

REGULATION OF OXIDATIVE STRESS AND HEPATIC FUNCTION IN ACUTE KIDNEY INJURY

By

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Abstract

Acute kidney injury (AKI) is characterized by a rapid decline in renal function over a short period. Despite adequate dietary intake, patients with AKI, chronic kidney disease (CKD), or end-stage renal disease (ESRD) frequently exhibit folate deficiency. Under normal conditions, the kidney reabsorbs folate through a transporter-mediated mechanism to prevent urinary loss. In our previous study, we found a negative correlation between plasma creatinine and folate concentrations in rats with ischemia–reperfusion (IR)-induced AKI. AKI also affects distant organs such as the liver, a key regulator of metabolism and lipid homeostasis. Previous reports have shown that AKI induces hepatic injury characterized by oxidative stress, inflammation, and steatosis. This study investigated the protective effects of folate on hepatic lipid accumulation following IR-induced AKI.

Renal ischemia (45 min) followed by 24 h of reperfusion in Sprague–Dawley rats significantly increased plasma creatinine and aspartate aminotransferase, confirming kidney and liver injury. Biochemical analysis revealed marked hepatic oxidative stress, with decreased glutathione (GSH) and increased oxidized glutathione (GSSG) and malondialdehyde (MDA) levels. IR injury also promoted hepatic lipid accumulation, evidenced by elevated triglyceride and cholesterol levels. These changes correlated with reduced phosphorylation of AMP-activated protein kinase (AMPK), a central regulator of lipid metabolism. Correspondingly, fatty acid oxidation genes carnitine palmitoyltransferase-1 α (CPT1 α) and peroxisome proliferator-activated receptor- α (PPAR α) were downregulated, while lipogenesis-related genes sterol regulatory element-binding protein-1c (SREBP-1c) and acetyl-coA carboxylase1 (ACC1) were upregulated.

Administration of 5-methyltetrahydrofolate (5-MTHF) significantly improved renal and hepatic function, mitigated oxidative stress, restored glutathione balance, reduced lipid peroxidation, and

decreased hepatic lipid accumulation via AMPK activation. Complementary *in vitro* experiments using HepG2 cells showed that hydrogen peroxide (H₂O₂)-induced suppression of AMPK phosphorylation increased intracellular lipid accumulation, downregulated fatty acid oxidation genes, upregulated lipogenic genes, and elevated oxidative stress, all of which were reversed by folate treatment.

In conclusion, renal IR injury caused kidney and liver dysfunction by reducing folate levels and disrupting AMPK signaling, leading to oxidative stress and hepatic lipid accumulation. Folate supplementation restored AMPK activity, enhanced antioxidant defense, and protected both organs, suggesting that targeting folate metabolism and AMPK signaling may help prevent multi-organ injury following AKI.

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List of abbreviations

- (5-MTHF) 5-Methyltetrahydrofolate
- (ACC1) Acetyl-coA carboxylase-1
- (AFLD) Alcoholic fatty liver disease
- (AKI) Acute kidney injury
- (AMPK) AMP-activated protein kinase
- (AREs) Antioxidant response elements
- (AST) Aspartate aminotransferase
- (BUN) Blood urea nitrogen
- (CaMKK β) Calcium/calmodulin-dependent protein kinase kinase β
- (CAT) Catalase
- (cDNA) Complementary DNA
- (ChREBP) Carbohydrate-responsive element-binding protein
- (CKD) Chronic kidney disease
- (CPT1 α) Carnitine palmitoyltransferase-1 α
- (DAMPs) Damage-associated molecular patterns
- (DFE) Dietary folate equivalents
- (DMEM) Dulbecco's modified eagle medium
- (DNL) De novo lipogenesis
- (dTMP) Deoxythymidine monophosphate
- (dUMP) Deoxyuridine monophosphate
- (ECL) Enhanced Chemiluminescence
- (ER) Endoplasmic reticulum
- (ESRD) End-stage renal disease
- (FBS) Fetal bovine serum
- (FFAs) Free fatty acids
- (FOLR1) Folate receptor 1
- (GCLC/GCLM) Glutamate-cysteine ligase catalytic/modifier subunits
- (GFR) Glomerular filtration rate
- (GPx) Glutathione peroxidase
- (GR) Glutathione reductase

