Neurobiological effect of 7-nitroindazole, a neuronal nitric oxide synthase inhibitor, in experimental paradigm of Alzheimer’s disease

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Received 4 June 2012; revised 2 August 2013

Nitric oxide plays a role in a series of neurobiological functions, underlying behaviour and memory. The functional role of nNOS derived nitric oxide in cognitive functions is elusive. The present study was designed to investigate the effect of specific neuronal nitric oxide synthase inhibitor, 7-nitroindazole, against intracerebroventricular streptozotocin-induced cognitive impairment in rats. Learning and memory behaviour was assessed using Morris water maze and elevated plus maze. 7-nitroindazole (25 mg/kg, ip) was administered as prophylactically (30 min before intracerebroventricular streptozotocin injection on day 1) and therapeutically (30 min before the assessment of memory by Morris water maze on day 15). Intracerebroventricular streptozotocin produced significant cognitive deficits coupled with alterations in biochemical indices. These behavioural and biochemical changes were significantly prevented by prophylactic treatment of 7-nitroindazole. However, therapeutic intervention of 7-nitroindazole did not show any significant reversal. The results suggests that 7-nitroindazole can be effective in the protection of dementia induced by intracerebroventricular streptozotocin only when given prophylactically but not therapeutically.

Keywords: Alzheimer’s disease, Nitrergic signaling, Nitric Oxide, 7-nitroindazole, nNOS, Streptozotocin

Alzheimer’s disease (AD) is the most common type of dementia in Western societies, and has been creating profound economic and social impact as the aging population continues to rise. Impaired cerebral glucose utilization and energy metabolism represent very early abnormalities in initial stages of cognitive impairment. Sporadic dementia of Alzheimer’s type (SDAT) in particular, is characterized by a progressive deterioration of both cognitive function and energy metabolism. Besides diminished glucose utilization, insulin receptor signal transduction cascade is severely impaired in hippocampus and hypothalamus of AD (Alzheimer's disease) brain. Another major feature of SDAT pathophysiology is oxidative damage. Oxidative stress can affect all classes of macromolecules (sugar, lipids, proteins, and DNA), leading inevitably to neuronal dysfunction.

The Intracerebroventricular streptozotocin (ICV STZ) injected rat has been described as an appropriate animal model for SDAT characterized by progressive deterioration of memory and cerebral glucose and energy metabolism, along with oxidative stress. The intracerebroventricular injection of STZ induced reactive gliosis and oxidative-nitrative stress before the induction of memory deficits. The reactive gliosis involved microglia and astrocytes in the cingulated and motor cortex around the area of cannula penetration, CA1 region of the hippocampus, and in the corpus callosum, medial and lateral septum close to the lateral ventricle.

In AD and other age-related neurodegenerative conditions recruitment and activation of microglia and astrocytes occur before pathological and clinical signs of the disease. Activated microglia and astrocytes can release cytokines, reactive oxygen species (ROS) and nitric oxide (NO) that may contribute to the memory deficits. Glial changes also play a role in the cognitive decline that occurs during normal human aging even in the absence of overt neuron loss. Activated microglia can release neurotoxic molecules, such as cytokines and toxic oxygenand nitrogen species. Activated microglia can damage neurons by generating ONOO- via NO/O2- pathways. It has been shown that neuron death in cultures of rat cortical microglia and neurons caused by peroxynitrite, as determined by electronparamagnetic spectroscopy.
With this background, the present study has been designed to explore the effects of 7-nitroindazole, in an experimental paradigm of Alzheimer’s disease in rats.

