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Original Research Article

Experimental Studies on the Possible Role of Nitric Oxide Signalling Pathways in Post-Traumatic Stress Disorder in Rats

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Abstract

Background: Post-traumatic stress disorder (PTSD) is associated with traumatic experiences for which treatment strategies are poorly defined. Nitric oxide (NO) is a neuromodulator and thus we examined the role of NO in PTSD by assessing behavioral and biochemical parameters in an experimental model in rats.

Materials and Methods: Wistar rats were exposed to time dependent sensitization (TDS, stress + re-stress) and the influence of NO modulators were assessed on neurobehavioral parameters and brain biochemistry. Rats were evaluated for anxiety behaviour in the elevated plus maze (EPM) test, and open arm entries/time were recorded. Post EPM test, animals were sacrificed, and brain homogenates were assayed for biochemical markers of lipid peroxidation markers (MDA) and NO metabolites (NOx). The effects of various drug treatments, viz. L-arginine, L-NAME and fluoxetine, were evaluated on behavioural and brain biochemical parameters, and compared with disease control (TDS) group.

Results: In the EPM test (for anxiety), TDS induced an anxiogenic response (decreased % open arm entries) which was inhibited by L-arginine (NO mimetic), but not with L-NAME (NOS inhibitor). Biochemical studies of brain homogenates showed that TDS induced increased MDA and NOx metabolites in brain, and these alterations were attenuated by L-arginine. The L-arginine effects were comparable with fluoxetine on both neurobehavioral and brain biochemical parameters.

Conclusion: The NO mimetic drug, L-arginine, attenuated behavioural manifestations and brain biochemical changes of PTSD by interacting with oxidative stress pathways and could be a potential therapeutic agent in this stress disorder.

Keywords: Post-traumatic stress disorder, Time Dependent Sensitization, Nitric Oxide, Oxidative stress

Introduction

Post-traumatic stress disorder (PTSD) is a neuropsychiatric condition associated with traumatic experiences and can develop years after exposure to the stressors/trauma. It is a

classic example of psychological stress and trauma, can trigger a cascade of neurohumoral and immunological dysfunction that damage the body over time [1,2]. It is not only a lifestyle disorder but can be seen in special situations eg. in armed forces, following natural disasters, terrorist acts, or domestic violence, etc [3]. Recently, the global COVID-19 pandemic which has had an impacting influence on health and disease has also been considered as one of the aetiological factors for PTSD [4,5]. Studies have showed that such PTSD individuals are potentially susceptible to cardiovascular and immunological disorders which can also contribute to increased morbidity and mortality [6].

PTSD also results in complex neurobehavioral alterations among which anxiogenesis and cognitive dysfunction are important clinical features [7,8]. Though the neurochemical basis of PTSD involves interacting signalling pathways [9], the pharmacological basis of this stress disorder is poorly understood. Treatment strategies for PTSD primarily utilize psychotherapeutic agents like anxiolytics and anti-depressants and long-term use of such centrally acting agents are not only inconsistent but also associated with adverse drug reactions [10]. Thus, there is an unmet need to explore newer therapeutic approaches which are safe and efficacious in the long term.

Nitric oxide (NO) is a ubiquitous, gasotransmitter signalling molecule in the brain and its role in neuropsychiatric disorders has been suggested. L-Arginine- NO synthase-NO pathway had made a considerable impact on disease biology and therapeutics [11]. The role of NO in stress and stress related biological effects has been documented – but its role in PTSD has not been investigated. In view of the above, in the present study, we experimentally evaluated the effects of NO modulators on PTSD induced neurobehavioural and biochemical changes in rats.

Materials and Methods

Animals

Wistar rats (200-250g) of either sex was used. Animal care was done as per CPCSEA guidelines (Govt. of India). The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the Jamia Hamdard, New Delhi, India. They were housed in standard polypropylene cages under controlled room temperature (24± 2˚C) in a 12 h light--12h dark cycle and was given standard laboratory diet and water ad libitum. The animals were adapted to lab environment for 5 days before experimental testing.

