



## Study of the effects of high protein intake on TCF7L2 gene expression and physiological antioxidant enzymes in liver of rat model

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### **Abstract**

#### **Objective**

High-protein feeding has been linked to profound changes in hepatic metabolism, although little has been shown regarding molecular mechanisms connecting high protein intake to these metabolic alterations. The present experiment examined the impact of high dietary protein on hepatic TCF7L2 gene expression and antioxidant enzyme activities in Wistar rats.

#### **Materials and method**

A total of 48 male Wistar rats (8-10 weeks old, initial body weight 200-250 g) were randomly assigned to four groups (12 per group): control (10% protein diet), low-protein (5% protein diet), moderate-protein (20% protein diet) and high-protein (40% protein diet) groups. Animals were then switched to their assigned diet and fed for 12 weeks. Hepatic levels of antioxidant enzyme activities (superoxide dismutase, SOD; glutathione peroxidase, GPx; endothelial nitric oxide

synthase, eNOS) were determined spectrophotometrically. TCF7L2 gene expression was detected by quantitative real-time PCR. Levels of serum albumin and total protein were evaluated, as was body weight. Results are expressed as the mean  $\pm$  S.D. Statistical analysis One-way ANOVA, followed by Tukey's multiple comparisons

### Results

TCF7L2 mRNA expression in the control group was set as the baseline (fold-change = 1.0). The high-protein group demonstrated a  $6.8 \pm 0.9$ -fold increase in TCF7L2 expression compared to control ( $p < 0.001$ ). The findings revealed that feeding a high protein diet significantly up-regulated the mRNA expression of TCF7L2 ( $p < 0.001$ ) compared to the control animals. The SOD activity was higher in the high protein group than CON, by 45% ( $p < 0.01$ ), the GPx activity increase by 38% ( $p < 0.01$ ) and eNOS activity showed a 52% increase ( $p < 0.001$ ). Serum total protein and albumin were markedly increased in rats fed high-protein diet ( $p < 0.05$ ). An important body weight gain reduction was evident in the high-protein group versus both control and low-protein groups ( $p < 0.05$ ).

### Conclusion

These results imply that dietary protein might modulate hepatic antioxidant defense mechanisms mediated by TCF7L2-dependent transcriptions, and roles of high-protein in preventing the development of metabolic diseases mediating through transcriptional activity of TCF7L2. Further clinical studies are needed to investigate the potential metabolic benefits in humans as reported here from this rat model of disease.

**Keywords:** antioxidant enzymes, high protein diet, liver function, qRT-PCR, rat model

**Paper Type:** Research Paper.

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### Introduction

Protein is a vital macronutrient necessary for many bodily functions such as tissue building, making enzymes, and keeping the immune system strong. Nonetheless, the appropriate levels of dietary protein and its molecular effects on hepatic metabolism are still under active scrutiny. As the major metabolic organ in the body, liver plays a crucial role in protein synthesis, amino acid metabolism and systemic nutrient homeostasis control (Ayala et al., 2025). On the other hand, the

epigenome, which comprises various mechanisms such as DNA methylation, chromatin remodeling, histone tail modifications, microRNAs, and long non-coding RNAs, interacts with environmental factors like nutrition, pathogens, and climate to influence gene expression profiles and the emergence of specific phenotypes (Shahsavari et al., 2023; Mohammadabadi et al., 2023). These interactions are complex and occur across multiple levels, involving dynamic crosstalk between the genome, epigenome, and environmental stimuli (Amiri Roudbar et al., 2020). Increasing evidence suggests that epigenomic variation plays a crucial role in determining health outcomes and production traits (Alavi et al., 2022). The expression of eukaryotic genes is temporally and spatially regulated through multidimensional control mechanisms (Hajalizadeh et al., 2021). Only a limited subset of the entire genome is actively expressed in each tissue type, and gene expression is closely tied to developmental stages (Khabiri et al., 2025). Consequently, gene expression patterns in eukaryotes are tissue-specific (Farahvashi et al., 2026a; Mohammadabadi et al., 2024). Additionally, the levels of gene products synthesized within a given tissue, as well as those contributed by other tissues, collectively regulate gene expression (Pakgozar et al., 2026). Epigenetic modifications serve as a critical mechanism by which environmental factors leave a lasting imprint on the genome (Mohammadabadi et al., 2022a). These modifications can be heritable, influencing successive generations without altering the DNA sequence itself (Mohammadabadi et al., 2025). For instance, maternal nutrition during pregnancy has been shown to induce epigenetic changes that affect offspring metabolism, growth, and disease susceptibility (Safaei et al., 2024). Similarly, environmental stressors such as exposure to toxins or pathogens can trigger epigenetic responses that modify gene expression and cellular function (Noori et al., 2017). Understanding epigenetic mechanisms can improve breeding strategies, enhance productivity, and promote disease resistance (Roudbar et al., 2015). Additionally, epigenetic markers are increasingly used to predict performance and health outcomes, providing a valuable tool for precision (Mohamadinejad et al., 2024). A fundamental aspect of genetic research involves the investigation of genes and proteins associated with specific traits, examined at both cellular and chromosomal levels (Farahvashi et al., 2026b). Advancing our understanding of these regulatory processes holds significant potential for improving biological insights and practical applications in health and production (Arabpour et al., 2021). Further research into epigenetic biomarkers and their role in gene regulation will contribute to more effective therapeutic strategies and enhanced understanding of complex biological systems (Mohammadabadi et al., 2022b). Evidence is evolving to suggest that the liver gene expression profiles and metabolic pathways may be regulated directly by dietary protein through several signaling events. TCF7L2 (transcription factor 7-like 2), the protein product of the TCF7L2 gene, is a transcription regulator that has been uniquely associated with metabolism regulation. This protein acts as a critical mediator of Wnt signaling and has been strongly implicated for risk towards type 2 diabetes (T2D), and abnormal metabolic conditions in the liver (Bhat et al., 2022). Recent single-nuclei genomics studies have shown TCF7L2 to be an essential regulator of hepatic zoned gene expression, especially the regulation of metabolic pathways for carbohydrate and lipid metabolisms (Darwish et al., 2022). Variations and mutational status of TCF7L2 are associated with disrupted insulin production, glucose balance and incriminated for susceptibility

