

Glyphosate Exposure to Wistar Rats Develops Diabetic Nephropathy via Nrf2/ Keap-1 - Mediated Signalling

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ABSTRACT

Background: Glyphosate (GLY), a widely used pesticide and endocrine disruptor, is frequently found in food and environmental sources. Long-term exposure may cause glucose resistance, the development of Type 2 Diabetes Mellitus (T2DM), and Diabetic Nephropathy (DN) by disrupting insulin signaling and causing oxidative stress. **Objectives:** The aim of this study was to examine how oral glyphosate exposure affected the Nrf2/Keap-1 signaling pathway and how it contributes to the development of DN by causing inflammation and oxidative stress. **Materials and Methods:** Male Wistar albino rats ($n=6/\text{group}$) were divided into three groups: a control group and two groups that were given oral glyphosate at low (50 mg/kg) and high (100 mg/kg) dosages for 16 weeks. Biochemical, gene expression, molecular docking, and dynamics studies were performed to assess renal and metabolic impacts. **Results:** Glyphosate exposure significantly increased kidney and liver function markers (65 ± 2 , 35 ± 2 , 70 ± 5 , 35 ± 2 , 0.7 ± 0.04), oxidative stress markers H_2O_2 (6 ± 0.3), LPO (16 ± 1.1), OH (20 ± 1.2), and decreased antioxidants SOD (32 ± 1.3), CAT (16 ± 0.9), GPx (18 ± 0.8), GSH (18 ± 0.8 , 13 ± 0.7). LDH (65 ± 4 , 90 ± 6), γ -GT (17 ± 1 , 19 ± 1), and total lipids (19 ± 1.8 , 45 ± 2.9) were elevated; Na^+/K^+ ATPases were significantly reduced ($p<0.05$). Gene expression revealed downregulation of IR, GLUT-2, AKT, Nrf2, and upregulation of IL-6, TNF- α , IKkB, Keap-1, KIM-1. Docking showed strong glyphosate binding with GLUT-1, IL-6, IKkB, and Nrf2 and MD simulations confirmed complex stability. **Conclusion:** Glyphosate exposure induces oxidative stress, inflammation, and insulin signaling impairment by suppressing Nrf2/Keap-1 signaling, contributing to DN development.

Keywords: Diabetic Nephropathy, Glyphosate, Inflammation, Insulin Signaling, Molecular Docking and Dynamics, Nrf2/Keap-1 Signaling.

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INTRODUCTION

Glyphosate is an organophosphate herbicide that is necessary for agriculture but can be harmful to human health. Its glycine-like chemical structure and varying half-life add to its adaptability in agriculture. It is composed of a strongly ionized 126-phosphate group and a basic amino group, similar to the natural amino acid glycine.^{1,2} Pesticide exposure disrupts circadian rhythms, which are closely linked to adipogenesis and metabolic regulation.³ The most used herbicide in the world, glyphosate, is toxic to organisms that are not intended targets and is often found in

aquatic habitats.⁴ The overall incidence of Metabolic Disorder (MetS) among adult Americans risen from 37.6% to 41.8% during 2011-12 and 2017-2018.⁵ Despite glyphosate's predicted market expansion until 2035, its broad use poses serious dangers to human health and the environment, requiring improved management techniques to minimize its negative effects.⁶ Many people believe glyphosate to be relatively safe because it is touted as being quickly biodegradable and is widely used.⁷ In human lymphocytes, glyphosate and its metabolite AMPA, in separate and equivalent amounts, produce genomic damage without any cooperative effects.⁸ By blocking the EPSPS enzyme, GLY disrupts the shikimate pathway and the production of aromatic proteins.⁹ At first, glyphosate exposure was believed to be safe for people, but new research has linked it to several detrimental health effects in both individuals. GLY exposure was originally thought to be harmless for humans, but recent studies have shown that it is now associated with a number of harmful health impacts in both occupational^{10,11} and non-occupational groups.¹²⁻¹⁶



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A diabetes-related condition known as DN occurs when chronically elevated blood sugar destroys renal blood vessels, which may eventually result in kidney failure.^{17,18} Hyper-glycemia and aberrant gene expression are caused by oxidative stress and disturbed signaling, which exacerbate T2DM.¹⁹ DN is one of the side effects of T2DM. It is brought on by chronic low-grade inflammation that triggers pro-inflammatory pathways and releases cytokines and chemokines. This inflammation exacerbates renal impairment in diabetes mellitus by promoting systemic insulin resistance and disrupting glucose homeostasis.^{20,21} Damage to the kidneys caused by diabetes called DN affects the metabolism of vitamin D by preventing its production into its physiologically active form through 1 α -hydroxylation.²² It is thought to induce oxidative stress and function as an endocrine disruptor, resulting in glucose intolerance, obesity, and insulin resistance. It has been related in animal research to oxidative damage, diabetes, and disturbances in metabolism and hormones.^{23,24} Exposure to maternal glyphosate-based herbicides changes the expression of genes linked to inflammation, oxidative stress, and lipid metabolism in offspring, which may have long-term neurological implications.²⁵ Also, the study showed that by changing the expression of KIM-1 and IGF-1, and GLY exposure causes hyperglycemia, insulin dysregulation, and kidney injury in rats, which exacerbates diabetic nephropathy.²⁶ DN is associated with neuroinflammation fueled by proinflammatory cytokines, which impair neuronal function and worsen cognitive impairment. By focusing on these cytokines, treatment approaches for diabetic neuropathy and cognitive decline may become available.²⁷⁻²⁹ Hepatotoxicity is caused by GLY, which suppresses the Nrf2/GSH/GPx axis to cause ferroptosis. It was demonstrated that ferroptosis inhibitors and GSH precursors may repair the liver damage caused by GLY, illuminating a crucial mechanism in GLY toxicity.³⁰

Through the Nrf2/Keap-1 pathway, cells are protected from damage caused by oxygen and swelling.³¹ It improves the antioxidant defenses of the cell by stimulating antioxidant response elements. It is normally regulated by Keap-1, which maintains Nrf2 in the cytoplasm. When Nrf2 is activated, it moves to the cytoplasm and promotes the replication of genes related to antioxidant and detoxification activities.³² When paired with other factors like paraquat, high temperatures, and various pollutants, glyphosate may significantly worsen persistent kidney disease of undetermined origin (CKDu) by disrupting defensive mechanisms and intensifying negative consequences, the study revealed. There's evidence that glyphosate exacerbates kidney impairment associated with CKDu in agricultural areas.³³ In young common carp, exposure to glyphosate causes hepatotoxicity via inflammatory reactions, oxidative stress, and abnormalities in lipid metabolism. Over time, these interrelated processes result in cholesterol buildup and liver damage.³⁴ Activating the Nrf2 signaling pathway is one of the antioxidative methods by which GLP-1 and its receptor agonists protect different organs from oxidative damage. This enhances GLP-1's ability to treat

long-term conditions like diabetes.³⁵ The Nrf2/Keap-1 pathway was significantly disrupted, with reduced Nrf2 activation and increased Keap-1 expression, contributing to the progression of nephropathy. It concludes that glyphosate exacerbates nephropathy through Nrf2/Keap-1-mediated oxidative stress, emphasizing the potential risks to kidney health and improving glyphosate's role in diabetic nephropathy and its implications for human health.

MATERIALS AND METHODS

Chemicals and experimental design

Sigma Chemical Company, Missouri, USA, provided the total RNA Isolation Reagents (TRIR) and glyphosate (#45521). Krishgen Bio-systems, Mumbai, provided the ELISA kits, and Eurofins Genomics, Bangalore, India, provided the gene-specific primers. Eight-week-old male Albino Wistar rats (IAEC No: BRULAC/SDCH/SIMATS/IAEC/02-2019/015) weighing 150-180 g was kept in a controlled setting with a 12 hr light/dark cycle and unlimited access to food and drink. Group 1 received normal water, Group 2 received 50 mg/kg of glyphosate by weight and Group 3 received 100 mg/kg of glyphosate by weight. The rats were randomly assigned to one of four groups, each with six rats. Rats were anesthetized, blood was drawn, and samples of the kidney tissues from normal and glyphosate-exposed groups were taken for molecular analysis after glyphosate was given orally for 16 weeks and diabetes was confirmed by FBG. The dose and study were designed based on our previous research.³⁶

Assessment of Kidney, Liver Function, and Lipid Profile

Kidney and liver function parameters were evaluated using biochemical assay kits from Spin React, Spain; the outcomes were represented in Units per Liter (U/L). Additionally, the lipid profile, which included the levels of lipoprotein and triglycerides (TG), were analyzed. The results were expressed in milligrams per deciliter (mg/dL).

