Glyphosate exposure exacerbate gluconeogenic enzymes in adult male Wistar rats.

Aashiq Mohamed P, Gayathri R*, V Vishnu Priya, J Selvaraj, Kavitha S

Department of Biochemistry, Saveetha Institute of Medical and Technical Sciences, Chennai, India

Abstract

Glyphosate is an herbicide. It is applied to the leaves of plants to slaughter both broadleaf plants and grasses. Glyphosate being water soluble enters the food chain by biomagnification. Increased concentration of glyphosate may lead to oxidative stress and thus can induce insulin resistance associated with diabetes mellitus. Oxidative stress is invaded in the dysregulation and increased gene expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, a key gluconeogenic enzyme.

Keywords: Glyphosate, Glucose-6-phosphatase, PEP carboxykinase, Diabetes mellitus, Innovative technology, Novel method.

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Introduction

Diabetes is considered as a major metabolic disorder. The global prevalence of diabetes among adults over 18 years of age increased from 4.7% in 1980 to 8.5% in 2014. The predominance of diabetes in the grown-up population of Ethiopia was 5.2% in 2018 [1]. Way of life adjustments including diet are significant conduct for diabetes, corpulence the executives, and lipid control, subsequently lessening the frequency of metabolic disorder in patients without diabetes and keeping up the glycemic control in those with both kind I and type II diabetes. Diabetic nephropathy is considered as the key cause of renal failure, but unfortunately the mechanism leading to this development and progression of renal injury are not yet fully known [2]. Gluconeogenesis is a metabolic pathway which results in the generation of glucose from certain non-carbohydrate substrates. It is present in plants, animals, fungi, bacteria and other micro-organisms [3]. Glucose is the only energy source used by the brain, testes, erythrocytes, kidney medulla, etc. In vertebrates, gluconeogenesis takes place mainly in the liver, and to the cortex of kidneys. There are two major enzymes that modulate gluconeogenesis, Glucose-6-phosphatase and Phosphoenol Pyruvate Carboxykinase (PEPCK). Glucose-6-phosphatase is an enzyme which hydrolyzes Glucose-6-phosphate, resulting in the removal of a phosphate group to release free glucose. This catalysis completes the final step in gluconeogenesis and thereby plays a major role in homeostasis of blood glucose levels [4]. Phosphoenol Pyruvate carboxykinase is an enzyme of the lyase family involved in gluconeogenesis. It converts oxaloacetate into carbon dioxide and phosphoenol pyruvate [5]. It is majorly found in two forms which are cytosolic and mitochondrial.

Glyphosate is a most commonly used herbicide which is basically available in chemical form. It consists of isopropylamine, diammonium salt, ammonium salt, potassium salt, etc [6]. The sodium salt form of glyphosate is majorly used in the regulation of plant growth and ripening in specific crops. The compounds are basically inert ingredients and it is commercially available as roundup, rangerpro, etc [7]. Glyphosate was first sold in 1974. The recent findings have led to the discovery of harmful effects of glyphosate in people who suffer from diabetes. Hence, glyphosate has been banned in at least 10 jurisdictions which include countries like Germany, Saudi Arabia, etc due to its ill effects like skin irritations, vomiting, and diarrhea [8]. But it is not yet banned in India because of its agricultural purpose. Around 670 tons of glyphosate was used in India in the year 2018-2019 [9]. Recent studies proved that glyphosate and its components may also spread by wind and water erosion In recent years, glyphosate has been reviewed by multiple authorities concluding it as “Probable human Carcinogen” [10]. The strongest evidence shows that glyphosate causes hemangiosarcomas, kidney tumours and malignant lymphomas in male Wistar rats, kidney adenomas, liver adenomas, skin kerato adenomas and skin basal cell tumours in male Wistar rats. Ingestion of large volumes causes systemic toxicity and death [11]. Individuals may be exposed to glyphosate through various means such as from drinking water, food, environmental settings, etc [12]. Our team has extensive knowledge and research experience that has translate into high quality publications [13-32]. Aim of the present study is to investigate the impact of glyphosate on the expression of gluconeogenic enzymes in experimental rats.

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 (27°C ± 28°C) with a 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: Glyphosate treated (dissolved in water at a dose of 50 mg/kg b.wt/day at 8 am) orally for 16 weeks; Group III: Glyphosate treated (dissolved in water at a dose of 100 mg/kg b.wt/day at 8 am) orally for 16 weeks; Group IV: Glyphosate treated (dissolved in water at a dose of 250 mg/kg b.wt/day at 8 am) 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. Tissues were collected from control and glyphosate induced rats and used for the assessment of various parameters.

mRNA Expression Analysis

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 xg 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 xg 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 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 a reaction mixture (45 μl) was 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 investigated statistically by a 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 the $p<0.05$ level were considered to be statistically significant.

Results

Impact of glyphosate on the mRNA expression of Glucose-6-phosphate and PEPCK in the adult male Wistar rats

mRNA expression of Glucose-6-phosphate and PEPCK were assessed by Real-Time-PCR. There is a significant dose-dependent increase ($p<0.05$) in the expression of Glucose-6-phosphate in the glyphosate treated rats compared to control rats (Figure 1) conversely PEPCK mRNA expression was also found to be significantly ($p<0.05$) down-regulated in glyphosate exposed rats compared to control in a dose-dependent manner indicating that glyphosate has detrimental changes in adult male Wistar rats (Figure 2).

