

ORIGINAL RESEARCH

Insulin-induced Hypoglycemia in a Mouse Model: Association of Inflammatory Markers with Liver and Kidney Injury

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ABSTRACT

The aim of this study was to evaluate the early histopathological and inflammatory effects of acute insulin-induced hypoglycemia in liver and kidney tissues. Acute hypoglycemia was experimentally induced by insulin administration, and liver and kidney samples were analyzed histologically. Tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and nuclear factor kappa B (NF- κ B) protein expression were assessed by immunohistochemistry, while TNF- α and IL-6 gene expression were evaluated using reverse transcription quantitative polymerase chain reaction. Acute hypoglycemia induced early structural alterations in both organs, with more pronounced and progressive changes observed in the kidney. Renal injury included tubular epithelial degeneration, tubular dilatation, and glomerular alterations, whereas hepatic changes were milder and mainly characterized by sinusoidal dilatation and hyperemia. No significant differences were observed in TNF- α , IL-6, or NF- κ B protein expression. However, renal TNF- α and IL-6 messenger RNA levels showed transient alterations over time. In conclusion, acute insulin-induced hypoglycemia leads to early structural injury in liver and kidney tissues, with greater susceptibility observed in the kidney. These changes occur in the absence of significant inflammatory protein activation, suggesting that early tissue injury is primarily driven by metabolic stress rather than overt inflammatory signaling, although transient transcriptional responses may precede detectable protein-level changes.

Keywords: Hypoglycemia. Liver. Kidney. Inflammation. Insulin.

Fare Modelinde İnsülin Kaynaklı Hipoglisemi: Karaciğer ve Böbrek Hasarı ile İnflamatuvar Belirteçler Arasındaki İlişki

ÖZET

Bu çalışmanın amacı, akut insülin kaynaklı hipogliseminin karaciğer ve böbrek dokularında erken histopatolojik ve inflamatuvar etkilerini değerlendirmektir. Akut hipoglisemi, yoğun insülin uygulaması ile deneysel olarak oluşturulmuş ve karaciğer ile böbrek dokuları histolojik olarak incelenmiştir. TNF- α , IL-6 ve NF- κ B protein ekspresyonları immünohistokimya ile, TNF- α ve IL-6 gen ekspresyonları ise RT-qPCR yöntemi ile analiz edilmiştir. Akut hipoglisemi her iki organda da erken yapısal değişikliklere yol açmış, böbrekte tübüler ve glomerüler hasar dahil olmak üzere daha belirgin histopatolojik bulgular gözlenirken karaciğer değişiklikleri daha hafif düzeyde kalmıştır. TNF- α , IL-6 ve NF- κ B protein düzeylerinde anlamlı bir değişiklik saptanmamıştır. Buna karşılık, böbrek dokusunda TNF- α ve IL-6 mRNA düzeylerinde geçici değişiklikler gözlenmiştir. Bu bulgular, akut insülin kaynaklı hipogliseminin erken dönemde organ hasarına yol açabileceğini ve bu etkinin özellikle böbrek dokusunda daha belirgin olduğunu göstermektedir. Elde edilen sonuçlar, hipoglisemiye bağlı doku hasarının belirgin inflamatuvar protein aktivasyonundan ziyade, metabolik stres mekanizmaları ile ilişkili olabileceğini düşündürmektedir.

Anahtar Kelimeler: Hipoglisemi. Karaciğer. Böbrek. İnflamasyon. İnsülin.

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Hypoglycemia is one of the most important acute metabolic complications encountered during diabetes treatment, particularly in patients receiving insulin therapy^{1,2}. A reduction in blood glucose levels below the physiological range not only causes neuroglycopenic and adrenergic symptoms but may also trigger systemic cellular stress responses³. During acute hypoglycemic episodes, impaired energy metabolism, increased oxidative stress, activation of inflammatory pathways, and disruption of tissue homeostasis may occur⁴. Therefore, hypoglycemia should not be considered merely a short-term clinical event in diabetes management, but also as a biological stress factor that may contribute to target organ injury.

The liver and kidney are two critical organs involved in the maintenance of glucose homeostasis and the regulation of metabolic responses. The liver represents a major site of the compensatory response to hypoglycemia through glycogenolysis and gluconeogenesis, whereas the kidney contributes to systemic glucose balance through renal gluconeogenesis and the elimination of metabolic waste products. The increased metabolic demand imposed on these tissues during glucose deprivation may promote cellular stress and oxidative imbalance, potentially leading to structural and functional alterations^{2,5}. However, the extent to which acute hypoglycemia induces inflammatory responses in hepatic and renal tissues, and how these responses are associated with histopathological alterations, remains to be further clarified.

Inflammation is a key mechanism that may contribute to hypoglycemia-associated tissue injury^{4,6}. In this context, nuclear factor kappa B (NF- κ B) is a key transcription factor involved in the regulation of inflammatory signaling pathways, which can influence the expression of downstream pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), thereby contributing to the progression of cellular stress responses and tissue inflammation^{7,8}.