Materials and Methods

Animals—Adult male Wistar rats (250–300 g) bred in Central Animal House facility of Panjab University were used. The rats were housed in polycrylic cages [38×23×10 cm] and maintained under standard laboratory conditions with natural dark and light (12:12 h) cycle and had free access to food (Ashirwad Industries, Chandigarh, India) and water ad libitum. Animals were acclimatized to laboratory conditions before the tests. All experiments were carried out between 0900 and 1700 hrs. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

Drugs—7-nitroindazole (7NI) and streptozotocin were purchased from Sigma Aldrich, St. Louis, MO, USA. Streptozotocin was dissolved in artificial CSF (ACSF) [(2.9 mM KCl, 147 mM NaCl, 1.7 mM CaCl₂, 1.6 mM MgCl₂, 2.2 mM D-glucose) in a 25 mg/mL solution]. 7-nitroindazole was dissolved in few drops of Tween 80 and volume was made with normal saline. The doses of 7-nitroindazole was selected on the basis of previous studies. Streptozotocin—streptozotocin in ACSF was adjusted so as to deliver the dose of 1.5 mg/kg each day. The concentration of streptozotocin in ACSF was adjusted so as to deliver 10 µL of the solution at the rate of 1µL/min. Control animals received intracerebroventricular injection of the same volume of ACSF on the first and third day. The skin was sutured after first and second injection followed by daily application of antiseptic powder (Neosporin®). Postoperatively, the rats were fed with glucose by oral gavage for 4 days. All drug solutions were freshly prepared immediately prior to injection.

Experimental procedure—Rats were randomly divided into following four groups containing 5–8 animals in each group Gr. 1: Control animals received an equivalent volume of vehicle for streptozotocin i.e. artificial CSF (ACSF) on day 1 and day 3; Gr. 2: animals received intracerebroventricular injection of streptozotocin (ICV-STZ) 3 mg/kg on day 1 and day 3; Gr. 3: Pretreatment group: ICV-STZ treated rats received 7NI (25 mg/kg; ip) on day 1, 30 min before the ICV administration of streptozotocin; Gr. 4: Post treatment group: ICV-STZ treated rats received 7NI (25 mg/kg; ip) on day 15th, 30 min before the assessment of memory by Morris Water Maze.

Behavioural estimation

Morris water maze test Animals were tested in a spatial version of Morris water maze test. The apparatus consisted of a circular water tank (180 cm in diameter and 60 cm high). A platform (12.5 cm in diameter and 38 cm high) invisible to the rats, was set 2 cm below the water level inside the tank with water maintained at 28.5±2 °C at a height of 40 cm. The tank was located in a large room where there were several brightly coloured cues external to the maze; these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for five consecutive days from 15th to 19th day. The rats received four consecutive daily training trials in the following 5 days, with each trial having a ceiling time of 90 sec and a trial interval of approximately 30 sec. For each trail, each rat was put into the water at one of four starting positions, the sequence of which being selected randomly. During test trials, rats were placed into the tank at the same starting point, with their heads facing the wall. The rat had to swim until it climbed onto the platform submerged underneath the water. After climbing onto the platform, the animal remained there for 20 sec before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the rat failed to reach the escape platform within the maximally allowed time of 90 sec, it was guided with the help of a rod and allowed to remain on the platform for 20 sec.
The time to reach the platform (escape latency in seconds) was measured.

Memory consolidation test: A probe trial was performed on day 19, wherein the extent of memory consolidation was assessed. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe trial, the rat was placed into the pool as in the training trial, except that the hidden platform was removed from the pool. The total time spent in target quadrant in a time period of 90 sec was recorded.

Elevated plus maze test: Memory acquisition and retention was tested using elevated plus maze test on days 19 and 20. The apparatus consisted of two crossed arms, one closed and the other, open. Each rat was placed on the open arm, facing outwards. The time taken by the rat to enter the closed arm in the first trial (acquisition trial) on 19th day was noted and was called as initial transfer latency. Cut-off time was fixed as 90 sec and in case a rat could not find the closed arm within this period, it was gently pushed in to one of the closed arms and allowed to explore the maze for 30 sec. Second trial (retention trial) was performed 24 h after the acquisition trial and retention transfer latency was noted. The retention trial latency was expressed as percentage of initial trial latency.

Closed field activity: Closed field activity was measured to rule out the interference of change in locomotor activity in the parameters of learning and memory. Spontaneous locomotor activity was measured on day 20 using digital photoactometer and values expressed as counts per 5 min. The apparatus was placed in a darkened, light and sound attenuated and ventilated testing room.