Lipid peroxidation and reactive oxygen species

In 1987, Devasagayam and Tarachand conducted an investigation of Lipid Peroxidation (LPO).³⁷ Using the spectrophotometric method, H₂O₂ activity was determined,³⁸ and the results were expressed as μ moles/min/mg protein. The amount of hydroxyl radicals was measured and recorded.³⁹

Antioxidant enzymes

Superoxide Dismutase (SOD)⁴⁰ and Catalase (CAT)⁴¹ activities were measured and expressed in units per milligram of protein. The amount of glutathione utilized per minute per milligram of protein was measured in micrograms for Glutathione Peroxidase (GPx) activity.⁴² μ moles of CDNB conjugated per minute per mg of protein are used to measure

glutathione-S-transferase activity.⁴³ The reduced Glutathione (GSH) levels were expressed as nmol/mg.⁴⁴

LDH activity

With minor adjustments, the method was employed to quantify Lactate Dehydrogenase (LDH) activity.⁴⁵ After centrifuging the serum samples, the supernatant was combined with INT, NAD, sodium lactate, and sodium phosphate buffer. The mixture had been heated for 30 min at 37°C after the tissue homogenate was added, and the reaction was halted using glacial acetic acid. The resulting formazan was extracted into toluene, and a spectrophotometer was used to measure its color at 495 nm. $\mu\text{mol/mg/h}$ were used to generate LDH activity.

Assessment of γ -GGTP

Using a substrate, γ -glutamyl-p-nitroanilid was used to measure the activity of gamma-glutamyl transferase (γ -GGTP). γ -GGTP was measured by standard method.

Lipid peroxidation

The Malondialdehyde (MDA) level was represented as small molecules of MDA generated per minute per milligram of protein (nmoles/min/mg), and LPO was evaluated.³⁷

Estimation of Membrane-Bound Adenosine Triphosphatase

Using somewhat modified techniques from Ferguson *et al.*, 1976⁴⁶ membrane-Bound Adenosine Triphosphatase (ATPase) enzymes, such as Na^+/K^+ -ATPase, Ca^{2+} -ATPase, and Mg^{2+} -ATPase, were examined in rat kidney tissue. The assay mixture consisted of 2 mL of kidney homogenate, ice-cold Tris-HCl buffer, ATP, CaCl_2 , MgCl_2 , NaCl, KCl, and protein enzyme. Cold Trichloroacetic Acid (TCA) was used to halt the process, and the amount of phosphorus released during incubation at 37°C was used to measure ATPase activity. Centrifugation was used to extract the proteins after 20 min on ice.

Fatty acid extraction method

Using the Folch method,⁴⁷ total lipids were extracted from preserved brain tissues using a chloroform/methanol ratio of 2:1, v/v. Following tissue homogenization, 3 g of each sample was combined with methanol and chloroform, and non-lipid impurities were eliminated using a KCl solution containing 0.88%. After being extracted, the lipids were stored after being evaporated.

Measurement of total phospholipids (TP)

The overall quantity of fatty acids was calculated using Bartlett's method.⁴⁸

Regulation of genes by q-RT-PCR

Isolation of total RNA, synthesis of cDNA, and q-RT-PCR from wound sample

Trizol reagent was used to extract total RNA from the wound sample, and an RT kit from Seraing, Belgium was utilized to synthesize cDNA from 2 μg of RNA. The following thermal conditions were used for DNA amplification using a Bio-Rad Real-Time PCR system: initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 sec at 95°C, 1 min at 59°C, and 30 sec at 72°C. The relative quantity of target genes was evaluated using amplification and melt curve studies.

Molecular docking

A computational technique called molecular docking helps in virtual screening and drug development by predicting the binding mechanism and affinity of a ligand to a target protein. Docking is done assuming the ligand is stiff after protein and ligand structures have been developed and optimized. Using AutoDock 1.5.6, docking was carried out, and Biovia discovery Studio was used for 2D and 3D visualization.

Protein preparation

Using the Protein records bank, 3-dimensional systems for the target molecules were obtained (PDB). A small regularization was carried out to display the missing aspect chains after the.pdb file was submitted to the "construct/take a look at/restore design" and "set up the database file for dock programs" modules. Further to correcting the water molecules' locations and symmetrical alignment, hydrogen molecules were additionally delivered. In an effort to prepare the pdbqt document, Autodock equipment (ADT ver. 1.5.4) was most effective given chain A of the repaired.pdb files. Furthermore, water molecules and irregular residues had been removed, simplest polar hydrogen turned into kept, and Gustier charges for protein atoms had been calculated the use of ADT.

Ligand training

GLY were constructed the usage of the software program ChemSketch-12.01. Advancements had been made to the geometry of the created compound, partial expenses had been also designed, and they have been saved as mol2 files, which had been then despatched, as normal, to ADT for the introduction of pdbqt files.

Docking method

For docking experiments, Autodock4 (ver. 4.2.6) was used. a total of a hundred runs of the Lamarckian genetic set of rules with neighborhood search (GALS) had been hired because the search engine. On the docked effects, cluster evaluation becomes additionally carried out.

Table 1: Effects of antioxidant enzymes and lipid oxidation markers. Each bar shows the mean±standard error of the effects of glyphosate exposure on antioxidants and inflammatory markers within the kidneys of rats with diabetic nephropathy. Significant variation between groups (a-control and b-glyphosate treatment at 50 mg) is indicated by a *p*-value <0.05.

Parameters	Control	GLY (50 mg)	GLY (100 mg)
SOD (U/mg)	50±3	32±1.3 ^a	20±1 ^{ab}
CAT (μmol/mg)	30±1.2	16±0.9 ^a	13±0.7 ^{ab}
Gpx (μg/mg)	40±2	18±0.8 ^a	14±0.8 ^a
GSH (nmol/mg)	10±0.4	5±0.2 ^a	3±0.1 ^{ab}
H ₂ O ₂ (μ mol/min/mg)	6±0.3	9±0.4 ^a	14±0.9 ^{ab}
LPO (nmol/min/mg)	16±1.1	29±1.2 ^{ab}	30±1.9 ^a
OH* (μmol/min/mg)	20±1.2	39±2 ^a	45±3 ^a

Software used for molecular docking

ACD/Chem Sketch 12.01 (Advanced Chemistry Development, Inc.) was used to prepare the ligands, Hyper chem 8.0.3 was utilised to optimise the geometrical representations, and Wizard of Autodock tools 1.5.4 was used to prepare the proteins for this work.

Molecular Dynamics simulations

Using molecular dynamics, protein-ligand interactions were simulated using GROMACS program.⁴⁹ To separate the docked protein-ligand complex into its component proteins and ligands for MD simulations, PyMOL was utilized.⁵⁰ Following the construction of separation protein topology in GROMACS using the Amber99sb-ildn force field, ligand topology was constructed using the ACPYPE package.^{51,52} Subsequently, the resulting topologies were combined to create the Protein-ligand complex topology, which allowed us to proceed to the next stage of MD. Boundary conditions were set to a dodecahedron sized 1.0 for the prepared complex, and the water model TIP3P (transferable intermolecular potential with three points) was used to solve it. NaCl ions neutralized the system, and MDP files from the Lekumal protein-ligand MD simulation tutorial were used to minimize energy for 1000 steps,^{53,54} it was equilibrated for 300 and 500 picoseconds (ps) at 300K temperature in the NVT step and NPT with 1 bar pressure steps in the NPT step. After that, a 10-nanosecond MD simulation was run on the equilibrated system (ns). Ultimately, the trajectory file (XTC) contains the MD simulation results that have been saved.

