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Glycine Mitigates Lipopolysaccharide Induced Neurotoxicity via Inhibition of TLR-4 Signaling Pathway in Developing Mice Brain

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ABSTRACT

Background: Lipopolysaccharide (LPS) is an endotoxin of the gram-negative bacteria, playing a crucial role in systematic toxicity and cause neurotoxicity in immature brains. Glycine is non-essential amino acid, acting as a neurotransmitter, antioxidant and immune function regulator.
Objective: To investigate the potential use of glycine inhibition of TLR-4 receptor to reduce LPS induced neurodegeneration in the developing brain of post-natal day 7 (PND-7) mice.
Materials and Methods: A total of 12 PND-7 mice were included in this experimental study from Feb. 2023 to May 2023 at NMMRC, Peshawar. PND-7 mice were randomly distributed into four groups, a control group, a LPS group, LPS+Gly group and Gly group. LPS (250µg /kg) was administered to LPS group. Glycine (1g/kg) was injected after LPS administration to LPS+Gly group and Gly group mice. After 4hr of the drug treatment, all the PND-7 mice were sacrificed for Western blot analysis. ImageJ software was used for the densitometry of the blots. One way ANOVA and post-hoc tukey tests through Prism graph-5 were applied for statistical analysis. P-value=0.05 was considered statistically significant.
Results: Significant differences were observed in the assessed markers in of PND 7 mice brains. Post hoc tukey test revealed significant changes ($p<0.01$) in the TLR-4 along with BAX, Casp-3, PARP-1and Bcl-2 levels in LPS group. However, significant decrease was observed in TLR-4 levels along with successful amelioration of BAX, Bcl-2, Casp-3 and PARP-1 level in LPS + Gly group as compared to LPS group.
Conclusion: Glycine administration significantly improved LPS induced neurodegeneration in brain of the PND-7 mice.
Keywords: Apoptosis, Caspase-3, Glycine, Lipopolysaccharide, Neurodegeneration, PARP-1, TLR-4 signaling pathway.

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INTRODUCTION

Neurodegenerative diseases are clinically a heterogeneous group of illness including Parkinson's disease (PD), Alzheimer's disease (AD), frontotemporal dementia, prion diseases, Huntington's disease and chronic traumatic encephalopathy which are commonly associated with the death of neuronal cell of brain.[1] Neurodegenerative diseases include a wide range of similar signs and symptoms like weakness, mood imbalance, psychosis, instability and cognitive impairment.[2] These diseases share some common features which can help to understand its mechanism like accumulation of endogenous protein in the misfolded form which are insoluble inclusion.[3]

Bacterial (gram negative bacteria) and viral infections (herpes simplex type-1, human immunodeficiency virus, influenza virus, and by Chlamydia pneumonia) have been reported as different sort of causative agents for neurodegenerative diseases including Parkinson, Alzheimer, Prion and Amyotrophic lateral sclerosis disease.[4] Besides bacteria and viruses some chemicals like alcohol are also involved in causing neurological disorders.[5]

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Inflammatory processes and host immune responses are activated after getting entry of the above causative agents in neuronal cells which result in altering of neuronal cell function of CNS.[6] These viral and bacterial agents are able to develop molecular hallmarks of neurodegenerative disease such as the creation and deposition of misfolded form of protein aggregates, insufficient autophagic process, oxidative stress, synaptopathies and neuronal cell death.[7]

Lipopolysaccharide (LPS) is a chronic compound found in the cell membrane of gram negative bacteria.[8] This endotoxin from the outer membrane of bacteria is very toxic in nature and it is known as a potent agent to trigger neurodegeneration and neuroinflammation.[9,10] The peripheral administration of LPS in mice via intraperitoneal induced neuroglial cells activation including (microglia and astrocytes) as well as inducible nitric oxide synthase, cyclooxygenase-2, chemokines and proinflammatory cytokines production in brain.[11] Intracellular accumulation and aggregation of amyloid precursor protein, amyloid β and hyper-phosphorylated tau protein and exacerbation of memory deficits are the main defects of LPS.[12]

