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Original Article

Neuroprotective Effect of p38 MAPKs Inhibitor (SB239063) and Vitamin B_{12} against Lipopolysaccharide Induced Neuroinflammation

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ABSTRACT

Objectives: To evaluate the effect of MAP kinase inhibitor (SB239063) on neuroinflammation along with Vitamin B_{12}. **Materials and Methods:** Male wistar albino rats divided in to eight groups each group having six animals. Neuroinflammation was induced in animals by the lipopolysaccharides/ LPS (100µg/ml) fallowed by treatment with MAPK inhibitor (SB239063) alone (5mg/kg) and along with Vit B_{12} (0.5mg/kg) for 30 days. Subsequently, animals were screened in the behavioral tests by using radial arm apparatus and actophotometer apparatus followed by biochemical and histopathological estimations. **Results:** Chronic LPS, 100 µg/kg i.p., caused physical and mental abnormalities in animals, including memory impairment, reduced locometer activity abnormal posture and cognitive deficit. Biochemical analysis of brain homogenate in LPS treated rats showed altered biochemical marker. Neurohistological alterations of various parts of brain of LPS treated rats exhibit severe neuronal space, irregular damaged cells. MAP Kinase inhibitor (SB239063) alone and along with Vit B_{12} administered for 15 days significantly improved motor performance and cognitive behavior task and restored the histopathological changes. **Conclusion:** MAP Kinase inhibitor (SB239063) alone show significant effect at dose 5 mg/kg, I.V and whenever it was used in combination at reduced concentration with Vitamin B_{12} provided slow neuroprotective effect.

Key words: p38 MAP Kinase, Neuroinflammation, Vitamin B_{12}, Neurodegenerative diseases, Lipopolysaccharide.

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INTRODUCTION

Neurodegenerative diseases are devastating conditions for which there is no cure so far. In general, the mechanisms involved in disease onset and development are still poorly understood. Therefore, increasing efforts are being made to better comprehend their pathogenesis. Among the different factors involved in these conditions, inflammation is considered a key contributor. Several lines of experimental evidence have demonstrated that neuronal cell death may induce an inflammatory process and inflammation by itself may lead to cell death.[1] Thus, it is necessary to induce inflammation in models of neurodegeneration in order to evaluate its intricate consequences. Induction of inflammation may be achieved in different manners and lipopolysaccharide (LPS) is an important tool for this purpose.

Protein kinases have become one of the important targets in drug discovery since the beginning of the 21st century, with marketing approvals for therapeutic applications, especially cancers.[2] However, compounds targeting protein kinases are still limited in other therapeutic areas, despite the crucial roles of these enzymes in various pathophysiological processes. Among such kinases, Mitogen-activated protein kinase (MAPK) has attracted tremendous attention due to its roles in Numerous cellular events, including differentiation, mitogenesis, cell survival and apoptosis.[3] p38 MAPK is a class of MAPKs responsive to stress stimuli such as inflammatory cytokines and reactive oxygen species (ROS). Many studies have revealed a central role of p38 MAPK in chronic inflammation, leading to preclinical or clinical trials for the application of p38 MAPK inhibitors in inflammatory diseases such as rheumatoid arthritis and asthma.

MAPKs are serine/threonine protein kinases that process and regulate cellular properties in Response to a wide range of extracellular stimuli. These enzymes phosphorylate the OH group of serine or threonine in proteins and play important roles in the regulation of cell proliferation, differentiation, survival and apoptosis. In mammalian cells, several distinct MAPKs have been identified, including p38 MAPK, c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK 1/2) and ERK 5/BMK-1. Among MAPKs, p38 MAPK is involved in a wide range of signaling pathways that stimulate different biological functions. In particular, p38 MAPK has been found to play an essential role in the MAPKs are serine/threonine protein kinases that process and regulate cellular properties in response to a wide range of extracellular stimuli. These enzymes phosphorylate the OH group of serine or threonine in proteins and play important roles in the regulation of cell proliferation, differentiation, survival and apoptosis. In mammalian cells, several distinct MAPKs have been identified, including p38 MAPK, c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK 1/2) and ERK 5/BMK-1. Among MAPKs, p38 MAPK is involved in a wide range of signaling pathways that stimulate different biological functions. In particular, p38 MAPK has been found to play an essential role in the regulation of pro-inflammatory signaling networks and in the biosynthesis of cytokines, including tumor necrosis factor-α (TNF- α) and interleukin-1 β (IL-1β) in γ immune cells.[4] p38 MAPK comprises four isoforms (α, β, γ and δ). P38alfa_ and beta_ are approximately 70% identical, whereas p38 γ and δ share approximately 60% sequence identity