Drug & Chemicals

L-Arginine HCl, L-NAME and Fluoxetine HCl were procured from Sigma-Aldrich (USA). ELISA kits for NOx were procured from Krishgen Biosystems (Mumbai). All other routine chemicals/biologicals for biochemical assays were procured from SRL Labs, New Delhi.

Experimental Animals

Wistar rats (150-200 gm) of either sex were used. The rats were maintained under standard laboratory conditions following CPCSEA guidelines. Animals was randomly assigned to the following groups (n=6 per group): a control group (vehicle, no TDS); Negative Control group (Vehicle + TDS); L-arginine (200 mg/kg) +TDS treated groups; L-NAME (50mg/kg) +TDS and Fluoxetine 10 mg/kg + TDS (Positive control).

The experimental groups were exposed to the TDS procedure and after the re-stress session on Day 7, the animals were allowed to remain undisturbed in their cages for a period of 7 days, during which they received either saline or one of the test drugs i.e. L-arginine and L-NAME by intraperitoneal injection.

Neurobehavioral studies

a. Experimental Model of PTSD -Time-dependent sensitization (TDS): It is a well-documented animal model of PTSD. Animal models of PTSD have utilized intense stressors, aversive challenges, and situational reminders of a traumatic stress in an attempt to model long-term effects on behavioral, autonomic, and hormonal responses seen in humans with PTSD. The TDS model emphasizes the role of prior trauma in predicting subsequent dysfunction, allows for the study of bidirectional expression of symptoms (enhanced or reduced responsiveness to environmental stimuli), and provides credible intra-subject variation [12].
b. Elevated plus-maze test (EPM; for Anxiety): The EPM consists of two sets of open arms and closed arms opposing each other of the same dimension (50 cm x 10 cm) at a height of 50cm from the floor. The central square (10 cm x 10 cm) gives the EPM the shape of (+) plus sign, in a dimly lit room. For testing anxiety, rats were placed singly in the centre of the maze and the number of open arm entries and open arm time were recorded for 5 min sessions [13].

Tissue extraction procedure

Following behavioural testing, rats were sacrificed by decapitation, brains removed and fixed in liquid nitrogen before storing at-86 ^{0}C. For assay, the brain tissue was homogenised in 1ml buffer (pH¼7.2) containing 25mM Tris, 1mM EDTA, homogenized with a Heidolph glass-teflon homogenizer (15 strokes on ice). The Samples were then centrifuged for 10 min at 14000rpm by a refrigerated centrifuge and the supernatant ware separated from the tissue pellet and kept on ice until used.

Biochemical Assays

a. Brain NOx (NO metabolites): The homogenised brain samples ware centrifuged at 10,000 g for 15 min at 4 °C and NOx was measured by the method of Tracey *et al* and protein estimation was done by Lowry *et al*. 1951 [14,15].
b. Brain MDA: MDA is a well-known marker of free radical induced lipid peroxidation and was assayed by the method of Okhawa *et al*. 1979 [16]. Specifically, the assay sample was made up of 0.2 ml of brain homogenate, 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid and. The sample volume was made 4ml by adding distilled water and heated at 95 ◦C for 60 min. Following this, the sample was cooled in tap water and 5 ml of n-butanol and pyridine (15:1, v/v) and 1ml of distilled water was added and centrifuged. The supernatant layer was collected, and its absorbance was measured at 532 nm using a UV-Vis spectrophotometer (Hitachi) and MDA content was expressed as nmol/mg protein.

Statistical Analysis

All data were expressed as Mean ± SE. The behavioural data were analyzed by using Kruskal-Wallis test followed by Mann-Whitney-U test for intergroup comparisons. The biochemical data was analysed by one-way ANOVA followed by Tukey's test. A p-value of at least 0.05 was considered as the level of significance in all statistical tests.