RESULTS

Effects of antioxidant enzymes and lipid oxidation markers in glyphosate induced diabetic nephropathy rats

GSH and other antioxidant enzyme levels decrease in dose-responsive way when exposed to glyphosate, which exacerbates the effect of oxidative stress. Decreased SOD and CAT activity inhibits the breakdown of hydrogen peroxide, which increases oxidative cell damage. Reduced cellular protection

against reactive oxygen species is indicated by lowering Glutathione (GSH) and GPx. Higher MDA generation indicates that glyphosate also raises hydrogen peroxide (H₂O₂) levels and lipid peroxidation, which may indicate membrane damage and increased oxidative stress, including hydroxyl radicals. At 50 mg, SOD levels dropped to (32±1.3), and further decreased to (20±1) at 100 mg. Similarly, CAT levels fell to (16±0.9) at 50 mg and to (13±0.7) at 100 mg. Gpx levels reduced to (18±0.8) and (14±0.8) at 50 mg and 100 mg, respectively, while GSH levels also decreased, reaching (5±0.2) at 50 mg and (3±0.1) at 100 mg. Conversely, H₂O₂ levels increased to (9±0.4) and (14±0.9), and LPO levels rose to (29±1.2) and (30±1.9) at 50 mg and 100 mg doses, respectively. Hydroxyl radicals (OH) increased significantly, reaching (39±2) at 50 mg and (45±3) at 100 mg, showed in Table 1. These changes indicate glyphosate's role in exacerbating oxidative stress in a dose-dependent manner.

Effects of liver and kidney enzymes, total lipids and membrane bound ATPase in glyphosate induced diabetic nephropathy rats

Glyphosate exposure significantly elevated the markers of renal function (creatinine and urea) and liver enzymes (ALT, AST and ALP), indicating kidney and liver toxicity. Increased LDH and γ-GT activities suggest liver damage, metabolic disruptions, and cellular stress. Lipid levels were elevated by both 50 and 100 mg dosages and phospholipid content, pointing to lipid oxidation and potential oxidative damage, particularly in diabetic nephropathy. Additionally, glyphosate lowers Na/K⁺ ATPase, Ca ATPase, and Mg-ATPase activities, indicating compromised ion transport, calcium homeostasis, and magnesium-dependent functions, suggesting possible cellular membrane malfunction. Table 2 showed the glyphosate exposure at 50 mg and 100 mg resulted significant elevations in various biochemical parameters. ALT levels rose to 100±6 IU/L at 50 mg and 110±5 IU/L at 100 mg, while AST levels increased to (65±4 IU/L) and (90±6 IU/L), respectively. ALP levels also went up to (100±8 IU/L) at 50 mg and (110±7 IU/L) at 100 mg. Urea and creatinine levels were elevated, with urea reaching (55±3 mg/dL) at 50 mg and (65±3 mg/dL) at 100 mg, and creatinine levels increasing to (1.4±0.07 mg/dL) and

(2.2 ± 0.1 mg/dL). LDH levels rose to (65 ± 4 μ mol) at 50 mg and (90 ± 6 μ mol) at 100 mg, while γ -GT increased to (17 ± 1 μ mol) and (19 ± 1 μ mol), respectively. Total lipids increased to (39 ± 1.8 mg/dL) at 50 mg and (45 ± 2.9 mg/dL) at 100 mg, with phospholipids also elevated to (25 ± 1.8 mg/g) wet tissue and (35 ± 2.1 mg/g) at the respective doses. Additionally, Na/K⁺ ATPase activity decreased significantly to (3 ± 0.1 mmol) and (1.9 ± 0.08 mmol) at 50 mg and 100 mg, while Ca ATPase and Mg-ATPase activities both dropped to (0.4 ± 0.02 μ mol) at the higher doses, indicating impaired cellular function due to glyphosate exposure.

Effects of inflammatory cytokines, kidney injury and oxidative stress markers in glyphosate induced diabetic nephropathy

Glyphosate exposure reduced the levels of anti-inflammatory protein IL-10, while raised IL-6 and TNF- α were found to be elevated in response to glyphosate indicating that it promotes the inflammation. Increased TGF- β 1 suggests potential tissue fibrosis. An increase in kidney injury molecule-1 levels suggests that kidney damage may have occurred. GLY also lowers Nrf2 levels, indicating compromised antioxidant defense, making tissues more vulnerable to oxidative stress. The decline in Keap-1 levels suggests further disruption in oxidative balance, potentially exacerbating renal impairment. Table 3 represents glyphosate exposure at 50 mg and 100 mg significantly affected several inflammatory and oxidative stress markers. Interleukin-10 (IL-10) levels decreased to (120 ± 8 pg/mL) at 50 mg and further to (110 ± 7 pg/mL) at 100 mg. Conversely, interleukin-6 (IL-6) levels increased to (29 ± 1.5 pg/mL) at 50 mg and (35 ± 2 pg/mL) at 100 mg, indicating heightened inflammation. Tumor necrosis factor- α showed an increase to (140 ± 12 pg/mL) at 50 mg but returned to (116 ± 9 pg/mL) at 100 mg. TGF- β 1 levels rose to (116 ± 12 pg/mL) at 50 mg and to (126 ± 10 pg/mL) at 100 mg,

suggesting a response to tissue injury. Kidney Injury Molecule-1 (KIM-1) levels increased to (60 ± 3 pg/mL) at 50 mg and (80 ± 3 pg/mL) at 100 mg, indicating renal stress. Keap 1 levels decreased significantly to (6 ± 0.4 ng/mL) at 50 mg and (4 ± 0.1 ng/mL) at 100 mg, while Nrf2 levels also dropped to (4 ± 0.2 ng/mL) at 50 mg and (3 ± 0.2 ng/mL) at 100 mg, reflecting impaired antioxidant response.

Glyphosate exposure attenuates the expression of insulin signalling molecules in kidney

The groups treated with glyphosate showed lower expression of Insulin Receptor (IR), GLUT-2 and AKT in the kidney, indicating a possible insulin resistance that could impede cells' ability to absorb glucose. Exposure to glyphosate decreases the renal cell expression of insulin-related proteins. As shown in Figure 1, IR mRNA expression was 0.7 ± 0.05 fold in group 2 (50 mg glyphosate-treated) ($p < 0.05$) and 0.6 ± 0.01 fold in group 3 (100 mg glyphosate-treated) ($p < 0.01$); GLUT-2 mRNA expression was 0.9 ± 0.05 fold in group 2 ($p < 0.05$) and 0.5 ± 0.02 fold in group 3 ($p < 0.002$); and AKT mRNA expression was 0.9 ± 0.05 fold in group 2 ($p < 0.05$) and 0.7 ± 0.02 fold in group 3 ($p < 0.002$).

Inflammatory markers in glyphosate-induced diabetic nephropathy rats

The decrease in IL-10 indicates diminished anti-inflammatory actions, which could worsen inflammatory damage in those treated with glyphosate. Elevated TNF- α , and IK κ B expression indicates a strong inflammatory reaction that can cause kidney damage and other glyphosate-related problems. Glyphosate administration causes a drop in IL-10 levels, which suggests that anti-inflammatory signaling is diminished in the groups. The glyphosate-treated groups have elevated expression of TNF- α and IK κ B, indicating enhanced fibrotic and inflammatory responses.

Table 2: Effects of liver and kidney enzymes, total lipids and membrane bound ATPase. Wistar rats with diabetic nephropathy and control rats are shown in this table to show glyphosate exposure affects both groups. Each bar shows the mean $\pm$ standard error of wistar rats. The three groups have the following labels: a- Control, b- Compound with 50 mg of glyphosate treatment.