Glycine is a non-essential amino acid isolated in 1820 by French chemist from acid hydrolysate.[13] It is required for the proper function and growth of fetus. Elastin and collage structural proteins are made up of glycine. Glycine provides plasticity to the enzyme

active site. It acts as neurotransmitter and can maintain homeostasis.[14] Cytokine production and immune function regulation is done by glycine by changing the level of calcium ions. There is effective absorption of the lipid and lipid soluble vitamins in the presence of glycine. Glycine is having a vital role in many reactions like haem formation, gluconeogenesis and immune system suppression after transplantation. Glycine is also is used as anti-cancer therapy.[15]

LPS can cause neurotoxicity and cause exacerbation of memory deficits[16] while glycine acts as an anti-inflammatory, anti-oxidant, immunomodulator and neuroprotective agent.[17,18] Now, whether Glycine can display the same anti-oxidant and anti-inflammatory properties against the possible neurotoxic effects of LPS, we intended to investigate the beneficial effects of Glycine against LPS induced neurotoxicity in PND-7 albino mice brain providing a potential useful therapeutic index for preventing neurodegeneration and its consequences.

MATERIALS AND METHODS

Animals

A total of 12 post-natal day 7 (PND-7) mice were purchased from Veterinary Research Institute, Peshawar and were included in this experimental study. Resource equation approach was used for sample size calculation. PND-7 mice were randomly distributed into four groups (n=3 each), a control group, a LPS group, LPS + Gly group and Gly group. These mice were labeled and kept in separate cages (Biobase China) at NMMRC, Peshawar. LPS was administered intraperitoneally (IP) at a dose of 250µg/kg to LPS group PND-7 mice. On the other hand, Glycine (1g/1000g) was injected after LPS administration to LPS+Gly group. Glycine (1g/1000g) alone was injected into the peritoneal cavity of Gly-group mice. Mice that did not receive Glycine were injected with an equivalent amount of saline. After 4 hours of the LPS and Glycine administration, all the PND-7 mice were sacrificed for laboratory investigations. It was an experimental study performed at Neuro Molecular Medicine Research Center (NMMRC), Peshawar from Feb. 2023 to May 2023. All the rules and regulations of animals grouping and their life standard were in accordance with the European Union regulations on animal research guidelines.

Western Blotting

Neurodegeneratory markers i.e.; BAX, Bcl-2, Caspase-3 and PARP-1 are commonly analyzed markers in the previous studies; hence we analyzed the same markers in our study. All animals were sacrificed in accordance with the method described earlier.[19] After decapitating the animals, we carefully and promptly removed the brain tissue and stored it on ice in a 1:1 mixture of RNA later solution and PBS. A total protein extraction solution (T-PER) was used to properly homogenize the brain tissue, which was then collected and kept at -20ºC for future analysis. Absorption at 595 nm was used to measure protein concentration in homogenates using a Bio-Rad protein estimation assay. SDS-PAGE at a concentration of 12-15% was used to electrophorese each sample's protein after it had been standardized to 30mg. Semi-dry Bio-Rad transblott technology was used to transfer proteins from gel to PVDF membranes (Santa Cruz, CA, USA). Different mouse derived primary antibodies such as anti-BCL-2 (SC-7382), anti-BAX (SC-7480), anti-CAS-3 (SC-7272), anti-PARP-1 (SC-8007), anti-TLR-4 (SC-13593), and β-actin (SC-47778) of Santa Cruz Biotech, CA, USA were used to detect and localize protein of interest. HRP conjugated secondary antibodies were procured from Promega, Melbourne, USA. The trans-blot results of electrophoresis were developed on X-rays films using chemiluminescence assay reagents. ImageJ software was used to perform the densitometry analysis of the bands in arbitrary units (A.U.) relative to the untreated control.

