Impact of glyphosate on IGF beta and TNF alpha exposure on male wistar rats.

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Abstract

Glyphosate is a herbicide that inhibits the plant enzyme. It is commercially available in the form of roundup. High exposure to roundup causes necrosis that damages the leydig cells and other cells of the rat. There is a decrease in the normal sperm count due to a decrease in the concentration of histone-1 and protamine-1. The insulin-like growth factor is produced by osteoblasts which are the bone-forming cells of the body. IGF is also important in maintaining the effect of systemic hormones on bone formation. TNF alpha is a tumor necrosis factor which is produced by macrophages that is responsible for the destruction of tumor cell lines.

Keywords: Diabetes, Wistar rats, Glyphosate, Necrosis, Growth factor, Innovative technology, Novel method.

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Introduction

Glyphosate is an organophosphate compound that acts as a herbicide, its action inhibits the plant enzyme. The molecular effects of glyphosate can be known by the acute exposure for the study of the effect on testicular and endocrine functions of male wistar rats which shows an increase of aromatase mRNA and aromatase protein. There is a decrease in the normal sperm count due to a decrease in the concentration of histone-1 and protamine-1. [1]. Roundup is glyphosate constituted herbicide which is a metabolite. The effect of roundup on the testicular cells of male wistar rats was tested by 48-hour exposure on it [2].

High exposure to roundup causes necrosis that damages the Leydig cells and other cells of the rat [3]. Diabetes is of two types, diabetes insipidus and diabetes mellitus which are caused due to impairment in the function of the body to process glucose. Diabetes mellitus is hyperglycemia which is impairment in the function of the body to process glucose. The deficiency of insulin causes polyuria, polydipsia, weight loss, and blurred vision [4]. The condition of diabetes mellitus is also popularly referred to as blood sugar. The insulin deficiency due to beta cells of islets of Langerhans causes hyperglycemia that causes diabetes which leads to failure of organs **[5]**. IGF-beta is insulin-like growth factor 1 which is usually involved in the regulation of the bone formation of bone. The insulin-like growth factor is produced by osteoblasts which are the bone-forming cells of the body. IGF is also important in maintaining the effect of systemic hormones on bone formation. It was also found in various other experiments that IGF-1 is considered to increase the formation of osteoclasts from osteoclast precursors of mice. IGF is the most abundant growth factor stored in the bone matrix. IGF released during resorption helps in the coupling of bone formation [6].

TNF alpha is a tumor necrosis factor that is produced by macrophages that is responsible for the destruction of tumor cell lines [7]. This research is needed to study the impact of glyphosate and compare its effect on diabetic wistar rats and normal wistar rats. Diabetes in rats leads to increased anxiety levels and cognitive deficits. Previous researchers were unable to explain the effect of glyphosate on the markers of inflammation. Our team has extensive knowledge and research experience that has translate into high quality publications [8-27]. This study aims to analyze the effect of glyphosate on IGF beta and TNF alpha exposure on male wistar 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.

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.

Experimental design

Controlled group- Normal rats fed with normal diet and drinking water.

Group 1: Was treated with 50mg glyphosate orally for 16 weeks.

Group 2: Was treated with 100mg glyphosate orally for 16 weeks

Group 3: Was fed with 250mg glyphosate 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. Visceral Adipose tissues from control and experimental animals were 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 the oral glucose tolerance test, 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.

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 mins. 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 the RNA pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 5min at 7,500xg (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

The diluted RNA sample was 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 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 & 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 hybridized 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 principle

The purpose of a Polymerase Chain Reaction (PCR) 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 [28]. 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 [29].

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.

Rat IGF-β:

FW- 5"- CTGAGCTGGTGGATGCTCT- 3"

RW- 5"- CACTCATCCACAATGCCTGT - 3"

Rat TNF-alpha: FW – 5" –CAG CGG CCG CAA CAC ATC TCC CTC CGG AAA GGA C – 3"

RW - 5" – GAC CGC ACA AGT AGG CAA GAG ATG GCG CCG GCG – 3"

Rat β-actin: FW – 5'- TACAGCTTCACCACCACAGC - 3'

RW– 5'- TCTCCAGGGAGGAAGAGGAT - 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 ℃ for 3 min, followed by 40 cycles of PCR, denaturation at 95 ℃ for 10 sec, annealing at 60 ℃ for 20 sec, and extension at 72 ℃ 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 ℃ 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 the control and treated rats were expressed as mean ± standard deviation. Results were analyzed statistically by A One-Way Test 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 and Discussion

Impact of glyphosate on the expression of IGF beta and TNF alpha in the adipose tissue of experimental rats

The mRNA expression of IGF beta and TNF alpha were assessed by Real-Time PCR. There is a significant dose-dependent increase ($P<0.05$) in the expression of IGF beta and TNF alpha in the glyphosate treated rats in comparison to the controlled group of rats who did not show any alteration in the gene expression.