TNF- α and IL-6 are among the most extensively studied pro-inflammatory cytokines involved in tissue injury. TNF- α contributes to cellular apoptosis, oxidative stress, and inflammatory cell recruitment^{9,10}, whereas IL-6 regulates acute-phase responses and immune activation¹¹. Elevated expression of these cytokines has been associated with the pathogenesis of various hepatic and renal disorders, including acute kidney injury, chronic kidney disease, hepatic inflammation, and metabolic dysfunction¹⁰⁻¹². Therefore, evaluation of TNF- α and IL-6 may provide important insights into the inflammatory responses associated with hypoglycemia-induced tissue injury⁴.

While the systemic consequences of hypoglycemia have been extensively investigated, organ-specific effects remain less well defined. In particular, limited

information is available regarding how acute insulin-induced hypoglycemia impacts tissue integrity and molecular inflammatory responses in hepatic and renal tissues, despite the central role of these organs in glucose homeostasis. Most existing studies focus on systemic markers of inflammation and oxidative stress, whereas direct describing tissue level injury and the expression of inflammatory mediators such as TNF- α and IL-6 at both the transcriptional and protein levels remain comparatively limited. This gap underscores the need for targeted investigations into molecular mechanisms to better understand how hypoglycemia contributes to tissue injury and metabolic dysfunction.

In the present study, we aimed to evaluate the effects of insulin-induced acute hypoglycemia on liver and kidney tissues by integrating histopathological assessment with the analysis of TNF- α and IL-6 expression at both gene and protein levels in an in vivo Balb/c mouse model.

This study provides a comprehensive evaluation of hypoglycemia-induced organ alterations using histopathological and molecular approaches. The findings contribute to a better understanding of tissue responses to acute metabolic stress and may help clarify the involvement of inflammatory mediators in hypoglycemia-associated liver and kidney injury.

Materials and Methods

Ethics Statement

All mice experiments were approved by the Erciyes University Animal Experiments Local Ethics Committee (approval date: 03.12.2025; decision no:25/273). The study was conducted at the Transgenic Mouse and Genome Units of the Betül-Ziya Eren Genome and Stem Cell Center (GENKÖK), Erciyes University.

All experimental procedures were performed in accordance with national and international guidelines for the care and use of laboratory animals.

Mouse Model

A total of 15 male Balb/c (8 weeks old, weighing 18-23g) were used in this study. Male were selected to minimize variability associated with hormonal fluctuations during the estrous cycle, which may influence glucose homeostasis and counter-regulatory responses to hypoglycemia. All mice were maintained under identical controlled environmental conditions (23°C temperature, 55% humidity, and a 12 h light/dark cycle with lights on from 06:00 to 18:00), to minimize environmental variability.

For induction of hypoglycemia, mice were randomly assigned to five experimental groups (n = 3 per each):

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healthy control and hypoglycemia groups euthanized at 0 (basal, prior to insulin injection), 10, 25, and 40-minutes after insulin administration. Hypoglycemic groups were fasted for 12 h starting at 21:00 with free access to water, and at 09:00 following the fasting period, the received an intraperitoneal injection of insulin (Humulin®, Cat. No. HI0210, USA) at a dose of 2 U/kg body weight. All experimental procedures were performed between 09:00 and 10:00 in a controlled and isolated environment to reduce external stress factors¹³. Healthy control mice were not fasted and did not receive any injection.

Tissue Collection

Blood glucose levels were measured from the tail vein, at basal levels (0 min) and following insulin administration, at 10, 25, and 40 minutes for each respective group. Mice in the 10-, 25- and 40-min groups that reached blood glucose concentrations between 20 and 50 mg/dL were considered hypoglycemic and euthanized at their designated time points. Mice in the 0-min group were euthanized by cervical dislocation immediately after baseline blood glucose measurement, before insulin administration¹³. Kidney and liver tissues were rapidly dissected, collected, and processed for downstream histological, immunohistochemical, and molecular analyses.

These tissues were used to assess hypoglycemia-induced changes in the expression of inflammatory markers, including TNF- α and IL-6.

Histochemical Analysis

Paraffin-embedded liver and kidney sections (5 μ m thickness) were stained with Hematoxylin-Eosin (H&E) and photographed using the ZEN3.1 computer-assisted imaging program under a ZEISS Axiocam 208 color model light microscope. In liver tissue, hepatocyte degeneration, sinusoidal dilatation and hyperemia, mononuclear cell infiltration, and necrosis were evaluated, while in kidney tissue, tubular epithelial degeneration, tubular dilatation, glomerular degeneration, dilatation of the Bowman's capsule, and mononuclear cell infiltration were evaluated¹⁴⁻¹⁶. The resulting histopathological changes were scored for each parameter as follows: 0, 1, 2 and 3 (none, mild, moderate, and severe, respectively)¹⁶.

