Chronic exposure of glyphosate modulates the expression of inflammatory signaling molecules in brain tissue in adult male rats.

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Abstract

Glyphosate is an active compound and non-selective widely used herbicide among farmers in all over developing countries. When glyphosate is applied to growing plants like broad leaf plants, weeds; it gets absorbed *via* tissue and kills the plants. Glyphosate -based herbicide exposed to rats causes oxidative stress. Glyphosate induces oxidative stress, leads to imbalance in production and accumulation of oxygen reactive species in cells and tissues which induce inflammation. To analyse the expression of inflammatory markers with chronic exposure of glyphosate among experimental rats. Total RNA isolation followed by conversion of RNA to cDNA with the help of reverse transcriptase and the mRNA expression level of TNFα, IL-1β was analyzed by RT-PCR. The triplicate statistical analysis results of the experiments performed on control and treated rats were expressed as mean ± SEM. Results were analyzed statistically by one-way Analysis Of Variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range tests using Graph Pad Prism version 5. The results with $p<0.05$ level were considered to be statistically significant. Total RNA isolation followed by conversion of RNA to cDNA with the help of reverse transcriptase and the mRNA expression level of TNFα, IL-1β was analyzed byRT-PCR. The triplicate statistical analysis results of the experiments performed on control and treated rats were expressed as mean ± SEM. Results were analyzed statistically by one-way analysis of variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range tests using Graph Pad Prism version 5. The results with $p<0.05$ level were considered to be statistically significant. Our present *in vivo* findings for the first time clearly demonstrate that glyphosate exposure leads to the development of diabetic neuropathy by modulating the expression of genes involved in the proinflammatory cytokines in the brain tissues. Further studies on the impact of glyphosate on downstream signaling molecules are warranted in order to ascertain the potential mechanisms of action of glyphosate.

Keywords: Glyphosate, Neuroinflammation, TNF α, IL-1β, Diabetic neuropathy, Rats, RNA, Innovative technology, Novel method.

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Introduction

Glyphosate is a non-selective and most commonly used herbicide all over developing countries. In a previous study, glyphosate was applied to growing plants like broadleaf plants, weed and it was absorbed via tissue and killed the plants [1,2]. Glyphosate-based herbicide exposed to rats causes oxidative stress. Sodium salt form of glyphosate is used to regulate plant thickening and ripen specific crops [3,4]. Glyphosate has both genotoxic and cytotoxic effects causing inflammation and affects interaction between microbes and the immune system. Glyphosate is banned in many countries but it is still not banned in India.

Roundup is a broad-spectrum glyphosate-based herbicide that is banned in an open environment and may cause nausea, vomiting, and irritation. Glyphosate is present in the blood and urine of humans and within a few days, it excretes from the body ROS play a crucial role within the living system through their helpful and prejudicial effects. ROS will suppress the hormone response and contribute to the event of hormone resistance, a key pathological feature of type2diabetes mellitus within the gift study, diabetic rats showed a rise within the LPO *OH and H2O levels in the skeletal muscle [5,6].

Through dermal pulmonary and oral roots glyphosate enters the human body and glyphosate accumulates in kidneys, liver, colon, and small intestine and within 48 hours it is eliminated through urine and feces [7]. Glyphosate herbicides also involve the inhibition part of biochemical reactions like the shikimate pathway in plants which is responsible for synthesis of proteins. This shikimate pathway does not occur in both animals and human beings [8]. According to IARC, glyphosate acts as a carcinogen for humans and it also affects the liver and kidney [9].

TNF-α is associated with the degree of inflammation and protein made by macrophages throughout acute inflammation and is liable for a variety of signaling events inside cells, resulting in apoptosis. The protein is additionally vital for resistance to infection and cancer. TNF being a prominent cytokine has 19 different members.TNF-α helps in homeostasis and defense response, neuro inflammation. TNF-α (-308) G/A and IL1β-511 C/T polymorphisms and their implications on

age are well reportable in numerous populations across the globe. Inflammatory markers like TNF α and IL-1β are also known as acute phase reactants.

TNF-α and IL-1β are required for supporting phagocytic cells and also activate innate immunity [10]. Effect of glyphosate can reduce TNF-α [11-14]. IL-1β (interleukin 1 beta) is a cytokine protein encoded by the IL1b gene and also released by many cells which acts in autocrine thereby stimulating a variety of signaling pathways. IL-1β as expressed in the hippocampus plays an important role in memory. IL-1β also acts as a peripheral messenger which communicates with CNS *via* passage across the blood brain barrier. Glyphosate also increases the IL-1b gene expression [15-20]. Our team has extensive knowledge and research experience that has translate into high quality publications [21-32]. The aim of the current research is to study the expression of inflammatory signalling molecule in brain tissue of male rodents after chronic exposure to glyphosate.