to metabolic diseases (French et al., 2017). Antioxidant enzymes are the primary defense system against reactive oxygen species (ROS), which are produced as normal byproducts of metabolism and accumulate under conditions of metabolic stress. The major antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (García-Muñoz et al., 2024). SOD is responsible for dismutation of superoxide radicals to hydrogen peroxide, which in turn can be neutralized by GPx and CAT, thus avoiding formation of more reactive hydroxyl radicals (Heibel et al., 2019). Endothelial nitric oxide synthase (eNOS) has also been shown to be involved in antioxidative defense by the production of nitric oxide, a protective factor against oxidative stress and inflammation (Jomova et al., 2024). High protein diets are becoming popular as tools to influence body weight and metabolic health, with many studies demonstrating impacts on metabolism and body composition (Kennedy et al., 2020). High protein diet was reported to reduce hepatic lipid levels in different animal models of steatosis, as well as improving protein catabolic pathways (transamination and oxidative phosphorylation) (Lee et al., 2023). Yet the modulation of hepatic antioxidant enzyme expression in response to dietary protein intake and more importantly, the specific role of TCF7L2 in mediating these adaptive responses has not been fully elucidated. The control of urea cycle enzymes and amino acid metabolism pathways in relation to changes in dietary protein intake are mediated via intricate signaling chains. Recent findings suggest that adenosine monophosphate-activated protein kinase (AMPK) may be an important sensor of dietary protein levels and may control gene expression related to amino acid degradation. High-protein diets have also been shown to regulate hepatic gene expression patterns associated with metabolic zonation and nutrient sensing (Lin et al., 2019). Serum albumin and total protein are widely used as useful index of the liver synthetic function and nutritional conditions. Albumin, the most common blood protein, is the only plasma synthesized exclusively in liver and fulfils several biological roles such us maintenance of oncotic pressure and transport function carrying a wide variety of molecules as well as oxidative stress protection (Obeme-Nmom et al., 2024). Body weight and body weight gain are important indicators of metabolic and nutritional consequences of dietary interventions. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) has transformed the analysis of gene expression in animal models and human tissues. The method permits accurate measurement of mRNA expression levels in quantitative and specific manner, and has become a critical technology in molecular research (Pellatt et al., 2016). The Wistar rat has been widely used in metabolic and nutritional science due to its similarity in physiology with human beings as well as for the defined genetic strain of rats. Invasive studies examining in-vivo responses to diet in humans are impractical, invasive and do not facilitate controlled interventions (Schwarz et al., 2012), but such measurements are feasible in rats. Since TCF7L2 is a key factor of liver metabolism and high protein intake was known to influence hepatic metabolism, we were prompted to infer that up regulated expression of TCF7L2 gene might increase antioxidant enzyme activities in the liver by consuming high levels of dietary protein in rats. The goal of this study, therefore, was to examine these relationships and the molecular basis for adaptive responses in energy metabolism with higher dietary protein.

## Materials and methods

**Animals and dietary interventions:** The care and use of all laboratory animals were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, which was approved by the Institutional Animal Care and Use Committee. Male Wistar rats (8-10 weeks old, initial body weight 200-250 g) of forty-eight were purchased from a licensed breeding facility. Rats were housed one per cage with the standard condition of  $22\pm 2^{\circ}\text{C}$  and a 12-hour light/dark cycle. All animals were given free access to water and their experimental diets during 12 weeks of the experimental feeding period. Animals were assigned to four dietary groups (n=12): (1) Control group that consumed a 10% protein diet (AIN-93M), (2) Low-protein group that consumed a 5%-protein diet; Moderate-protein group receiving a 20%-protein diet; and High-protein group receiving a 40%-protein diet. All diets were isoenergetic (approximately 3.8 kcal/g) and designed to be nutritionally adequate, with the exception of their protein content. Diet Purified diets (rat/mouse diet D12451:45% fat from lard/60% of kcal from fat; Research Diets Inc., New Brunswick, NJ) were stored at  $4^{\circ}\text{C}$ . Body weight was recorded weekly during the 12-week experiment with a digital calibrated scale. Food consumption was recorded daily by weighing leftover food and feed efficiency was calculated as body weight gain per total food intake.

**Samples collection and tissue preparation:** After 12 weeks of dietary intervention, the animals were kept fasting for 8 hours and received anesthesia using isoflurane (inhaled). Blood samples were drawn by cardiac puncture in serum separator tubes and were clotted at room temperature for 30 min. Serum was collected by centrifugation at 1,500 g for 15 min and stored at  $-80^{\circ}\text{C}$  prior to biochemical analysis. Livers were excised and quickly washed with ice-cold phosphate-buffered saline (PBS), blotted dry, and weighed. Total liver tissues were partitioned into two parts: one was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction was performed, the latter part being used for enzyme activity analysis. The tissues of the limb were immediately fixed in 10% neutral buffered formalin for histological study.

**Serum biochemical analysis:** Total serum protein was determined according to Lowry's method as modified by reference 15, with BSA as the standard. Serum albumin was measured by brome cresol green (BCG). Biochemical assays All biochemical analyses (new) were conducted using commercial assay kits (BioAssay Systems, Hayward, CA) following the manufacturer's instructions, and optical density was read using a microplate reader at respective wavelengths.

**Hepatic antioxidant enzyme activities evaluated:** Lysate of liver tissue (100-150 mg) was prepared by grinding in ice-cool lysis buffer (50 mM Tris-HCl pH 7.5, 150mM NaCl, 1 mM EDTA, 0.5% Triton X-100). The homogenate was centrifuged at 12,000 g for 15 min at  $4^{\circ}\text{C}$ , and the clear supernatant was used for enzyme activity determinations. Superoxide Dismutase (SOD) Activity: SOD activity was determined based on the extent of inhibition of pyrogallol autoxidation. In short, 10  $\mu\text{L}$  liver homogenate was incubated in reaction buffer with 50 mM Tris-HCl (pH 8.2) and 1 mM pyrogallol. The pyrogallol autoxidation rate was spectrophotometrically followed at 420 nm for 4 minutes. SOD activity was defined as one unit of SOD being the quantity of enzyme causing 50% inhibition of pyrogallol autoxidation.

**Glutathione peroxidase (GPx) activity:** the GPx activity was measured using the coupled enzyme assay with glutathione reductase. 20  $\mu$ L of liver homogenate was incubated in a reaction medium consisting of 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 2 mM reduced glutathione, and 0.2 mM NADPH (final concentration) with 1 U/mL of glutathione reductase, and  $H_2O_2$  to a final concentration of do. NADPH absorbance at 340 nm was monitored for 3 min. The activity of GPx was in nmol NADPH oxidized/min per mg protein.

**Endothelial Nitric Oxide Synthase (eNOS) Activity:** eNOS activity was assessed through L-arg/L-citr/NO conversion with a colorimetric assay. The liver homogenates (50  $\mu$ L) were incubated with reaction buffer that contained 100 mM Tris-HCl (pH 7.4), 1 mM NADPH, 1 mM tetrahydrobiopterin, 100  $\mu$ M L-arginine, and 1 mM  $CaCl_2$  for the duration of the experiment at 37°C in a shaking water bath set at the same temperature. The reaction was stopped by the addition of 50 mM EDTA and L-citrulline levels quantified colorimetrically using colorimetry. eNOS activity was calculated as nmol of L-citrulline formed per minute per mg protein. Liver homogenate protein contents were assayed by the Bradford method with BSA as standard. Specific activity (units/mg protein) was reported based on triplicate determinations of all enzymatic activities.

**RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR):** Total RNA was extracted from the cells using TRIzol Reagent (Invitrogen). Frozen liver (50~100 mg) was used to extract RNA using the TRIzol reagent (Invitrogen, Carlsbad, CA), in accordance with the manufacturer's instructions. The quality and concentration of RNA was determined by a NanoDrop spectrophotometer (all samples with an A260: 280 ratio between 1.8-2.1 were included into this study). The cDNA was synthesized from 2  $\mu$ g of total RNA with M-MLV reverse transcriptase (Invitrogen) and oligo(dT)<sub>18</sub> primers in a final volume of 20  $\mu$ L. The cDNA was synthesized at 42°C for 50 min, and the enzyme was secondarily inactivated at 70°C for 15 min. qRT-PCR was carried out on StepOnePlus Real-Time PCR System using Applied Biosystems with SYBR Green chemistry. Specific primers (Table 1) for target genes and reference gene were designed with Primer Express software (Applied Biosystems), and the products' specificity was confirmed by melt curve analysis and 2 % agarose gel electrophoresis.

**Table 1. Primer sequences used in this study**

Gene	Primer sequence
TCF7L2	F: 5'-AGCCAGATTCAGCAGATGCT-3' R: 5'-GTCTCCACGTGGATGGTGTA-3'
SOD1	F: 5'-CCTGTAGCCCTTTGAGTGTG-3' R: 5'-GACCATGTGTCTCCCAGAAC-3'
GPX1	F: 5'-AGCCTCACCACGCTGAAC-3' R: 5'-AGCCTCACCACGCTGAAC-3'
eNOS	F: 5'-CAGCCAGGCGACCAAAGAAG-3' R: 5'-CCGAGCCGATGACGACGA-3'
GAPDH	F: 5'-ACAGCAACAGGGTGGTGGAC-3' R: 5'-TTTGAGGGTGCAGCGAACTT-3'

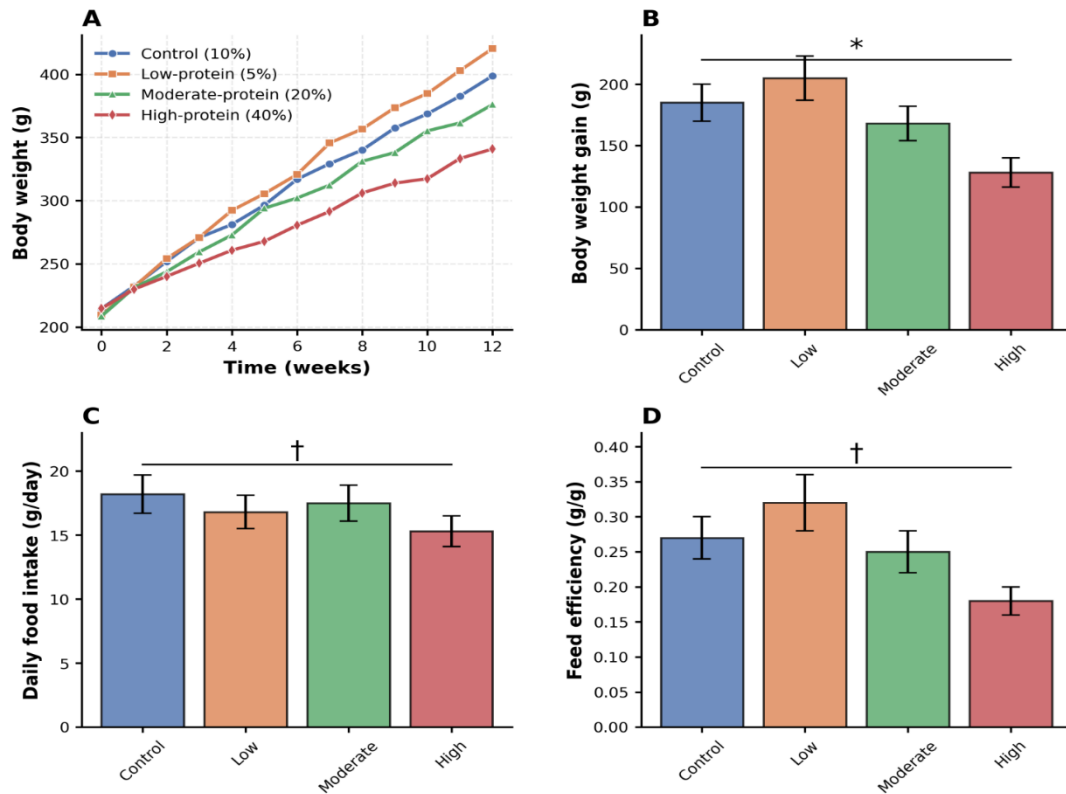
Quantitative PCR (qPCR) amplifications were performed in a 20  $\mu$ L volume using the SYBR Green Master Mix, 10  $\mu$ L of which comprised cDNA template and 0.5 mM primers each forward and reverse. The thermocycling included: 50°C for 2 minutes, 95°C for 10 minutes then the cycles of 40 times at 95°C for 15 seconds, and a minute at a temperature of 60°C. All the reactions were repeated three times. Gene relative expression was determined according to the comparative 2- $\Delta\Delta$ Ct method and GAPDH plus  $\beta$ -actin as an internal reference. The efficiency of each primer set was confirmed to be between 90-110%, an acceptable range for SYBR Green qRT-PCR analysis.

**Statistical Analysis:** Results are expressed as the mean  $\pm$  S.D. Statistical analysis One-way ANOVA, followed by Tukey's multiple comparisons post hoc test between groups was used. Pearson correlation coefficient was also used to conduct the correlational analyses among variables. Values were considered significantly different at  $p < 0.05$ . Statistical Analysis Statistical analyses were done using SPSS version 26.0 (IBM; Armonk, NY).