Parameters	Control	GLY (50 mg)	GLY (100 mg)
ALT (IU/L)	65 ± 2	100 ± 6^a	110 ± 5^a
AST (IU/L)	35 ± 2	65 ± 4^a	90 ± 6^{ab}
ALP (IU/L)	70 ± 5	100 ± 8^a	110 ± 7^{ab}
Urea (mg/dL)	35 ± 2	55 ± 3^a	65 ± 3^{ab}
Creatinine (mg/dL)	0.7 ± 0.04	1.4 ± 0.07^a	2.2 ± 0.1^{ab}
LDH (μ mol/mg)	50 ± 3	65 ± 4^a	90 ± 6^{ab}
γ -GT (μ mol/min/mg)	10 ± 0.5	17 ± 1^a	19 ± 1^a
Total Lipids (mg/dL)	29 ± 1.8	39 ± 1.8^a	45 ± 2.9^a
Phospholipids- Total Lipids (mg/g)	15 ± 0.8	25 ± 1.8^a	35 ± 2.1^{ab}
Na/K ⁺ ATPase (mmol/h/min/mg)	4.8 ± 0.2	3 ± 0.1^a	1.9 ± 0.08^{ab}
Ca ATPase (μ mol/h/mg)	1.6 ± 0.06	0.4 ± 0.02^a	0.4 ± 0.02^a
Mg-ATPase (μ mol/h/mg)	1.2 ± 0.09	0.5 ± 0.04^a	0.4 ± 0.01^a

Table 3: Effects of inflammatory cytokines, kidney injury and oxidative stress markers. In diabetic nephropathy rats, glyphosate's effect on inflammatory cytokines, KIM-1, Nrf2, and Keap-1 levels was evaluated using immunoassay. The results showed significant differences ($p < 0.05$) between the diabetic nephropathy group treated with control and 50-100 mg glyphosate exposure.

Parameters	Control	GLY (50 mg)	GLY (100 mg)
IL-10 (pg/mL)	200±14	120±8 ^a	110±7 ^a
IL-6 (pg/mL)	19±1.1	29±1.5 ^a	35±2 ^{ab}
TNF- α (pg/mL)	115±8	140±12 ^a	116±9 ^a
TGF- β 1 (pg/mL)	80±6	116±12 ^a	126±10 ^{ab}
KIM-1 (pg/mL)	40±2	60±3 ^a	80±3 ^{ab}
Keap 1 (ng/mL)	8±0.4	6±0.4 ^a	4±0.1 ^{ab}
Nrf2 (ng/mL)	6±0.3	4±0.2 ^a	3±0.2 ^a

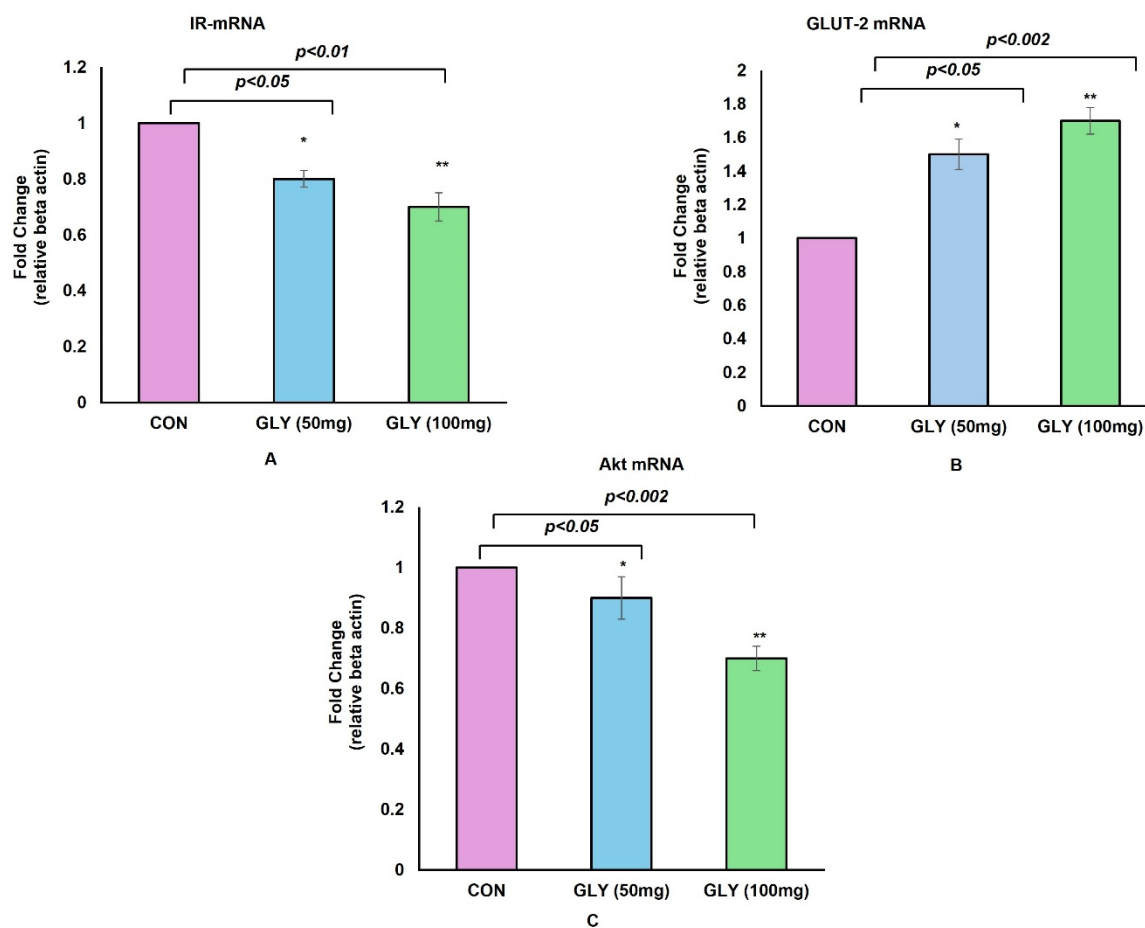


Figure 1: A-C: Impact of glyphosate exposure the mRNA expression of insulin signalling molecules in kidney of glyphosate-exposed rats. Insulin Receptor (IR), GLUT-2 and Akt mRNA expression were measured by Real-Time RT-PCR analysis using gene specific primers. Significance was considered to be $p < 0.05$.

As shown in Figure 2, Glyphosate exposure significantly affected gene expression related to inflammatory markers. In group 2, treated with 50 mg of glyphosate, IL-10 mRNA expression was measured at 0.7 ± 0.05 fold ($p < 0.05$), while in group 3, treated with 100 mg, it was reduced to 0.5 ± 0.02 fold ($p < 0.02$). For TNF- α mRNA expression, group 2 showed a value of 1.3 ± 0.01 fold ($p < 0.05$), and group 3 exhibited a higher level at 1.6 ± 0.0001 fold ($p < 0.0001$). Additionally, IK κ B mRNA expression in group 2 was

recorded at 1.2 ± 0.01 fold ($p < 0.01$), increasing to 1.5 ± 0.002 fold in group 3 ($p < 0.002$) (Figure 2).

Effects on TNF- β 1 and KIM-1 in glyphosate induced diabetic nephropathy rats

A substantial inflammatory response, shown by elevated KIM expression, may result in kidney injury and other problems associated with glyphosate exposure. The increase in TGF- β 1 expression in groups treated with glyphosate suggests that it may

have a part in kidney injury and the onset of fibrosis. Figure 3 illustrates the detrimental effects of glyphosate exposure on TNF- β and KIM-1 expression. The study indicated that the group treated with 50 mg of glyphosate had considerably lower TNF- β mRNA expression, reducing it by 0.7 ± 0.01 fold ($p < 0.01$) and further decreased to (0.6 ± 0.001) fold in the group treated with 100 mg of glyphosate ($p < 0.001$). Conversely, KIM-1 mRNA expression increased to (1.5 ± 0.001) fold in the 50 mg GLY-treated group ($p < 0.001$) and further increased to (1.7 ± 0.0001) fold in the 100 mg GLY-treated group ($p < 0.001$).

