Impact of glyphosate alters the expression of insulin receptor and GLUT-4 molecule in cardiac tissue: an *in vivo* analysis.

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Abstract

Background: Glyphosate, a herbicide, and desiccant, is one of the most widely used herbicides all around the world. It has teratogenic, tumorigenic, and hepatorenal effects which can be explained by the endocrine disruption and oxidative stress causing metabolic alterations conditioned by the dose and time of exposure. Glucose Transporter (GLUT-4) activity alterations are considered responsible for the decrease of glucose uptake in muscle and adipose tissues in obesity and diabetes. Insulin Receptors (IR) is a receptor important for metabolic regulations and its alteration causes insulin resistance. Insulin resistance promotes impaired fasting blood glucose level which increases the prevalence of atherogenic lipid profile.

Aim: The aim of the study is to study the effect of glyphosate on the expression of both insulin receptor and GLUT-4 molecule.

Materials and Methods: Healthy male albino rats were divided into four groups and each consisting of 6 animals. Group I-Control; Group II-50mg; Group II-100mg; Group II-250mg exposed rats. After exposure with glyphosate, the rats were sacrificed, cardiac tissues from control and treated rats were dissected out and used for the assessment of gene expression analysis of Insulin Receptor (IR) and Glucose Transporter-4 (GLUT4). The data were analyzed statistically by a one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range tests were used to see the statistical significance among the group. The results with the $p < 0.05$ level were considered to be statistically significant.

Results: There was a decrease in the IR mRNA and GLUT-4 mRNA expression, leading to hyperinsulinemia and hyperglycemia eventually leading to diabetes. There was a statistical significance with the value $P < 0.05$,

Conclusion: The present study revealed the role of glyphosate in inducing hyperinsulinemia and hyperglycemia, which can cause diabetes by altering IR and GLUT4.

Keywords: Glyphosate, Insulin receptor, GLUT-4 molecules, diabetes. Innovative technologies; Novel methods.

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Introduction

Glyphosate, a herbicide, and desiccant, is one of the most widely used herbicides all around the world. Glyphosate was first sold in 1974 and since then it has become the most commonly and assiduously used herbicide worldwide [1]. Glyphosate-based herbicides including roundup are one of the most widely used herbicides all around the world. Its residue level in the water and food has increased extensively as well as its exposure to human beings. It has teratogenic, hepatorenal, and tumorigenic effects. All of these issues can be explained by the endocrine disruption and oxidative stress causing metabolic alterations conditioned by the dose and time of exposure [2]. Agricultural workers exposed to glyphosate suffer from pregnancy problems and can have several other enzymatic effects. Experimental animals exposed to glyphosate reported depressed function of cytochrome P450 and two other enzymes which are essential for the proper processing of toxicants [3].

Metabolic syndrome specified by insulin resistance is closely related to the content of Glucose Transporter Type-4 (GLUT-4) in insulin-sensitive tissues. GLUT-4 is the insulin-sensitive transporter that plays an important role in insulin-stimulated glucose uptake by adipose tissue, heart tissue, skeletal muscles, tissues that especially express this protein [4]. Alterations in GLUT-4 activity are considered as one of the reasons responsible for the decrease in glucose uptake in muscle and adipose tissues in obesity and diabetes. A large amount of GLUT-4 is present in the intracellular vesicles until the stimulation of the cell by insulin. Studies done on both rodents and humans reported that there is a decrease in *in-vivo* and *in-vitro* insulin-mediated glucose transport in diabetic subjects in comparison to the control subjects, both lean and obese [5].

Insulin resistance is described as an important biological response to insulin. Insulin resistance promotes impaired Fasting Blood Glucose Level (FBGLs) which increases the prevalence of atherogenic lipid profile molecules [6]. In a study conducted by Aparamita et al. exposure to glyphosate showed elevation in cytokines in liver tissue in male rats to various doses, at higher doses was also reported an increase in fibrosis in the exposed liver tissue [7]. A study conducted by Daruich et al. showed a disruption of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and malic dehydrogenase activity in the heart of female Wistar rats and fetuses after 21 days of glyphosate treatment in water during gestation [8]. A study

conducted by Chan P and Mahler J showed B6C3F1 male mice marked towards the heart weight increase after 13 weeks of glyphosate treatment [9]. In a study conducted by Owagboriaye F et al. it was reported that glyphosate-based herbicide, roundup, can induce reproductive toxicity in the male reproductive system of the exposed animals [10]. The present study aimed to study the impact of glyphosate exposure on the expression of insulin receptors and GLUT-4 molecules in cardiac tissue [11-30]. The effect of glyphosate on the expression of Insulin receptors and GLUT-4 molecule was not studied so far. Hence the present study aims to study the effect of glyphosate on the expression of both insulin receptor and GLUT-4 molecule.