Materials and Methods

Chemicals

All chemicals and reagents used in this study were purchased from Sigma Chemical Company St. Louis, MO, USA; Invitrogen, USA; Eurofins Genomics India Pvt Ltd, Bangalore, India; New England Biolabs (NEB), USA; Promega, USA. glyphosate was procured from Sigma Chemical Company St. Louis, MO, USA; Total RNA isolation reagent (TRIR) was purchased from Invitrogen, USA. The reverse-transcriptase enzyme (MMuLv) was purchased from Genet Bio, South Korea purchased from Promega, USA. TNF α and IL-1β and β-actin primers were purchased from Eurofins Genomics India Pvt Ltd, Bangalore, India.

Animals

The present experimental study was approved by the institutional animal ethics committee (IAEC no.: BRULAC/SDCH/SIMATS/IAEC/02-2019/015). Adult male Wistar albino rats, weighing 180–200g, were obtained and maintained in clean propylene cages at the Biomedical Research Unit and Laboratory Animal Centre (BRULAC), Saveetha dental college and hospitals, Saveetha university, India) in an air-conditioned animal house, fed with standard rat pelleted diet (Lipton India Ltd., Mumbai, India), and clean drinking water was made available ad libitum. Rats were divided into 3 groups, each consisting of 6 animals.

Group 1: Control.

Group 2: Glyphosate-exposed rats (50 mg/kg b.wt).

Group 3: Glyphosate-exposed rats (100 mg/kg b.wt).

Group 4: Glyphosate-exposed rats (250 mg/kg b.wt).

At the end of the treatment, animals were anesthetized with sodium thiopentone (40 mg/kg b.wt), blood was collected through cardiac puncture, sera were separated and stored at −80°C, and 20 ml of isotonic sodium chloride solution was perfused through the left ventricle to clear blood from the organs. Brain tissues from control and experimental animals was immediately dissected out and used for assessing the various parameters.

Gene Expression Analysis

Isolation of total RNA from adipose tissue

Total RNA was isolated from control and experimental samples using TRIR (total RNA isolation reagent) kit. Briefly, 100 mg fresh tissue was homogenized with 1 ml TRIR and the homogenate was transferred immediately to a microfuge tube and kept at -80°C for 60 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed for 1 min and placed on ice at 4°C for 5 min. The homogenates were centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 sec and placed on ice at 4°C for 10 min. The samples were centrifuged at 12,000 xg for 10 min at 4°C. The supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 xg (4°C). The supernatant was removed and RNA pellets were mixed with 50 µl of autoclaved Milli-Q water and dissolved by heating in a water bath for 10 min at 60°C.

Quantification of RNA

Diluted RNA samples were quantified spectrophotometrically by measuring the absorbance (A) at 260/280 nm. 40 µg of RNA in 1 ml gives one absorbance at 260 nm. Therefore, the concentration of RNA in the given sample can be determined by multiplying its A260 by 40 and dilution factor. The purity of RNA preparation can be calculated using the ratio between its absorbance at 260 and 280 nm. A ratio of absorbance at 260/280 nm>1.8 is generally considered as good quality RNA. The purity of RNA obtained was 1.8.

cDNA conversion and real time PCR

By using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2 µg of total RNA as stated in the manufacturer's protocol. To perform real-time PCR, the reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), Forward and reverse primers of the target gene and house-keeping gene, water and β-actin.

Primers: Rat IL-1β:

FW- 5”-CAGCGGCCGCCTTGTGCAAGTGTCTGAAGCAG-3”

RW- 5”-GAACAGCTCTTACCCGTCAGAGGTCCAGATCTCGCCGGCGAC-3”;

Rat TNF-alpha:

FW – 5”-CAG CGG CCG CAA CAC ATC TCC CTC CGG AAA GGA C-3”:

RW - 5”-GAC CGC ACA AGT AGG CAA GAG ATG GCG CCG GCG-3”;

Rat β-actin-

FW – 5’-TACACCTTGGCGACGACT-3’

RW– 5’-TCTC GAGAGAGAGAGAGA-3’

In total volume of 45 µl expect the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 µl of control DNA for positive control, 5 µl of water for negative control and 5 µl of template cDNA for samples were taken and reaction mixture (45 µl) were added. 40 cycles (95°C for 5 min, 95°C for 5 s, 60°C for 20 s and 72°C for 40 s) was set up for the reaction and obtained results were plotted by the PCR machine (CFX 96 Real Time system Bio-Rad, USA) on a graph. Relative quantification was calculated from the melt and amplification curves analysis.

Statistical Analysis

The triplicate analysis results of the experiments performed on control and treated rats were expressed as mean ± SEM. Results were analyzed statistically by one-way Analysis Of Variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range tests using Graph Pad Prism version 5. The results with $p<0.05$ level were considered to be statistically significant.

Results

Impact of glyphosate on TNF α mRNA expression in brain tissue

Compared to the control group, expression of IL-1β increases when it is exposed to glyphosate impact in a dose-dependent manner (Figure 1).