INSILICO ANALYSIS RESULTS

Docking studies of glyphosate with targeted proteins

The effects of glyphosate substances on AKT, IL-1 β , GLUT-1, IL-10, GLUT-2, IKK β , IL-17, IR, KIM-1, Nrf2, TGF- β , IL-6, and TNF- α proteins using molecular docking in Figure 4A. According to these results, the compound that was chosen was more effective against the following protein targets: GLUT-1 with residues ASN A:415, ASN A:411, ASN A:28; IL-6 with residues THR A:134, THR A:130, ARG A:128, GLY A:127, GLU A:10; IKK β with residues ASN A:161, LEU A:159, TRP A:167, ARG

A:156, LEU A:122; and Nrf2 with residues VAL X:463, VAL X:465, VAL X:418, VAL X:512, ILE X:416 showed in Table 4.

MOLECULAR DYNAMICS

The first step in analyzing the MD simulation data is to take out the Periodic Boundary Conditions (PBC) from the trajectory file (XTC). Generally, RMSD and RMSF have been used to examine the structural alterations of a protein-ligand complex in MD simulation. Whereas RMSF illustrates how the residues fluctuate over the course of the simulation, RMSD demonstrated how the complex's structure has deviated from its starting point. In addition to that hydrogen bond analysis and energy calculations has been used to examine the strength of interaction between protein and compounds during md simulation. The results analysis methodology was referred from research article.⁵⁵ The RMSD for IKK β complexes (A&B) and Nrf2 Complexes (C&D) has been separately shown in Figure 4B. The high deviation was due to a loop region at the end of the proteins. Then a RMSF graph has been plotted separately and it shows that both proteins show high fluctuations at initial residues. From the RMSD and RMSF calculations analysis, we were able to confirm that all the complexes were stable.

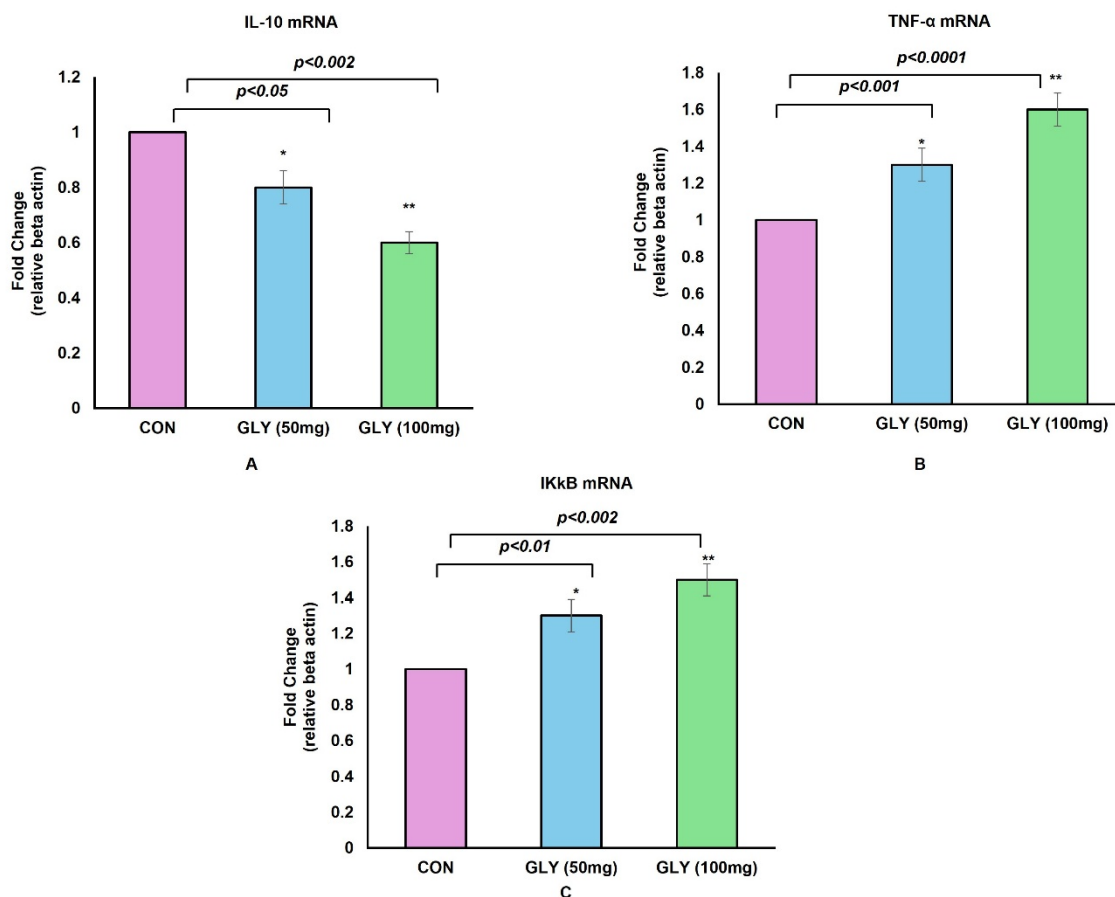


Figure 2: A-C: Inflammatory markers in glycosate induced diabetic nephropathy rats. Impact of glycosate exposure on the mRNA expression of inflammatory signalling molecules in kidney of glycosate-exposed rats. IL-10, TNF- α , and IKK β mRNA expression were measured by Real-Time RT-PCR analysis using gene specific primers. Significance was considered to be $p < 0.05$.

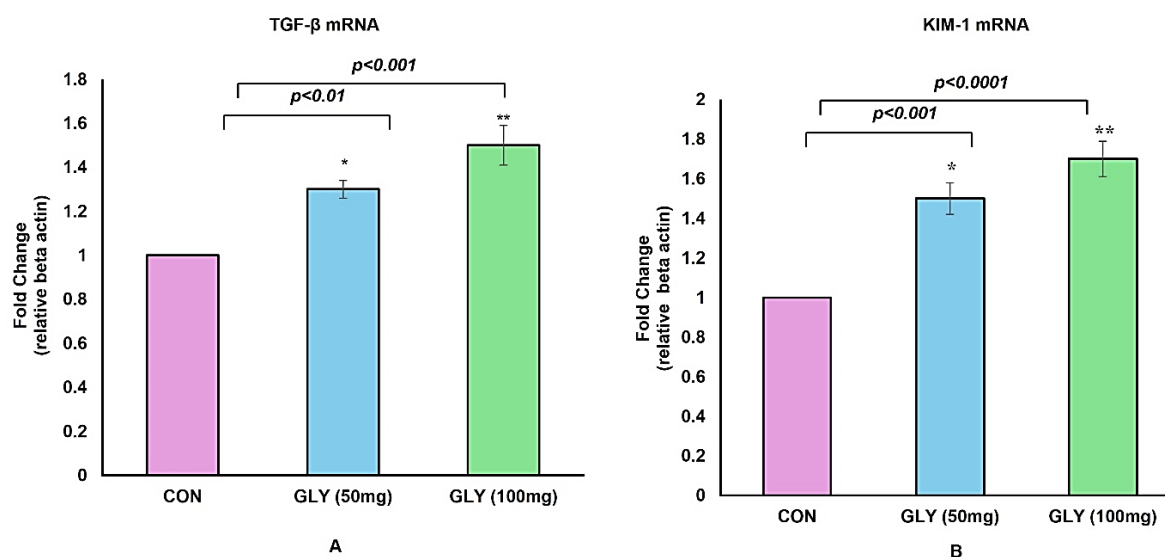


Figure 3: A and B: mRNA expression of TNF-β1 and KIM-1 in glycosylated wistar rats. mRNA expression were measured by Real-Time RT-PCR analysis using gene specific primers. Significance was considered to be $p < 0.05$.

Table 4: Docking studies of glycosylated with targeted proteins. Molecular docking results of glycosylated compound with target proteins IL-1β, AKT, IL-10, GLUT-1, IL-17, GLUT-2, IKKβ, IR, KIM-1, Nrf2, TGF-β, IL-6, and TNF-α.

