) in both the paradigms and TL was observed on day 1.

Passive avoidance performance test (step through test)

The step through test was performed according to the method of Casamenti et al. [5]. The apparatus (UGO BASILE, Italy) consisted of two compartments with grid floor 50 × 50 cm and 35 cm high walls, separated by a wall with a guillotine door 6 × 6 cm. One of the two chambers was illuminated with 100 V bulb placed at 150 cm height and the other was dark. The test was conducted on 2 consecutive days at the same time of the day. On the day 0 (learning trial) each mouse was placed in the illuminated compartment of the apparatus. After 60 s the guillotine door was raised, allowing access to the dark compartment and a mouse placed in the illuminated chamber rapidly moved to the dark chamber. Once the mouse entered the dark compartment, it received an electric shock on the feet (2 mA, 2 s) through the stainless steel grid floor. The time when mouse entered in the dark chamber was recorded automatically and described as step-through latency (STL). On the second day (testing trial or TL), the same test procedure was followed. The TL was recorded in testing trial.

Elevated plus maze test

The elevated plus maze was used to evaluate spatial long-term memory, following the procedure as described earlier [19]. Briefly, the apparatus consisted of two open arms (50 cm × 10 cm × 40 cm) and two closed arms (50 cm × 10 cm × 40 cm). The maze was elevated to a height of 50 cm from the floor. On the first day, each rat was placed at the end of an open

arm. TL, i.e., the time taken by the rat to move into one of the closed arms, was recorded on the first day. If the animal did not enter any one of the closed arms within 90 s, it was gently pushed into one closed arm and the TL was assigned as 90 s. The rat was allowed to explore the maze for 20 s, and then was returned to its home cage. Retention was examined 24 h after the first-day trial. Each rat was again placed at the end of one of the open arms of the maze and TL was recorded as described above. A long latency period to reach closed arm indicated poor retention as compared to significantly shorter latencies.

Locomotor activity test

The animal's locomotor behavioral pattern was monitored using activity meter (IMCORP, India). The animals were individually placed in a Plexiglas cage (40 × 15 × 15 cm) and the total activity count was registered for 5 min. The locomotor activity was expressed in terms of total photo beam counts/5 min per animal [21].

Non-invasive blood pressure recorder in rats

Blood pressure was recorded in rats using non-invasive tail-cuff method (UGO BASILE, Italy). The blood pressure was recorded at 30 min, 1, 2 and 4 h after the treatment with the drug at the highest dose.

Statistical analysis

The results are expressed as the mean ± SEM. The data for STL, TL and locomotor activity were subjected to one-way ANOVA followed by Dunnett's test. In all the tests, the criterion for statistical significance was $p < 0.05$.

Results

Effect of sildenafil on diabetic rats in step-through and elevated plus maze procedures

Diabetic rats showed a significant change in transfer latency (STL and TL) as compared to non-diabetic, indicating diabetes-induced cognitive dysfunction. Acute administration of sildenafil (0.25–1 mg/kg, *ip*)

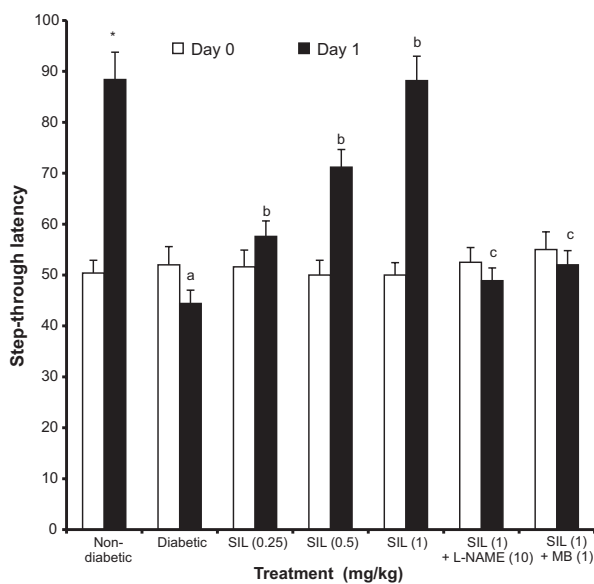


Fig. 1. Effect of sildenafil (SIL; 0.25, 0.5 and 1 mg/kg, *ip*), on transfer latency in diabetic rats during retention test in passive avoidance (step-through) task. The transfer latency of each group of rats is expressed as the mean \pm SEM (n = 6–8). $p < 0.05$ compared with step-through latency of non-diabetic rats on day 0; ^a $p < 0.05$ compared with step-through latency of non-diabetic on day 1; ^b $p < 0.05$ compared with step-through latency of diabetic rats on day 1; ^c $p < 0.05$ compared with step-through latency of sildenafil-treated rats (1 mg/kg, *ip*) on day 1

