# Effect of glyphosate on the mRNA expression of TGF beta, IL-6, and IL-1 in experimental rats.

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## Abstract

**Glyphosate is a herbicide that was previously unknown of its toxic qualities and effects. Now, due to technological advancements, it is known to cause endocrine receptor blockade in humans. The residues of this herbicide may be deposited on soil, water, plants, and other natural areas and be consumed by humans. TGF beta is an inflammatory marker and is majorly involved in host defenses. Both IL-6 and IL-1 are pro-inflammatory cytokines markers with both immune and non-immune functions.**

**Keywords:** Glyphosate, Gene, PCR, TGF beta, IL6, IL1, Inflammatory markers, Innovative technology, Novel method.

*Accepted on November 03, 2021*

## Introduction

Glyphosate, which is chemically N-(phosphonomethyl) glycine, is a broad-spectrum non-selective and synthetically manufactured herbicide. It is the only herbicide on the market that targets the enzyme 5-Enolpyruvyl-3-Sikhimate-Phosphate Synthase (EPSPS) [1]. It generally translocates into metabolic sites/sinks and thus, causes necrosis of meristematic tissues. In humans, glyphosate is known to disrupt the endocrine receptors, and thus, may have a role to play in the development of Breast cancer [2]. Glyphosate-based herbicides are also extensively used in cultivating vegetarian meat crops (soybean). The residues of glyphosate can be found in soil, water, and other natural spots. Previous studies have further deleterious effects of glyphosate on human health, however, most of these toxic effects are extensively based on the cell type, chemical structure, as well as exposure duration, and dosage [3]. This herbicide has been used for decades under the illusion that its side effects were minimal. However, its toxicity and other ill effects have surfaced recently. Upon breakdown, Glyphosate breaks into aminomethylphosphonic acid, and their deposition and effect on terrestrial life are still under survey [4]. In-plant life, most of the glyphosate is used in transgenic glyphosate-resistant crops. These varieties of transgenic crops provided the most effective and inexpensive weed destruction method for decades. However, recent studies have found that extensive use of GRCs has ultimately led to alteration of plant nutrition through minerals and has also made the plant system much more susceptible to a host of plant pathogens [5].

TGF beta or transforming growth factor-beta or a multifunctional group of essential genes that play a major and significant role in growth, inflammatory response, and host immunity. In mammalian cells, the transforming growth factor-beta has three isoforms namely TGF beta 1 beta two, and beta 3. This family of factors is coded by a sum of 33 genes and comprises of homo and heterodimers [6].

IL-6 or Interleukin 6 is another family of inflammatory cytokines that play a substantial role in both immune and non-immune cells. Recent studies have shown that IL-6 is inversely related to disease prognosis in patients with cancer. It also plays a major role in homeostasis upon disruption of homeostasis by infections, IL-6 is secreted to act as host defens [7]. The IL1 or interleukin one family consists of several proinflammatory proteins. Among the entire genes IL1 beta can be characterized as its best and most inflammation-inducing protein. However, its secretion is very tightly regulated. Exaggerated production can lead to a variety of diseases with varying and unknown intensities [8,9]. Our team has extensive knowledge and research experience that has translate into high quality publications [10-32]. There is no previous literature about the toxic effects of Glyphosate on TGF-Beta, IL-6, and IL-1. This study helps bring about awareness about the toxic effects of Glyphosate on the mRNA expression of TGF-beta, IL-6 and IL-1.

## Materials and Methods

### *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 180–200 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 4 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. Brain tissues from control and experimental animals were immediately dissected out and used for assessing the various parameters.

## *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 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 Principle*

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 2-5 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 (10 µM).

3. Reverse primer (10 µM).

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 IL-6*

FW- 5’- GAGGATACCACCCACACCAGACCAGTA- 3′

RW- 5’-GGTTTGCCGAGTAGACCTCATAGTGAC- 3’

### *Rat TGF beta*

FW- 5” - CTT CAG CTC CAC AGA GAA GAA CTG - 3”

RW- 5" – CAC GAT CAT GTT GGA CAA CTG CTC C – 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°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 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

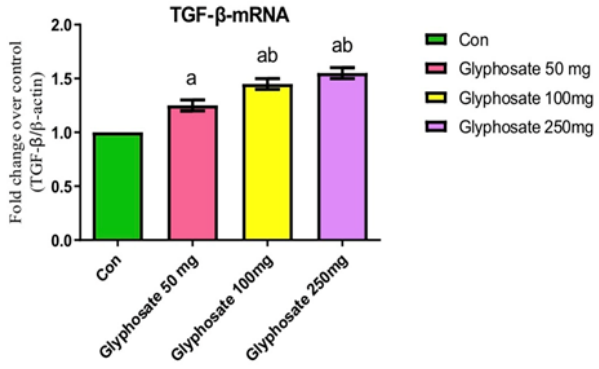


***Figure 1**: Impact of glyphosate on the mRNA expression of TGF-beta, in adult male Wistar rats. The X-axis represents the dose-dependent exposure of Glyphosate to the Wistar rats compared with the control. Y-axis represents the mRNA expression of TGF-beta expressed in fold change over control. Light Green represents the Control Rats. Red represents Group 1 Rats exposed to about 50 mg Glyphosate. Yellow represents Group 2 Rats exposed to about 100 mg of Glyphosate. Purple represents Group 3 Rats exposed to about 250 mg of Glyphosate. The mRNA expressions were assessed by Real Time-PCR. Each bar represents mean ± SEM (n=6). Significance at P<0.05.*

