

Original Research Article

Impact of glyphosate exposure on glycogen metabolic enzymes in the liver of adult male rats

Running title: Impact of glyphosate exposure on glycogen metabolic enzymes

ABSTRACT

Background: Glyphosate is a broad spectrum herbicide and desiccant. Diabetes is a group of metabolic diseases resulting due to deficiency in insulin secretion. Chronic hyperglycemia will lead to long term damage and failure of different organs like eyes, kidneys, nerves etc. Liver is the major site for gluconeogenesis and a lot of glycolytic enzymes will be involved. Expression of Glycogen synthase and glycogen phosphorylase, the glycolytic enzymes are studied in this research.

Aim: To determine whether glyphosate exposure is detrimental to the glycogen metabolic enzymes (Glycogen synthase and phosphorylase) in the liver of adult male rats.

Materials and Methods: The following study was done on albino rats of wistar strain, and was approved by the institutional animal ethics committee. They were fed with a rat pellet diet. In our study the rats were divided into 4 groups with 6 rats in each and were subjected to glyphosate orally with different dosage in each group and mRNA expression analysis of glycogen related enzymes was done after a span of 16 weeks. The data were analyzed statistically by a one way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to see the statistical significance among the group. The results with $p < 0.05$ level were considered to be statistically significant.

RESULT: The present result showed that the mRNA expression of glycogen synthase significantly reduced ($P < 0.05$) and mRNA expression of glycogen phosphorylase activity increased significantly with an increased dose of glyphosate ($P < 0.05$) to that of control.

CONCLUSION: Exposure to glyphosate causes detrimental changes in the glycolytic enzymes glycogen synthase and glycogen phosphorylase leading to diabetes.

KEYWORD: - Glyphosate, Diabetes, Glycogen synthase, Glycogen phosphorylase, Innovative technology, Novel method

INTRODUCTION: -

Glyphosate, also known as N-phosphonomethyl glycine is one of the most widely used herbicides worldwide, but was banned in several countries due to its non-target impacts on long-term usage(1). This herbicide was commonly used for crops such as soybean, wheat, corn etc. This chemical affects the shikimate pathways in the plants thereby inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), EPSPS is an enzyme which catalyzes the penultimate stage in the shikimate pathway . The inhibition of EPSPS results in a decrease of the three essential amino acids in the plant, tyrosine, phenylalanine, and tryptophan, and also leading to a decrease of secondary metabolites such as flavonoids, lignin, and phytoalexins. EPSPS is an enzyme present in all plants, both herbaceous and arboreal(2,3). The advantage of this herbicide was, it acted as a non- selective herbicide and was achieved in preventing weeds in crops(4). Despite all the positive effects of glyphosate, some studies showed that they induce cytotoxic and genotoxic effects in humans (5). In 2015 the IARC (International Agency for Research on Cancer, Lyon, France), stated its organization as the specialized cancer agency of the World Health Organization (WHO, Geneva, Switzerland), stated glyphosate as a carcinogenic substance and maybe harmful to human beings (2). This led to a drastic downfall of the company “Roundup” which had high contents of glyphosate in its products and were banned in several countries like Thailand, Sri Lanka and also states in India like Andhra Pradesh.

Diabetes is also one among the non-target effects caused by glyphosate. Diabetes was identified 3000 years ago by Egyptians. It was coined by Aractus of Cappadocia in 81-133 AD(6). Diabetes disrupts the function of conversion of carbohydrate to energy (6) leading to heterogeneous disturbance of metabolism. The main findings is chronic hyperglycemia which causes impaired action of insulin (7) and can be classified into type1 and type 2 diabetes.

Glycolysis is a central metabolic pathway and is present in all parts in an organism, the enzymes in the pathway are exceptionally well characterised, both in terms of enzymic properties and also in terms of structure (8).Glycogen breakdown and synthesis are reciprocally regulated by a hormone triggered cAMP cascade acting through protein kinase(9).Insulin is an hormone which usually upregulates glycogen synthesis and downregulates glycogen breakdown(10). In chronic diabetic conditions , either due to the

lack of insulin or due to insulin resistance, there is continuous upregulation (11),(12),(13) of glycogen phosphorylase and downregulation of glycogen synthase. Evaluating the enzymes related to glycogen metabolism reveals the diabetic state of the experimental animal. Our team has extensive knowledge and research experience that has translate into high quality publications (14),(15),(16),(17),(18),(19),(20),(21),(22),(23),(24),(25),(26),(27),(28),(29),(30) Thus this study aims to evaluate the detrimental effects of exposure of glyphosate in glycogen metabolism.