Results

Effects of NO modulators in TDS induced anxiety behavior on elevated plus maze test

Exposure of the rats to the TDS procedure (stresses + re-stress) resulted in the development of an anxiogenic response. The % no. of entries in open arms were markedly reduced in comparison to the control (no TDS) group of rats. A nearly 50% reduction in the OAE were seen in the TDS group which was statistically significant ($p < 0.05$ vs

controls; Mann-Whitney U test). Such changes were, however, not seen in the OAT time data of the TDS group, and the differences with the control group were not statistically significant ($p > 0.05$). L-arginine (200 mg/kg) reversed the effects of TDS on anxiety behavior in the EPM test, i.e., the OAE and OAT data were significantly higher when compared with both TDS and control groups ($p < 0.05$). However, the NO depletory, L-NAME (30 mg/kg) was not able to influence the EPM data of the TDS group by any appreciable extent, and though approx. 33% increases were seen in OAE, and OAT data as compared to the TDS group, which were not statistically significant ($p > 0.05$). On the other hand, pretreatment of rats with fluoxetine (10 mg/kg) also caused an increase in the OAE and OAT responses when compared to the disease control (TDS) group and were comparable to the results seen in the L-arginine group of rats (Table 1).

Table 1: Effects of NO modulators on TDS induced changes in Elevated Plus Maze (EPM) test in rats

Treatment Groups(mg/kg)	EPM - Open arm entries (%)	EPM - Open arm Time (%)
Controls (no TDS)	42.29 ± 7.0	16.0 ± 3.5
TDS	25.0 ± 4.6*	26.0 ± 5.5*
L-Arginine (200mg/kg) + TDS	62.9 ± 3.8[a]	68.6 ± 4.3[a]
L-NAME (30mg/kg) + TDS	33.8 ± 4.0	34.1 ± 2.3
Fluoxetine (10) + TDS	61.7 ± 3.8[a]	69.5 ± 3.8 [a]

All data are Mean ± SE; V – Vehicle; TDS – Time Dependent Sensitization; * $P < 0.05$ (vs controls); [a]$p < 0.05$ (vs V + TDS)

Effects of TDS on brain biochemical parameters

Assay of brain homogenates of the TDS exposed and EPM tested rats showed that TDS induced substantial rise (nearly 2-fold) in the brain MDA levels (an index of lipids peroxidation) when compared to control values, which were statistically significant ($p < 0.05$).

L-arginine (200 mg/kg) appreciably reduced the elevated MDA levels seen in the TDS group (nearly half fold reduction was seen) and the data of the L-arginine treated group was markedly different when compared to TDS group of rats. However, L-NAME (30 mg/kg) induced inconsistent effects on the MDA response, and though there was an overall reduction in the mean values, the differences were not statistically significant. Marked reductions in the brain MDA levels were also seen after pretreatment with the comparator drug, fluoxetine (10 mg/kg) and these differences were comparable with those seen in the L-arginine group treated rats.

NO metabolites are good indices of tissue NO activity and accordingly we assayed brain NOx levels in different treatment groups exposed to TDS and EPM. Our results showed that brain NOx levels were raised significantly in the TDS exposed group of rats (though not as marked as the changes seen in MDA) ($p < 0.05$, vs controls). L-arginine treatment lowered the raised levels of NOx seen in the TDS group, but L-NAME treatment was ineffective in this regard, i.e. no significant differences were seen between the TDS and L-NAME + TDS treated groups ($p > 0.05$). As seen earlier with MDA, the fluoxetine + TDS treated rats showed lowered brain NOx levels in comparison with the TDS alone, and these changes were comparable with L-arginine + TDS group (Table 2).