Immunohistochemical Analysis

Immunoreactivity changes of IL-6, TNF- α , and NF- κ B proteins in 5 μ m thick liver and kidney sections obtained from paraffin blocks were evaluated by immunohistochemical analysis¹⁵. For this purpose, sections deparaffinized under appropriate conditions were rehydrated and washed with PBS (Phosphate Buffer Saline, pH: 7.4). Then, antigen retrieval was performed in a microwave oven with a citrate buffer

(pH: 6.0). Hydrogen peroxide was used to inhibit endogenous peroxidase activity. Subsequently, protein blocks (Ref: PL-125-HL, Lot: DB2411, PatoLab, Turkey) were administered to the sections to prevent non-specific binding. Following the blocking step, both liver and kidney sections were incubated overnight at 4°C with primary antibodies prepared by diluting antibody diluent (Ref: TA-125-UD, Lot: UD45348, Thermo Scientific, USA) at a 1/50 dilution ratio for IL-6 (Cat: E-AB-40073, Elabscience, USA), at a 1/100 dilution ratio for TNF- α (Cat: E-AB-22159, Elabscience USA) and at a 1/100 dilution ratio for NF- κ B p65 (Cat: AP05984, Bioassay Technology Laboratory, China). The sections were then incubated first with biotinylated anti-polyvalent (Ref: PL-125-HL, Lot: DB2411, PatoLab, Turkey) and then with streptavidin peroxidase (Ref: PL-125-HL, Lot: DB2411, PatoLab, Turkey). PBS was used for washing between steps until the chromogen incubation stage. To visualize immunoreactivity, the sections were incubated with diaminobenzidine (DAB) chromogen (Ref: TA-125-HD, Lot: HD47396, Thermo Scientific, USA) and then washed with distilled water. After counterstaining with Gill hematoxylin, the sections were washed with tap water. Subsequently, the sections, which were passed through increasing alcohol series and xylene, were mounted with entellan. Under a ZEISS Axiocam 208 color light microscope, 10 random areas were selected from each liver and kidney section at 400X magnification and photographed using the ZEN3.1 computer-assisted imaging program. The density of immunopositive areas was determined as a percentage using the ImageJ program (National Institutes of Health, Bethesda, Maryland, USA). At the end of the evaluations, statistical data was generated for the immunohistochemical staining density of each primary antibody.

Deparaffinization of FFPE Tissue Samples

Formalin-Fixed Paraffin-Embedded (FFPE) tissue cores were deparaffinized by two sequential Xylene washes (1 mL each), followed by incubation at 50°C for 3 min and centrifugation at maximum speed for 2 minutes. The residual paraffin was removed after cooling the samples on ice. The tissue pellets were subsequently washed twice with 100% ethanol, homogenized in 700 μ L ethanol using a motorized tissue homogenizer (SCILOGEX, USA), and the volume was adjusted to 1 mL with 100% ethanol. Following centrifugation at maximum speed for 15 min, the ethanol was discarded, and the pellets were air-dried prior to RNA extraction¹⁷.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted using PureZol™ Reagent (Bio-Rad, USA; Cat. No. 7326890) according to the

manufacturer's instructions. Cells were washed with PBS (pH 7.5), lysed in 500 µL PureZol™, and mixed with 200 µL chloroform. Following centrifugation at $12,000 \times g$ for 20 min at 4°C, the aqueous phase was transferred to a fresh tube, and RNA was precipitated with an equal volume of isopropanol overnight at -20°C. The RNA pellet was collected by centrifugation, washed twice with 75% ethanol at $7,500 \times g$ for 5 min at 4°C, air-dried, and resuspended in 40 µL nuclease-free water. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and samples were stored at -20°C until further analysis¹⁸.

Following total RNA extraction, complementary DNA (cDNA) was generated through reverse transcription using the iScript™ High-Fidelity cDNA Synthesis Kit (Bio-Rad, USA; Cat. No. 1708891) according to the manufacturer's recommendations. For each reaction, 6 µL of RNA template was mixed with 4 µL of reaction buffer, 2 µL of reverse transcriptase, and 8 µL of nuclease-free water, yielding a final reaction volume of 20 µL. Reverse transcription was performed under the following thermal cycling conditions: primer annealing at 25°C for 5 min, cDNA synthesis at 64°C for 20 min, and enzyme inactivation at 95°C for 1 min. Upon completion of the program, samples were maintained at 4°C until further use¹³.

The expression levels of *TNF-α* and *IL-6* genes were quantified by real-time quantitative polymerase chain reaction (RT-qPCR) using a LightCycler® 480 System (Roche, Germany). Amplification reactions were performed with SYBR Green I Master Mix (Roche, Germany; Cat. No. 04707516001) following the manufacturer's protocol. Each 20 µL reaction mixture consisted of 10 µL SYBR Green Master Mix, 0.5 µL of each forward and reverse primer (10 pM) (Table I), 4 µL of cDNA template, and 5 µL of nuclease-free water. The experiment was performed using three independent biological replicates, each analyzed in triplicate as technical replicates. *β-Actin* was used as the endogenous reference gene for normalization. Relative gene expression levels were determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method¹⁹.