with p38 α among p38 isoforms, α and β are ubiquitously expressed in most tissues, including the brain,[5] whereas γ and δ exhibit tissue specific variations in expression.[6-7] Since discovering p38 α is primarily responsible for regulating inflammation, most studies have intensively focused on p38α.[8]

Vitamin B_{12} (Mecobalamin, MeCbl) is an important micronutrient that is required in numerous biological processes.[9] It is considered a coenzyme in folate metabolism and nucleotide biosynthesis, which makes it crucial in the metabolism of fatty acids and some amino acids and normal nervous system function.[10] Furthermore, vitamin B12 deficiency results in methionine deficiency, leading to the dyes-synthesis of both phospholipids and myelin.[11] Currently, combination therapy with Vitamin B_{12} is widely combined and used in clinical patients with nerve diseases. It has been reported that systemic administration of vitamin B12 promoted the recovery process from peripheral nerve damage in experimental rats.[12] Additionally, Vitamin B_{12} was recently shown to be a superoxide scavenger contributing to neuronal cells axonal growth.[13] Thus, we hypothesized that Vitamin B_{12} could enhance axon formation after TBI via stabling microtubule and reducing neuronal apoptosis.

In this study, we investigated the possible protective effect of p38 MAP Kinase inhibitor alone and in combination with Vit B12 LPS induced neuroinflammation in brain. Neuronal. Thirty days of study was carried out in Wistar rats, following which behavioral, histological and oxidative stress-related biochemical parameters were studied under different treatment conditions.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used which were of analytical grade were products of Sigma Aldrich Ltd., Medchem express and are prepared in volumetric flask using glass wares with distilled water.

Animals

Male albino wistar rats (2.5 month old, weighing about 100-200gm) were used for the study. The animal were housed in standard polypropylene cages and maintained under controlled room temperature (22+2°C) and relative humidity (55+5%) with 12:12 hr light and dark cycle. All the animals were provide with commercially available normal pellet diet and water. The animals were acclimatized to laboratory conditions before behavioral experiments that were carried out between 09:00 and 17:00 h. The experimental protocol was approved by the institutional animal ethics committee, the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) Registration no. 1446/PO/a/11/CPCSEA of the Govt. of India was followed and prior permission will be granted from the Institutional Animal Ethics Committee for conducting the experimental studies.

Induction of Neuroinflammation

Male wistar albino rats divided in to eight groups each group having six animals. Neuroinflammation was induced in animals by the LPS (100 µg/kg) by intraperitoneal (i.p.).

Group 1- Normal control received normal saline (1ml/kg,P.O.)

Group 2-Diseased control received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study.

Group 3-Test group received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study +SB239063 (5 mg/kg, I.V.) on 21st to 30 th day of study.

Group 4-Test group received received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study + Vit B12 alone (0.5 mg/kg P.O.) on 21st to 30th day of study.

Group 5-Test group received Test group received received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study + SB239063 (2.5mg/kg,I.V.) + Vit B_{12} (0.5 mg/kg P.O.) on 21st to 30th day of study.

Group 6- Test group received received received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study + SB239063 (2.5mg/kg, I.V.) + Vit B12 (0.5 mg/kg P.O.) on 21st to 30th day of study.