Statistical Analysis

Protein bands of the Western blot were analysed through Image J software. Arbitrary units (A.Us) as Mean ± S.E.M were used to express proteins' Integral optical density (IOD). One-way analysis of variance (ANOVA) was used to determine significant difference, followed by Post-hot tukey's test of the Prism graph-5. Less than 0.05 ($p<0.05$) p-value was considered to be statistically significant.

RESULTS

Glycine inhibited TLR-4 to reduce LPS induced neurodegeneration in PND-7 mice brain

In the current study, one way ANOVA showed significant differences ($p<0.01$) in the expression levels of various apoptotic neurodegeneratory markers in the brain homogenates of PND-7 mice brain. Post-hoc tukey analysis revealed that LPS caused significant increase ($p<0.01$) in the TLR-4 receptor protein expression. However, Glycine administration significantly inhibited ($p<0.05$) the protein expression of TLR-4 in LPS+Gly group mice brain as compared to LPS group mice (Figure 01).

One way analysis showed significant differences ($p<0.001$) in the expression levels of all the assessed neurodegeneratory markers i.e.; BAX, Bcl-2, Caspase-3 and PARP-1 in the brain homogenates of mice of various groups. Post-hoc tukey test revealed that LPS significantly increased ($p<0.001$) BAX, Bcl-2, Caspase-3 and PARP-1 levels in mice brain. However, Glycine administration significantly inhibited ($p<0.01$) the expression of BAX (Figure 02), Bcl-2 (Figure 03), Caspase-3 (Figure 04) and PARP-1 proteins (Figure 05) in the LPS+Gly group mice as compared to Gly group. These findings ascertained Glycine role as an anti-apoptotic agent.

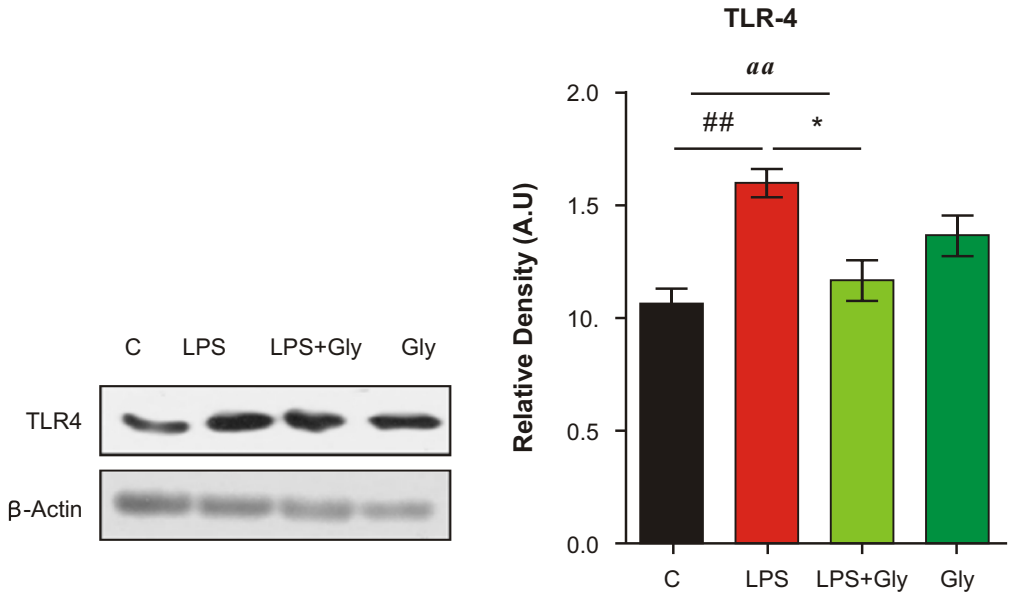


Figure 01. Glycine successfully ameliorated the expression level of TLR-4 protein in the LPS+Gly group mice brains.