Table I. Sequences of primers for real-time PCR analysis

Gene	Primer Sequence	
<i>TNF-α</i>	Forward	5'ATGGGCTCCCTCTCATCAGTTCC3'
	Reverse	5'GCTCCTCCGCTTGGTGGTTTG3'
<i>IL-6</i>	Forward	5'ACTTCAGCCAGTTGCCTTCTTG3'
	Reverse	5'TGGTCTGTTGTGGGTGGTATCCTC3'

Table II. Thermal Cycling Parameters Used for qPCR Amplification and Melt Curve Analysis

Step	Temperature (°C)	Time	Cycles	Description
Initial Denaturation	95	5 minutes	1	DNA denaturation
Denaturation	94	20 seconds	40	Strand separation
Annealing	55	20 seconds	40	Primer binding
Extension	72	45 seconds	40	DNA synthesis

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 10 and R software (version 4.5.1). Data distribution was assessed using normality tests to determine the suitability of parametric or non-parametric statistical approaches. Quantitative data obtained from histochemical, immunohistochemical, and RT-qPCR analyses were evaluated using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Graphical representations of the data were generated using R software (version 4.5.1). Statistical significance was defined as $p < 0.05$.

Results

Histochemical Results

Liver sections stained with H&E were evaluated and scored for hepatocyte degeneration, sinusoidal dilatation and hyperemia, mononuclear cell infiltration, and necrosis, and it was observed that the liver tissues of the control group had a normal histological structure. No significant histopathological changes were detected in the liver tissues of the 0-min and 10-min experimental groups; however, compared to the control group, hepatocyte degeneration and mild sinusoidal dilatation were observed in some areas. Hepatocyte degeneration was more pronounced in the liver tissues of the 25-min experimental group compared to the control group, but there was no significant difference between the two groups. Compared to the control group, focal necrotic hepatocytes and a marked degree of hepatocyte degeneration with sinusoidal dilatation were detected in the liver tissues of the 40-min experimental group. Hyperemia was negligible in the liver tissues of all groups. Statistical analysis revealed that only sinusoidal dilatation and hyperemia were significantly increased in the experimental group at 40-min compared to the control group. Photographs showing H&E staining of liver sections from the groups and graphs comparing the scoring data for each group are presented in Figure 1.

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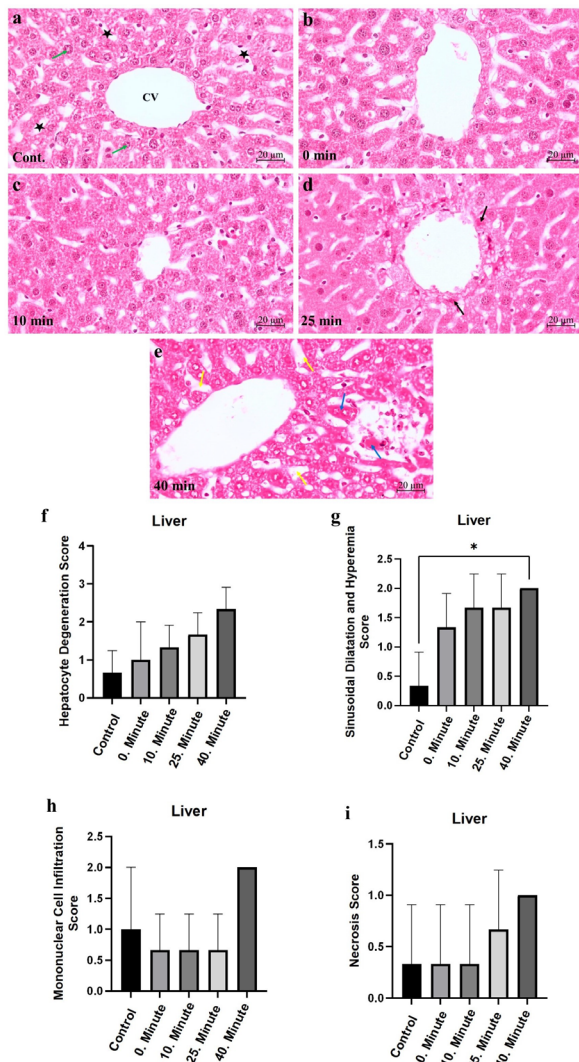


Figure 1:

H&E stained images of liver tissues from the groups. Histological image of liver tissue from (a) the control group, CV: central vein, green arrows: hepatocytes, black stars: sinusoids, (b) the group. (c) the 10-min group, (d) the 25-min group, black arrows: degenerated hepatocytes. (e) The 40-min group, yellow arrows: dilated sinusoids, blue arrows: necrotic hepatocytes. Microscope magnification 40x, scale bar 20µm. (f, g, h, i) A significant difference ($*p=0.0180$) was detected between the control group and the 40-min experimental group in terms of sinusoidal dilatation and hyperaemia. No significant differences were found between the groups in terms of hepatocyte degeneration, mononuclear cell infiltration, and necrosis ($p=0.0969$, $p=0.1094$, $p=0.4516$, respectively).