Group 7- Test group received received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study + SB239063 (2.5mg/kg, I.V.) + Vit B_{12} (0.5 mg/kg P.O.) on 21st to 30th day of study.

Group 8- Standard control received received LPS (100 µgm/kg, I.P.) on 1st, 7th, 14th, 21st and 30th day of study + Donepazil (1 mg/kg, P.O.).

Behavioral methods

In this study we were using two behavioral models namely radial arm apparatus and actophotometer.

Radial arm maze test

Locally fabricated wooden radial arm maze elevate 50 cm above the floor consist of an octagonal central hub 36cm in diameter with eight radial arms. Each arm is 43 cm long, 15 cm wide with 12 cm sides and has small black plastic cups mount at 30cm from the central hub. Each rat maintain at 85% of its total diet, is exposed to the maze daily with the food pellet in a fix arm followed by respective drug treatment for the period of 07 days. The apparatus is cleaned with damp cloth after each trial to avoid place preference and the influence of olfactory stimuli. The evaluation is carried out on 7th day, 60 minutes after the respective drug treatment where in a food pellet is placed in a variable arm for evaluation of working memory. Each mouse place on the central hub is allowed to choose any of the arms freely to get the food. Latency to find food is recorded as a measure of working memory evaluation.[11] The comparison is made against the vehicle treated control group and the data is expressed as mean ± SEM.[14]

Actophotometer test

The animal locomotor behavior was monitored using Actophotometer, described by Dews P.B. (1953). Actophotometer (Dolphin make) provided with a digital counter, photocell and a light source were used to measure locomotor activity (horizontal movement) of animals. Each animal was placed in Actophotometer for 5 minutes and basal activity score was recorded for all animals. Each animal was treated with respective drug and activity score was recorded after 30 min and 1hr. Deceased activity score was taken as index of CNS depression.[15]

Biochemical estimations

Brain was isolated from the normal and neuroinflammed rats. It was washed in tris buffer (pH 7.4), weighed and then placed in tris buffer and homogenized in the homogenizer. The homogenate was centrifuged at 3000 rpm in the centrifuge machine and the supernatant was taken for the study.

Estimation of acetylcholinesterase levels

The quantitative measurement of acetylcholinesterase (AchE) activity in the brain was performed according to the method described by Ellman et al. The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01M sodium phosphate buffer (pH 8), 0.10 ml of acetylthiocholine iodide,and 0.10 ml of DTNB (Ellman's reagent). The change in absorbance was measured immediately at 412 nm spectrophotometrically. The enzymatic activity in the supernatant was expressed as µM/mg protein.[16]

Table 1: Assessment of memory by using radial arm apparatus.

Treatment	Time taken to collect food on day 1 (Sec.)	Time taken to collect food on day 7 (Sec.)	Time taken to collect food on day 14 (Sec.)	Time taken to collect food on day 21 (Sec.)	Time taken to collect food on day 30th (Sec.)
Group 1	52.4±1.67	53.0±1.58	52.4±1.67	52.8±1.78	52.6±1.81
Group 2	51.8±0.83	55.6±1.8**	m59.2±2.1**	65.0±2.34**	70.6±1.9**
Group 3	52.6±1.51	53.8±2.16**	60.4±2.07**	65.0±2.34**	55.6±1.94**
Group 4	29.0±1.00	55.0±2.70**	61.6±2.07**	64.2±2.58**	63.0±1.87**
Group 5	53.2±1.78	57.0±2.12**	63.2±1.30**	59.2±1.64**	57.4±2.07**
Group 6	52.6±1.51	56.4±1.14**	62.2±1.92**	62.0±2.34**	61.6±2.07**
Group 7	52.6±1.51	55.6±1.81**	59.8±1.92**	64.0±1.87**	59.2±2.16**
Group 8	52.6±1.5**	55.8±1.92**	59.6±2.07**	55.4±2.30**	51.0±2.34**

The statistical significance of difference between means was calculated using one-way Analysis of Variance (ANOVA) followed by tukey multiple comparison test *$N = 6$
Values were expressed as Mean ± SEM *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 2: Assessment of locomotor activity by using actophotometer apparatus.

