Impact of glyphosate on nuclear factor erythroid 2 - related factor 2 and heme - oxygenase expression in liver of adult male rats.

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Abstract

Glyphosate is a compound which is a herbicide and it is harmful to animals and also to plants when used in large amounts. Nrf2 is an emergency regulator of cellular resistance towards oxidants. Heme-oxygenase catalyses heme through heme oxygenase system. To analyse the impact of glyphosate on Nrf2 and heme-oxygenase expression in the liver of adult male rats. Male albino rats of Wistar strain weighing 180g-210g were used in this study. The rats were divided into 4 groups, each consisting of 6 animals. The animals were sacrificed and the liver tissue was taken to do real time PCR analysis for Nrf2 and HO. Data were analysed by ANOVA and DUNCAN'S multiple range test to check the statically significance among the groups. The results with the $p<0.05$ level were considered to be statistically significant. Glyphosate-induced rats showed a significant ($p<0.05$) reduction in the expression of Heme oxygenase-1 and Nrf2 mRNA compared to normal control rats in a dose-dependent manner suggesting that glyphosate induces diabetes by downregulating Nrf2 mediated signaling in liver. Glyphosate exposure has detrimental changes in downregulating the expression of Nrf2 signaling molecules and thereby it induced diabetes by disrupting antioxidant signaling.

Keywords: Glyphosate, Diabetes, Erythroid 2-related factor 2, Liver, Heme - oxygenase, Innovative technology, Novel method.

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Introduction

Glyphosate is an organophosphorus compound. It was discovered in 1970 and for the first time sold in 1974 [1]. Glyphosate is also named as N-phosphonomethyl (glycine) [2]. Different forms of glyphosate obtained such as propyl amine salt, ammonium salt, diammonium salt. On an average 526 tons of glyphosate is utilized in India over the past 5 years. It is a non-selective and broad - spectrum herbicide [3]. Individuals may be exposed to glyphosate through inhibition or absorption. It is also present in the wind and water which is the cause for a lot of diseases [4]. Glyphosate was banned in many other countries.But, it isn't banned in India yet. The Indian government didn't take any initiative to ban this chemical due which India has become one of the predominant countries that uses glyphosate in large amounts. Glyphosate is usually a weed killer. But as the years passed scientists have found that it is actually non selective which kills most of the plants and causes damage to the farmers to a large extent. Glyphosate is considered as probably carcinogenic [4].

The nuclear factor erythroid-2 related factor-2 (Nrf2) and heme oxygenase are the two enzymes chosen for this study. Nrf2 is an emergency regulator of cellular resistance towards oxidants. Nrf2 activation provides protection against chronic diseases of lung and liver, oxidative stress, cancer initiation [6]. (Nrf2) controls the antioxidant response. This regulates psychological and patho physiological outcomes of oxidant exposure. Heme oxygenase is an enzyme that catalyses the degradation of heme through heme oxygenase system. The heme oxygenase system can degrade the heme into Biliverdin (BV), ferrous iron (fe2+) and Carbon Monoxide (CO). Heme oxygenase is divided into two isomers. They are HO-1 and HO-2. Heme oxygenase-1(HO-1) is a stress response protein [7]. It may also play a vital role in cellular homeostasis maintenance. Oxidative stress is an imbalance between free radicals and antioxidants present in the body or it is an imbalance between production and accumulation of Oxygen Reactive Species (ORS) [8]. This imbalance leads to change in the levels of respective enzymes. The prevention for oxidative stress is by obtaining enough antioxidants in the diet. Our team has extensive knowledge and research experience that has translate into high quality publications [9-28]. On impact of glyphosate on diabetic complications in this study, we have shown that glyphosate exposure negatively influences the antioxidant signaling molecules such as Nrf2 and heme oxygenase mRNA expression in the liver of experimental animals.

Materials and Methods

Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics Committee (IAEC no.: BRULAC/SDCH/SIMATS/IAEC/02-2019/015). Healthy male albino rats of Wistar strain (Rattus norvegicus) weighing 180–210 g (150–180 days old) were used in this study. Animals were obtained and maintained in clean polypropylene cages under specific humidity (65 ± 5%) and temperature (272°C) with constant 12 h light and 12 h dark schedule at the Central animal house facility, Saveetha dental college and hospitals, Chennai-77. They were fed with a

standard rat pellet diet (Lipton India, Mumbai, India), and clean drinking water was made available ad libitum.