(GSH) Glutathione
(GSSG) Oxidized glutathione
(GTP) Guanosine triphosphate
(H₂O₂) Hydrogen peroxide
(HO-1) Heme oxygenase-1
(HOCl) Hypochlorous acid
(ICU) Intensive care unit
(IR) Ischemia-reperfusion
(Keap1) Kelch-like ECH-associated protein 1
(LKB1) Liver kinase B1
(MASLD) Metabolic dysfunction–associated steatotic liver disease
(MDA) Malondialdehyde
(MODS) Multiple organ dysfunction syndrome
(MPT) Mitochondrial permeability transition
(NEFAs) Non-esterified fatty acids
(Nrf2) Nuclear factor erythroid 2
(O₂) Singlet oxygen
(O₂^{•−}) Superoxide anion
(OH) Hydroxyl radical
(PABA) Para-aminobenzoic acid
(PBS) Phosphate-buffered saline
(PCFT) Proton-coupled folate transporter
(PPAR α) Peroxisome proliferator-activated receptor- α
(qPCR) Quantitative real-time polymerase chain reaction
(RFC) Reduced folate carrier
(RNS) Reactive nitrogen species
(ROS) Reactive oxygen species
(SAM) S-adenosylmethionine
(SCFAs) Short-chain fatty acids
(SDS-PAGE) SDS-polyacrylamide gel electrophoresis
(SOD) Superoxide dismutase

(SREBP-1c) Sterol regulatory element-binding protein-1c

(TAK1) Transforming growth factor- β -activated kinase 1

(TBARS) Thiobarbituric acid reactive substances

(THF) Tetrahydrofolate

(VLDL) Very low-density lipoproteins

CHAPTER I: LITERATURE REVIEW

1.1. Physiological function of kidneys

The kidneys are vital paired organs responsible for maintaining internal homeostasis. Specifically, they regulate fluid and electrolyte balance, remove metabolic waste, control acid-base status, and produce hormones such as erythropoietin and renin. Notably, each human kidney contains approximately 1 million nephrons, the functional unit of the kidney (Denic A. et al., 2016). These nephrons, in turn, perform three main functions: glomerular filtration (plasma is filtered through the glomerular capillaries into Bowman's space), tubular reabsorption (essential substances such as glucose, sodium, and water are reabsorbed into the bloodstream), and tubular secretion (additional waste products are secreted into the tubular fluid). Furthermore, the kidneys also play crucial roles in regulating extracellular fluid volume, maintaining electrolyte concentrations, excreting nitrogenous waste (urea and creatinine), producing active vitamin D (calcitriol), which is critical for calcium homeostasis (Hruska K. et al., 2009). In addition, blood flow to the kidneys is high, receiving 20–25% of the resting cardiac output via the renal arteries (Hall J. E. et al., 2011). Consequently, glomerular filtration rate (GFR) is an essential indicator of kidney function, normally averaging 90–120 mL/min/1.73 m² in healthy adults (Levey A. S. et al., 2009). To evaluate renal function, both blood urea nitrogen (BUN) and creatinine are routinely used to assess kidney function; however, creatinine is generally considered a more reliable indicator. Creatinine is generally considered a more reliable indicator of the GFR because it is produced at a relatively constant rate, filtered freely by the kidneys, and is less influenced by other factors (Levey A. S. et al., 2009). By contrast, BUN is useful as a complementary test but can be misleading on its own due to its sensitivity to hydration status, dietary protein, and liver function (Yeh T.-H. et al., 2024). The concept of kidney lifespan refers to the functional longevity of the kidneys over a person's lifetime (Denic A. et al., 2016). Although nephron number is fixed at birth and does not regenerate,