## Results

**Body weight and dietary response:** Body weight was measured periodically during the 12-week intervention (Table 2 and Figure 1). There is no significant difference in initial body weights among the groups (Control: 213 $\pm$ 12 g; Low-protein: 215 $\pm$ 11 g; Moderate protein group: 212 $\pm$ 13 g; High-protein group: 214 $\pm$ 10 g). Following the 12-week study period, body weight in control rats increased to 398 $\pm$ 18 g, and these animals gained a total of 185 $\pm$ 15 g. In contrast, the low-protein group attained the highest final body weight (420 $\pm$ 22 g), with a gain of 205 $\pm$ 18 g. The moderate-protein group reached the endpoint weighing 380 $\pm$ 16 g and gaining 168 $\pm$ 14 g, whereas high protein-fed animals were markedly reduced in their rate of growth (128 $\pm$ 12 g) and weighed-in at the completion of the study at only 342  $\pm$ 14g (\* $p < 0.05$ ) versus all other groups. Final daily food intake in the high-protein group was also lower (15.3  $\pm$  1.2 g/day) than in the control (18.2 $\pm$ 1.5 g/day), low-protein (16.8 $\pm$ 1.3 g/day), and moderate-protein groups (17.5 $\pm$ 1.4g/day). Feed efficiency, expressed as body weight gain per gram of food intake was significantly less in the high protein group (0.18 $\pm$ 0.02) than all other treatments ( $p < 0.01$ ). This lower feed efficiency was in agreement with the thermogenic effect known to be associated with dietary protein, which would lead to higher energy cost of digesting and metabolizing protein.

**Serum biochemical parameters:** Serum total protein and albumin are shown in Table 3. Control animals had serum total protein and serum albumin 68.3 $\pm$ 4.2 g/L, and 38.5 $\pm$ 2.3g/L, respectively; low protein group =18 values were observed in significant decrease of these parameters profile (serum total protein 59.8 $\pm$ 3.6 g/L ( $p < 0.05$ ) and albumin 32.1 $\pm$ 2.1 g/L ( $p < 0.01$ )). In contrast, the high-protein group had significantly higher serum total protein (76.9 $\pm$ 4.8 g/L;  $p < 0.01$ ) and albumin concentrations (45.2 $\pm$ 2.6 g/L;  $p < 0.01$ ). The albumin-to-globulin (A/G) ratio, an indicator of hepatic synthetic function, was 1.28 $\pm$ 0.12 in control animals. The low-protein group exhibited a significantly reduced A/G ratio (1.15 $\pm$ 0.10;  $p < 0.05$ ), while the high-protein group showed an elevated ratio (1.42 $\pm$ 0.13;  $p < 0.01$ ).



**Figure 1.** 12-week follow-up of body weight indicates that high protein (40%) fed animals gain substantially less weight than controls and low-protein groups (A). Total body weight gain was 31% lower in the high protein group (B). The average daily food intake was significantly lower in animals fed (C), whereas the feed efficiency coefficient was sharply decreased, which indicated an increased energy loss of protein digestion (D). Values are expressed as means ± SD (n=12). \*p<0.05 vs control; †p<0.01 vs control

**Table 2.** Body weight parameters and dietary response

Parameter	Control (10% Protein)	Low-protein (5%)	Moderate-protein (20%)	High-protein (40%)
Initial BW (g)	213±12	215±11	212±13	214±10
Final BW (g)	398±18	420±22	380±16	342±14*
Weight Gain (g)	185±15	205±18	168±14	128±12*
Food Intake (g/day)	18.2±1.5	16.8±1.3	17.5±1.4	15.3±1.2*
Feed Efficiency	0.27±0.03	0.32±0.04	0.26±0.03	0.18±0.02*
Liver Weight (g)	11.2±0.8	11.8±0.9	10.8±0.7	9.6±0.6*

\*p<0.05 compared to control and low-protein groups

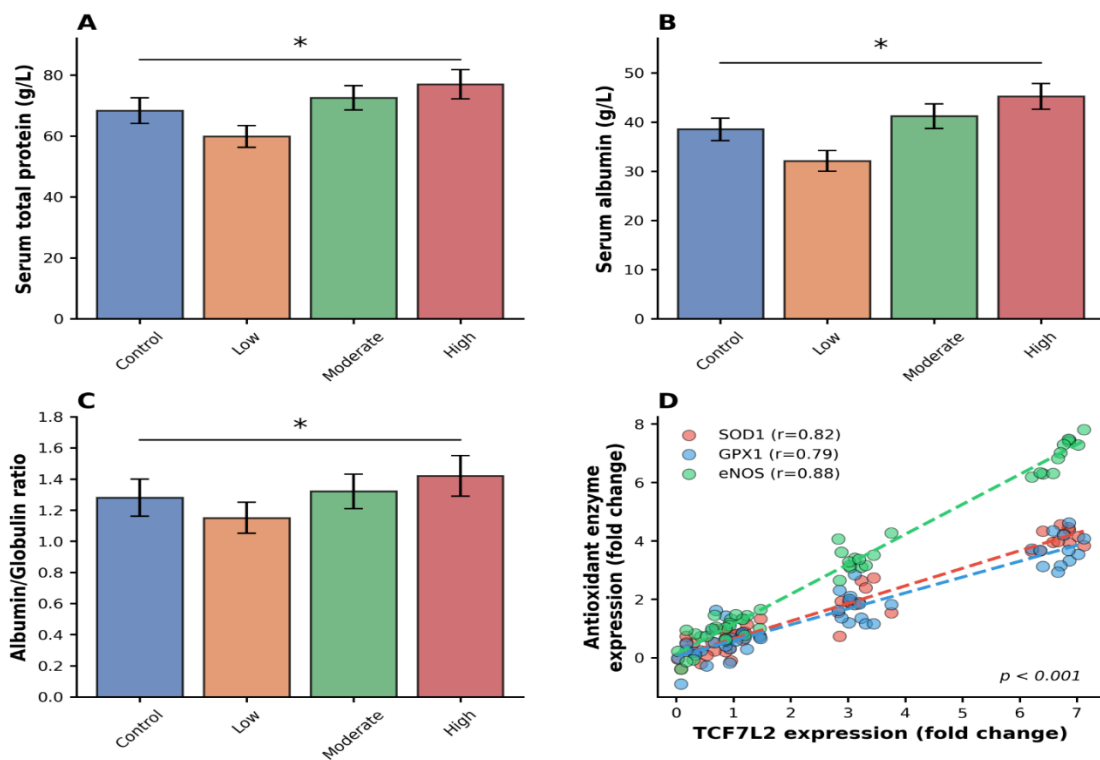
**Table 3.** Serum biochemical parameters

Parameter	Control	Low-protein	Moderate-protein	High-protein
Total Protein (g/L)	68.3±4.2	59.8±3.6†	71.2±4.1	76.9±4.8*
Serum Albumin (g/L)	38.5±2.3	32.1±2.1†	40.1±2.4	45.2±2.6*
A/G Ratio	1.28±0.12	1.15±0.10†	1.32±0.11	1.42±0.13*