Experimental design

Healthy adult male albino rats were divided into four groups consisting of six animals each. In the present study Group I: Normal control rats fed with normal diet and drinking water; Group II-IV: Glyphosate treated rats (50, 100 and 250 mg/kg b.wt respectively) orally for 16 weeks. At the end of the experimental period, animals were subjected to ether anesthesia; blood was collected from retro orbital plexus and serum was separated by centrifugation. Liver tissues were collected from control and glyphosate induced rats and used for the assessment of various parameters.

mRNA Expression Analysis

Total RNA isolation, cDNA conversion and real-time PCR

Using a TRIR kit (Total RNA Isolation Reagent Invitrogen), total RNA was isolated from control and experimental samples. In brief, to 100 mg fresh tissue, 1 ml of TRIR was added and homogenized. The content was transferred to a microcentrifuge tube instantly and 0.2 ml of chloroform was added, vortexed for 1 min then kept at 4°C for 5 min. Later, the contents were centrifuged at 12,000 ×g for 15 min at 4°C. The aqueous phase (upper layer) was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 S and placed on ice for 10 min. After centrifugation of the content at 12000 ×g for 10 min at 4°C, the supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol by the vortex. The isolated RNA was estimated spectrometrically by the method of Fourney et al. The RNA concentration was expressed in micrograms (µg). 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 (the primer sequences were listed in Table 1) 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 (CFX96 Touch Real-Time PCR Detection System) 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 ± standard deviation. Results were analysed statistically by one-way Analysis of Variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range test using Graph Pad Prism Version 5. The results with the $p<0.05$ level were considered to be statistically significant.

Results

Impact of glyphosate on the mRNA expression of Nrf2 in adult male rats

In the present study, Nrf2 mRNA expression was significantly reduced in glyphosate-induced rats in a dose-dependent manner compared to control rats (Figure 1).

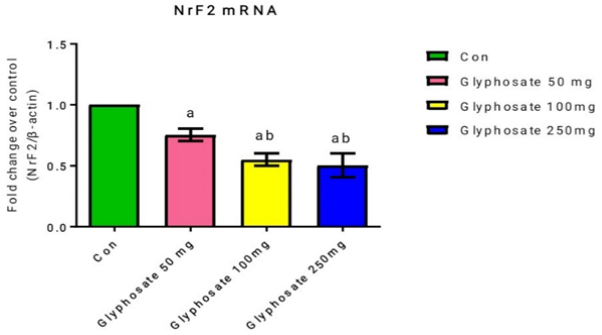

Figure 1. *Impact of glyphosate on the mRNA expression of nrf2in adult male rats.The x -axis represents dose-dependent exposure of glyphosate to the Wister rats compared with control. Y -axis represents the mRNA expression of nrf2 expressed in fold change over control. Light green represents the controlled rats, pink represents Group 1 rats exposed to about 50mg glyphosate, yellow represents the Group 2 rats exposed to about 100mg glyphosate, blue represents the Group 3 rats exposed to about 250mg glyphosate assessed by real time PCR. The mRNA expressions were assessed by Real Time-PCR using gene-specific primers. Each bar represents ± SEM (n=6). p value of <0.05 is considered significant. a: Compared to control; b: Compared to 50mg glyphosate exposed rats.*

Impact of glyphosate on the mRNA expression of HO in adult male rats

In the present study, HO mRNA expression was significantly reduced in glyphosate-induced rats in a dose-dependent manner compared to control rats (Figure 2).