their functional capacity can adapt to physiological demands and pathological insults (Chevalier R. L., 2017). Nevertheless, several factors can shorten the lifespan of the kidneys: high blood pressure gradually damages the delicate filtering capillaries, diabetes places a chronic burden on the glomeruli through high blood sugar, chronic glomerulonephritis causes inflammation and scarring, nephrotoxic drugs and environmental toxins quietly erode function, and obesity increases filtration stress that ultimately harms the kidneys (Kuma A. & Kato A., 2022). Conversely, kidney health can be preserved through healthy lifestyle habits such as maintaining a balanced weight, exercising regularly, moderating salt and protein intake, and avoiding smoking; by keeping blood pressure under control with or without medications that reduce stress on the glomeruli; through careful glucose management in diabetes; by minimizing exposure to harmful drugs and toxins; and by monitoring kidney function in those at higher risk so that problems can be detected early (Luyckx V. A. et al., 2017). Ultimately, kidneys are given to us with a finite and nonrenewable supply of nephrons, and their longevity depends on whether damaging influences outweigh protective strategies or vice versa (Charlton J. R. et al., 2021).

Moreover, in experimental animal models (e.g. mice and rats), similar patterns of age-related renal decline have been observed, which makes them valuable in studying the mechanisms of renal aging, oxidative stress, and fibrosis (Hruska K. A. et al., 2009).

1.1.1. Pathophysiology of kidney failure

Kidney failure occurs when the kidneys lose their ability to adequately filter waste products, maintain fluid and electrolyte balance, and regulate acid–base homeostasis, leading to the accumulation of toxins and metabolic disturbances that can affect multiple organ systems.

Depending on the onset and progression, kidney failure is broadly classified into two categories (Chawla L. S. et al., 2014; Hoste E. A. J. et al., 2018).

Kidney injury can be classified into two main categories: acute kidney injury (AKI) and chronic kidney disease (CKD). AKI refers to a sudden decline in kidney function that develops over hours to days. It is often reversible if detected and managed promptly and can result from conditions such as severe dehydration, sepsis, obstruction of the urinary tract, or exposure to nephrotoxic drugs (Kellum J. A. et al., 2021; Thongprayoon C. et al., 2020). AKI is typically characterized by a rapid increase in serum creatinine, reduced urine output, and disturbances in fluid and electrolyte balance (Kellum J. A. et al., 2013). In contrast, CKD represents a gradual, progressive, and typically irreversible loss of kidney function for more than 3 months (Pethő Á. G. et al., 2024). CKD is commonly associated with long-standing conditions such as diabetes mellitus, hypertension, and chronic glomerulonephritis. It is characterized by a persistent decline in glomerular filtration rate, progressive nephron loss, and the gradual development of complications such as anemia, mineral and bone disorders, and cardiovascular disease (Luyckx V. A. et al., 2017). Therefore, the distinction between AKI and CKD is clinically important because it influences management strategies, prognosis, and the potential for renal recovery (Yeh T.-H. et al., 2024). As a consequence, reduced glomerular filtration rate (GFR) in AKI and CKD causes nitrogenous waste, mainly urea and creatinine, to build up in the blood, leading to a condition called azotemia (Chawla L. S., 2022). This retention of waste, in turn, can disrupt normal metabolic and electrolyte balance, leading to complications such as fatigue, nausea, anorexia, and in severe cases, uremic encephalopathy (Mercado M. G. et al., 2019). The underlying causes of reduced GFR are diverse and include three main mechanisms. First, glomerular damage whether from immune-mediated injury, diabetic nephropathy, hypertension, or toxins reduces the kidneys' filtering surface area

and efficiency. Second, decreased renal perfusion caused by hypovolemia, hypotension, or heart failure limits the delivery of blood to functioning nephrons, impairing filtration. Third, obstruction to urine flow anywhere along the urinary tract, such as from kidney stones, tumors, or enlarged prostate, increases intratubular pressure and reduces effective GFR. Often, these mechanisms may act in combination, compounding renal injury and accelerating the progression of kidney dysfunction. (Humphreys B. D., 2018; Kellum J. A., et al., 2021).

