Original Research Article

IMPACT OF GLYPHOSATE ON THE CHANGES IN THE mRNA EXPRESSION OF NEUROTRANSMITTERS IN EXPERIMENTAL RATS

Running title: Impact of glyphosate in the mRNA expression of neurotransmitter in rats

ABSTRACT

Introduction: Glyphosate, an N-(phosphonomethyl) glycine is the active ingredient in the pervasive herbicide, Monsanto Roundup. It is widely used herbicide worldwide to eliminate unwanted plants both on agricultural and non agricultural landscapes. Glyphosate is used in different formulations and applied in diverse forms including isopropylamine salt, potassium salt, ammonium salt, diammonium salt and dimethyl ammonium salt. Glyphosate can display endocrine –disrupting activity, promote carcinogenicity in mouse skin and affect human erythrocyte. In humans the effects of glyphosate on metabolic health research has not been done to a larger extent.

Aim: To analyse the impact of glyphosate on the changes in the mRNA expression of neurotransmitters in experimental rats.

Materials and Methods: Male albino rats were classified into three groups. Group I: Normal rats; Group II: glyphosate induced rats with 50 mg/ kg of glyphosate for 16 weeks; Group III:. The glyphosate induced rats with 100 mg/ kg of glyphosate for 16 weeks;Group IV:. The glyphosate induced rats with 250 mg/ kg of glyphosate for 16 weeks. After 16 weeks of glyphosate exposure , the control and induced animals were anesthetized and brain tissue were dissected to analyse the gene expression of serotonin and GABA alpha .The data were statistically analysed and tabulated.

Results: mRNA expressions of neurotransmitters such as serotonin receptor and gamma-aminobutyric acid alpha (GABA a) were significantly ($p<0.05$) down regulated in glyphosate-exposed rats in a dose-dependent manner (50, 100 and 250 mg/kg b.wt) suggesting that glyphosate exposure causes detrimental changes in the brain tissues in rats.

CONCLUSION: Our present study for the first time proves that glyphosate leads to diabetic neuropathy modulating expression of the neurotransmitters such as serotonin and GABA apha.

KEYWORDS

Innovative technology, Glyphosate, diabetic neuropathy, serotonin, GABA alpha, novel method

INTRODUCTION

Glyphosate [N-phosphonomethyl-glycine] (GBHs) are broad spectrum herbicides and crop desiccants . It is an organophosphorus compound, specifically a phosphonate which acts by inhibiting the plant enzyme 5- enolpyruvylshikimate -3- phosphate synthase on pathways in bacteria, fungi and plants. It is available in a variety of chemical forms, such as isopropylamine salt, ammonium salt, diammonium salt, dimethylammonium salt, and potassium salt .The widespread application of glyphosate and GBH to crops has spurred the spread of tolerant and resistant weeds worldwide, which in turn has created the need for more frequent applications at higher concentrations Individuals may be exposed to glyphosate through various routes such as food and drinking water, both in the occupational and environmental settings(1) . Glyphosate has also been found in dust within non-agricultural homes, suggesting that the exposure is not only occupational (2). Glyphosate levels in human beings can be quantified by measuring the levels of either glyphosate or its metabolite, AMPA(3). Glyphosate is used in agriculture and forestry as well as for weed killing in non agricultural areas such as water systems.(4). Hence the identification of glyphosate in water is becoming frequent in both median and maximum concentrations and 0.03 and 73 µ / L found in large rivers and ponds respectively (5). Glyphosate based herbicides were initially considered safe for animals since glyphosate specifically targets aromatic amino acids synthesis, a metabolic pathway

(6). It may seem implausible that glyphosate could be toxic to humans, given the fact that government regulators appear nonchalant about steadily increasing residue limits, and that the levels in food and water are rarely monitored by government agencies,(7). Many recent studies support the neuro toxic potential of glyphosate including related effects in different animal species(5). Oxidative stress of neurotransmitters profile, neuro inflammation glutamate excitotoxicity and changes in the neurotransmitters and changes in behaviour are some of the reported effects on the central nervous system(3,8).Serotonin has been implicated in practically every type of behavior, such as appetitive, emotional, motor, cognitive and autonomic shows a gradual decline as the animal becomes drowsy and enters slow-wave sleep. A decrease in the

regularity of firing accompanies this overall slowing of neuronal activity. In most of the cases neurotoxic effects were obtained using concentration of glyphosate showing a gradual decline as the animal becomes drowsy and enters slow-wave sleep. A decrease in the regularity of firing accompanies this overall slowing of neuronal activity.(9) . GABAa receptors are expressed in the rat central nervous system (CNS)GABA interneurons are highly diverse and operate with a corresponding diversity of GABAA receptor subtypes in controlling behaviour(10). Our team has extensive knowledge and research experience that has translate into high quality publications [11-30]. This study was aimed to analyse the impact of glyphosate in the changes in the mRNA expression of neurotransmitters in experimental rats .