Proteins	Binding energy (kcal/mol)	No. of hydrogen bonds	Residues
Glyphosate compound			
AKT	-4.8	4	ARG A:367, LEU A:347, PRO A:348, ARG A:346
GLUT-1	-5	3	ASN A:415, ASN A:411, ASN A:288
GLUT-2	-4.8	3	TYR A:325, TRP A:97, ALA A:451
IKKβ	-5.5	5	ASN A:161, LEU A:159, TRP A:167, ARG A:156, LEU A:122
IL- 1β	-4.8	4	LYS A:162, ILE A:81, HIS A:78, ILE A:82
IL-6	-5	5	THR A:134, THR A:130, ARG A:128, GLY A:127, GLU A:105
IL-10	-4	3	PHE L:111, CYS L:62, GLY L:61
IL-17	-4.3	4	SER A:49, SER A:47, THR A:48, CYS A:121
IR	-4.9	3	HIS A:1058, SER A:1270, GLN A: 1111
KIM-1	-4.2	2	HIS A:109, PHE A:113
Nrf2	-5.5	5	VAL X:463, VAL X:465, VAL X:418, VAL X:512, ILE X:416
TGF-β	-4.2	3	SER A:276, MET A: 283, LEU A:286
TNF-α	-4.7	4	LEU A:142, PRO A:139, GLY A:24, PHE A:144

DISCUSSION

GLY, known as N-(phosphonomethyl) glycine, is the active ingredient in the world's most widely used herbicides, Glyphosate-Based Herbicides (GBHs).⁵⁶ Resistance management is critical to preserving the efficacy of glyphosate, as it encounters increasing competition from resistant weeds.⁵⁷ Globally, glyphosate usage ranges from an estimated 600,000 to 750,000 tonnes annually, projected to increase to between 740,000 and 920,000 tonnes by 2025.⁵⁸ Its use has increased significantly over the last 25 years, and this increase has been related to a

number of different medical problems, such as oxidative stress and decreased glucose absorption, both of which are linked to diabetes mellitus.⁵⁹⁻⁶² Insulin is essential for controlling protein, lipid, and carbohydrate metabolism as well as for preserving the stability of glucose. In hyperglycemic circumstances, cellular oxidative stress is defined by the production of Reactive Oxygen Substances (ROS).⁶³ Previous studies have indicated that GLY exposure alters crucial signaling pathways and reduces antioxidant levels in a dose-dependent manner, disrupting insulin signaling in the gastrocnemius muscle, causes T2DM.³⁶

Our research demonstrates that glyphosate induces insulin resistance by modulating the Nrf2/Keap-1 signaling pathway in the adipocytes of experimental rats. Adipocytes are crucial for insulin signaling as they mobilize intracellular transport proteins in response to insulin, significantly enhancing glucose uptake.⁶⁴ In our study, we investigated the impact of glyphosate on various biomarkers, including inflammatory cytokines, kidney injury markers, oxidative stress indicators, antioxidant enzymes, lipid oxidation markers, liver and kidney function parameters, total lipids, membrane-bound ATPase activities, and mRNA levels of insulin signaling molecules and inflammatory markers, including TNF- α and KIM-1. Our findings reveal that glyphosate exposure adversely affects the Nrf2/Keap-1 and insulin signaling pathways, contributing to insulin resistance. Furthermore, significant changes were observed in oxidative stress markers, lipid profiles, liver and kidney functions, and inflammatory markers. Molecular docking studies corroborated these results, demonstrating glyphosate's detrimental effects on the Nrf2/Keap-1 pathway.

GLY exposure generates ROS, leading to oxidative stress and tissue damage through processes such as lipid peroxidation and DNA damage.⁶⁵⁻⁶⁷ Our study indicates reduced activities of key antioxidant enzymes, including CAT and SOD in glyphosate-treated rats, reflecting a compromised defense against oxidative damage potentially due to iron deficiency and increased ROS levels. It may interfere with insulin production and blood glucose regulation, resulting in further oxidative stress and

metabolic disturbances.^{68,69} Notably, significant alterations in oxidative stress markers were observed in response to glyphosate exposure, evidenced by increased amount of LPO, OH $\cdot$, and H $_2$ O $_2$. For instance, in the GLY 100 mg group, H $_2$ O $_2$ levels rose from 6 μ mol/min/mg protein in the control group to 14 μ mol/min/mg protein, while OH $\cdot$ levels increased from 20 μ mol/min/mg protein to 45 μ mol/min/mg protein. These findings support the hypothesis that glyphosate disrupts insulin production and blood glucose regulation, exacerbating oxidative stress and metabolic abnormalities. According to earlier research, exposure to glyphosate dramatically lowers skeletal muscle's levels of enzyme antioxidants such as GST, GPx, and GSH, which causes oxidative stress and metabolic dysregulation linked to hyper-glycemia.²⁷ Environmental contaminations with glyphosate can markedly elevate oxidative stress and disturb metabolic balance in biological systems.⁷⁰ With GLY exposure, GSH levels decreased from 10 nmol/mg protein in the control group to 3 nmol/mg protein in the highest treatment group, according to our consistent results. The activities of antioxidant enzymes such as GPx, SOD, and CAT also considerably decreased; in the group that received 100 mg of glyphosate, GPx activity dropped from 40 μ g of glutathione utilized/mg protein in the controls to 14 μ g. This suggests that elevated ROS levels induce oxidative stress, compromising the antioxidant defenses essential for cellular function in adipocytes.

Our study revealed a significant decrease in membrane-bound ATPase activities, including Mg-ATPase, Ca $^{2+}$ ATPase, and

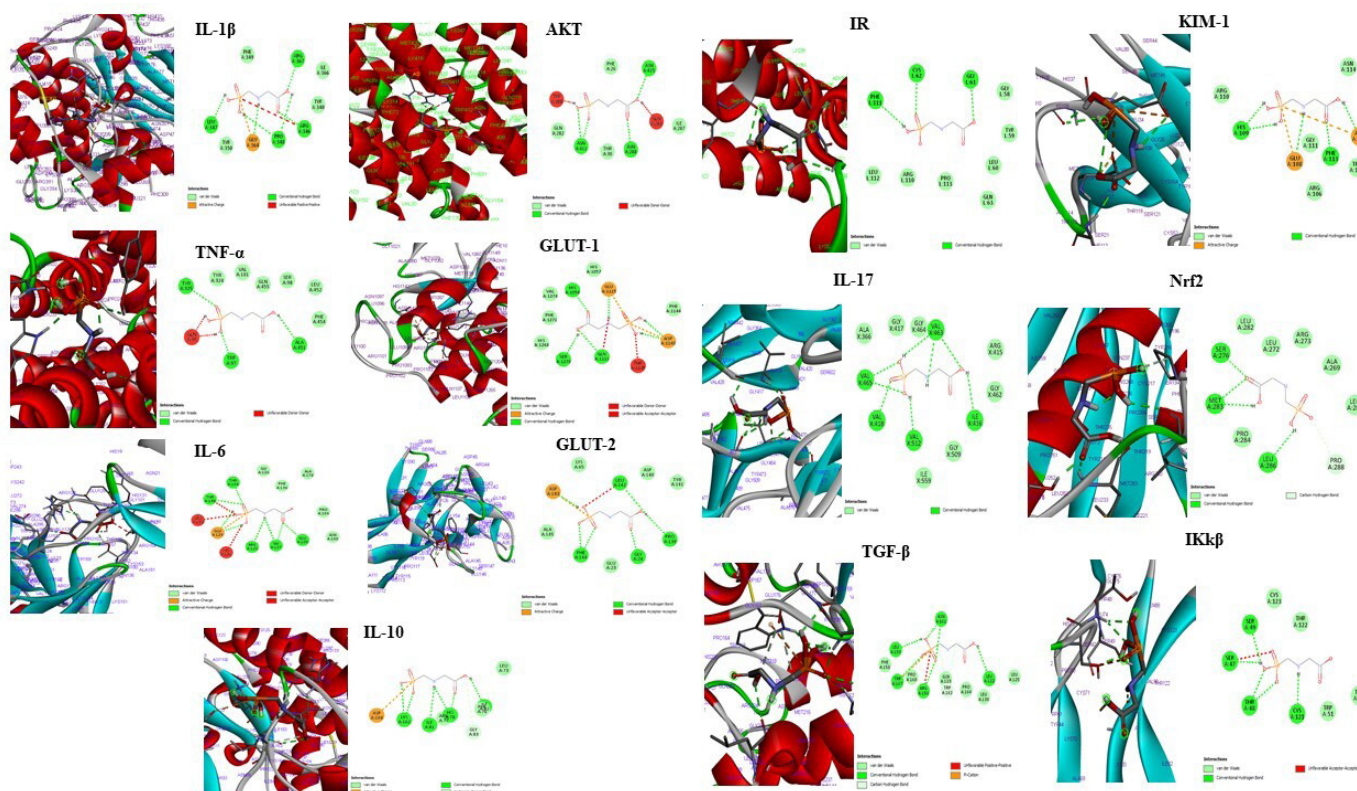


Figure 4A: A: Molecular docking analysis. Molecular docking analysis showing the binding interactions of glyphosate with target signaling molecules (IL-1 β , AKT, TNF- α , GLUT-1, IL-6, GLUT-2, IL-10, IR, KIM-1, IL-17, Nrf2, TGF- β , and IKK β).