Furthermore, tubular injury and dysfunction are central features of AKI, where ischemia, nephrotoxic substances, or inflammatory processes damage the renal tubular epithelial cells. As a result, this cellular injury disrupts the tubules' ability to reabsorb essential substances such as sodium, water, glucose, and amino acids, while also impairing the secretion of waste products and hydrogen ions (Bonventre J. V. & Yang, C., 2011; Zuk A. & Bonventre J. V., 2016). Consequently, tubular dysfunction contributes significantly to the electrolyte imbalances, fluid retention, and acid–base disturbances observed in AKI. Moreover, tubular injury can trigger downstream effects, including activation of inflammatory pathways, endothelial dysfunction, and further compromise of renal perfusion, which can exacerbate kidney damage if not promptly managed (He J. et al., 2025)

Similarly, in CKD, similar tubular processes occur but evolve gradually. Over time, progressive tubular atrophy develops alongside ongoing nephron loss, and interstitial fibrosis accumulates in response to sustained injury and maladaptive repair mechanisms. This fibrosis further impairs tubular function, reducing the kidney's ability to concentrate urine, maintain electrolyte balance, and excrete metabolic wastes. Ultimately, the combination of tubular atrophy, interstitial fibrosis, and glomerulosclerosis leads to irreversible loss of renal function and contributes to the clinical

manifestations of CKD, including chronic azotemia, electrolyte disturbances, anemia, and metabolic bone disease (Humphreys B. D., 2018; Schelling J. R., 2016).

A key pathophysiological mechanism in CKD is compensatory hyperfiltration, whereby the remaining functional nephrons increase their filtration rate to offset lost renal function. However, this adaptive response leads to glomerular hypertension, further glomerular injury, and ultimately glomerulosclerosis (scarring), perpetuating the cycle of nephron loss and disease progression (Levey A. S. et al., 2009).

1.2. Acute kidney injury

AKI refers to a sudden loss of kidney function, which is identified by elevated serum creatinine levels and reduced urine output (oliguria). By definition, AKI is limited to a duration of up to 7 days (Kellum J. A. et al., 2013). It represents one category within a broader spectrum of functional kidney conditions known as acute kidney diseases and disorders (AKD), which can range from mild and self-limiting to severe and persistent forms (Kellum J. A. et al., 2021). AKD is characterized by abnormalities in kidney function and/or structure lasting between 7 and 90 days, with implications for health. This definition encompasses a range of clinical scenarios, including progressive kidney dysfunction without prior AKI and persistent dysfunction following AKI. In cases where AKI does not resolve within 7 days, the condition transitions into AKD. This persistent dysfunction may be due to incomplete recovery or ongoing structural damage, highlighting the importance of monitoring renal function beyond the acute phase. Furthermore, if kidney abnormalities persist beyond 90 days, the condition is reclassified as CKD (Hsu R. K. & Hsu C., 2016; Thongprayoon C. et al., 2020). This progression underscores the continuum of

kidney disease and the need for early detection and management strategies for AKI (Yeh T.-H. et al., 2024).

Since serum creatinine levels and urine output primarily reflect loss of kidney function rather than direct structural injury, the term can be somewhat misleading. In the absence of specific injury markers, individuals experiencing transient episodes of volume depletion may meet the diagnostic criteria for AKI without actual kidney damage. For example, a few hours of hypovolemia in an otherwise healthy person may temporarily raise serum creatinine without causing lasting harm (Kellum J. A. et al., 2013). However, when AKI persists despite appropriate volume resuscitation, it often indicates underlying structural damage to the kidneys. To clarify this distinction, it is helpful to briefly describe the pathological features of AKI. Histologically, AKI is typically characterized by tubular epithelial cell injury, loss of brush border, cell necrosis or apoptosis, and intratubular cast formation. Biochemical changes include increased markers of oxidative stress, inflammation, and endothelial dysfunction. Recognizing these pathological features helps differentiate transient functional changes from true kidney injury, which carries a higher risk of long-term renal impairment (Thomas M. E. et al., 2015).

In the absence of specific injury markers, individuals experiencing transient episodes of volume depletion may meet the diagnostic criteria for AKI without actual kidney injury. For instance, a few hours of volume depletion in an otherwise healthy person may have no lasting health consequences. Likewise, medications such as renin–angiotensin system inhibitors or other agents that affect glomerular filtration may cause modest, reversible increases in serum creatinine that do not indicate true kidney injury. However, when AKI persists despite appropriate volume resuscitation, it likely signals the presence of structural damage to the kidneys (Thomas M. E. et al., 2015).