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. Dopamine Receptor, Serotonin receptor (The serotonin 1A receptor) and β-actin primers were purchased from Eurofins Genomics India Pvt Ltd, Bangalore, India and.

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.

CHART 1. Experimental Design

Group I	Normal control rats fed with normal diet and drinking water

Group II	Glyphosate treated (dissolved in water at a dose of 50 mg/kg body weight/day at 8 to AM) orally for 16 weeks
Group III	Glyphosate treated (dissolved in water at a dose of 100 mg/kg body weight/day at 8 to AM) orally for 16 weeks
Group IV	Glyphosate treated (dissolved in water at a dose of 250 mg/kg body weight/day at 8 to AM) orally for 16 weeks

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 by Real Time PCR

Isolation of total RNA

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 1min and placed on ice at 4°C for 5min. 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 x g 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 5min at 7,500 x g (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 (Fourney et al., 1988). The purity of RNA obtained was 1.8.

Reverse Transcriptase – Polymerase Chain Reaction (RT – PCR)

RT-PCR is an approach for converting and amplifying a single stranded RNA template to yield abundant double stranded DNA products. 1. First strand reaction: Complementary DNA (cDNA) is made from the mRNA template using Oligo dT, dNTPs & reverse transcriptase. 2. Second strand reaction: After the reverse transcriptase reaction is complete, standard PCR (called the "second strand reaction") is initiated. Principle RT-PCR is a method used to amplify cDNA copies of RNA. It is the enzymatic conversion of mRNA into a single cDNA template. A specific oligodeoxynucleotide primer hybridizes to the mRNA and is then extended by an RNAdependent DNA polymerase to create a cDNA copy. First strand DNA synthesis The RT kit was purchased from Eurogentec (Seraing, Belgium). Reagents 1. 10X RT buffer: One vial containing 1.4 ml of 10X RT buffer. 2. EuroScript reverse transcriptase: One tube containing 75 µl of Moloney Murine leukemia virus reverse transcriptase (3750 U at 50 U/µl).

Quantitative Real Time PCR Principle

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. There are three major steps in a PCR, which are as follows: Denaturation at 94°C for 3 min: During the denaturation at 94°C for 2-5 min, the double strand melts open to single stranded DNA, all enzymatic reactions stop. Annealing at 54°C- 65°C for 30 sec: Ionic bonds are constantly formed and broken between primer and the single stranded template to ensure the extension process. Extension at 72°C for 30 sec: Primers that are in positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP from 5' to 3', reading the template from 3' to 5' side; bases are added complementary to the template). Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene.

Reagents:

1. 2X Reaction buffer: The PCR master mix kit was purchased from Takara Bio Inc., Japan. Contains TaKaRa Ex Taq HS (a hot start PCR enzyme) dNTP Mixture, Mg2+, Tli RNase H (a heat-resistant RNase H that minimizes PCR inhibition by residual mRNA), and SYBR Green I.
2. Forward primer (10µM)
3. Reverse primer (10µM)

4. cDNA- Template
5. Autoclaved milli Q water
6. Primers: The following gene specific oligonucleotide primers were used.

Details of primers used in the present study

Rat serotonin receptor (5HT1A)

FW-5-TCCGACGTGACCTTCGGCTACC-3’
RW: 5-AGTTCCTGCTCCCCGATTCTCC-31

Rat GABA a

5'-GGATTGGGAGAGCGTGTAACC-3'
5'-TGAAACGGGTCCGAAACTG-3’

Rat β-actin

FW – 5’- TACACCTTGGCGACGACT - 3’

RW– 5’- TCTCGAGAGAGAGAGAGAGA - 3’

Procedure

Procedure Real Time PCR was carried out on CFX 96 Real Time system (Bio-Rad). The reaction mix (10 µl) was prepared by adding 5 µl of 2X reaction buffer, 0.1 µl of sense and antisense primer, 1 µl of cDNA and 3.8 µl of sterile water. The thermal cycler protocol was as follows: Initial denaturation at 95˚C for 3 min, followed by 40 cycles of PCR, denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 20 sec. All reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50-95˚C for each sample to determine the presence of multiple amplicons, non-specific products and contaminants. The results were analysed using CFX 96 Real Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin.