Given are the results of Western blot of TLR-4 expression levels, along with their bar-chart representation in various experimental mice groups respectively. Analysis of the results was determined through Image J software. and bar-chart show mean in A.U ± SEM. Significance of one way ANOVA is stated as á, significance of control vs LPS is stated as #, while significance of LPS vs LPS+Gly is stated as*. Significance: ***, ###p<0.001.

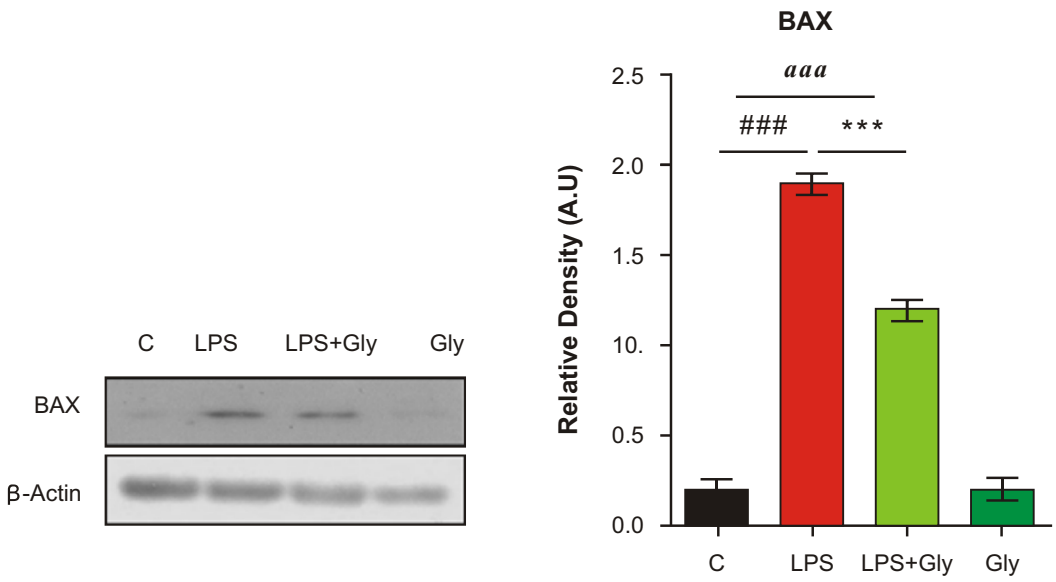


Figure 02. Glycine successfully ameliorated the expression level of BAX protein in the LPS+Gly group mice brains.

Given are the results of Western blot of BAX expression level, along with their bar-chart representation in various experimental mice groups respectively. Analysis of the results was determined through Image J software. and bar-chart show mean in A.U ± SEM. Significance of one way ANOVA is stated as á, significance of control vs LPS is stated as #, while significance of LPS vs LPS+Gly is stated as*. Significance: ***, ###p<0.001.