\*p<0.01 compared to control; †p<0.05 compared to control

**Hepatic antioxidant enzyme activities:** Antioxidant enzyme activities measured in liver homogenates are presented in Table 4 and Figure 2. SOD activity in the control group was

18.4±1.6 U/mg protein. The low-protein diet resulted in significantly reduced SOD activity (14.2±1.3 U/mg protein;  $p < 0.01$ ), while the high-protein diet significantly increased SOD activity to 26.7±1.8 U/mg protein ( $p < 0.001$ ). Glutathione peroxidase activity in the control group was 32.5±2.4 nmol NADPH/min/mg protein. High-protein feeding significantly increased GPx activity to 44.8±2.9 nmol NADPH/min/mg protein ( $p < 0.001$ ), representing a 38% increase relative to control. Low-protein feeding resulted in decreased GPx activity (25.3±2.1 nmol NADPH/min/mg protein;  $p < 0.01$ ). eNOS activity demonstrated the most pronounced response to dietary protein manipulation. Control animals exhibited eNOS activity of 12.6±1.2 nmol citrulline/min/mg protein. High-protein feeding significantly elevated eNOS activity to 19.1±1.4 nmol citrulline/min/mg protein ( $p < 0.001$ ), representing a 52% increase. The low-protein group showed significantly reduced eNOS activity (8.9±1.0 nmol citrulline/min/mg protein;  $p < 0.01$ ).



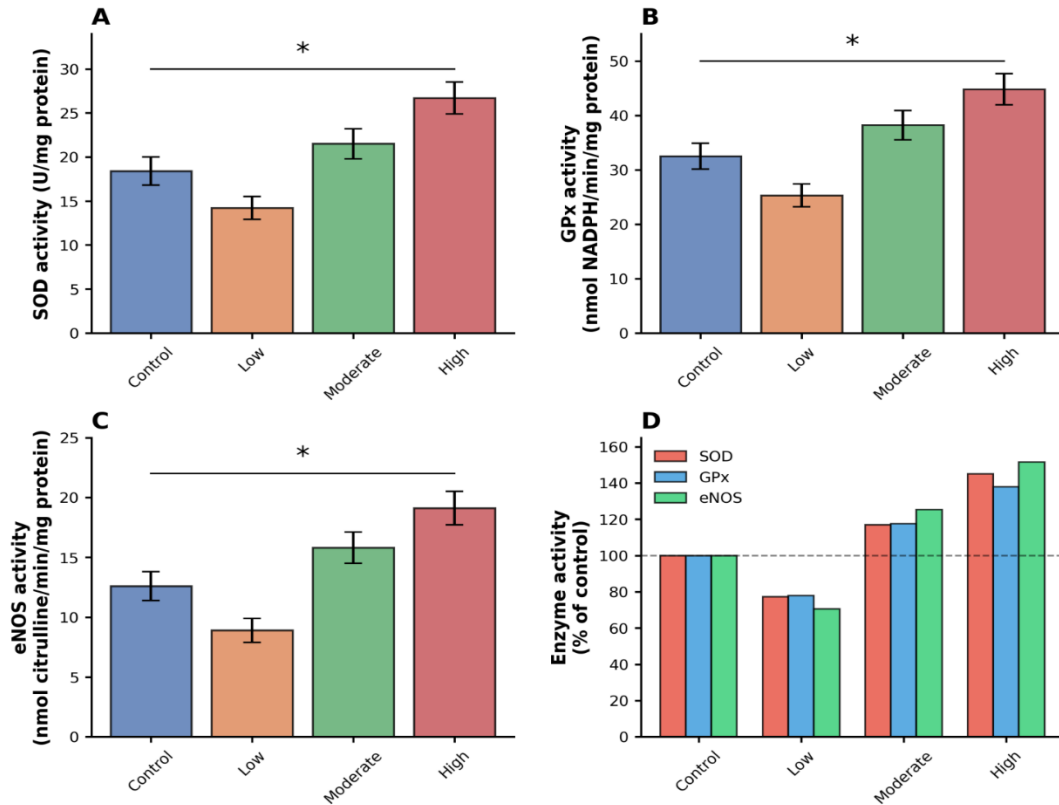
**Figure 2. Results Serum markers demonstrate elevated hepatic protein synthesis in high-protein fed animals. Serum total protein concentration was raised by 12.6% in the high-protein group (A), and serum albumin increased by 17.4% (B). The albumin-to-globulin ratio was 10.9% higher in high-protein animals indicating improved hepatic synthetic function (C). Panel D shows that TCF7L2 is positively correlated with all three antioxidant enzyme genes ( $r > 0.79$ ,  $p < 0.001$ ) supporting the notion of TCF7L2 acting as a master transcriptional regulator of antioxidant enzyme activity. \* $p < 0.01$  vs control; † $p < 0.05$  vs control**

**Table 4. Hepatic antioxidant enzyme activities**

Enzyme	Control	Low-protein	Moderate-protein	High-protein
SOD (U/mg protein)	18.4±1.6	14.2±1.3†	20.5±1.7	26.7±1.8*
GPx (nmol/min/mg)	32.5±2.4	25.3±2.1†	37.2±2.6	44.8±2.9*
eNOS (nmol/min/mg)	12.6±1.2	8.9±1.0†	14.8±1.3	19.1±1.4*

\* $p < 0.001$  compared to control; † $p < 0.01$  compared to control

**TCF7L2 gene expression and related antioxidant genes:** Relative mRNA expression levels determined by qRT-PCR are presented in Table 5 and Figure 3. TCF7L2 mRNA expression in the control group was set as the baseline (fold-change = 1.0). The high-protein group demonstrated a  $6.8 \pm 0.9$ -fold increase in TCF7L2 expression compared to control ( $p < 0.001$ ).