Statistical analysis

The data were subjected to statistical analysis to check the significance of individual variance within the control and treated groups using one-way analysis of variance (ANOVA) and Duncan's multiple range test, computer-based software (Graph Pad Prism version 5). The significance was presented at the level of $P < 0.05$ in Duncan's test.

Results

Effect of glyphosate on the mRNA expression of serotonin receptor in adult male rats

Serotonin mRNA expression was significantly reduced in the expression of serotonin in a dose-dependent manner when compared to control (P<0.05). However, 100 and 200mg dose exposure drastically decreased the expression of serotonin receptor (Fig.1).

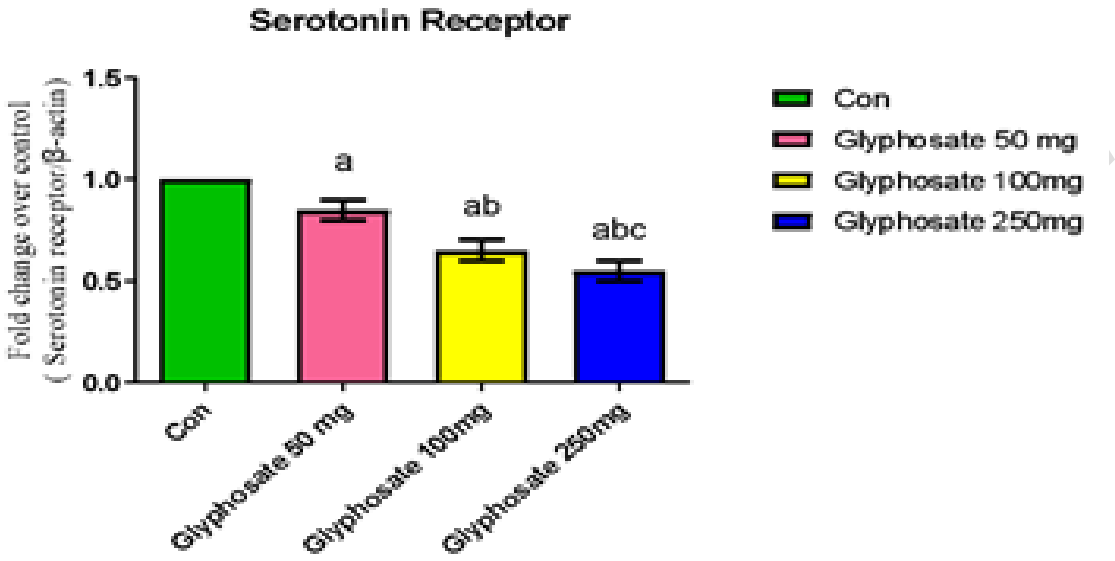

Fig. 1: Showing the impact of glyphosate on the serotonin receptor mRNA expression in adult male rats. Each bar represents mean ± SEM 6 animals. Significance was considered at the levels of p<0.05.a-compared with control; b-compared with 50mg glyphosate exposed rats; c-compared with 100 mg exposed rats.

Effect of glyphosate on the mRNA expression of GABAa in adult male rats

Serotonin mRNA expression was significantly reduced in the expression of GABAa in a dose-dependent manner when compared to control (P<0.05). However, 100 and 200mg dose exposure drastically decreased the expression of GABAa (Fig.2).