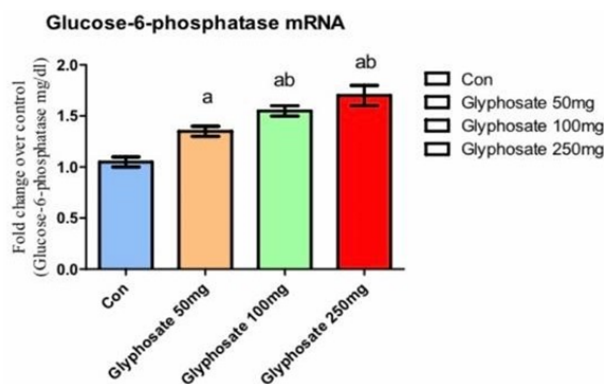

Figure 1. *Impact of glyphosate on the mRNA expression of glucose-6-phosphate in adult male rats. The X-axis represents dose-dependent exposure of glyphosate to the Wistar rats compared with control. Y-axis represents the mRNA expression of glucose-6-phosphate expressed in fold change over control. Light Green represents the controlled rats, orange represents Group 1 rats exposed to about 50 mg glyphosate, blue represents Group 2 rats exposed to about 100 mg glyphosate and purple represents Group 3 rats exposed to about 250 mg glyphosate. The mRNA expressions were assessed by Real Time-PCR using gene-specific primers. Each bar represents mean ± SEM (n=6). Significance at P<0.05, a: Significantly different from the control group, b: Significantly different from diabetic control.*

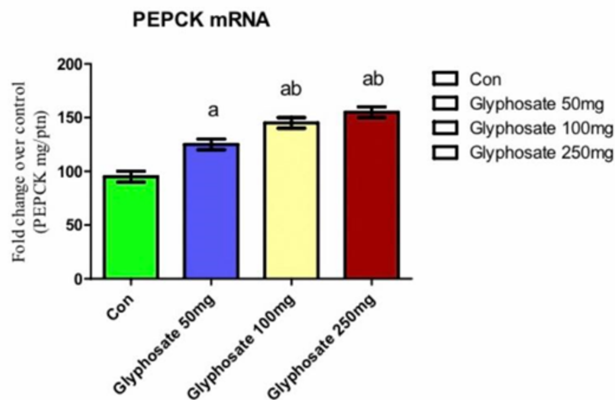

Figure 2. *Impact of glyphosate on the mRNA expression of PEPCK in adult male rats. The -axis represents dose-dependent exposure of glyphosate to the Wistar rats compared with control. Y-axis represents the mRNA expression of PEPCK expressed in fold change over control. Light Green represents the controlled rats, orange represents Group 1 rats exposed to about 50 mg glyphosate. Blue represents Group 2 rats exposed to about 100 mg glyphosate and purple represents Group 3 rats exposed to about 250 mg glyphosate. The mRNA expressions were assessed by Real Time-PCR using gene-specific primers. Each bar represents mean ± SEM (n=6). Significance at P<0.05, a: Significantly different from the control group, b: Significantly different from diabetic control.*

Discussion

This study was aimed to determine the effect of glyphosate is causing diabetes by investigating the expression of gluconeogenic enzymes, glucose-6-phosphatase and phosphoenol pyruvate carboxykinase. The gluconeogenic enzyme PEPCK was discovered by [33]. Oxaloacetate is one of the several important intermediates in krebs cycle that are withdrawn for biosynthetic pathways. Therefore, it plays an important anaplerotic role in numerous biological processes [34,35]. The metabolic link between gluconeogenesis and the TCA cycle is represented by PEP-pyruvate/oxaloacetate. Under gluconeogenic conditions, the TCA cycle intermediates malate or oxaloacetate which is later converted to pyruvate and PEP by means of decarboxylation and thus it provides the direct precursors for gluconeogenesis. Previous studies proved the increased expression of PEPCK enzyme in diabetes. Thus our results indicate the effect of glyphosate in inducing diabetogenic effects [36].

Studies clearly indicated that diabetes and starvation induce two to three fold increases in glucose-6-phosphatase activity in the liver [37]. Glucose-6-phosphatase is the enzyme found mainly in the liver and kidneys which play an important role in providing glucose during starvation and it is also associated with the endoplasmic reticulum [38]. Our study indicates the increased glucose-6-phosphatase level in all the glyphosate induced rats. The increased level of both the gluconeogenic enzymes might lead to hyperglycemia. Hyperglycemia and hypertension are important factors predisposing patients to nephropathy, however accumulating evidence points out the association of abnormal lipid metabolism also with diabetes [39]. From the study, it is evident that glyphosate with an increased concentration contributes to increased expression of both PEPCK and glucose-6-phosphatase mRNA. Several evidence indicates oxidative stress as a major contributor in regulation of key gluconeogenic enzymes. This study is of first sort in studying the effect of glyphosate in gluconeogenesis. It would be further expanded with molecular study on signaling molecules involved in gluconeogenesis and other enzymes associated with the pathways.

Conclusion

The present study indicates that glyphosate induces diabetes by altering the expression of gluconeogenic enzymes. Further detailed studies on in silico and *in vivo* models need to be conducted to study the mechanism of glyphosate action.

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Statement of Conflict of Interest

The author declares that there was no conflict of interest in the present study.

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*Corresponding to:

Gayathri R

Department of Biochemistry

Saveetha Institute of Medical and Technical Sciences

Chennai

India

E-mail: gayathri.sdc@saveetha.com