Biochemical estimation

**Brain homogenate preparation**—Whole brain samples were rinsed with ice cold saline (0.9% NaCl) and homogenized in chilled phosphate buffer (pH 7.4). The homogenates were centrifuged at 800 g for 5 min at 4 °C to separate the nuclear debris. The supernatant thus obtained was centrifuged at 10,500 g for 20 min at 4°C to get the post mitochondrial supernatant, which was used to assay acetylcholinesterase activity, lipid peroxidation, reduced glutathione, nitrite, catalase and superoxide dismutase activity.

Acetylcholinesterase activity: Cholinergic dysfunction was assessed by acetylcholinesterase activity. The quantitative measurement of acetylcholinesterase levels in the whole brain homogenate were estimated according to the method of Ellman et al. The assay mixture contained 0.05 mL of supernatant, 3 mL of 0.01 M sodium phosphate buffer (pH 8), 0.10 mL acetylthiocholine iodide and 0.10 mL 5, 5-dithiobis (2-nitro benzoic acid) (Ellman reagent). The change in absorbance was measured at 412 nm for 5 min. Results were calculated using molar extinction coefficient of chromophore (1.36x10^4 M^-1 cm^-1) and expressed as percentage of control.

Estimation of lipid peroxidation: The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills. Briefly, 0.5 mL of post-mitochondrial supernatant and 0.5 mL of Tris–HCl were incubated at 37 °C for 2 h. After incubation 1 mL of 10% trichloro acetic acid was added and centrifuged at 1000 g for 10 min. To 1 mL of supernatant, 1 mL of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling, 1 mL double distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56x10^5 M^-1 cm^-1 and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Biuret method and the brain malondialdehyde content expressed as percentage of control.

Estimation of reduced glutathione: Reduced glutathione was assayed by the method of Jollow et al. Briefly, 1.0 mL of post-mitochondrial supernatant (10%) was precipitated with 1.0 mL of sulphosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4 °C. The assay mixture contained 0.1 mL supernatant, 2.7 mL phosphate buffer (0.1 M, pH 7.4) and 0.2 mL 5, 5-dithiobis (2-nitro benzoic acid) (Ellman's reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 mL. The yellow color developed was read immediately at 412 nm.

Estimation of superoxide dismutase: Cytosolic superoxide dismutase activity was assayed as per Kono. The assay system consisted of 0.1 mM EDTA, 50 mM sodium carbonate and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 mL of above mixture was taken and to it 0.05 mL of post mitochondrial supernatant and 0.05 mL of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added. The auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30/60 sec intervals.
Estimation of catalase: Catalase activity was assayed as per Claiborne. Briefly, the assay mixture consisted of 1.95 mL phosphate buffer (0.05 M, pH 7.0), 1.0 mL hydrogen peroxide (0.019 M) and 0.05 mL post mitochondrial supernatant (10%) in a final volume of 3.0 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of K min \(^{-1}\) and expressed as percentage of control.

Nitrite estimation: Nitrite was estimated in the whole brain using the Greiss reagent and served as an indicator of nitric oxide production. A measure of 500 µL of Greiss reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylenediameinedihydrochloric acid in water) was added to 100 µL of post mitochondrial supernatant and absorbance was measured at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitrite. Nitrite levels were expressed as percentage of control.

Statistical analysis—Results were expressed as mean ± SE. The intergroup variation was measured by one way analysis of variance (ANOVA) followed by Tukey’s test. Statistical significance was considered at \(P<0.05\). The statistical analysis was done using the SPSS Statistical Software version 16.

Results

Behavioural observations

Effect of 7-nitroindazole on performance in Morris water maze task—The change in escape latency was observed onto a hidden platform produced by training trials. Although latency to reach the submerged platform decreased gradually in all the groups during 5 days of training in Morris water maze test the mean latency (from 2\(^{nd}\) to 5\(^{th}\) day) was significantly \(P<0.05\) prolonged in ICV-STZ group, as compared to control group, showing a poorer learning performance due to ICV-STZ injection. This disrupted performance of ICV-STZ group was significantly \(P<0.05\) improved by the single administration of 7-NI (25 mg/kg) intraperitoneally on day 1, 30 min before the administration of ICV-STZ. However, there was no significant alteration in rats treated with 7-NI (25 mg/kg) on day 15, 30 min before the assessment of memory by Morris water maze (Fig. 1a).