Table 2: Effects of Time dependent sensitization (TDS) and NO modulators on brain MDA and NOx levels in rats

Treatment Groups (mg/kg)	Brain MDA levels (uM/ml)	Brain NOX levels ((uM/ml)
Controls (no TDS)	55.2 ± 5.5	14.98 ± 2.48
TDS	123.2 ± 17.0*	41.74 ± 4.64*
L-Arginine (200mg/kg) + TDS	76.1 ± 6.2 [a]	22.29 ± 6.46[a]
L-NAME (30mg/kg) + TDS	133.9 ± 12.5	54.11 ± 3.93
Fluoxetine (10mg/kg) + TDS	42.59±5.95	25.98 ± 3

All data are Mean ± SE. *p< 0.05 (vs controls); [a]p < 0.05 (vs TDS)

Discussion

PTSD is a unique and complex disorder with multisystem involvement and for which therapeutic strategies are poorly defined [17]. Our present study attempted to evaluate the influence of nitric oxide (NO) and NO signalling pathways in an experimental model of PTSD in rats in order to explore newer safe and effective newer therapeutic approaches for PTSD. Time Dependent sensitization (involves stress + re-stress) emphasizes the role of prior trauma exposure in predicting subsequent neurobehavioural dysregulation which is commonly seen in PTSD patients.

In our study, we used this TDS model to simulate PTSD in animals as shown by the anxiogenic response of TDS group of animals in EPM test. In the EPM, reduction in open Arm Entries (OAE) and/or Open Arm Time (OAT) is well documented as an indicator of anxiogenesis in rodents [18]. The results of our study with TDS group confirmed the expression of anxiety in that group of rats. The role of NO in stress and anxiety has been documented [19] but its role in PTSD has not been clearly defined. NO modulators are used in experimental situations to evaluate the role of NO in disease biology and our present results showed that L-Arginine, a NO precursor attenuated TDS induced anxiogenesis as evidenced by the increased number of OAE (%) and OAT (%) in the EPM test. However, L-NAME, a NOS (Nitric oxide synthase) inhibitor and NO depletor did not showed similar effects in the EPM test. This could have been due to the fact that TDS would have produced maximum anxiogenesis which could not be influenced further by the NOS inhibitor. Similar attenuations in stress-induced neurobehavioral changes by NO modulators and L-arginine in particular, have been reported earlier [20,21].

Biochemical assay of brain homogenates was performed to support neurobehavioural data seen in experimental PTSD. NO has a very short half-life (<10 sec) and NO metabolites (Nitrates, Nitrites, NOx) are effectively used to measure NO activity in the brain (20). TDS induced elevations in brain NOx levels suggesting a possible association between PTSD and brain NO. Interestingly, L-Arginine attenuated TDS induced changes in brain NOx indicates the involvement of NO independent pathways in the effects of L-Arginine. On the other hand, similar reduction in brain NOx levels were not seen with L-NAME.

The susceptibility of brain tissue to oxidative stress is well known and stressful experiences are known to disrupt the redox balance in favour pro-oxidant forces. Role of oxidative stress in stress and anxiety has been proposed [21,22]. Interaction between reactive oxygen species and NO are well known in a variety of pathophysiological states including stress and antioxidant effects of L-arginine are reported [23]. Our present study is an agreement with the same. TDS induced rise in brain MDA levels, which is a consistent marker of lipid peroxidation and oxidative stress- suggesting the association between PTSD and brain oxidative stress. The fact that L-Arginine markedly lowered brain MDA levels as compared to the disease control (TDS) group

suggested the possible involvement of antioxidant effect of L-Arginine in this response. L-NAME induced reductions in brain MDA levels (comparatively marginal reduction) also suggested that NO independent mechanisms also responsible for the PTSD attenuating and anxiolytic effects of L-Arginine.

Taken together our preclinical studies suggested that L-Arginine has a potential of being a therapeutic agent for the treatment of PTSD and interactions of NO with reactive oxygen species could be involved in such effects. The role of L-Arginine as a nutraceuticals/dietary supplement has been suggested for cardiovascular diseases and could be beneficial in stress related disorders like PTSD.

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