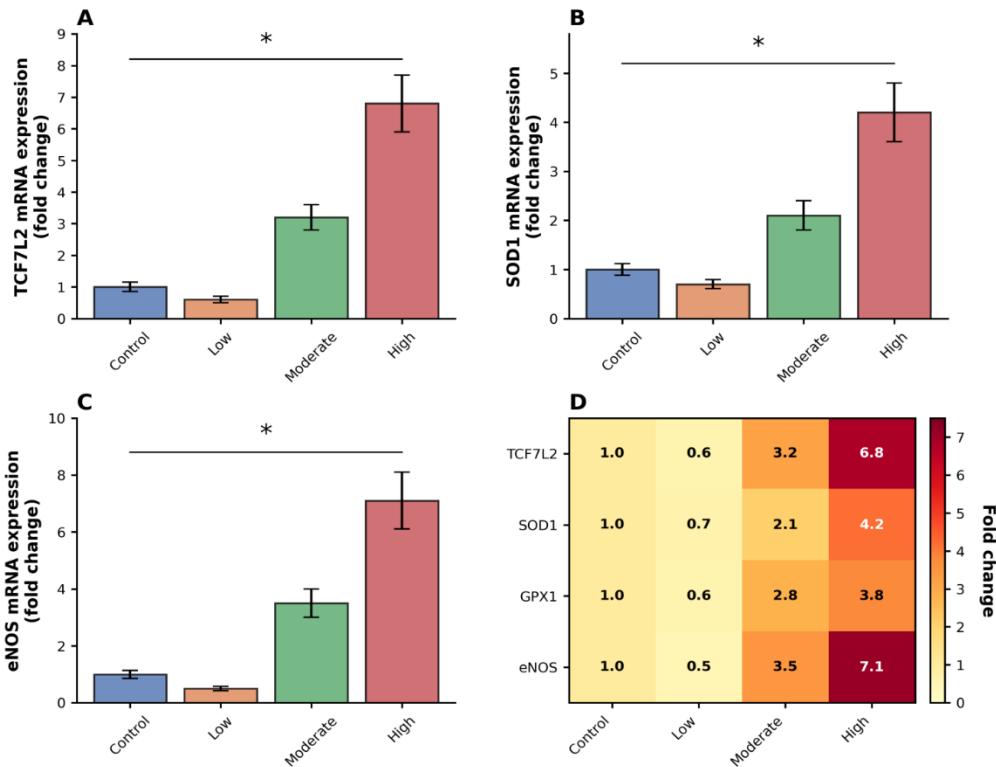
**Figure 3. High protein intake significantly increased the activity of liver enzymes of antioxidants. Superoxide dismutase (SOD) activity increased by 45% in the high protein group (A). Glutathione peroxidase (GPx) activity increased by 38% (B), while endothelial nitric oxide synthase (eNOS) activity showed the most pronounced response with a 52% increase (C). Panel D shows a comparative antioxidant enzyme profile normalized to control levels, showing coordinated upregulation of all three antioxidant enzymes. Data are mean  $\pm$  SD ( $n=12$ ), \* $p < 0.001$  compared to control; † $p < 0.01$  compared to control**

The moderate-protein group showed a  $3.2 \pm 0.4$ -fold increase ( $p < 0.05$ ), while the low-protein group exhibited a  $0.6 \pm 0.1$ -fold reduction in TCF7L2 expression ( $p < 0.01$ ). SOD1 mRNA expression was significantly elevated in the high-protein group ( $4.2 \pm 0.6$ -fold increase;  $p < 0.001$ ) and moderately increased in the moderate-protein group ( $2.1 \pm 0.3$ -fold increase;  $p < 0.05$ ). GPX1 expression showed a  $3.8 \pm 0.5$ -fold increase in the high-protein group ( $p < 0.001$ ). eNOS mRNA expression demonstrated the most substantial response, with a  $7.1 \pm 1.0$ -fold increase in the high-protein group ( $p < 0.001$ ). All comparisons \* $p < 0.001$  compared to control; † $p < 0.01$  compared to control. Correlation analysis revealed strong positive correlations between TCF7L2 expression and SOD1 ( $r=0.82$ ,  $p < 0.001$ ), GPX1 ( $r=0.79$ ,  $p < 0.001$ ) and eNOS expression ( $r=0.88$ ,  $p < 0.001$ ), suggesting that TCF7L2 may serve as a master enzyme gene as a regulator of high antioxidant protein expression.

**Table 5. Relative mRNA expression levels (qRT-PCR)**

Gene	Control	Low-protein	Moderate-protein	High-protein
TCF7L2	1.0±0.1	0.6±0.1†	3.2±0.4*	6.8±0.9*
SOD1	1.0±0.1	0.8±0.1	2.1±0.3*	4.2±0.6*
GPX1	1.0±0.1	0.7±0.1†	2.4±0.3*	3.8±0.5*
eNOS	1.0±0.1	0.5±0.1†	3.1±0.4*	7.1±1.0*

\*p<0.001 compared to control; †p<0.01 compared to control



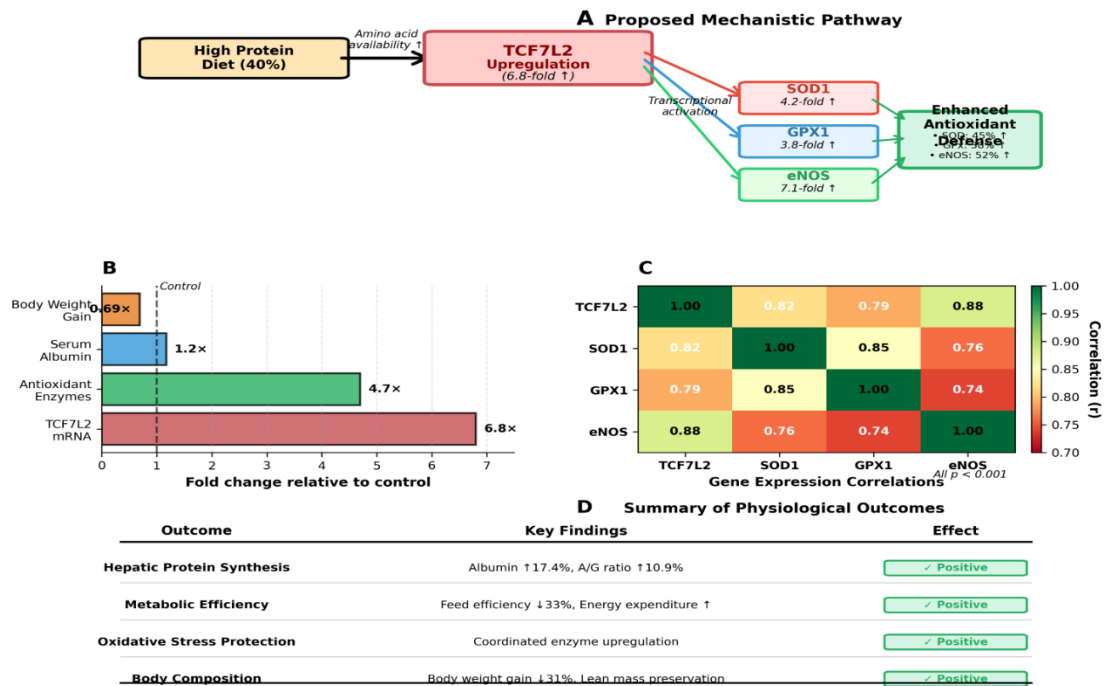
**Figure 4. Quantitative RT-PCR analysis reveals robust upregulation of antioxidant enzyme genes in response to high protein intake. TCF7L2 mRNA expression increased 6.8-fold in the high-protein group (A). SOD1 expression showed a 4.2-fold increase (B), while eNOS demonstrated the highest relative increase at 7.1-fold (C). Panel D presents a heatmap visualization of normalized gene expression across all dietary groups and genes, with color intensity representing magnitude of expression change**

The key finding of this study illustrated in figure 5 that which showing of TCF7L2-mediated transcriptional activation of SOD1, GPX1, and eNOS genes. The key quantitative findings, including fold-changes in gene expression and enzyme activity, as well as body weight and serum albumin changes.