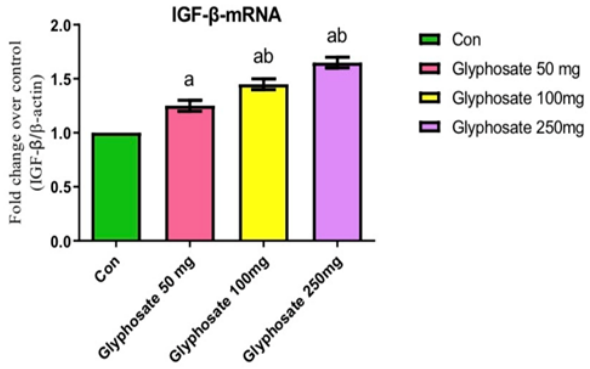

Figure1. *Impact of glyphosate on mRNA expression on IGF-beta in adult male wistar rats. The X-axis represents a controlled group of rats in comparison to rats exposed to subsequent doses of glyphosate. Y-axis represents a change in expression of IGF beta/ Beta-actin on exposure to glyphosate in the rats. Green represents controlled group of rats. Red represents rats who were treated with 50 mg of glyphosate orally for 16 weeks. Yellow represents rats who were treated with 100 mg of glyphosate orally for 16 weeks. Purple represents rats who were treated with 250 mg of glyphosate orally for 16 weeks. The mRNA expressions were assessed by real-time PCR. Each bar represents mean SEM (n=6). Significance at P< 0.05. Significantly different from the controlled group of rats who were fed with normal diet and drinking water. In comparison to controlled rats, when glyphosate was induced, the gene expression kept increasing subsequently with the increase of dosage at 50 mg, 100mg, and 250 mg.*

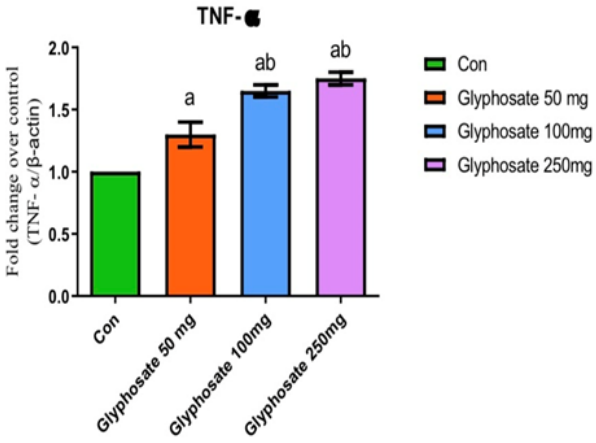

Figure 2. *Impact of glyphosate on mRNA expression on TNF-alpha. The X-axis represents a controlled group of rats in comparison to rats who are exposed to subsequent doses of glyphosate. Y-axis represents the change in expression of TNF alpha/ Beta-actin on exposure to glyphosate. Green represents a controlled group of rats. Red represents rats treated with 50*

mg of glyphosate orally for 16 weeks. Blue represents rats treated with 100 mg of glyphosate for 16 weeks. Purple represents rats treated with 250 mg of glyphosate for 16 weeks. In adult male wistar rats, the mRNA expressions were assessed by real-time PCR. Each bar represents mean SEM (n=6). Significance at P< 0.05. Significantly different from the controlled group of rats who were fed with normal diet and drinking water.

In comparison to control, at a subsequent increase of glyphosate dosage at 50 mg, 100 mg, and 250 mg, increased gene expression of TNF alpha was induced, which thereby increased the inflammatory response. Figure 1 impact of glyphosate on mRNA expression of IGF beta. The mRNA expressions were assessed using the Real-time PCR technique. In comparison to the controlled group of rats when glyphosate was induced in the rats, there was a significant increase in the gene expression of IGF-beta which kept elevating on subsequent exposure of glyphosate at 50 mg, 100 mg, 250 mg. (Figure 2) Impact of glyphosate on mRNA expression on TNF-alpha. The mRNA expressions were assessed using the Real-Time PCR technique. In comparison to the controlled group of rats, at a subsequent increase of glyphosate dosage at 50 mg, 100 mg, and 250 mg, increased gene expression of TNF alpha was induced, which thereby increased the inflammatory response.

In this study, it was found that there was no significant alteration of gene expression in the controlled group of rats. Exposure to glyphosate altered the IGF beta and TNF alpha gene expression in the rats which showed an inflammatory response that can lead to arteriosclerosis and other disorders in the rat. IGF-1, a single polypeptide with 70 amino acids, was widely expressed in mammalian tissues **[30]**

In a study conducted by Thomas A link hart, it was found that IGF is an exciting therapeutic potential agent and helped in the formation of bone when released during resorption [31]. IGF beta also called an Insulin-like growth factor showed a significant increase in glyphosate exposure that lead to diabetes in the rats. A previous study conducted by Narayanan Parames Saran showed that TNF alpha produced by macrophages was responsible for the regulation of survival, cell differentiation, and proliferation. TNF alpha signaling has been associated with a pathogenesis that is related to various diseases that are induced in the body such as psoriasis, diabetes, rheumatoid arthritis, and sepsis. TNF alpha expression increased when the rats were treated with subsequent doses of glyphosate at 50 mg, 100 mg, and 250 mg. In another study conducted by Bonavida B, it was found that TNF increased the expression of HLA-DR antigens and high-affinity IL 2 receptors. As a consequence, TNF-treated T cells showed an enhanced proliferative response to IL 2 **[32]**. The action of IGF-1 peaks around the puberty period and decreases gradually with aging. The results of this study indicate the role of glyphosate in the development of diabetes by increasing the expression of IGF1 and TNF alpha exposure.

Even though the results were promising, only 2 parameters were used. Furthermore, the serum levels of glyphosate and other diabetic profiles were not analyzed. Further studies on downstream signaling molecules of proinflammatory signaling mechanisms are warranted to better understanding glyphosate towards the development of new drugs.

Conclusion

From this study, it was concluded that glyphosate exposure leads to the development of diabetes by modulating the expression of IGF beta and TNF Alpha in male wistar rats.

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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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