MATERIALS AND METHODS:-

Animals:

Animals were maintained as per 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 180-210g (150-180 days old) were used in the study animals were obtained and maintained in clean polypropylene cages under specific humidity(65+ or -5%)and temperature (27+or-2'c) with constant 12 hr light and 12hr dark schedule at the central animal house facility, Saveetha dental college and hospitals-Chennai. 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 male albino rats were divided into 4 groups consisting of 66 animals each in the present study.

GROUP 1:- Normal control rats fed with control diet and drinking water.

GROUP 2:- Glyphosate treated [dissolved in water at a dose of 50□g/kg/body wt/day at 8am]orally for 10 weeks.

GROUP 3:- Glyphosate treated [dissolved in water at a dose of 100□g/kg/body wt/day at 8am]orally for 10 weeks.

GROUP 4:- Glyphosate treated [dissolved in water at a dose of 250□g/kg/body wt/day at 8am]orally for 10 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 tissue was collected from control and glyphosate induced rats were used for the assessment of various parameters.

Activity of glycogen synthase

To 5 µl of tissue extract, 30 µl of glycogen – buffer – G-6-P solution, 5 µl of cysteine and 10 µl of UDPG were added and incubated at 37°C for 15 min. This was added to 25 µl of PEP and 25 µl of pyruvate kinase and incubated at 37°C for 15 min. To this, 150 µl of DNPH was added and incubated for 5 min. Then 200 µl NaOH and 1.1 ml of ethanol were added and the tubes are mixed and centrifuged. The optical density of the supernatant fluid at 520 nm is measured. The protein concentration in the tissue extract was measured by method described by Lowry et al. (1951). The enzyme activity is expressed as µmoles of uridine diphosphate (UDP) formed/min/mg protein.

Activity of glycogen phosphorylase

To 100 µl of tissue extract, 190 µl of incubation mixture was added and incubated for 15 min at 37°C. Then the reaction was arrested by adding 10% TCA 50 solution. It was incubated at 25°C for 5 min and centrifuged at 1,600 x g for 10 min. 2 ml of supernatant was taken and to this 1 ml of ammonium molybdate solution was added and incubated for 10 min at room temperature. Then, 0.4 ml of ANSA was added. After 20 min incubation, OD was measured at 640 nm. Standards of various concentrations were also prepared. The standard graph was drawn and the phosphate liberated was calculated accordingly. The protein concentration in the tissue extract was measured by method described by Lowry et al. (1951). The results are expressed as µmoles of orthophosphate liberated / min / mg protein

Statistical analysis

The triplicate analysis result of the experiments performed on control and treated rats were expressed as a 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 $p < 0.05$ level were considered to be statistically significant.

RESULTS: -

Effect of Glyphosate on Glycogen Synthase:

In this study it was observed that the enzyme activity for glycogen synthase was significantly decreased at 50 µg, when compared to control. At the dose of 100 µg and 250 µg, the glyphosate concentration was similar to each other and is significantly reduced in comparison

to control, since there is a decrease in glycogen synthase, it is evident that glycogen has not formed and remains as an indicator of diabetes(fig1)