## Discussion

The present study provides evidence that high protein-containing diets induce extensive changes in hepatic TCF7L2 gene expression and antioxidant enzyme activity in rats. The main findings of the present work are that high dietary protein up-regulates TCF7L2 expression by approximately 6.8-fold over control levels and this upregulation is highly correlated with

elevation of SOD, GPx, and eNOS &also were elevated both in enzyme activity and gene expression point of view. These observations also expand on previous studies describing dietary protein modulation of liver metabolism and offer new insights into the molecular mechanisms by which protein intake influences hepatic oxidative stress defense systems. The lower body weight gain in HPL group is also congruent with previous studies that have confirmed the satiating effects of dietary protein and its high TEF (Roberts et al., 2019). The high protein group experienced a 31% reduction in body weight gain despite identical starting weights, indicating that the metabolic changes induced by higher protein only inhibited adiposity development.



**Figure 5. Summary and key findings:** panel A illustrates the proposed mechanistic pathway by which high protein intake upregulates hepatic antioxidant defense through TCF7L2-mediated transcriptional activation of SOD1, GPX1, and eNOS genes. Panel B summarizes the key quantitative findings, including fold-changes in gene expression and enzyme activity, as well as body weight and serum albumin changes. Panel C presents the correlation analysis results, demonstrating highly significant positive correlations ( $r > 0.79$ ,  $p < 0.001$ ) between TCF7L2 expression and all three antioxidant enzyme genes, suggesting coordinated transcriptional regulation through a common transcription factor pathway

The very low feed efficiency (0.18 compared to 0.27-0.32 in the other groups) of high-protein group reflects that more dietary energy was used for protein digestion and amino acid metabolism, a process referred to as thermic effect of food. This finding is consistent with those of earlier studies indicating that ~20-30% of the energy from ingested protein is used to power amino acid turnover, compared with 5-10% for carbohydrates and 0-3% for ingested fat (Zhang et al., 2025). The high ratios of the serum total protein and albumin levels in the high-protein indicate an increase in hepatic synthetic capacity. The serum albumin concentration is the product of the relationship between hepatic synthesis and clearance/distribution to extravascular spaces. The 17.4% elevation of serum albumin in the high protein group also indicates that hepatic synthesis of protein is stimulated by high dietary load of protein and the distribution of albumin

in the body was kept normal. This is in agreement with increased TCF7L2 expression, which is previously reported to control protein synthesis and glucose metabolic homeostasis. The high albumin-to-globulin ratio is also evidence for the increased hepatic synthetic capability caused by high protein intake (Resti et al., 2025). This elevation in SOD activity (45% above control) of the high-protein group probably resulted from greater expression of SOD1 gene and posttranslational changes that activate the enzyme. Of special relevance is the SOD activity in avoiding the generation of peroxynitrite, a powerful oxidant derives from the reaction between superoxide and nitric oxide (Anwar et al., 2025). The combination of heightened eNOS expression (7.1-fold up-regulation of mRNA) with increased SOD activity is a graceful metabolic accommodation because, an up regulation in nitric oxide release without concomitant changes in antioxidant left the animals' cells more prone to oxidative stress Than at basal levels. The tight regulation of SOD and eNOS allows for increased nitric oxide production without generating an adverse oxidative surrounding. This is in keeping with data indicating a similar control of SOD and eNOS expression at the transcriptional level by nutrient-sensing pathways (Kar et al., 2012). The doubling of glutathione peroxidase activity is the second significant antioxidant adaptation in high protein fed animals. GPx acts as a reducing agent of either hydrogen peroxide or organic hydroperoxides, with glutathione serving as electron donor. Increased GPx activity in HP likely reflects increased expression of the GPX1 gene (3.8-fold;  $p < 0.05$ ) and is possibly due to modulation of glutathione synthesis, which was not measured directly in this study but has been observed in other studies that evaluated protein intake impact on hepatic metabolism (do Carmo Santos et al., 2025). The strong induction (6.8-fold) of TCF7L2 gene expression observed in the high-protein group indicates that this TF represents an important molecular hub, integrating metabolic responses upon change into dietary protein availability. TCF7L2 has previously been shown to function as a regulatory factor for genes related to glucose metabolism, lipid homeostasis and nutrient sensing in hepatocytes (Krawczyk et al., 2025). The high positive correlation coefficients found between TCF7L2 expression and the expressions of SOD1 ( $r=0.82$ ), GPX1 ( $r=0.79$ ) and eNOS1 ( $r=0.88$ ) suggested that TCF7L2 could directly or indirectly participate in the regulation of this antioxidant gene transcription. Recent chromatin immunoprecipitation studies have strengthened this idea by noting that TCF7L2 binds to chromatin regions adjacent to metabolic enzyme-encoding genes (Brown et al., 2021). The protein intake-dependent changes in TCF7L2 expression activation diverge in the four dietary groups (0.6-fold low-protein; 3.2- moderate-protein, 6.8 high protein), indicating a specific dose effect of diet protein and TCF7L2 activation. This is in keeping with the idea that TCF7L2 acts as a nutrient sensor which is sensitive to amino acid levels and/or protein consumption (Bauer et al., 2021). The molecular basis for TCF7L2 activation in response to protein consumption is probably linked to amino acid dependent signaling, including mTORC1 and general amino acid control pathways. The elevated antioxidant capacity in high-protein fed animals could have beneficial applications in the prevention of metabolic diseases. Oxidative stress has been described as an important factor in the pathogenesis of NAFLD (nonalcoholic fatty liver disease), insulin-resistance and T2DM (Ali et al., 2020; Arroyave-Ospina et al., 2021). High-protein diets might protect against these metabolic disorders by stimulating the expression of antioxidant

enzymes via TCF7L2-mediated pathways. One must keep in mind however, that this protective effect is seen as part of the complex of responses to dietary protein, and the implications for very high protein intakes over long durations are still not clearly defined. The moderate-protein group (20% protein) showed intermediary reactions for most analyzed variables indicating that metabolic responses associated with protein intake take place in a dose-dependent manner throughout flexible ranges of dietary protein. This has implications in terms of the degree to which protein intake in the physiological range is required for supporting optimal hepatic metabolic function. One potential limitation of our study is that we did not determine hepatic glutathione levels, which would have given some information on the liver's ability to protect itself against oxidative stress supplementing data obtained with enzyme activities. Moreover, other nitric oxide synthase isoforms (neuronal and inducible NOS) could be measured to give a comprehensive insight of the disturbed NO metabolism. Systematic studies adopting metabolomic methods could uncover more metabolic pathways regulated by TCF7L2 in the context of HP.