In the probe trial, with the hidden platform removed, ICV-STZ group failed to memorise the precise location of the platform, spending significantly \(P<0.05\) less time (20.40±1.03 sec) in the target quadrant than control group (68.33±0.88 sec). The total time spent in the target quadrant was significantly \(P<0.05\) increased by the single dose of 7-NI (25 mg/kg) intraperitoneally on day 1, 30 min before the administration of ICV-STZ. However, there was no significant increase in total time spent in target quadrant in rats treated with 7-NI (25 mg/kg) on day 15, 30 min before the assessment of memory by Morris water maze (Fig. 1b).

Effect of 7-NI on initial transfer latency in elevated plus maze test—Initial transfer latency (ITL) did not differ significantly in any of the groups. Retention transfer latency (RTL) of control group was significantly less than that of ICV-STZ injected group which has lowered the %ITL, which is calculated by (RTL/ITL x 100). Treatment with 7-NI (25 mg/kg) on day 1 significantly \(P<0.05\) lowered the RTL in ICV-STZ injected rats. However, 7-NI (25 mg/kg) treatment on day 15 did not show any effect (Fig. 2a).

Fig. 1—Effect of 7-NI (25 mg/kg) on the performance of spatial memory acquisition phase (a) and on time spend in target quadrant (b) using Morris water maze in intracerebroventricular streptozotocin treated rats. Data is expressed as mean ± SE. \(P\) values :<0.05, (*) compared to Control from the second day of the training sessions; (**) compared to STZ. CTRL: Control; STZ: Intracerebroventricular streptozotocin; 7-NI: 7-Nitroindazole.
Effect of 7-NI on the locomotor activity—The spontaneous locomotor activity did not differ significantly between the control, ICV-STZ group, 7-NI and L-NAME treated ICV-STZ groups on 20th day. The mean values in the control vehicle treated group, ICV-STZ group, 7-NI (25 mg/kg) on day 1 and 7-NI on day 15 were 281.00±10.11, 271.80±33.00, 245.5±13.33, 267.75±13.6 counts respectively.

Biochemical observations

Effect of 7-NI on acetylcholinesterase activity—Acetylcholinesterase activity was increased in ICV-STZ treated rat brain as compared to control group.
7-NI (25 mg/kg) on day 1 treatment significantly decreased acetylcholinesterase activity in the brain of ICV-STZ injected rats ($P<0.05$). However, 7-NI (25 mg/kg) treatment on day 15 did not show any effect (Fig. 2b).

**Effect of 7-NI on antioxidant profile**—The enzymatic activities of catalase (Fig. 2c) and superoxide dismutase (Fig. 2d) and reduced glutathione levels (Fig. 2e) were significantly decreased in the brain of ICV-STZ treated rats as compared to control group. The endogenous antioxidant profile was significantly (p<0.05) restored by 7-NI (25 mg/kg) administration on day 1.

**Effect of 7-NI on lipid peroxidation**—Malondialdehyde (MDA) levels were significantly increased in the brain of ICV-STZ treated rats as compared to control group. Single dose treatment with 7-NI (25 mg/kg) on day 1 produced significant ($P<0.05$) reduction in MDA levels in STZ-treated rat brain. However, 7-NI (25 mg/kg) on day 15 did not show any effect (Fig. 2f).

**Effect of 7-NI on nitrergic stress**—Nitrite levels were significantly elevated in the brain of ICV-STZ treated animals as compared to control group. 7-NI (25 mg/kg) treatment on day 15 did not show any effect (Fig. 2f).

**Discussion**

The present study demonstrated the prophylactic effect of neuronal nitric oxide synthase inhibitor, 7-nitroindazole in the intracerebroventricular streptozotocin-induced cognitive impairment. Several studies previously showed that 7-nitroindazole, a selective inhibitor of nNOS, could protect against ischemia and kainate-induced neuronal death\textsuperscript{34,35}. On the contrary, Guevara et al\textsuperscript{36} demonstrated that treatment with 7-nitroindazole increases the cholinergic damage induced by direct injection of kainic acid into the striatum.