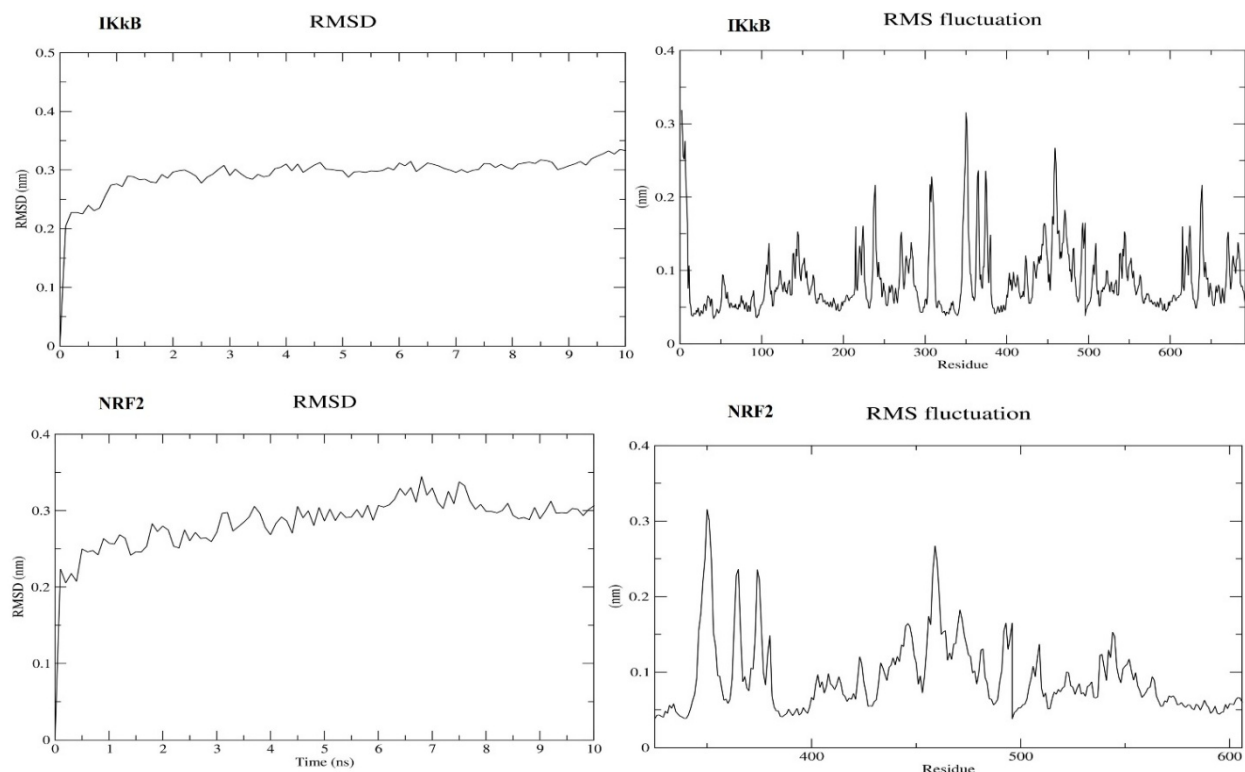


Figure 4B: The RMSD profile of the protein-ligand and protein complexes over 100 ns of MD simulation, along with a graph showing the RMSF values of the IKkB and Nrf2 proteins in complex with Glyphosate the 100 ns trajectories.

Na⁺/K⁺ ATPase, in glyphosate-treated groups. Specifically, Na⁺/K⁺ ATPase activity decreased from 4.8 mmol of phosphorus liberated/h/min/mg protein in the control group to 1.9 mmol in the glyphosate 100 mg group. This reduction indicates a disruption of ion transport systems critical for maintaining cellular homeostasis. Additionally, we observed notable decreases in Ca²⁺ ATPase and Mg²⁺ ATPase activities, reflecting disturbances in calcium and magnesium homeostasis. These disruptions may exacerbate cellular dysfunction and impair energy-dependent processes, particularly insulin signaling, thereby contributing to insulin resistance. Furthermore, decreased HDL cholesterol (HDL-c) may result from the exchange of triglycerides for cholesterol esters, facilitated by cholesteryl ester transfer proteins. Elevated levels of LDL cholesterol (LDL-c), Total Cholesterol (TC), and Triglycerides (TG) in glyphosate-treated rats suggest impaired LDL receptor function.^{71,72} Reduced glucose absorption in insulin-sensitive organs correlates with these lipid abnormalities, indicating compromised insulin signaling and insulin resistance.^{73,74} Our study identified a significant increase in phospholipid levels, from 15 mg/g of wet tissue to 35 mg/g, and total lipids from 29 mg/dL in the control group to 45 mg/dL in the glyphosate 100 mg group. Moreover, the levels of LDH increased from 50 mg/dL to 90 mg/dL, while those of γ -GT increased from 10 mg/dL to 19 mg/dL. These results imply that exposure to glyphosate may cause lipid buildup, which in turn may result in lipid peroxidation and further impairing lipid metabolism, which could cause metabolic abnormalities and insulin resistance.

Benedetti *et al.* 2004 found that Wistar rats treated with glyphosate-Biocarb® for 75 days exhibited elevated ALT and AST levels, indicating liver damage.⁷⁵ Similarly, after Roundup exposure, Nesrine Djaber *et al.* 2020 found higher indicators of renal function combined with significant increases in ALT, AST, ALP, LDH, and GGT.⁷⁶ Our study corroborates these findings, demonstrating hepatotoxicity and liver injury through significantly higher levels of serum in glyphosate treatment groups compared to controls. Specifically, the 100 mg glyphosate group exhibited an increase in ALT from 65 IU/L in the control group to 110 IU/L, indicating a dose-dependent response associated with escalating liver damage. Additionally, we observed notable increases in urea and creatinine levels, rising from 35 mg/dL and 0.7 mg/dL in the control group to 65 mg/dL and 2.2 mg/dL, respectively, in the glyphosate 100 mg group, suggesting possible impairment of kidney function. Based on a variety of biochemical markers of damage and malfunction, these studies collectively illustrate the significant consequences of glyphosate exposure on liver and kidney health. Hyper glycemia causes oxidative stress by generating Advanced Glycation End products (AGEs) and Reactive Oxygen Intermediates (ROIs). These compounds activate NF- κ B through their receptor, RAGE, exacerbating diabetic complications and suggesting that antioxidants such as α -lipoic acid could be beneficial in treatment.⁷⁷ By raising the mRNA levels of pro-inflammatory, glyphosate exposure interferes with insulin signaling and may lead to inflammation and insulin resistance.^{27,63} Pandey *et al.* also found that glyphosate-containing