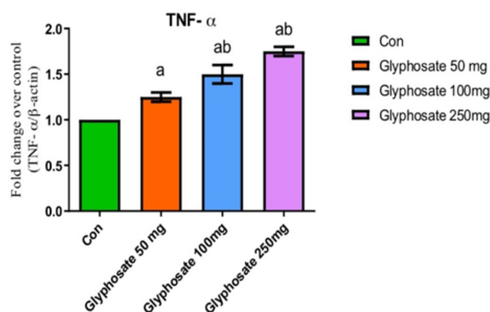

Figure 1. *Effect of glyphosate on TNF α mRNA expression in brain tissue of adult male rats. The expression of TNF α mRNA was assessed by real time-PCR. Each bar represents Mean ± S.E.M of 3 observations representing 6 animals. Significance at p<0.05. a: Compared with control; b: Compared with 50mg glyphosate-induced rats. Green bar represents expression level of control rats, orange bar represents expression level of glyphosate -induced rats at a dose of 50 mg, blue bar represents expression levels of glyphosate-induced rats at a dose of 100mg and purple bar represents expression levels of glyphosate-induced rats at a dose of 250mg.*

Impact of glyphosate on IL-1β mRNA expression in brain tissue

Compared to the control group, expression of IL-1β increases when it is exposed to glyphosate impact in a dose-dependent manner (Figure 2).

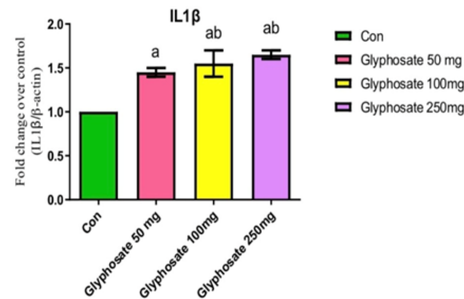

Figure 2. *Effect of glyphosate on IL-1β mRNA expression in brain tissue of adult male rats.The expression of IL-1β mRNA was assessed by Real Time-PCR. Each bar represents Mean ± S.E.M of 3 observations representing 6 animals. Significance at p<0.05. a: Compared with control; b: Compared with 50 mg glyphosate-induced rats. Green bar represents expression level of control rats, orange bar represents expression level of glyphosate -induced rats at a dose of 50 mg, blue bar represents expression levels of glyphosate-induced rats at a dose of 100mg and purple bar represents expression levels of glyphosate-induced rats at a dose of 250mg.*

Discussion

Increased accumulation of oxidative stress leads to an increased expression of inflammatory markers such as TNF α and IL-1β [33-35]. Previous research findings suggest that chronic inflammation is associated with various disorders and conditions. In the current study, it is evident that there is increased expression of TNF α and IL-1β mRNA in the groups exposed to glyphosate. Expression increases in dose dependent manner. In a previous study on toxicity, it was observed that the tendency to associate the cytotoxic action of glyphosate with aerophilic stress, led to the breakdown product of glyphosate AMPA, in HepG2 cell line [36]. In the current study, it is evident that environmental exposure of herbicides may lead to conditions such as diabetes **[37]**. In previous research reported that the activity of antioxidant enzymes were decreased in different segments of the small intestine in rats. In the current study also antioxidant levels decreased with increase in glyphosate in male adult Wistar albino rats. Another previous research done by Cindy et al. where glyphosate and GBHs impart a dose dependent genotoxic and cytotoxic effects, increases oxidative stress and correlates to develop some tumors and disorders. In the current study,

glyphosate has both genotoxic and cytotoxic effects causing inflammation and *vice versa*.

In previous research done by Martin, schwann cells secrete pro-inflammatory cytokines such as TNF α. TNF-α is an associate degree inflammatory protein made by macrophages throughout acute inflammation and is liable for a variety of signaling events inside cells, resulting in cell death. TNF-α measures into broad spectroscopic analysis assays, like multiplex technology, that measures varied cytokines (e.g. interleukins). In the current study, inflammatory markers like TNF- α increases when it is exposed to glyphosate and results in inflammation and also causes diseases and disorders like cancer, diabetes mellitus etc.

A study conducted by Richard, inflammatory markers like IL-1β are also known as acute phase reactants. IL-1β is required for supporting phagocytic cells and also activates innate immunity. IL-1β (interleukin 1 beta) is a cytokine protein encoded by the IL1b gene and also released by many cells which acts in autocrine thereby stimulating a variety of signaling pathways. IL-1β also acts as a peripheral messenger which communicates with CNS via passage across the blood brain barrier. Glyphosate also increases the IL-1b gene expression. In the current study, IL-1β inflammatory markers like increases when it is exposed to glyphosate and results in inflammation and also causes diseases and disorders like cancer, diabetes mellitus etc [38].

Conclusion

Many conditions such as cancer, diabetes mellitus are linked to environmental exposure of herbicide and various toxins. In the current study there was a marked increase in the expression of inflammatory genes (TNF α, IL-1β) in the glyphosate induced experimental animals. Inflammation is the root cause for various stress related disorders such as cancer, diabetes etc. Thus long term studies are necessary to understand the toxicity and chronic influence of glyphosate on humans.

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Author Contribution

Author A: Venkatakarthikeswari GV- contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

Author B: Gayathri R- contributed in study design, guiding the research work, manuscript correction.

Author C, D, E: Selvaraj J, Vishnu Priya V, Kavitha S study design, statistical analysis, manuscript proof reading and correction.

Conflict of Interest

The authors declare no conflict of interest.

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