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.

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 g–200 g, 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.

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 the 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. Cardiac tissue from control and experimental animals was immediately dissected out and used for assessing the various parameters.

Assessment of Fasting Blood Glucose (FBG)

After the overnight fasting, the blood glucose was estimated using On-Call Plus blood glucose test strips (ACON Laboratories Inc., USA). From the rat tail tip, the blood was collected and results were expressed as mg/dl.

Oral Glucose Tolerance Test (OGTT)

For oral glucose tolerance tests, animals fasted overnight. After giving the oral glucose load (10 ml/kg; 50% w/v). blood glucose level was estimated at various periods (60, 120, and 180 min) by using On-Call Plus blood glucose test strips. Before giving a glucose load, the value of blood glucose is considered as 0 min value. Results were marked as mg/dl.

Gene Expression Analysis

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 1 min, and placed on ice at 4°C for 5 min. The homogenates were centrifuged at 12,000 xg 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 the 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 the dilution factor. The purity of RNA preparation can be calculated using the ratio between its absorbance at 260 nm 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.

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 and 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 RNA-dependent 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

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 min - 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 in 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

The 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 -insulin receptor

FW: 5′- GCC ATC CCG AAA GCG AAG ATC-3′

RW: 5′- TCT GGG TCC TGA TTG CAT-3′

Rat GLUT4

FW: 5′- GGG CTG TGA GTG AGT GCT TTC - 3′

RW: 5′- CAG CGA GGC AAG GCT AGA - 3′

Rat β-actin

FW : 5'- TACAGCTTCACCACCACAGC - 3'

RW– 5'- TCTCCAGGGAGGAAGAGGAT - 3'

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 anti-sense primer, 1 µl of cDNA, and 3°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°C-95°C for each sample to determine the presence of multiple amplicons, non-specific products, and contaminants. The results were analyzed using CFX 96 Real-Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin.

Statistical Analysis

The triplicate analysis results of the experiments performed on control and treated rats were expressed as a mean ± standard deviation. 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

Due to glyphosate exposure, there was a decrease in the IR mRNA expression in the animals exposed to glyphosate compared to control rats. Compared to control rats' value of 1 IR/β-actin, group 1 rats showed a decreased expression to approximately 0.8 IR/β-actin, while group 2 and 3 showed a decreased expression to approximately 0.6 IR/β-actin. This signifies a decreased expression of IR in the glyphosate-exposed group of rats. The greater the exposure the greater was the impaired expression though not necessarily consistent. This causes impairment of insulin action in the experimental animals due to insulin resistance and leads to hyperinsulinemia.

Similarly, there was a decrease in the expression of GLUT-4 mRNA in the rats exposed to glyphosate compared to control rats. Compared to control group rats' value of 1 GLUT4/β-actin, group 1 rats showed a decreased expression to approximately 0.5 GLUT4/β-actin, while group 2 showed a

decreased expression to approximately 0.3 GLUT4/β-actin and group 4 showed a decrease to approximately 0.4 GLUT4/β-actin. The greater the exposure the greater was the impaired expression though not necessarily consistent. Due to the decreased expression of GLUT 4 mRNA, there was a decreased or no transport of glucose into the cells, causing it to get.

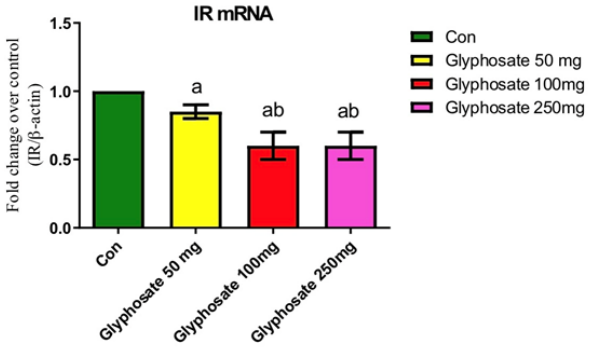

Figure1: *Impact of glyphosate on the mRNA expression of IR mRNA in adult male rats. The X-axis represents the amount of glyphosate which was exposed to the rats. Y-axis represents the fold change in the insulin receptor expression in experimental rats in comparison to controlled rats. Dark green represents the control rats, light yellow color represents Group 1 rats exposed to about 50 mg of glyphosate, red color represents Group 2 rats exposed to about 100 mg of glyphosate, and pink represents Group 3 rats exposed to about 250mg of glyphosate. The mRNA expression was assessed by real time-PCR. 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.*