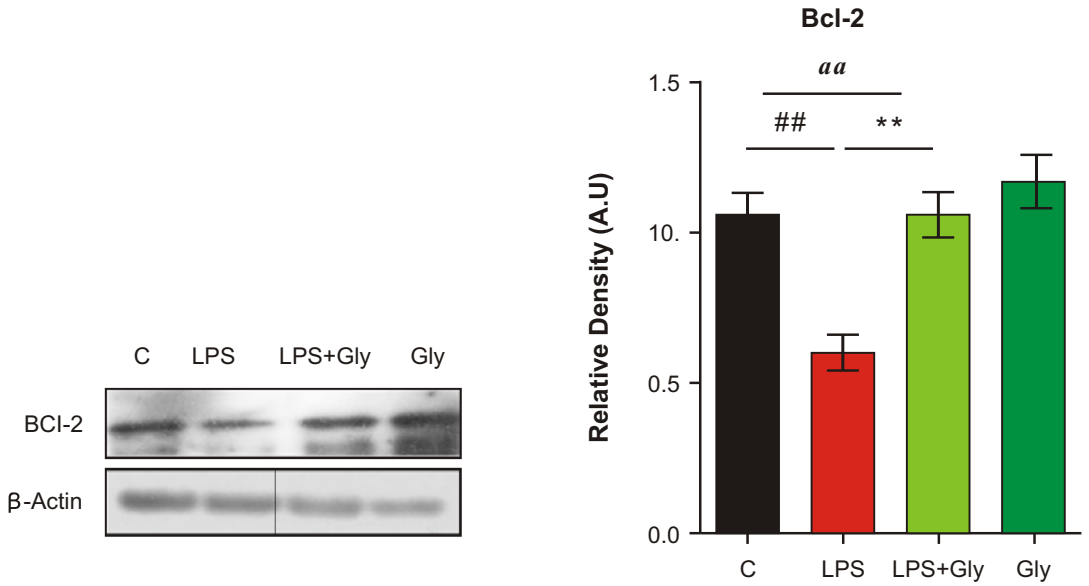


Figure 03. Glycine significantly rectified the expression level of Bcl-2 protein in the LPS+Gly group mice brains.

Given are the results of Western blot of Bcl-2 expression level, along with their bar-chart representation in various experimental mice groups respectively. Analysis of the results was determined through Image J software. and bar-chart show mean in A.U ± SEM. Significance of one way ANOVA is stated as á, significance of control vs LPS is stated as #, while significance of LPS vs LPS+Gly is stated as*. Significance: ***, ###p<0.001.

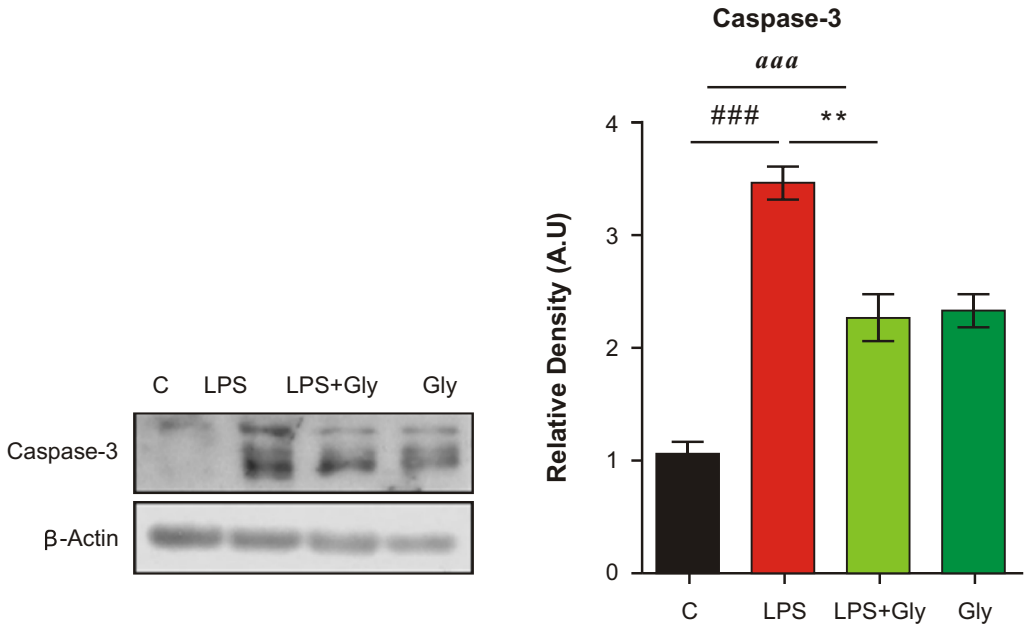


Figure 04. Glycine demonstrated substantial amelioration in the expression level of Caspase-3 protein in the LPS+Gly group mice brains.