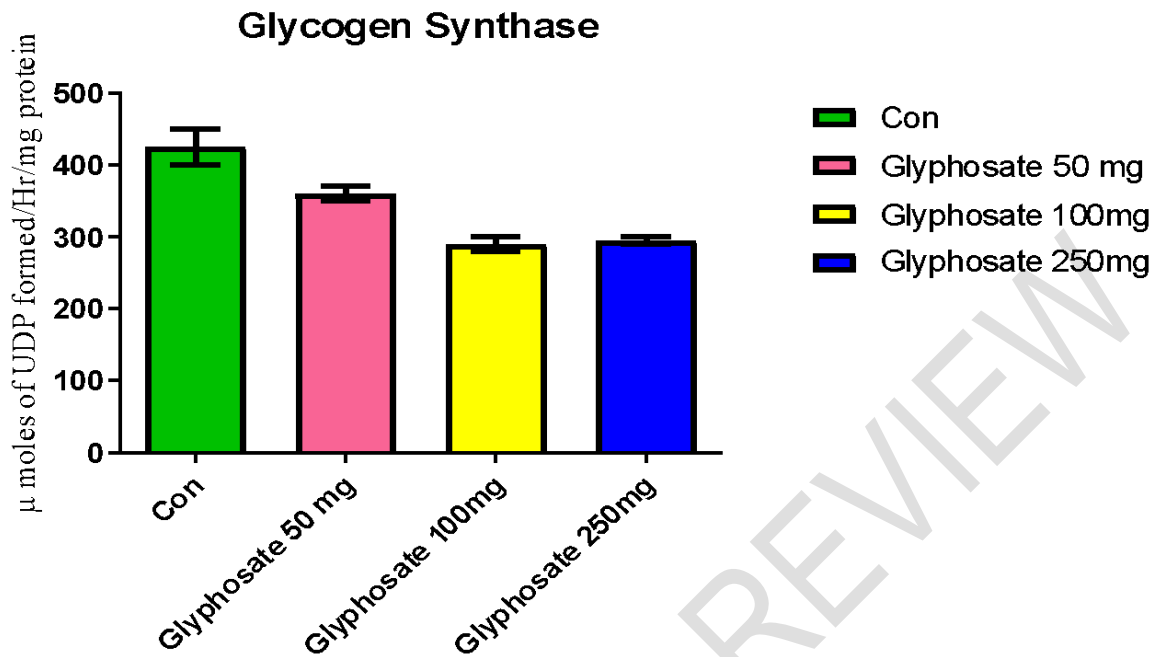


Fig 1: The graph represents a decrease in the expression of glycogen synthase in all glyphosate induced rats. Bar graph represents the differential expression of glycogen synthase. Each bar represents mean \pm SEM (n=6). Significance at $P < 0.05$

Effect of Glyphosate on Glycogen Phosphorylase:

In this study it was observed that the enzyme activity for glycogen phosphorylase increased in a dose dependent manner from 50 g, 100 g, 250 g, as the function of phosphorylase is to break glycogen it may lead to diabetes due to dysfunction in insulin secretion(fig2).