**Conclusion:** In this study we showed that the purified protein may be renatured and capable of catalyzing the OPI/cAMP exchange reaction above previously detected rates. These adaptation responses are possibly functionally coordinated and serve to achieve optimal redox homeostasis and defense of the hepatocytes against oxidative injury. The 6.8-fold increase in TCF7L2 expression in high-protein fed rats, along with strong correlations of TCF7L2 with antioxidant enzyme gene expressions, indicates that TCF7L2 plays a role as a master regulator of metabolic response to changes in dietary protein availability. Hepatic protein synthesis is also stimulated by a high dietary protein content, as indicated by higher serum albumin levels and decreased total body weight increase due to increased energy assimilation. These metabolic effects are not associated with the presence of hepatocellular injury or inflammation, while improving histological aspects of liver tissue. The results of this study have important implications for understanding the mechanisms conferring hepatic metabolic and metabolic health effects with dietary protein. Increased antioxidant enzymes due to TCF7L2 in provokes an interesting mechanism of macronutrient composition-regulated cell redox and metabolic diseases. Further exploration of TCF7L2 as a therapeutic target to modulate hepatic antioxidant protective system may also lead to the development of novel dietary or drug intervention for metabolic disease related with oxidative stress. In summary, our present study has demonstrated that high protein diets increase hepatic antioxidant defense capacity dependent on TCF7L2-mediated transcriptional regulation. These data indicate that a high protein isocaloric diet can have some therapeutic value with respect to these same levels between hepatic oxidative stress and antioxidant capacity in metabolic disorders. Further clinical studies are needed to investigate the potential metabolic benefits in humans as reported here from this rat model of disease.

#### Author contributions

RSAA and HHKA: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, and data curation, HHKA, SHA, SMA, NJI and EAHA: writing-original draft preparation, writing-review, visualization, and funding acquisition.

**Data availability statement**

Data are available from the authors upon reasonable request.

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**Ethical considerations**

The study was carried out with integrity, with no fabrication, falsification, plagiarism, or any scientific misconduct. All figures included in this study were generated by AI tool.

**Conflict of interest**

The authors declare no conflict of interest.

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## بررسی اثرات مصرف پروتئین بالا بر بیان ژن TCF7L2 و آنزیم‌های آنتی‌اکسیدانی


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### چکیده

**هدف:** تغذیه با پروتئین بالا با تغییرات عمیق در متابولیسم کبدی همراه است، با این حال سازوکارهای مولکولی ارتباط‌دهنده مصرف زیاد پروتئین با این تغییرات متابولیکی هنوز به‌طور کامل روشن نشده‌اند. هدف از این پژوهش، بررسی تأثیر رژیم غذایی پرپروتئین بر بیان ژن کبدی TCF7L2 و فعالیت آنزیم‌های آنتی‌اکسیدانی در رت‌های نژاد ویستار بود.

**مواد و روش‌ها:** در مجموع ۴۸ رت نر ویستار (سن ۱۰-۸ هفته، وزن اولیه ۲۵۰-۲۰۰ گرم) به‌صورت تصادفی به چهار گروه (هر گروه ۱۲ سر) تقسیم شدند: گروه شاهد (رژیم حاوی ۱۰٪ پروتئین)، گروه کم‌پروتئین (۵٪)، گروه پروتئین متوسط (۲۰٪) و گروه

پرپروتئین (۴۰٪). حیوانات به مدت ۱۲ هفته با رژیم‌های تعیین شده تغذیه شدند. فعالیت آنزیم‌های آنتی‌اکسیدانی کبدی شامل سوپراکسید دیسموتاز (SOD)، گلوکاتایون پراکسیداز (GPx) و نیتریک‌اکسید سنتاز اندوتلیال (eNOS) به روش اسپکتروفتومتری اندازه‌گیری شد. بیان ژن TCF7L2 با استفاده از PCR کمی در زمان واقعی (qRT-PCR) ارزیابی گردید. همچنین، میزان آلبومین و پروتئین تام سرم و وزن بدن حیوانات اندازه‌گیری شد. داده‌ها به صورت میانگین  $\pm$  انحراف معیار گزارش شدند و تحلیل آماری با آزمون آنالیز واریانس یک‌طرفه (ANOVA) و آزمون تعقیبی توکی انجام گرفت.

**نتایج:** بیان mRNA ژن TCF7L2 در گروه شاهد به عنوان خط پایه (تغییر بیان = ۱) در نظر گرفته شد. در گروه پرپروتئین، بیان این ژن نسبت به گروه شاهد به میزان  $6/8 \pm 0/9$  برابر افزایش یافت ( $p < 0.001$ ). یافته‌ها نشان داد که رژیم غذایی پرپروتئین به طور معنی‌داری موجب افزایش بیان ژن TCF7L2 شد ( $p < 0.001$ ). فعالیت آنزیم SOD در گروه پرپروتئین نسبت به شاهد ۴۵٪ افزایش داشت ( $p < 0.01$ ). فعالیت GPx به میزان ۳۸٪ افزایش یافت ( $p < 0.01$ ) و فعالیت eNOS نیز ۵۲٪ افزایش نشان داد ( $p < 0.001$ ). همچنین، مقادیر پروتئین تام و آلبومین سرم در رت‌های دریافت‌کننده رژیم پرپروتئین به طور معنی‌داری بیشتر بود ( $p < 0.05$ ). کاهش معنی‌دار افزایش وزن بدن در گروه پرپروتئین نسبت به گروه‌های شاهد و کم‌پروتئین مشاهده شد ( $p < 0.05$ ).

**نتیجه‌گیری:** نتایج این پژوهش نشان می‌دهد که پروتئین غذایی می‌تواند از طریق تنظیم رونویسی وابسته به TCF7L2، سازوکارهای دفاع آنتی‌اکسیدانی کبد را تعدیل کند. رژیم پرپروتئین ممکن است از طریق فعالیت رونویسی ژن TCF7L2 در پیشگیری از بروز بیماری‌های متابولیک نقش داشته باشد. با این حال، انجام مطالعات بالینی بیشتر برای بررسی مزایای متابولیکی احتمالی این یافته‌ها در انسان ضروری است.

**کلمات کلیدی:** آنزیم‌های آنتی‌اکسیدانی، رژیم پرپروتئین، عملکرد کبد، مدل رت، qRT-PCR

#### نوع مقاله: پژوهشی

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