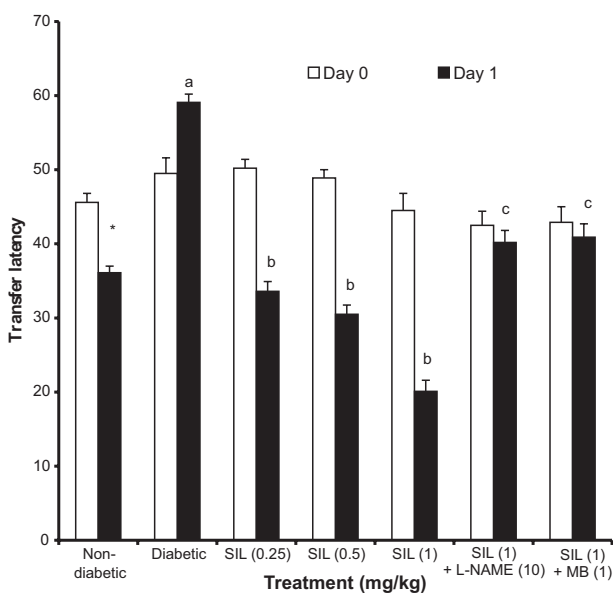


Fig. 2. Effect of sildenafil (SIL; 0.25, 0.5 and 1 mg/kg, *ip*) on transfer latency in diabetic rats during retention test in elevated plus maze task. The transfer latency of each group of rats is expressed as the mean \pm SEM (n = 6–8). $p < 0.05$ compared with transfer latency of non-diabetic rats on day 0; ^a $p < 0.05$ compared with transfer latency of non-diabetic on day 1; ^b $p < 0.05$ compared with transfer latency of diabetic rats on day 1; ^c $p < 0.05$ compared with transfer latency of sildenafil (1 mg/kg, *ip*) on day 1

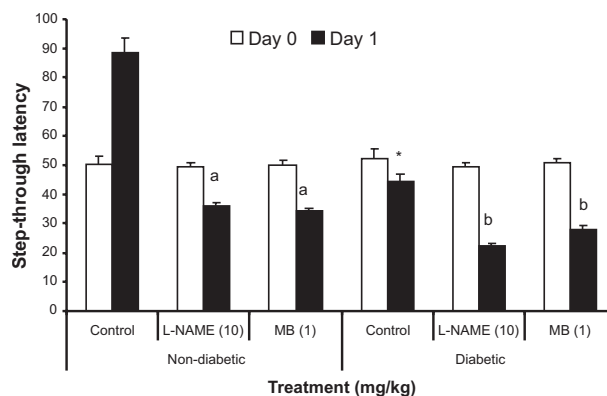


Fig. 3. Effect of L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*), on transfer latency in non-diabetic and diabetic rats during retention test in passive avoidance (step-through) task. The transfer latency of each group of rats is expressed as the mean \pm SEM (n = 6–8). * $p < 0.05$ compared with step-through latency of non-diabetic rats on day 0; ^a $p < 0.05$ compared with step-through latency of non-diabetic on day 1; ^b $p < 0.05$ compared with step-through latency of L-NAME- and MB-treated rats (non-diabetic) on day 1

produced a significant increase in cognitive performance in step-through and elevated plus maze task as compared to diabetic group (Fig. 1 and Fig. 2). The effect of sildenafil (1 mg/kg, *ip*) was reversed by L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*), which *per se* aggravated the cognitive performance in diabetic and non-diabetic groups in both the paradigms (Fig. 3 and Fig. 4).