NO is a diffusible free radical, and as a biological messenger, it is involved in several physiological and pathological functions\textsuperscript{37}. However, it is not yet certainly known whether NO is neuroprotective or neurotoxic. The proposed mechanism of action of NO have generated much debate in the field of neurode generation and repair in the central nervous system (CNS). NO has been suggested to mediate cytotoxicity by N-methyl-D-aspartate (NMDA) in cultured cerebellar granular cells\textsuperscript{38}, in cultured cortical neurons\textsuperscript{39}, and in hippocampal cells\textsuperscript{40}. An involvement of NO has been shown in the neuronal damage by β-amyloid peptide and glutamate in vitro\textsuperscript{41}. On the other hand, NO may react with superoxide to generate peroxynitrite\textsuperscript{42}, which may promote nitration of tyrosine\textsuperscript{43} and produce hydroxyl radicals. Thus, the generation of the free radical nitric oxide followed by production of peroxynitrite has been implicated in cell death. nNOS was originally thought to be a constitutively expressed enzyme. It now becomes increasingly clear, however, that its levels are dynamically regulated in response to neuronal development, plasticity and injury\textsuperscript{44,45}. Although the exact molecular mechanisms regulating nNOS expression are unknown, recent experimental evidence indicates that transcriptional regulation of nNOS occurs under the control of neurotrophins and other growth factors\textsuperscript{46}. Reactive expression of these growth-promoting factors induced by neuronal injury\textsuperscript{47} might thus explain the dynamic regulation of nNOS in response to a variety of neuronal injury paradigms in man\textsuperscript{48} and rodent\textsuperscript{49}. The transcriptional induction of nNOS that in turn is involved in regulating the expression of immediate early genes in neurons\textsuperscript{50}, thereby controlling neuronal growth and differentiation\textsuperscript{51}, might thus be part of the neuronal reparative/regenerative response to injury.

The intracerebroventricular streptozotocin model appears to produce cognitive deficits similar to those seen in Sporadic dementia of Alzheimer's type\textsuperscript{52}. However, Aβ aggregation is rarely present in this in vivo Sporadic dementia model. On the other hand, Sporadic dementia is a late onset disease and usually develops after the age of 65. Therefore, adult animals were used intentionally to approach a model that more resembles late onset type AD and that is representative of AD dementia. Streptozotocin is thought to be responsible for the pathogenesis of the neurodegenerative changes observed in this in vivo model of AD, even in the absence of Amyloid β (Aβ) aggregation\textsuperscript{53}. The neurodegeneration in AD is associated with oxidative stress, mitochondrial dysfunction, impaired energy metabolism, progressive Aβ agglutination and formation of neurofibrillary tangles\textsuperscript{54}. Intracerebroventricular injection of streptozotocin in sub-diabetogenic dose reduced energy metabolism, leading to cognitive dysfunction by inhibiting the synthesis of adenosine triphosphate and acetyl-CoA. This ultimately results into cholinergic deficiency supported by reduced cholineacetyltransferase activity in hippocampus.
and an increased acetylcholinesterase activity in the brain of intracerebroventricular streptozotocin administered rats.\textsuperscript{55} Aggregation of misfolded proteins that contain Aβ has been shown to initiate oxidative-nitriergic stress and inflammation. This seems to be responsible for the neurodegenerative changes and clinical characteristics of AD\textsuperscript{56}.

In the present study, prior administration (30 min before ICV-STZ on day 1) of 7-NI has been shown to attenuate behavioural (transfer latency) and biochemical alterations in the intracerebroventricular streptozotocin treated rats. However, it has been observed that the rats treated with 7-nitroindazole on day 15 did not produce any effect on behavioural and biochemical alterations in intracerebroventricular streptozotocin treated rats. This shows that nNOS-mediated neuronal damage may occur in early stages of the disease and its inhibition at later stage may not prove beneficial.

Taken together, it can be concluded that blockade of neuronal nitric oxide synthase by 7-nitroindazole improves memory and acts as a neuroprotective agent only when it is given as a prophylactic agent against intracerebroventricular streptozotocin-induced cognitive impairment and thus may find a place in therapeutic armamentarium for the treatment of patients with Alzheimer’s disease.

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