Treatment	Photo cell count on day 1	Photo cell count on day 7	Photo cell count on day 14	Photo cell count on day 21	Photo cell count on day 30th
Group 1	269.8±1.84	270.4±1.94	271.0±2.34	269.0±2.34	272.8±1.78
Group 2	270.0±1.87	267.2±2.1**	228.6±3.2**	175.4±1.9**	160.6±2.3**
Group 3	270.0±2.0	266.0±2.96**	227.8±2.38**	222.0±1.22**	231.8±5.16**
Group 4	269.8±2.04	268.4±2.51**	228.6±2.40**	179.8±2.04**	190.8±2.04**
Group 5	270.0±2.00	268.4±2.07**	229.6±2.07**	215.4±4.98**	225.0±2.34**
Group 6	269.6±1.81	267.2±2.16**	229.4±1.94**	209.6±2.51**	215.4±1.94**
Group 7	268.6±0.89	267.0±2.23**	228.4±3.50**	206.0±2.00**	211.6±1.51**
Group 8	269.6±2.07	267.6±2.51**	228.4±3.20**	232.2±2.86**	253.4±2.60**

The statistical significance of difference between means was calculated using one-way Analysis of Variance (ANOVA) followed by tukey multiple comparison test *$N = 6$
Values were expressed as Mean ± SEM *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Histological Examination

Histopathology of brain tissues was performed by Humanson method.[14] All sections of brain tissue were fixed with with 10% neutral buffered formalin. All tissues of brain were processed and embedded in paraffin wax and sectioned at 4 µm thickness. The sections were stained with hematoxylin and eosin. The dried sections were seen under Leica microscope. All images were captured at 10x and 40x.

Data analysis

Data were expressed as the mean ± SD of 9 determinations. Statistical analysis was performed using one-way Analysis of Variance (ANOVA) followed by tukey multiple comparison test. All these analyses were done using Graph prism pad 7.03 Software.

RESULTS

In the present study initially behavioral models (radial arm apparatus, actophotometer) were used to evaluate the memory of neuroinflammed rats which were followed by biochemical analysis and histopathology.

Behavioral assay

Assessment of memory by using radial arm apparatus: Results of present study are summarized in the Table 1 Results of the table reveal that administration of LPS(1mg/kg,i.p.) on 1st, 7th, 14th, 21st, 30th days caused significant increase ($p<0.01$) in time taken to collect food in all group. Treatment of the neuroinflammed rats by compound SB239063 from 21st to 30th day of study caused marked decrease in time taken to collect food as compared to the LPS treated rats. Administration of the

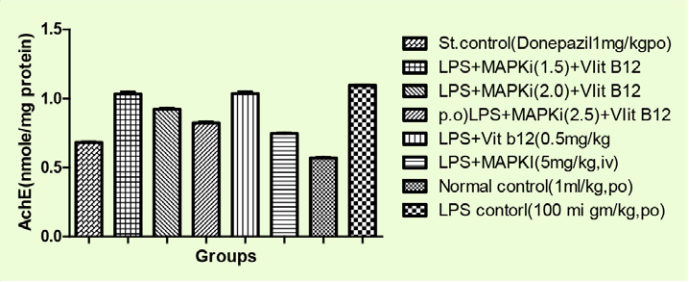

Figure 1: Assessment of acetyl cholinesterase.

combination of SB239063 at a dose of 4.5 mg/kg along with vit. B12 at a dose of 0.5 mg/kg also caused significant reduction as compared to LPS treated animals. Vitamin b12 given alone and combination of compound SB239063 and Vit B12 at a dose of 3.5mg/kg and 0.5 mg/kg also caused moderate decrease in the time taken to collect food in neuroinflammed rats however combination of compound SB239063B12 at a dose of 2.5 mg/kg does not cause any significant decrease in the time taken to collect food in neurotonic rats. The standard drug donepezil also provided maximum protection in neuroinflammation in LPS treated rats. Results indicates that the compound SB239063 at a dose of 5 mg/kg,i.p., alone and combination of SB239063 at a dose of 4.5mg/kg,i.p. +0.5mg/kg,.p.o., caused maximum improvement in time taken to collect food and memory in neuroinflammed rats.