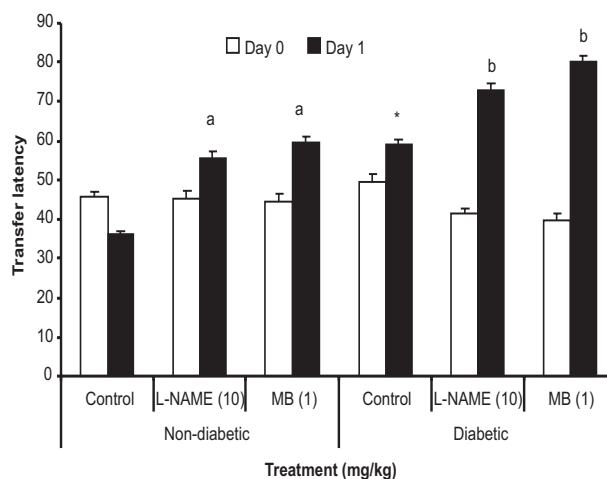


Fig. 4. Effect of L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*), on transfer latency in non-diabetic and diabetic rats during retention test on elevated plus maze task. The transfer latency of each group of rats is expressed as the mean \pm SEM (n = 6–8). * $p < 0.05$ compared with transfer latency of non-diabetic rats on day 0; ^a $p < 0.05$ compared with transfer latency of non-diabetic rats on day 1; ^b $p < 0.05$ compared with transfer latency of L-NAME- and MB-treated rats (non-diabetic) on day 1

Effect of sildenafil on ECS-treated rats in step through and elevated plus maze procedures

Chronic ECS treatment produced a significant change in transfer latency (STL and TL) in control (ECS-treated) animals as compared to normal (saline) group, indicating that ECS produced cognitive dysfunction in both the paradigms. Acute administration

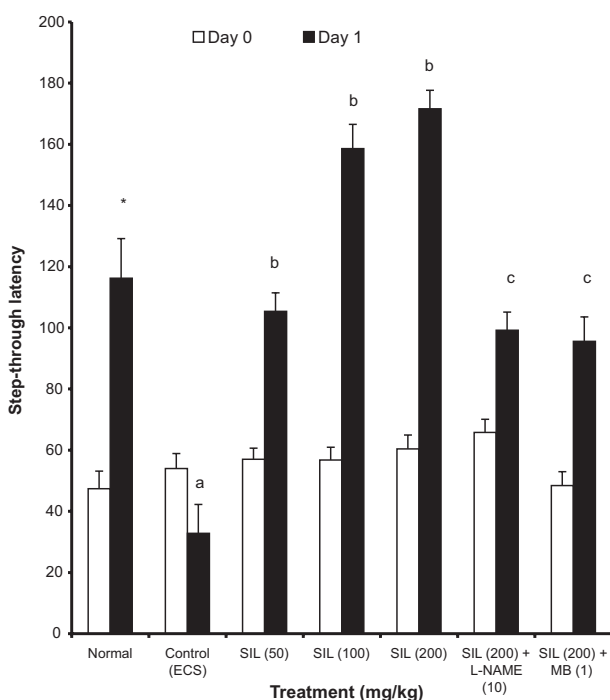


Fig. 5. Effect of sildenafil (SIL; 50, 100 and 200 μ g), and its reversal by L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*) on transfer latency in ECS-treated rats during retention test in passive avoidance (step-through) task. The transfer latency of each group of rats is expressed as the mean \pm SEM ($n = 6-8$). * $p < 0.05$ compared with step-through latency of control (saline) rats on day 0; ^a $p < 0.05$ compared with step-through latency of control (saline) rats on day 1; ^b $p < 0.05$ compared with step-through latency of control (ECS) on day 1; ^c $p < 0.05$ compared with step-through latency of sildenafil-treated rats (200 μ g) on day 1

of sildenafil (50, 100 and 200 μ g/rat) produced a significant increase in cognitive performance in step through and elevated plus maze task as compared to control (ECS-treated) group (Fig. 5 and Fig. 6). Further, the effect of sildenafil (200 μ g/rat) was reversed by L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*), which *per se* aggravated the cognitive performance in control (ECS) and saline-treated animals in both the paradigms (Fig. 7 and Fig. 8).

Effect of sildenafil on the locomotor activity in diabetes and ECS-treated rats

In order to check the general locomotor performance, animals were subjected to a 5 min activity test. Sildenafil did not produce any significant alteration in the locomotor activity of both diabetes and ECS-treated rats (data not shown).