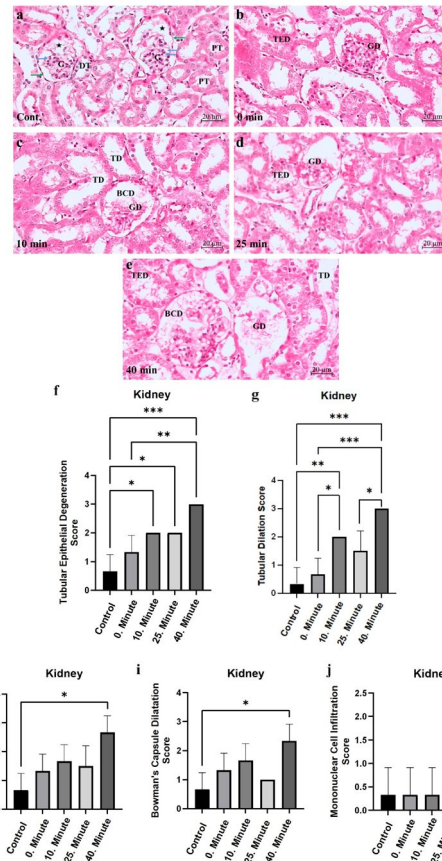


Figure 2:

H&E stained images of kidney tissues from the groups. Histological image of kidney tissue from (a) the control group, G: glomerulus, PT: proximal tubule, DT: distal tubule, green arrows: parietal layer of Bowman's capsule, blue arrows: capillaries in the glomerulus, black stars: Bowman's space; (b) the 0-minute (basal) group, GD: glomerular degeneration, TED: tubular epithelial degeneration; (c) the 10-minute group, TD: tubular dilatation, BCD: Bowman's capsule dilatation, GD: glomerular degeneration; (d) the 25-minute group, GD: glomerular degeneration, TED: tubular epithelial degeneration; (e) the 40-minute group, TD: tubular dilatation, BCD: Bowman's capsule dilatation, GD: glomerular degeneration, TED: tubular epithelial degeneration. Microscope magnification is 40x, the scale bar is 20µm. (f, g, h, i, j) Significant differences in tubular epithelial degeneration were observed between the control and experimental groups at 10, 25, and 40 minutes ($*p=0.0141$, $*p=0.0267$, and $***p=0.0003$, respectively). Additionally, a difference was detected between the 0-(basal) and 40-min groups ($**p=0.0034$). For tubular dilatation, the control group differed significantly from the 10- and 40-min groups ($**p=0.0095$ and $***p=0.0004$, respectively). Comparisons among time points also revealed differences between 0- and 10-min, 0 (basal)- and 40-min, and 25- and 40-min ($*p=0.0344$, $***p=0.0010$, and $*p=0.0333$, respectively). Regarding glomerular degeneration and Bowman's capsule dilatation, significance was reached only between the control and 40-min groups ($*p=0.0165$ and $*p=0.0284$, respectively). In contrast, mononuclear cell infiltration did not differ among groups ($p=0.7370$).

Kidney sections stained with H&E were evaluated and scored for tubular epithelial degeneration, tubular dilatation, glomerular degeneration, Bowman's capsule dilatation, and mononuclear cell infiltration. Histopathological evaluation revealed that kidney tissues from the control group had a normal histological structure compared to the experimental groups. Tubular epithelial degeneration, tubular dilatation, glomerular degeneration, and Bowman's capsule dilatation were observed to be increased in the experimental groups compared to the control group, but no statistically significant difference was found between the control group and the 0-min experimental group for any of these parameters. These increases were particularly significant between the control group and the 40-min experimental group. As in liver tissue, although mononuclear cell infiltration increased in the 40-min experimental group in kidney tissue, there was no significant difference between the groups. Photographs showing H&E staining of kidney sections from the groups and graphs comparing the scoring data between the groups are given in Figure 2.

Immunohistochemical Findings

No significant difference was found between the groups in terms of IL-6, TNF- α and NF- κ B immunoreactivity in liver and kidney tissues (Figure 3 and Figure 4). Photographs showing the IL-6, TNF- α and NF- κ B immunoreactivity in the liver and kidney tissues of the groups, and graphs comparing the immunoreactivity density according to the groups are given in Figure 3 and Figure 4, respectively.

Differential Expression of TNF- α and IL-6 Gene Expression in Liver and Kidney Tissues

To investigate molecular responses to insulin-induced acute hypoglycemia, the expression levels of pro-inflammatory cytokines, TNF- α , and IL-6, were analysed by RT-qPCR in both liver and kidney tissues. In liver tissue, TNF- α expression showed no statistically significant differences among the experimental groups. In contrast, significant alterations in TNF- α expression were detected in kidney tissue. The highest expression level was observed in the 10-min group, which exhibited significantly elevated TNF- α expression compared with the 0, 25, and 40-min groups (Figure 5). No other pairwise comparisons reached statistical significance. These findings indicate a transient increase in renal TNF- α expression shortly after the induction of hypoglycemia, followed by a decline.