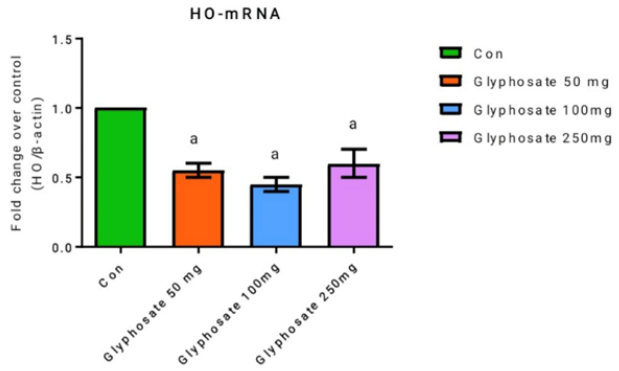

Figure 2. *Impact of glyphosate on the mRNA expression of HO in adult male rats. The x -axis represents dose-dependent exposure of glyphosate to the Wistar rats compared with control. Y -axis represents the mRNA expression of HO expressed in fold change over control. Light green represents the controlled rats, orange represents Group 1 rats exposed to about 50mg glyphosate, blue represents the Group 2 rats exposed to about 100mg glyphosate, violet represents the Group 3 rats exposed to about 250mg glyphosate assessed by real time PCR. The mRNA expressions were assessed by Real Time-PCR using gene-specific primers. Each bar represents ± SEM (n=6). p value of <0.05 is considered significant. a: Compared to control; b:Compared to 50mg glyphosate exposed rats.*

Discussion

This study is to analyse the impact of glyphosate on Nrf2 and heme oxygenase mRNA expression in the liver of adult male rats. Glyphosate has been found as harmful to soil adsorption capacity [29]. Glyphosate is not considered as harmful until its usage is judicious [30]. Glyphosate, a weed killer, is also used in different studies to get aware of its deleterious effects. Glyphosate was considered as probably carcinogenic by the International Agency for Research on Cancer (IARC) [31]. Glyphosate usage in large amounts kills the plant. The effect of large amounts of glyphosate is not only limited to destruction of plants, glyphosate also shows its effects on the soil functioning and the organisms growing in the soil. So, degradation of glyphosate is menacing [32]. One of such studies is induction of glyphosate on enzymes like cytochrome P450. Induction of glyphosate suppresses the level of cytochrome P450 enzyme [33].

Nrf2 gene expression was down regulated on induction of 50mg, 100mg and 250mg doses of glyphosate into the body. So, glyphosate shows significance towards Nrf2 in comparison with the control. In comparison with the previous studies, this study shows similar effects that Nrf2 decreases on induction of glyphosate [34]. Glyphosate acts as an inhibitor which inhibits Nrf2. The Nrf2 mRNA gene expression is decreased on induction of glyphosate due to increased oxidative stress based on the previous studies.

Heme oxygenase gene expression on induction of 50mg, 1pp mg and 250mg doses of glyphosate into the body. So, glyphosate shows significance towards heme oxygenase in comparison with the control [35]. Increase in oxidative stress in the body leads to decrease in the level of heme oxygenase. Increase in oxidative stress in the body leads to decrease in heme oxygenase [36]. The heme oxygenase mRNA expression is decreased on induction of glyphosate due to increased oxidative stress based on the previous studies. Hemeoxygenase discloses the properties like anti - oxidation and anti - inflammation. Heme oxygenase is helpful in the treatment of cancer [37].

Nrf2 exhibits multiple protective effects against toxicity, oxidative stress and chronic diseases, inhaled irritants, environmental toxicants and chemical carcinogens. Nrf2 mRNA gene expression surprisingly plays a protective role against age-related diseases, neurodegeneration and cancer [38]. Decreased expression of HO-1 mRNA is usually associated with the progression of coronary atherosclerosis correlated to increase in the oxidative stress. Increased exposure of glyphosate to the body leads to increase in oxidative stress which results in decreased expression of both Nrf2 and HO-1 mRNA in a dose dependent manner. So, the oxidative stress affects animals and humans to an extent due to exposure to glyphosate. Through the result obtained by this study, it can be concluded that glyphosate not only affects plants but also shows deleterious effects on animals and humans. So, the Indian government should take necessary measures to ban glyphosate for human welfare.

Conclusion

Glyphosate is a synthetic compound. It is a non-selective systemic herbicide which harms the animals as well as the plants. Heme oxygenase is an enzyme that catalyses the degradation of heme. For the first time our present findings showed that glyphosate exposure has detrimental changes in down regulating the expression of Nrf2 signaling molecules and thereby it induced diabetes by disrupting antioxidant signaling. Further *in vivo* studies on signalling pathways may serve as a platform to develop drugs for treatment of glyphosate toxicity.

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Conflict of Interest

The authors declare no conflict of interest.

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