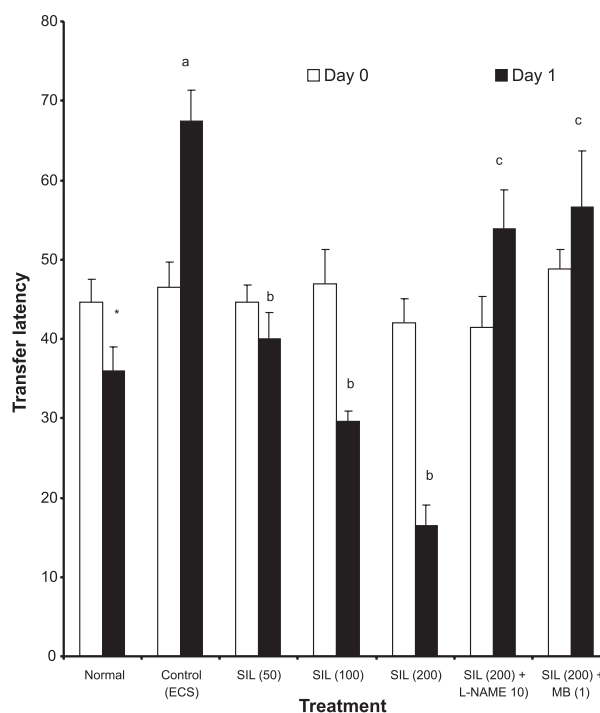


Fig. 6. Effect of sildenafil (SIL; 50, 100 and 200 μ g), and its reversal by L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*) on transfer latency in ECS-treated rats during retention test on elevated plus-maze task. The transfer latency of each group of rats is expressed as the mean \pm SEM ($n = 6-8$). * $p < 0.05$ compared with transfer latency of control (saline) rats on day 0; ^a $p < 0.05$ compared with transfer latency of control (saline) rats on day 1; ^b $p < 0.05$ compared with transfer latency of control (ECS) rats on day 1; ^c $p < 0.05$ compared with transfer latency of sildenafil-treated rats (200 μ g) on day 1

Discussion

PDEs play a key role in the regulation of cAMP and cGMP signaling. The cAMP and cGMP, the key regulatory molecules of cAMP/cGMP signaling cascade, are produced by adenylate cyclases and guanylyl cyclase regulated by G proteins, respectively [18, 24], and degraded by cyclic nucleotide phosphodies-

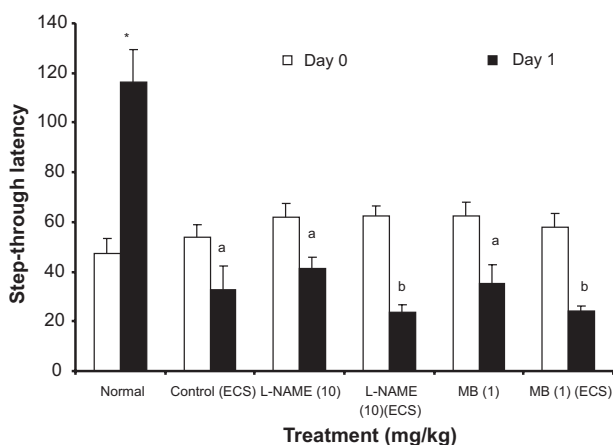


Fig. 7. Effect of L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*), on transfer latency in control and ECS-treated rats during retention test in passive avoidance (step-through) task. The transfer latency of each group of rats is expressed as the mean \pm SEM. ($n = 6-8$). * $p < 0.05$ compared with step-through latency of control (saline) rats on day 0; ^a $p < 0.05$ compared with step-through latency of control (saline) rats on day 1; ^b $p < 0.05$ compared with step-through latency of L-NAME- and MB-treated rats on day 1

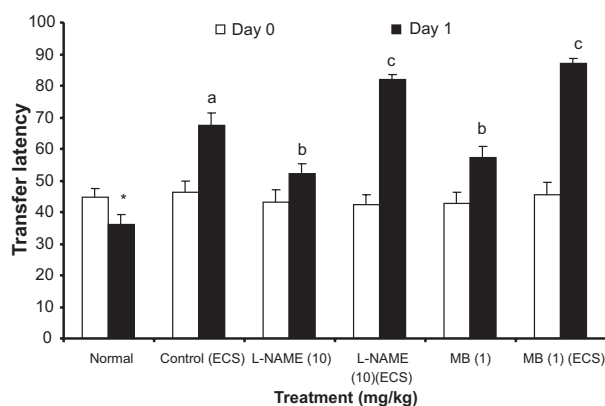


Fig. 8. Effect of L-NAME (10 mg/kg, *ip*) and MB (1 mg/kg, *ip*) on transfer latency in control and ECS-treated rats during retention test in elevated plus maze task. The transfer latency of each group of rats is expressed as the mean \pm SEM ($n = 6-8$). * $p < 0.05$ compared with transfer latency of control (saline) on day 0; ^a $p < 0.05$ compared with transfer latency of control (saline) rats on day 1; ^b $p < 0.05$ compared with transfer latency of L-NAME- and MB-treated rats on day 1