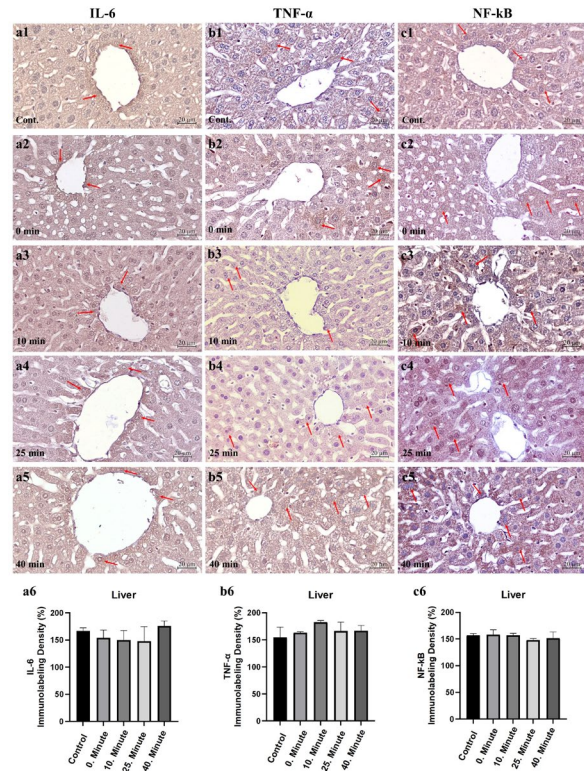


Figure 3:

Immunohistochemical staining images of IL-6, TNF- α , and NF- κ B in liver tissues from the groups, and graphs showing the density of immunopositive areas. In liver tissues from the groups expression of (a1-a5) IL-6, (b1-b5) TNF- α , (c1-c5) NF- κ B. Red arrows indicate immunopositive areas. Microscope magnification is 40x, scale bar is 20 μ m. (a6, b6, c6) No significant differences were found between groups in terms of IL-6, TNF- α , and NF- κ B immunoreactivities in liver tissues ($p=0.2507$, $p=0.1482$, $p=0.4137$, respectively).

Similar to TNF- α , no significant differences in IL-6 expression were observed among the experimental groups in liver tissue. In kidney tissue, however, IL-6 expression was significantly altered by acute hypoglycemia. The 10-min group exhibited the highest IL-6 expression level, showing significantly higher expression than the 0-min, 25-min, and control groups. In contrast, IL-6 expression in the 40-min group was significantly lower than that in both the 0-min and control groups (Figure 6). These results demonstrate a pronounced but transient induction of renal IL-6 expression during acute hypoglycemia, followed by suppression at later time points.

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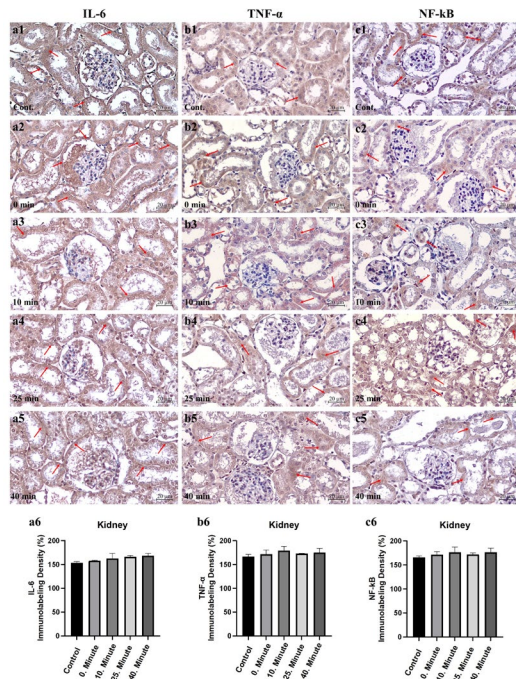


Figure 4:

Immunohistochemical staining images of IL-6, TNF- α , and NF- κ B in kidney tissues from the groups, along with graphs showing the density of immunopositive areas. Expression in kidney tissues of the experimental groups: (a1-a5) IL-6, (b1-b5) TNF- α , (c1-c5) NF- κ B. Red arrows indicate immunopositive areas. Microscope magnification is 40x, scale bar is 20 μ m. (a6, b6, c6) No significant differences were found between groups in terms of IL-6, TNF- α , and NF- κ B immunoreactivities in kidney tissues ($p=0.1123$, $p=0.3912$, $p=0.4944$, respectively).