Given are the results of Western blot of Caspase-3 expression level, along with their bar-chart representation in various experimental mice groups respectively. Analysis of the results was determined through Image J software. and bar-chart show mean in A.U ± SEM. Significance of one way ANOVA is stated as á, significance of control vs LPS is stated as #, while significance of LPS vs LPS+Gly is stated as*. Significance: ***, ###p<0.001.

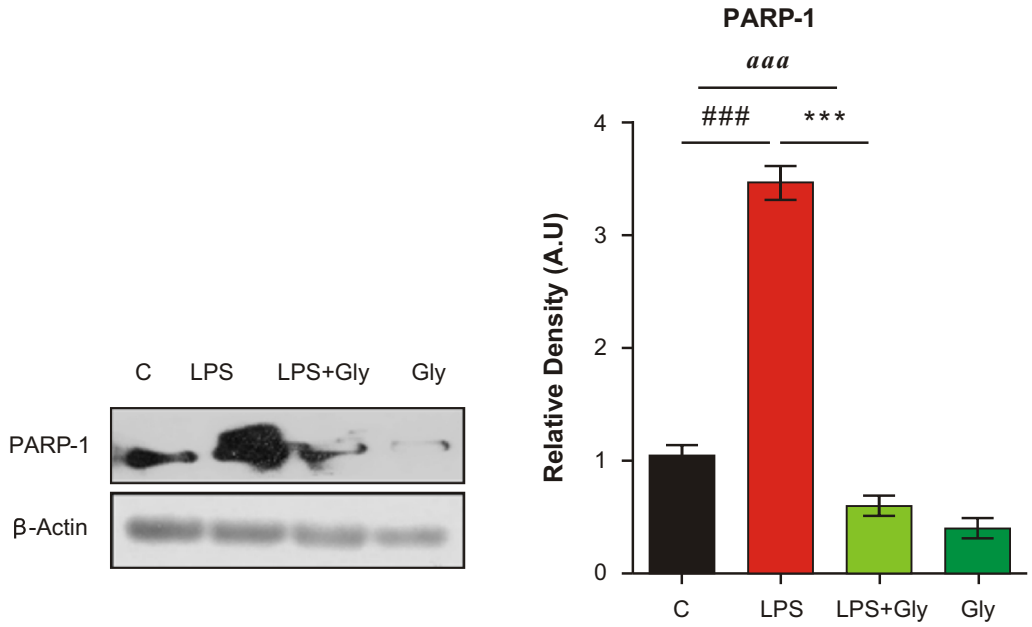


Figure 05. Glycine successfully ameliorated the expression level of PARP-1 protein in the LPS+Gly group mice brains.

Given are the results of Western blot of PARP-1 expression level, along with their bar-chart representation in various experimental mice groups respectively. Analysis of the results was determined through Image J software. and bar-chart show mean in A.U ± SEM. Significance of one way ANOVA is stated as á, significance of control vs LPS is stated as #, while significance of LPS vs LPS+Gly is stated as*. Significance: ***, ###p<0.001.

DISCUSSION

The current study demonstrated the neurotherapeutic efficacy of glycine against LPS induced neurodegeneration in the developing mice brain. Here we showed that Glycine has the potential to rescue PND-7 mice brain against LPS induced neurodegeneration. Glycine caused down-regulation of the TLR-4 receptor protein and resultantly rectified BAX, Bcl-2, Caspase-3and PARP-1 protein expression in the PND-7 mice brain.