terases. In mammals, PDE are encoded by at least 19 different genes, and each PDE isoform has different kinetic properties, such as substrate specificity and it responds differentially to diverse regulators, such as calcium/calmodulin and cyclic GMP [7]. In addition, the PDE isoforms are expressed differently in different tissues [1]. Such multiplicity of PDs has been suggested to play an important role in the regulation of

the cAMP/cGMP signaling system in intracellular targeting, crosstalk between a wide variety of other signaling systems, and flux-controlled sensitivity [12].

NO released centrally modulates the synaptic plasticity as the retrograde messenger which co-ordinates the enhancement of both pre- and post-synaptic mechanisms causing long-term potentiation (LTP) and long-term depression (LTD). LTP is the most pronounced in higher brain centres involved in cognitive functions, particularly in the cerebral cortex and hippocampus [2, 17, 23]. Gene targeting has revealed that both nNOS and eNOS are implicated in LTP. Animals deficient in one or both NOS isozymes exhibit a substantially decreased LTP [27, 31]. Guanylate cyclase is the main effector of NO in the induction of LTP [2, 11], however, ADP-ribosylation [4] and activation of calmodulin-dependent kinases [27] have also been implicated in LTP.

In the present study, diabetic rats showed a significant alteration in cognitive performance which was improved by sildenafil. Earlier, cell emulsion and immunoblot analysis revealed decreased nNOS mRNA and protein levels in hippocampal interneurons following STZ [20]. In contrast, Watkins et al. [30] observed no alteration of nNOS in the brains of diabetic animals. Despite this controversy, the results suggested that sildenafil may have compensated the NO deficit caused by loss of nNOS in diabetes. PDE5 is expressed in several tissues [13] including brain [14, 15]. The lipophilic [9] nature of sildenafil enables it to reach the brain and modulate the cognitive performance. Further, the effect of sildenafil was reversed by L-NAME and MB, which *per se* aggravated the cognitive performance in diabetic and non-diabetic rats. Previous studies have established nNOS and NO as the essential components in induction and maintenance of LTP. The NO donor, sodium nitropruside, evoked LTP while non-selective and selective NOS inhibitors, such as L-NAME and 7-nitroindazole (7-NI) blocked LTP. Additionally, the role of nNOS, calcium-calmodulin dependent protein kinase II, protein kinase C, and arachidonic acid have been reported in LTP.

In another study, we observed that acute administration of sildenafil significantly reversed the chronic ECS treatment-induced cognitive dysfunction in both the paradigms. This effect was reversed by L-NAME and MB, which *per se* aggravated the effect in ECS treated animals. A possible explanation of the loss of cognitive function upon chronic ECS treatment may

be linked to the increased expression of PDE isoforms. Cho et al. [6], reported differential changes in the expression of PDE isoforms 1, 2, 3, 4, 5 and 7 in the rat hippocampus and striatum following chronic ECS treatment [6]. These results are indicative of increased expression of PDE5 enzymes in the brain, which may cause rapid degradation of cGMP; the main secondary mediator for LTP.

The results of the present experiments indicate that drugs that enhance the effect of NO and its effector, cGMP, might improve the cognitive performance in diabetic conditions. Sindrup et al. have found a decreased motor latency, cognitive dysfunction and diminished rapid thinking in diabetic patients [25]. Recently, Sommerfield et al. demonstrated a significant impairment in information processing, aspects of working memory and attention [29]. An implication of nNOS in cognitive impairment was demonstrated by Sole-Padullés et al. who reported a role of nNOS gene during impairment of learning and memory [28].

We found that the treatment of diabetic rats with sildenafil, a PDE5 inhibitor reversed the diabetic and ECS-induced cognitive impairment. These findings are consistent with earlier reports demonstrating the down-regulation of nNOS-cGMP pathway in the hippocampus and wide distribution of PDE5 enzymes in several tissues including brain. Hence, sildenafil causes accumulation of cGMP that compensates for the NO deficit due to nNOS loss in diabetes whereas in ECS, sildenafil inhibits the up-regulation of PDE5 activity.

Furthermore, sildenafil-induced cognitive improvement in the tested paradigms suggested a protective role of PDE5 inhibitors in neurodegenerative disorders.

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