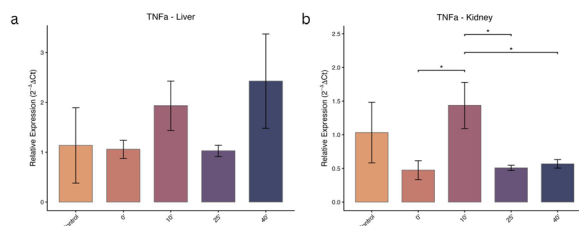


Figure 5:

Relative TNF- α mRNA expression levels in liver and kidney tissues. Gene expression was quantified by RT-qPCR, normalized to β -actin expression, and calculated using the $2^{-\Delta\Delta C_t}$ method. No significant differences in TNF- α expression were observed among the experimental groups in liver tissue. In contrast, significant alterations in TNF- α expression were detected in kidney tissue. Analysis revealed significant differences between the 10-min and 0-min (basal) groups ($*p = 0.0261$), the 10-min and 25-min groups ($*p = 0.0487$), and the 10-min and 40-min groups ($*p = 0.0438$). Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

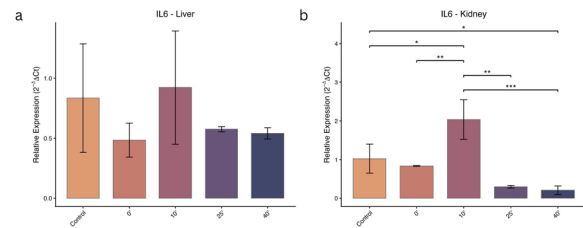


Figure 6:

Relative IL-6 mRNA expression levels in liver and kidney tissues. Gene expression was quantified by RT-qPCR, normalized to β -actin expression, and calculated using the $2^{-\Delta\Delta C_t}$ method. No significant differences in IL-6 expression were observed among the experimental groups in liver tissue. In contrast, significant alterations in IL-6 expression were detected in kidney tissue. Specifically, significant differences were observed between the 0-min and 10-min groups ($**p = 0.0076$), the 10-min and 25-min groups ($**p = 0.00126$), the 0-min and 40-min groups ($***p = 0.00048$), the control and 10-min groups ($*p = 0.0201$), and the control and 40-min groups ($*p = 0.0336$). Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

Discussion and Conclusion

Hypoglycemia is a common and potentially serious complication of intensive insulin therapy and represents a significant metabolic stressor capable of affecting multiple organ systems^{1,2}. Beyond its neurological consequences, accumulating evidence suggests that acute hypoglycemia may induce systemic physiological disturbances including oxidative stress, endothelial dysfunction, hormonal counter-regulation, and inflammatory activation^{4,20-22}. These responses are thought to contribute to tissue injury and may partly explain the adverse outcomes associated with recurrent or severe hypoglycemic episodes. Nevertheless, the mechanisms through which acute hypoglycemia affects peripheral organs remain incompletely understood, particularly at the tissue level.

The liver and kidney play central roles in maintaining glucose homeostasis during hypoglycemic conditions. The liver serves as the primary source of endogenous glucose production through glycogenolysis and gluconeogenesis, while the kidney contributes substantially to gluconeogenesis and systemic metabolic regulation^{2,5}. Consequently, both organs are directly exposed to the metabolic consequences of glucose deprivation and may be particularly susceptible to hypoglycemia-induced cellular stress. Previous studies have demonstrated that hypoglycemia induces significant hepatic metabolic

adaptations, including stimulation of glycogenolysis and gluconeogenesis, reflecting the liver's central role in glucose homeostasis^{23,24}. The kidney similarly contributes to systemic glucose regulation through renal gluconeogenesis and metabolic control, and hypoglycemia has been shown to challenge renal mitochondrial function and oxidative homeostasis^{21,25,26}. Nevertheless, the tissue-specific structural and inflammatory consequences of acute insulin-induced hypoglycemia in these organs remain insufficiently defined.

In the present study, acute insulin-induced hypoglycemia resulted in detectable histopathological alterations in both liver and kidney tissues. Notably, renal alterations were more prominent than hepatic changes and exhibited a progressive pattern throughout the experimental period. Tubular epithelial degeneration, tubular dilatation, glomerular degeneration, and Bowman's capsule dilatation increased gradually over time and were most pronounced in the 40-min group. In contrast, liver tissue demonstrated relatively mild alterations, with significant changes primarily limited to sinusoidal dilatation and hyperemia at later time points. These findings indicate that acute hypoglycemia is capable of inducing early structural tissue injury and suggest that the kidney may be more vulnerable to short-term glucose deprivation than the liver under the experimental conditions employed in this study.

The observed histopathological alterations are consistent with the concept that hypoglycemia acts as a metabolic stressor capable of disrupting cellular homeostasis. Previous experimental studies have demonstrated that hypoglycemia may impair mitochondrial function, alter antioxidant defense systems, and increase oxidative stress in both hepatic and renal tissues.²⁷ Reported significant alterations in glutathione metabolism, antioxidant enzyme activities, and mitochondrial electron transport chain complexes following insulin-induced hypoglycemia, supporting the notion that glucose deprivation may trigger metabolic disturbances capable of compromising tissue integrity. Such mechanisms may contribute to the structural alterations observed in the present study and suggest that early tissue injury during hypoglycemia may arise primarily from metabolic and oxidative stress rather than direct inflammatory damage.