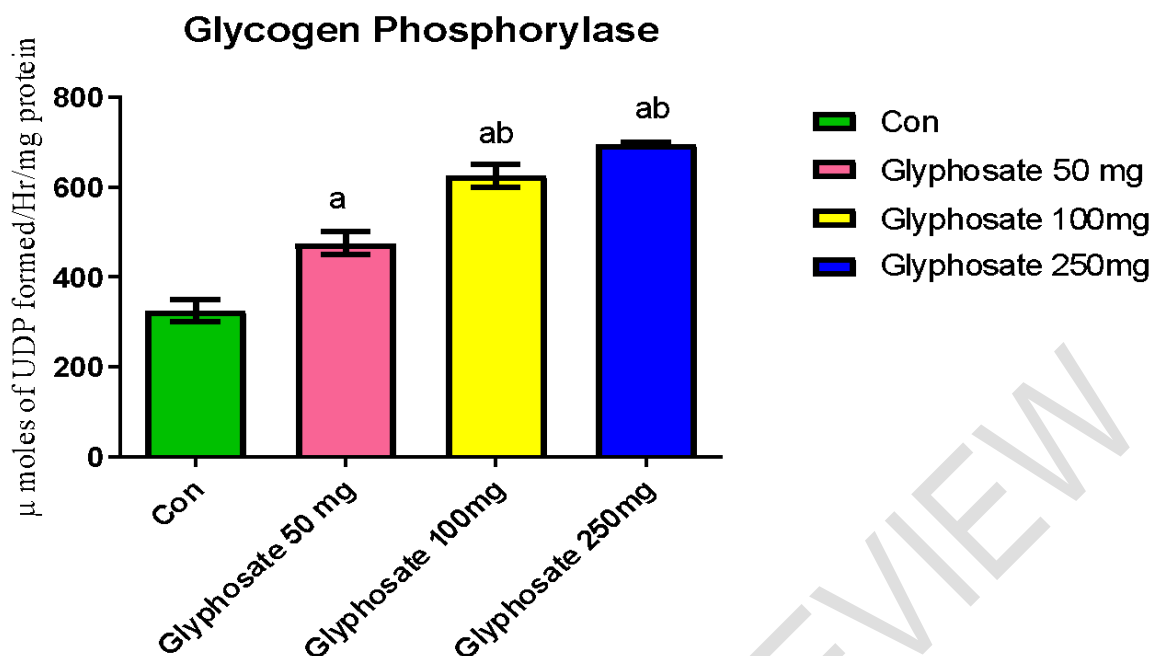


Fig 2: The graph represents an increase in the expression of glycogen synthase in all glyphosate induced rats. The bar graph represents the differential expression of glycogen phosphorylase. Each bar represents mean \pm SEM (n=6). Significance at $P < 0.05$, ^aSignificantly different from control group, ^bSignificantly different from diabetic control. This bar graph represents an increase in Glucose-6-phosphatase expression in glyphosate induced rats in a concentration dependent manner.

DISCUSSION:-

This study aims to determine the detrimental effect of glyphosate on glycolytic enzymes. In the current study, correlation between the increased dose of glyphosate (50, 100, 250 \square g/kg/wt) with insulin resistance and glycogen synthase was studied. It was evident that, with increase in glyphosate dose, there was a decrease in expression of glycogen synthase activity (glycogenesis) and increase in expression of glycogen phosphorylase activity (glycogenolysis). It clearly depicts the condition of Insulin resistance which is the prevailing reason for inactivation of glycogenesis, indirectly a metabolic disorder known as diabetes mellitus.

Previous Research experimented with silver catfish showed an increase in hepatic glycogen, but a reduction in muscle glycogen thus concluded that glyphosate changes the AChE activity, metabolic parameters and these parameters can be used as an indicator to measure

herbicide toxicity(31). Another study relates the lethal effects of glyphosate where glyphosate related products were subjected to tadpoles growth suppression, decrease in body mass, body width and decrease in total length was observed and concluded with the lethal effects of glyphosate (32). In a study (33) showed that glyphosate is a potent neurotoxic agent which implies oxidative stress. Studies also show their hazardous effects in reproductive cells (34). Apart from diabetic effects, glyphosate also have carcinogenic effects, In a research aimed in finding the carcinogenic effects of glyphosate and concluded that glyphosate has a large number of tumorigenic effects leading to breast cancer, pancreatic cancer, liver cancer and myeloid leukaemia . A recent study stated that increase in usage of glyphosate also lead to increase in diagnosis of various autoimmune diseases like autism, inflammatory bowel disease, multiple sclerosis and explained the aetiology of various autoimmune diseases(35) Stimulation of glycogen synthesis in skeletal muscle by insulin is a key feature of type two diabetes which leads to 50% decrease in fractional activity of glycogen synthase (36). Malfunction of Glycogen synthase is not only responsible for causing diabetes but also for cancer study (37) . The potential role of glycogen synthase in skeletal muscle resistance of type 2 diabetes concluded that increase in GSK-3 expression in diabetic muscle may contribute to the impaired Glycogen Synthase activity and Skeletal muscle Insulin resistance present in type 2 diabetes (38). As we know Glycogen phosphorylation is the first step in glycogenolysis. In a following study it was stated and aimed that glycogen breakdown can also inhibit cancer cell proliferation and induce apoptosis and it was concluded that, the observed inhibition of glycogen phosphorylation induces cell cycle arrest and apoptosis which supported the metabolic hypothesis by the regulation of cell growth and cell death of human cancers occurring through various substrate level regulation of macromolecule synthesis, which determines cell cycle progression and apoptosis formation. Talking about cancers another study conducted on ovarian cancer and aimed to explore the expression pattern of glycogen phosphorylase in ovarian cancer and to understand the potential mechanism in regulating ovarian cancer and concluded that in increase in dosage of glycogen phosphorylase on ovarian cancer showed poor prognosis hence it was concluded that glycogen phosphorylase played a important role in ovarian cancer prevention(39).

Our study is first of its sort to study the effect of glyphosate in glycogen metabolism. Further studies involving many enzymes in the glycolytic pathway and quantification of mRNA expression could add more effectiveness to our study.

CONCLUSION: -

Glyphosate being a herbicide has a definite role in inducing oxidative stress. Prolonged oxidative stress can lead to inflammation and other conditions like diabetes mellitus. Decreased glycogen synthesis and increased glycogen breakdown is an indicator of insulin resistance and diabetes mellitus which may be due to prolonged exposure of glyphosate.

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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