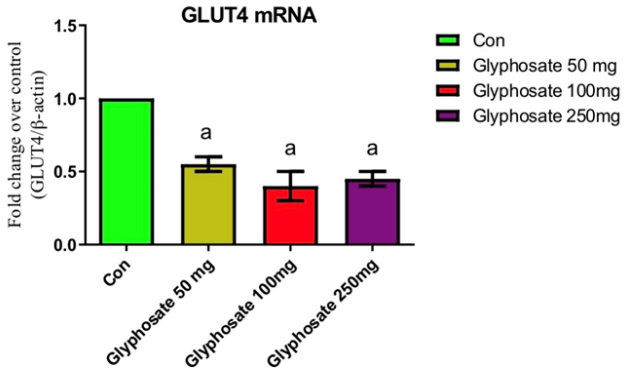

Figure 2: *Impact of glyphosate on the mRNA expression of GLUT4 mRNA in adult male rats. The X-axis represents the amount of glyphosate which was exposed to the rats. Y-axis represents the fold change in the GLUT-4 molecule expression in experimental rats in comparison to control to about 50 mg glyphosate, red color represents Group 2 rats exposed to about 100 mg of rats. Light green color represents the controlled rats, dark yellow color represents Group 1 rats exposed glyphosate, and purple represents Group 3 rats exposed to about 250 mg glyphosate. The mRNA expression was assessed by Real Time-PCR. 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

Decreased expression in the insulin receptor leads to a decrease in insulin action which causes insulin resistance leading to an impairment in the function of GLUT-4. In a study, it was observed that hyperinsulinemia is a predictor of Coronary Artery Diseases (CAD). The clearest association of hyperinsulinemia with CAD has been reported in Finland in a population with a relatively high frequency of CAD. Investigation of 2103 men from Quebec distinctively showed that high fasting insulin concentration is an independent predictor of CAD [31]. Another study suggests that a high ratio of estrogen to testosterone combined with hyperinsulinemia makes men vulnerable to premature CAD and related mortality in men [32]. In a previous study, it was reported that the potential of a high FRC-GP diet causes conditions of rats Type2 diabetes which induces hyperglycemia, hepatotoxicity, and lipid peroxidation in the treated rats [33]. Studies have also reported a decrease in GLUT-4 mRNA content to a very large extent than GLUT-4 protein response to diabetes and fasting. The effect of diabetes and fasting was found to be almost identical and lead to changes in the expression of GLUT-4 that are tissue-specific [34]. Studies also suggest that there is a primary defect in the transport of glucose in the muscle that leads to secondary defects in insulin action due to glucose toxicity in adipose tissue and the liver. These secondary defects lead to insulin resistance and also diabete [35].

In the present study, the results suggest that the mRNA expression of insulin receptors is decreased significantly in glyphosate-induced rats compared to the control rats. The same pattern was obtained for the expression for GLUT-4 stating that glyphosate negatively regulates insulin signaling molecules in cardiac tissue. Insulin resistance is a key element in many diabetic-related complications. In insulin resistance created by the changes in the insulin, receptor signaling pathway may lead to increased complications associated with diabetes. In this condition, though the insulin is synthesized in the body, it is not utilized due to the alteration in its receptors, so treatment modalities of such conditions have to be taken with extra care, as insulin cannot be replaced for the patient.

The study was done with just three varied doses of glyphosate. Studying more varied doses will help with obtaining more accurate effects on the expression with more time and sample.In the future, further studies need to be done to take care of the treatment modalities and other side effects created by the long-term exposure of the non-specific herbicide.

Conclusion

Glyphosate and glyphosate based herbicides like RoundUp have their impact on the expression of insulin receptors and GLUT-4 molecules on cardiac musculature causing hyperinsulinemia and hyperglycemia leading to diabetes in the

rats. It causes many adverse effects on several other tissues like adipose, skeletal, liver, etc. It causes many coronary artery diseases affecting the heart of the rats. Hence glyphosate, though used widely, is one of the most toxic elements present around in the environment which has many adverse effects.

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Statement of Conflict of Interest

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

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