Inflammation has been proposed as one of the mechanisms linking hypoglycemia to tissue injury. Several clinical and experimental studies have reported increases in circulating inflammatory mediators following acute hypoglycemic episodes. A systematic review by²⁸ demonstrated that acute hypoglycemia is associated with elevations in leukocyte counts and pro-inflammatory cytokines, including TNF- α and IL-6. In a rat sepsis model,

lipopolysaccharide-induced hypoglycemia was associated with elevated plasma concentrations of TNF- α , IL-1 β , and IL-6, while treatment that prevented hypoglycemia reduced these inflammatory markers²⁹. It was also demonstrated in humans that hypoglycemia leads to alteration in more than 15 inflammatory proteins, particularly TNF- α and IL-6³⁰. In a prospective case-control study, hyperinsulinemic hypoglycemia induced a transient increase in IL-6 in both healthy individuals and patients with type 2 diabetes, while TNF- α and other pro-inflammatory cytokines remained unchanged, and the inflammatory response returned to baseline within 24 hours³¹. Collectively, these findings suggest that hypoglycemia may provoke a systemic inflammatory response; however, this response appears to be dynamic, time-dependent, and may not involve sustained activation of all classical inflammatory mediators.

Based on this evidence, TNF- α and IL-6 were selected in the present study as representative inflammatory mediators because of their established roles in tissue injury and their documented association with both hepatic and renal disorders. TNF- α contributes to apoptosis, oxidative stress, and inflammatory cell recruitment^{9,10}, whereas IL-6 is a key regulator of acute-phase and immune responses¹¹. Moreover, elevations in these cytokines have been associated with various forms of liver and kidney injury³²⁻³⁴. Therefore, evaluation of TNF- α and IL-6 expression at both transcriptional and protein levels was considered an appropriate approach for investigating whether inflammatory signaling contributes to hypoglycemia-associated tissue alterations.

Interestingly, despite the presence of histopathological changes, immunohistochemical analyses revealed no significant differences in TNF- α , IL-6, or NF- κ B protein expression in either liver or kidney tissues. Furthermore, mononuclear cell infiltration was not significantly altered among groups. These findings suggest that the structural alterations observed following acute hypoglycemia were not accompanied by overt activation of local inflammatory pathways at the protein level within the experimental time frame. This observation may indicate that tissue injury develops before detectable inflammatory protein accumulation occurs, or alternatively, that inflammatory activation requires a longer duration or greater severity of hypoglycemic exposure. Therefore, the present findings suggest that early histopathological alterations may precede detectable changes in the selected inflammatory proteins rather than excluding a role for inflammation.

In contrast to the immunohistochemical findings, RT-qPCR analyses revealed significant alterations in TNF- α and IL-6 gene expression in kidney tissue. Although protein levels remained unchanged, transcriptional responses demonstrated a distinct

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temporal pattern characterized by an initial reduction, followed by transient upregulation at 10 minutes and subsequent downregulation at later time points. This finding suggests that acute hypoglycemia may induce rapid and dynamic molecular adaptations that are not necessarily reflected at the protein level during the early stages of tissue injury. One possible explanation is that the initial response reflects the direct anti-inflammatory effects of insulin, which has been shown to suppress inflammatory signaling pathways in several experimental models³⁵⁻³⁷. The transient increase observed at 10 minutes may correspond to activation of counter-regulatory stress responses triggered by declining glucose availability, whereas the subsequent reduction at later time points may reflect adaptive cellular mechanisms aimed at conserving energy during severe glucose deprivation. Although this interpretation remains speculative, it may explain the dissociation observed between transcriptional and protein-level findings.

An additional finding of interest was the greater susceptibility of kidney tissue compared with liver tissue. While previous studies have frequently emphasized hepatic metabolic adaptations during hypoglycemia, relatively limited information is available regarding direct renal tissue injury associated with acute glucose deprivation. In the present study, renal histopathological alterations were more pronounced and demonstrated clearer time-dependent progression than those observed in the liver. These findings raise the possibility that the kidney may be particularly sensitive to acute disturbances in glucose availability and warrant further investigation into the mechanisms underlying hypoglycemia-associated renal injury.

Several limitations should be considered when interpreting the present findings, including the limitation of experimental period at the early phase of acute hypoglycemia, which may not have been sufficient to detect protein-level inflammatory responses, and there is a need to evaluate a wider range of inflammatory mediators.

In conclusion, acute insulin-induced hypoglycemia produced early histopathological changes in liver and kidney tissues, with the kidney showing greater vulnerability. These alterations occurred without significant changes in TNF- α , IL-6, or NF- κ B protein expression, suggesting that early histopathological injury may precede detectable alterations in the selected inflammatory proteins during acute hypoglycemia. Given that metabolic and oxidative stress pathways were not directly evaluated, the mechanisms responsible for early tissue alterations remain to be determined. Nevertheless, the transcriptional upregulation of renal TNF- α and IL-6 indicates that hypoglycemia initiates rapid molecular responses that may precede detectable protein

changes. Together, these findings highlight tissue-specific susceptibility to hypoglycemia and underscore the need for further studies clarifying the temporal interplay between metabolic stress, inflammatory signaling and organ injury.

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