1.2.1. Risk factors for AKI

Diverse factors contribute to the development and outcomes of acute kidney injury (AKI), influencing not only its onset but also its short-term severity and long-term prognosis (Hsu R. K. & Hsu C. 2016).

Environmental factors play a critical role, including exposure to nephrotoxins, infectious agents, dehydration due to heat or limited water access, and occupational hazards (Thongprayoon C. et al., 2020). Socioeconomic factors such as income level, education, and access to resources can determine the timeliness and quality of medical care, thereby affecting AKI outcomes (Gomes C. L. R. et al., 2023). Cultural factors may influence health-seeking behaviors, dietary practices, and adherence to medical advice, which in turn impact kidney health (Lewington A. J. P. et al., 2013). Healthcare-related factors encompass the availability and quality of medical services, hospital infrastructure, timely recognition of AKI, and the appropriate use of interventions such as fluid resuscitation, dialysis, or nephroprotective medications (AlSahow A. et al., 2024). Finally, Individual patient characteristics, including age, underlying chronic diseases (e.g., diabetes, hypertension, and CKD), genetic susceptibility, and physiological resilience, significantly influence both the risk of developing AKI and the subsequent trajectory toward recovery or chronic kidney disease (Mallamaci F. & Tripepi G., 2024).

Together, these interconnected factors underscore the multifactorial nature of AKI, highlighting the need for personalized prevention strategies and comprehensive management approaches that address both medical and social determinants of health (Kellum J. A. et al., 2021; Thongprayoon C. et al., 2020).

Less common causes of AKI involve genetic predispositions, which may lead to conditions such as myoglobinuria, resulting from rhabdomyolysis; hemoglobinuria, often associated with

hemolysis; or kidney stones (urolithiasis) that obstruct urinary flow and compromise renal function. In addition to these rarer causes, several other significant risk factors increase susceptibility to AKI (Mercado M. G. et al., 2019).

Severe illnesses and acute infections, including sepsis and malaria, can induce systemic inflammatory responses and hypotension, reducing renal perfusion and causing tubular injury. Significant trauma, low blood volume (hypovolemia), and hypotension also impair renal blood flow, while advanced age and pre-existing CKD decrease the kidneys' physiological reserve, making them more vulnerable to injury (Gameiro J. et al., 2020). Moreover, acute organ failure, major surgeries particularly cardiac surgery and stays in the intensive care unit (ICU) expose patients to additional risks, such as nephrotoxic medications, contrast agents, and opportunistic infections, which can precipitate AKI. Other contributing factors include chemotherapy for leukemia or other cancers, delayed graft function following kidney transplantation, autoimmune disorders that cause rapid renal damage, cholesterol crystal embolism, and urinary tract obstructions, all of which can directly impair kidney function (Kellum J. A. et al., 2021).

Overall, epidemiological studies indicate that the most common and clinically relevant risk factors for AKI are advanced age, pre-existing CKD, diabetes mellitus, sepsis, hypovolemia or dehydration, hypotension, exposure to nephrotoxic drugs, major surgery especially cardiac surgery, and critical illness or ICU admission (Hsu R. K. & Hsu C., 2016; Kellum J. A. et al., 2021; Thongprayoon C. et al., 2020). Recognizing these risk factors is essential for both preventive strategies and early intervention to mitigate the severity and complications of AKI.

1.2.2. AKI and micronutrient deficiency

Kidney ischemia-reperfusion is a leading cause of AKI. It occurs when blood flow to the kidney is abruptly reduced or completely halted, followed by the restoration of blood flow to the affected area (Zuk A. & Bonventre J. V. 2016). This process initiates a complex cascade of cellular and molecular events. Initially, oxygen and nutrient deprivation compromise cellular metabolism, leading to energy deficit and disruption of normal cellular function. Consequently, oxidative stress increases due to the accumulation of reactive oxygen species (ROS), resulting in lipid peroxidation, DNA damage, and protein oxidation. Simultaneously, the inflammatory response is activated, involving immune cell infiltration, upregulation of cytokines and chemokines, and complement activation. Together with mitochondrial dysfunction and impaired energy production, these processes ultimately cause apoptosis, necrosis, and widespread renal tissue injury, which collectively drive the onset and progression of AKI (Park S. W. et al., 2011; Wijerathne C. U. B. et al., 2022).