Assessment of locomotor activity by using actophotometer apparatus: Results of present study are summarized in the Table 2. Results of the

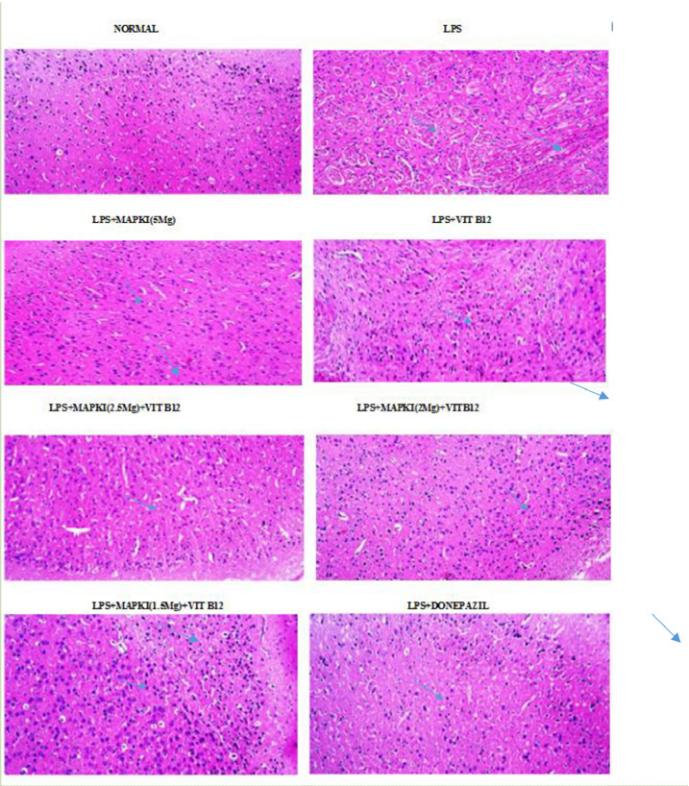

Figure 2: Effect of SB239063 alone and their combination at different doe's levels on the brain histology in LPS induced neuroinflammation in rats.

table reveal that administration of LPS(1mg/kg,i.p.) on 1st, 7th, 14th, 21st, 30th days caused significant decreases (p<0.01) in photo cell count in all group. Treatment of the neuroinflammed rats by compound SB239063 from 21st to 30th day of study caused marked increase in photo cell count (222.0±1.22), (231.8±5.16) as compared to the LPS treated rats (270.0±1.87), (267.2±2.16), (228.6±3.20), (175.4±1.94), (160.6±2.30). Adminstration of the combination of SB239063 at a dose of 4.5 mg/kg along with vit. B12 at a dose of 0.5 mg/kg also caused significant increase (215.4±4.98), (225.0±2.34) as compared to LPS treated animals. Vit b12 given alone and combination of compound SB239063 and Vit B12 at a dose of 3.5mg/kg and 0.5 mg/kg also caused moderate increase in the photo cell count in neuroinflammed rats however combination of compound SB239063B12 at a dose of 2.5 mg/kg dose not cause any significant increase in the photo cell count in neurotonic rats. The standard drug donepezil also provided maximum protection in neuroinflammation in LPS treated rats. Results indicates that the compound SB239063 at a dose of 5 mg/kg,i.p., alone and combination of SB239063 at a dose of 4.5mg/kg,ip.,+0.5mg/kg,.po.,caused maximum improvement in photo cell count and memory in neuroinflammed rats.

Biochemical assay

Assessment of acetyl cholinesterase: Figure 1 shows that level of acetylcholinesterase on administration of LPS on 1st, 7th, 14th and 21day of study caused significant increase in brain acetylcholinesterase activity as compared to normal animals.