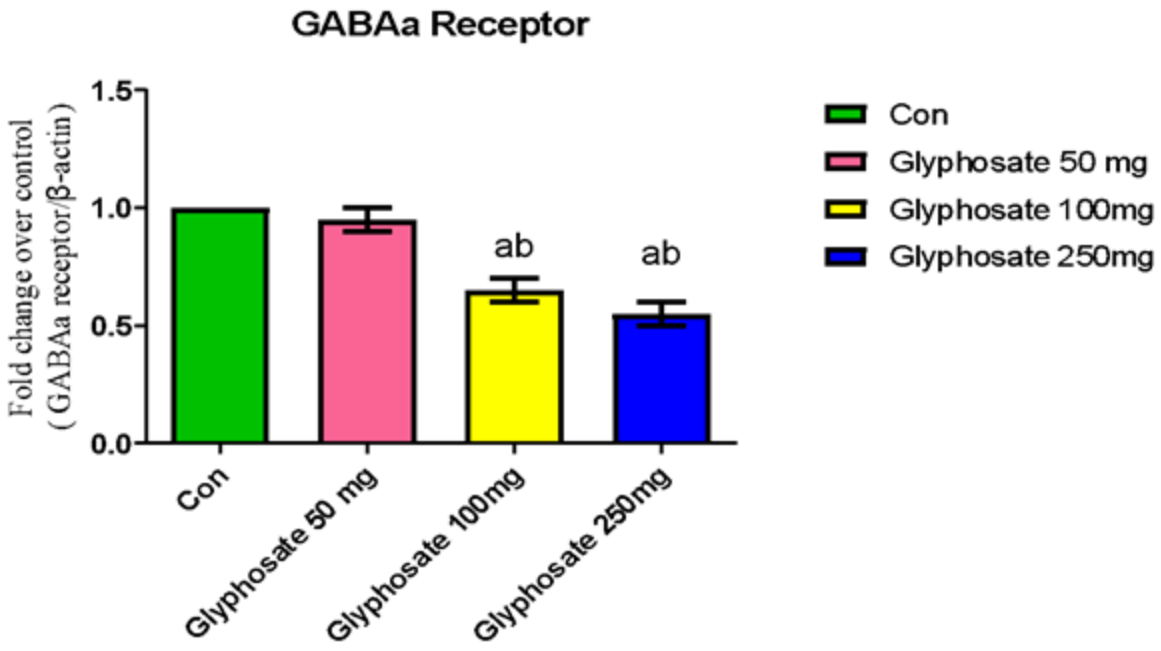

Fig. 2: Showing the impact of glyphosate on the GABAa mRNA expression in adult male rats. Each bar represents mean ± SEM 6 animals. Significance was considered at the levels of $p<0.05$.a-compared with control; b-compared with 50mg glyphosate exposed rats; c-compared with 100 mg exposed rats.

DISCUSSION

Glyphosate is a potent toxic agent which has been used as herbicide world wide. It's toxic effect has been studied in various tissues correlating the disturbance in protein expression. Exposure to pesticides may result in acute and chronic ill effects. Among the most commonly encountered side effects includes nausea, headache, dizziness etc., it has also been shown that cognitive disorders and hyper excitability were also encountered by the subjects.

This study assesses the impact of glyphosate on the changes in mRNA expression of serotonin and Gabapentin in experimental rats. It has been observed that there is a significant decrease in the serotonin expression when compared to control and at a dose of 50 mg (fig1). A significant decrease in GABAa expression was also observed in comparison to control and at a dose of 50 mg.

Recent studies have demonstrated various toxic aspects of glyphosate. A study (31) showed increases in nuclear aberrations indicating DNA damage after 20 minutes exposure to 10 to 20 mg/L of glyphosate. They also found that Roundup was, under all conditions, more active than glyphosate and that there were geno-toxic effects in short exposures at a concentration of a 450 dilution of spraying used in agriculture. It was also concluded that inhalation of glyphosate could cause DNA damage in agricultural workers.(31)ller et al., 2012). Study by (priyadharshini etal.,2000) showed that specific neurodegenerative effect and causal effect relationship between glyphosate and parkinson's disease has been revealed by a case report.

In our current study the mRNA expression of serotonin has been decreased when the experimental rats were exposed to glyphosate in a dose dependent manner. This shows the capability of the herbicide to cross blood brain barrier and accumulate significantly. The possible neurotoxic effects due to decrease in serotonin includes dizziness, headache, depression, mood swings (32). Previously(33)showed that there is no effect on serotonin levels on exposure to glyphosate. Inability to connect the two regions of the brain has been explained as cognitive disorders which is one of the side effects of decreased serotonin production (34) which results in confusion and poor decision making.

In our current study there is significant reduction in mRNA expression of GABAa in comparison to control. GABAa is known for its calming effect and anxiety controlling properties. They also result in dystonia and spasticity. GABA plays a major role in the HBG axis. GABAergic stimulation results in decreased catecholamine availability as well regulated gonadotropin hormones. Further detailed studies on GABA and glyphosate would decipher its role in the GABAergic system.

This is the first study to be done on glyphosate in mRNA expression of neurotransmitters in experimental rats. This study clearly dictates the possible hazardous effects of exposure to toxic herbicide, glyphosate. Further analysis of multiple transmitters will be of much use.

Conclusion:

In this study it is evident that when the exposure dose of glyphosate increases sequentially results in significant decrease in the major neurotransmitters. These neurotransmitters play a major role in stress and anxiety management and as well hold an important role in hormonal homeostasis. Our present study for the first time proves that glyphosate leads to diabetic neuropathy

modulating expression the neurotransmitters such as serotonin and GABA apha.Thereby this study dictates that glyphosate is a potential neurotoxic agent that may lead to development of diabetic neuropathy

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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