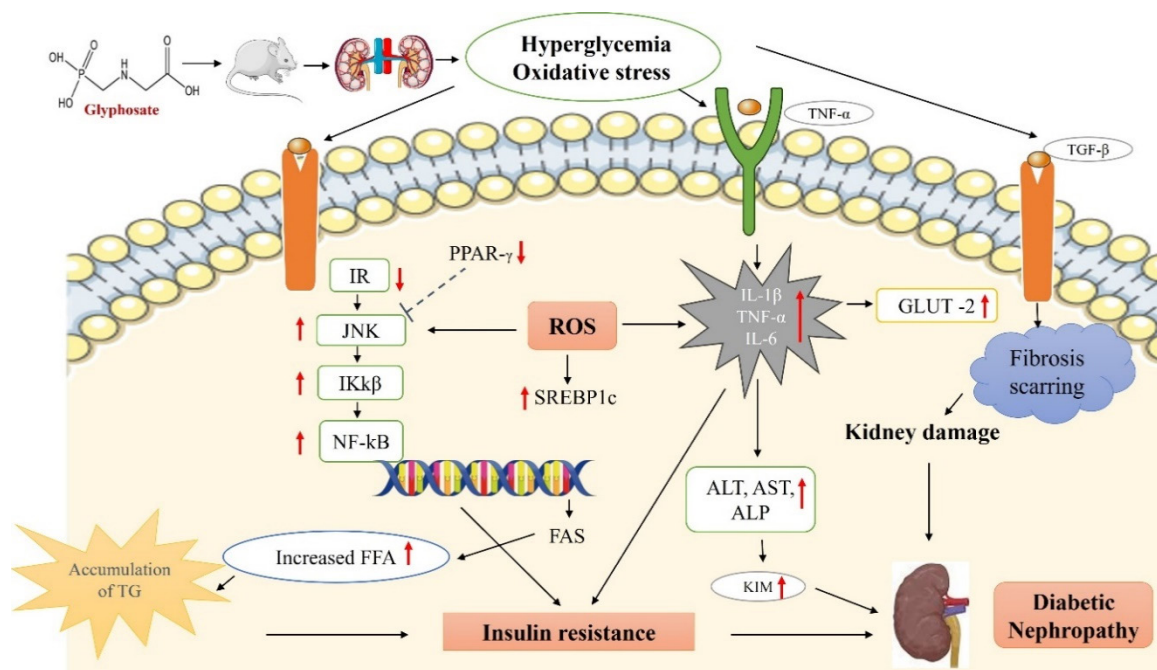


Figure 5: This study evaluated the effects of chronic oral glyphosate exposure on oxidative stress, inflammation, and insulin signaling in male Wistar rats. Results showed that glyphosate disrupted metabolic and renal function, elevated oxidative stress and inflammatory markers, and impaired antioxidant defenses. Gene expression analysis indicated suppression of insulin signaling (IR, AKT, GLUT-2) and Nrf2/Keap-1 antioxidant pathway, with increased pro-inflammatory genes (IL-6, TNF- α , IKK β , Keap-1, KIM-1). Molecular docking and dynamics confirmed stable binding of glyphosate to key signaling proteins. Overall, glyphosate exposure was linked to oxidative damage, inflammation, and diabetic nephropathy progression through disruption of Nrf2/Keap-1 signaling.

herbicides induce chronic inflammation in the liver and adipose tissues.⁷⁸ While reactive oxidants can contribute to oxidative stress, their controlled synthesis is essential for modulating signaling pathways. Nrf2, a major regulator of the body's antioxidant defenses, is crucial for inducing genes that reduce oxidative damage and influence physiological outcomes.⁷⁹ Normally low in healthy kidneys, KIM-1 is markedly increased in response to renal injury, making it a valuable marker for identifying and tracking kidney damage, especially in diabetic nephropathy. Elevated urinary KIM-1 levels correlate with kidney lesions and may serve as early indicators of renal damage in diabetes.^{80,81} By mitigating oxidative stress, Nrf2 holds promise as a treatment for DN. However, the potential cardiovascular implications of Nrf2 activators such as bardoxolone methyl necessitate careful therapeutic adjustments.⁸²

Our study revealed high amount of TGF- β 1, TNF- α , IKK β , and IL-6, indicating a pro-inflammatory state that promotes tissue damage and fibrosis. Reduced IL-10 levels reflect weakened anti-inflammatory responses. Increased KIM-1 levels confirm kidney damage associated with glyphosate exposure, while decreased Nrf2 expression and higher Keap-1 levels suggest compromised antioxidant defenses, increasing oxidative stress risk. The diminished expression of GLUT-2, AKT, and IR further underscores glyphosate's adverse impact on insulin signaling, contributing to insulin resistance and potentially leading to

diabetic nephropathy. Understanding the binding interactions and affinities between GLY and proteins involved in the Nrf2/Keap-1 pathway is crucial for developing therapeutic strategies and elucidating underlying mechanisms.^{83,84} Our *in vivo* work prompted us to extend our investigation into the molecular interactions between glyphosate and relevant proteins. Molecular docking studies revealed that glyphosate directly binds to proteins including IL-1 β , AKT, IL-10, GLUT-1, GLUT-2, IKK β , IR, KIM-1, IL-6, Nrf2, TGF- β , IL-17, and TNF- α . It exhibits strong hydrogen-bonding affinity with GLUT-1, IL-6, IKK β , and Nrf2. Docking studies revealed that glyphosate exhibits strong binding to Nrf2 (-5.5 kcal/mol) and IKK β (-5.5 kcal/mol) through multiple hydrogen bonds, implicating potential interference with the Nrf2/Keap-1 signaling pathway. The interactions involve key amino acid residues critical to the structure and function of these proteins, emphasizing glyphosate's complex pathogenic impact and its possible contribution to diabetic nephropathy. IKK β and Nrf2 complexes with glyphosate were further shown to be stable in structure throughout a 100 ns trajectory by molecular dynamics simulations. RMSD analysis highlighted deviations linked to loop regions at the protein termini, while RMSF analysis showed notable fluctuations in initial residues. Consistent hydrogen bonding and energy profiles throughout the simulation reinforce the stability of these complexes, suggesting that glyphosate could significantly affect the functional roles of IKK β and Nrf2 within their respective signaling pathways (Figure 5).

CONCLUSION

This study showed that GLY disrupted the Nrf2/Keap-1 signaling pathway, leading to diabetic nephropathy in Wistar rats. The damage is evident through increased urea, creatinine, and liver enzymes, along with disrupted ATPase activity, which harms kidney function. The elevated oxidative stress markers and reduced antioxidants suggested a redox imbalance, worsening inflammation and cellular damage. Changes in cytokine levels point to chronic inflammation. Simulations using docking and dynamics verified that glyphosate strongly binds to key proteins in the Nrf2/Keap-1 pathway, confirming its role in inflammation and oxidative stress, contributing to diabetic nephropathy. Future studies should validate these findings in human models and explore therapies targeting this pathway. However, the study's limitations include its use of animal models, which may not fully reflect human health, and the need for further research into the detailed molecular effects of glyphosate.

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ABBREVIATIONS

MetS: Metabolic Syndrome; **T2DM:** Type 2 diabetes mellitus; **DN:** Diabetic nephropathy; **GLY:** Glyphosate; **Nrf2:** Nuclear factor erythroid 2-related factor 2; **Keap1:** Kelch-like ECH-associated protein 1; **KIM-1:** Kidney Injury Molecule-1; **IGF-1:** Insulin-like Growth Factor 1; **TNF- α :** Tumor Necrosis Factor-alpha; **IL-6:** Interleukin-6; **IL-1 β :** Interleukin-1 beta; **GSH:** Reduced Glutathione; **GPX4:** Glutathione Peroxidase 4; **CKDu:** Chronic Kidney Disease of Unknown etiology; **GLP-1:** Glucagon-Like Peptide-1; **AKT:** Protein Kinase B; **IR:** Insulin Receptor; **GLUT-1:** Glucose Transporter Type 1; **GLUT-2:** Glucose Transporter Type 2; **IL-10:** Interleukin-10; **IL-17:** Interleukin-17; **IKK β :** I-kappa-B kinase beta; **CAT:** Catalase; **SOD:** Superoxide Dismutase.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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