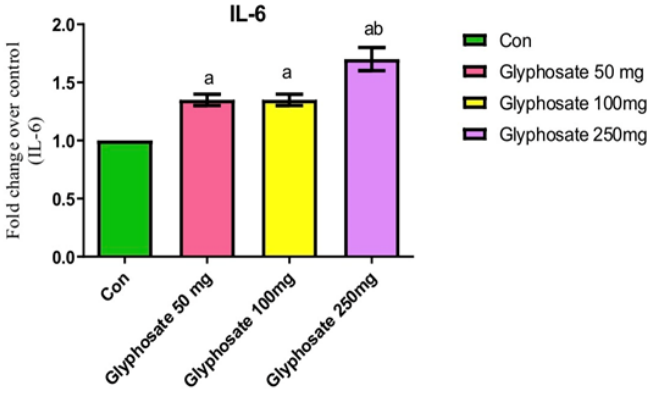


***Figure 2:** Impact of glyphosate on the mRNA expression of IL-6, in adult male Wistar rats. The X-axis represents the dose-dependent exposure of Glyphosate to the Wistar rats compared with the control. Y-axis represents the mRNA expression of IL-6 expressed in fold change over control. Light Green represents the Control Rats. Red represents Group 1 Rats exposed to about 50 mg Glyphosate. Yellow represents Group 2 Rats exposed to about 100 mg of Glyphosate. Purple represents Group 3 Rats exposed to about 250 mg of Glyphosate.*

The mRNA expressions were assessed by Real Time-PCR. Each bar represents mean ± SEM (n=6).

Significance at $P<0.05$.

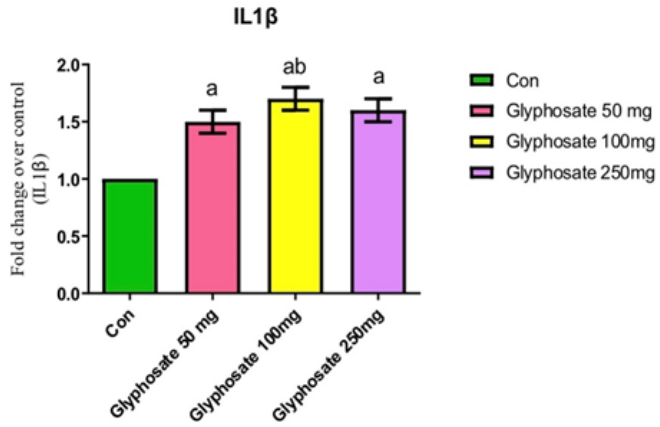


***Figure 3:** Impact of glyphosate on the mRNA expression of IL-1, in adult male Wistar rats. The X-axis represents the dose-dependent exposure of Glyphosate to the Wistar rats compared with the control. Y-axis represents the mRNA expression of IL-1 expressed in fold change over control. Light Green represents the Control Rats. Red represents Group 1 Rats exposed to about 50 mg Glyphosate. Yellow represents Group 2 Rats exposed to about 100 mg of Glyphosate. Purple represents Group 3 Rats exposed to about 250 mg of Glyphosate. The mRNA expressions were assessed by Real Time-PCR. Each bar represents mean ± SEM (n=6). Significance at P<0.05.*

The results showed in (Figures 1-3) that in comparison to the control when the glyphosate was induced, the gene expression of TGF beta increased from 1.0. Subsequently, as the dosage kept increasing, the expression also kept increasing. At a dosage of 50 mg, the fold change was observed to be 1.25, at a

dosage of 100 mg, the fold change was observed to be 1.4, at a dosage of 250 mg, the fold change was observed to be 1.5. The results also revealed that when the glyphosate was induced, the gene expression for IL-6 increased at 50 mg and 100 mg compared to the control. But at 250 mg, there is more increase in the expression. At a dosage of 50 mg and 100 mg, the fold change was observed to be 1.5, at a dosage of 250 mg, the fold change was observed to be 1.75. In comparison to the control, when glyphosate was introduced the gene expression for IL-1 kept increasing till 100 mg but decreased at 250 mg. At a dosage of 50 mg, the fold change was observed to be 1.4, at a dosage of 100 mg, the fold change was observed to be 1.75, at a dosage of 250 mg, the fold change was observed to be 1.5.

In the case of TGF beta, our study found a positive deviation (increase in inflammation) when glyphosate was induced, when compared to the control. Even though the results were promising, only 3 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 understand glyphosate towards the development of new drugs.

## Conclusion

From the conducted study, it was found that Glyphosate positively affected the expression of TGF-Beta, IL-6, and IL-1. Thus, they induced inflammation in the tissues. Furthermore, it can be said that increased inflammation can lead to diabetes.

## Acknowledgement

The authors would like to thank Saveetha Dental College and Hospitals, Saveetha Institute of medical and technical Sciences, Saveetha University for providing research laboratory facilities to carry out the study.

## Source of Funding

The present study was supported by the following agencies: Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha dental college, Saveetha university and orthodontic care, Chennai.

## Statement of Conflict of Interest

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

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