The advent and progression of neurodegenerative diseases are linked to inflammation. Different studies have revealed the relationship between neurodegenerative diseases and hippocampal neuronal loss due to apoptosis.20 The active (cleaved) form of caspase-3 is translocated to the nucleus, where it cleaves poly (ADP-ribose) polymerase (an enzyme involved in DNA repair), and thus leads to apoptosis.21 This further leads to mitochondrial translocation of the pro-apoptotic protein Bax and subsequent release of cytochrome c from intermembrane space followed by caspase-3 activation, whereas Bcl-2 protein inhibits apoptosis.22 LPS is a well-known agent to induce neurotoxicity in the developing mice brains. LPS administration increase in BAX proteins, inhibit BCl-2 and ultimately cause BAX/Bcl-2 ratio upregulation in PND-7 pup's brain.22 Bax-knock-out mice showed LPS-induced apoptosis in the developing brain through upregulation of BAX/Bcl-2 ratio. Decreased levels of Bcl-2 cause movement of Bax to mitochondria by disrupting mitochondrial membranes, releasing cytochrome C and hence cause caspase-3 activation. These events show that apoptosis is induced by LPS through the intrinsic apoptotic pathway in the developing brain.23

The current study is in agreement with the previous published literature, which also reveals that LPS induces BAX upregulation, Bcl-2 inhibition and increase in BAX/Bcl-2 ratio. These results suggest that LPS is a toxic agent and have deleterious effects on the immature brain.[19] Moreover, Bcl-2 pool can be restored by application of taurine and thus inhibit apoptosis.[24] As apoptosis induced by LPS is a BAX dependent procedure, we recommend Bcl-2 level restoration to be likely apoptosis preventive mechanism in our study.

Caspase-3 is one of the important executioners of apoptosis.[25] The activation of caspase-3 plays an important part in the induction of apoptosis. Elegant studies reveal that inhibiting caspase-3 with an agent can reduce the deleterious effects of LPS on the immature brain.[26] These authors have observed that by using Anthocyanins, melatonin and even glycine can exerts neuroprotection against ethanol-induced neurodegeneration via caspase-3 inhibition respectively.[27] Our western blot results reveal that a single injection of LPS after four hours significantly induced an increase in the expression of capase-3

protein. Glycine treatment slightly after the administration of LPS significantly inhibited the caspase-3 protein expressions.

LPS can enhance the protein expression of the PARP-1 protein.[28] Studies have confirmed that LPS intoxication can induces DNA fragmentation in the PND-7 mice brain.[29,30] Furthermore, it has been also confirmed that some natural products are involved in the neuroprotective effects against LPS induced DNA fragmentation.[31] Here too one injection of LPS significantly enhanced the protein expression of PARP-1 in the brain homogenates of PND-7 pups. Likewise, Glycine potentially reduced the expression level of PARP-1 proteins in the brains of the mice pups.

CONCLUSION

In conclusion, we provide evidence that glycine, decreased LPS-induced neurotoxicity in the PND 7 mice brain. The neuroprotective effect observed was associated with suppression of TLR-4 receptor protein along with its downstream BAX, Caspase-3, PARP-1 protiens and upstream Bcl-2 protein in the brain homogenates of PND 7 mice. However, it remains to be determined whether this is the cause or a consequence of protection. Glycine could prove useful as an adjuvant in the treatment of LPS-induced apoptotic neurodegeneration.

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Ethical consideration

This study was approved by Institutional Review Board of Neuro Molecular Medicine Research Center, Peshawar, Pakistan.

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Conflict of Interest

Authors declared no conflict of interest.

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DATA SHARING STATEMENT: The data that support the findings of this study are available on request from the corresponding author. The data is not publicly available due to privacy or ethical restrictions.

AUTHORS' CONTRIBUTION

The following authors fullfill authorship criteria as per ICMJE guidelines;

Zubair S, Jahan R: Substantial contribution to conception and design, drafting the manuscript, final approval, agreed to be accountable for all the work.

Jan A: Substantial contribution to conception and design, data acquisition, drafting the manuscript, final approval, agreed to be accountable for all the work.

Zeb S: Analysis and interpretation of data, drafting the manuscript, final approval, agreed to be accountable for all the work.

Shah SA: Analysis and interpretation of data, critical revision,final approval, agreed to be accountable for all the work.

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