Treatment with SB239063 (5.0mg/kg) for 21st to 30 days of study caused significant decrease in the level of acetylcholinesterase activity. At lower dose level (2.5mg/kg) SB239063 along with 0.5 mg/kg of vit B12 caused no changes in brain acetylcholinesterase activity in neuroinflammed mice. However donepezil caused maximum decrease in the activity of brain acetycholinesterase in neuroinflammed rats.

In the present study increase in the brain acetylcholinesterase level after administration of LPS indicating loss of cholinergic neurons decline in acetylcholinesterase level and loss of memory. SB239063 protected the cholinergic neurons by decreasing the level of acetylcholinesterase which indirectly reflected increase in brain acetylcholinesterase level.

Histology

In Figure 2 it was observed that the LPS treated rats caused neuronal injury induced cell damage on Hippocampal slices were observed clearly when compared with control because of microglial activation results in neuroinflammation. After treatment with the MAPK inhibitor alone and along with Vitamin B12 showed increase in the regeneration of neuronal cells with composite nature and same process obtained from Donepazil treated group. This clearly showed that MAPK inhibitor alone and along with Vitamin B12 protects the cells from get damage by built up of neuronal cell structure and reduces the cell edema. MAPK inhibitor at a dose 5 mg/kg expressed better protection when compared to other combinations and doses.

DISCUSSION

Neuroinflammation is a type of immune response in the CNS and is observed in diverse neurodegenerative disorders, such as AD, depression, multiple sclerosis and Parkinson's disease. Accumulating evidence indicates that neuroinflammatory processes also directly contribute to p38 MAPK, including the activation of astrocytes and microglia, which can increase the expression of cytokines or chemokines.[17] In our present study we use LPS for induction of neuroinflammation. LPS-stimulated p38 MAPK cascades in murine cells have been associated with the production of IL-1β[18] Interestingly, Aβ fibril, a representative pathological marker in AD, has also been identified as a stimulus activating p38 MAPK cascades for the production and upregulation of pro-inflammatory cytokines in microglia.[19] Giovanni *et al.* validated these findings in *in vivo* experiments by showing that p38 MAPK signal transduction in microglial cells is crucial for Aβ-induced neuroinflammation.[20]

In the CNS, p38 MAPK is highly expressed in regions that are crucial for learning and memory and is likely a key component in higher brain functions.[21] Therefore, dysfunction of this pathway might be related to the pathology of some neurological disorders, such as AD, ischemia, neuropathic pain, epilepsy and depression. P38 MAPK has also been implicated in inhibition of embryonic stem cell differentiation into neurons, regulation of synaptic plasticity and modulation of neuronal excitability.[22] Inhibition of p38 MAPK has been assessed in in vivo and in vitro experiments using various models of neurological disorders, and, in most cases, these p38 MAPK inhibitors have been shown to be effective, indicating that they could be used for the treatment of various inflammatory disease.[23]

CONCLUSION

In the present research work MAP Kinase inhibitor (SB239063) alone show significant effect at dose 5 mg/kg, I.V and whenever it was used in combination at reduced concentration With Vitamin B12 provided slow neuroprotective effect, which was confirmed by behavioral paradigms and biochemical test. It restored the behavioral and biochemical alteration caused by LPS and confirmed the strong neuroprotective mechanism of SB239063 with Vit B12 in LPS intoxicated memory and cognitive abnormalities.

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CONFLICT OF INTEREST

The authors declare no Conflict of interest.

ABBREVIATIONS

LPS: lipopolysaccharide; **TNF:** tumor necrotic factor; **CNS:** central nervous system; **AD:** Alzheimer's disease; **PD:** Parkinson's disease; **MAPK:** Mitogen activated protein kinase; **CSAID:** cytokine-suppressive anti-inflammatory drugs; **RA:** Rheumatoid arthritis; **IBD:** inflammatory bowel disease; **CD:** crohn's disease.

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