Beyond the kidney, AKI disrupts systemic homeostasis, producing metabolic disturbances that affect protein, carbohydrate, and lipid metabolism (Kellum C. et al., 2021). Skeletal muscle proteins undergo accelerated catabolism, amino acid turnover increases, and nitrogen balance becomes negative, contributing to malnutrition and muscle wasting (Doyle J. F. & Forni L. G., 2016). Moreover, AKI is often accompanied by hyperglycemia and insulin resistance, which may exacerbate metabolic stress, as well as alterations in lipid metabolism, including dyslipidemia and abnormal free fatty acid levels (Au-Yeung K. K. W. et al., 2023; Di Mario F. et al., 2025). In parallel, patients with AKI often exhibit dysregulated inflammatory responses, characterized by elevated levels of both pro- and anti-inflammatory cytokines, a phenomenon that occurs regardless

of the presence of sepsis. These inflammatory changes are strongly linked to an increased risk of mortality (Bihorac A. et al., 2013).

Furthermore, micronutrient deficiencies are common in AKI patients due to multiple interrelated mechanisms (Rabb H. et al., 2016). Increased nutrient loss occurs during renal replacement therapies, where water-soluble vitamins (e.g., B-complex, vitamin C) and trace elements (e.g., zinc, selenium) are removed through the dialysis membrane. Reduced nutrient intake, resulting from nausea, vomiting, altered mental status, or dietary restrictions during critical illness, further contributes to deficiencies. In addition, inflammatory and metabolic disturbances can alter micronutrient metabolism and distribution, while critical illness and sepsis (frequent complications in AKI) can cause micronutrients to become sequestered in tissues or redistributed away from plasma, lowering measurable blood levels. Oxidative stress and inflammation increase the body's demand for antioxidants, including vitamins C and E and selenium, which are essential for immune function, tissue repair, and mitigation of cellular injury (Berger M. M., 2006; Ostermann M. et al., 2020).

Overall, the combination of metabolic, inflammatory, and micronutrient disturbances underscores the systemic impact of AKI and highlights the need for early recognition, targeted supportive care, and careful management of nutrition and micronutrient status to improve patient outcomes.

1.2.3. Multiple organ dysfunction syndrome

AKI can trigger dysfunction in other organs, including the lungs, heart, liver, intestines, and brain. This harmful interplay between the kidneys and other organs, often referred to as the kidney organ crosstalk, can create a vicious cycle that amplifies systemic injury, leading to progressive multi-

organ damage (Husain-Syed F. et al., 2020; Wijerathne C. U. B. et al., 2020). In patients with preexisting conditions affecting the lungs, heart, liver, or intestines, deterioration of kidney function frequently exacerbates Multiple Organ Dysfunction Syndrome (MODS), which is typically associated with poor outcomes (Grams M. E. & Rabb H., 2012).

Importantly, AKI can induce distant organ injury even in the absence of preexisting comorbidities. This phenomenon is primarily mediated by systemic inflammation, oxidative stress, and immune dysregulation, which collectively disrupt the function of organs such as the heart, lungs, liver, and brain. Specifically, the inflammatory cascade triggered by AKI involves the release of cytokines, chemokines, and other pro-inflammatory mediators, which can damage endothelial cells, increase vascular permeability, compromise tissue integrity, and impair microcirculatory perfusion (Husain-Syed F. et al., 2020; Manrique-Caballero C. L. et al., 2021). In parallel, oxidative stress generated during AKI exacerbates cellular injury through the accumulation of ROS, lipid peroxidation, DNA damage, and mitochondrial dysfunction, further compromising distant organ function (Tomás-Simó P. et al., 2021).

Moreover, AKI is associated with immune dysregulation, including impaired innate and adaptive immune responses, which renders patients more susceptible to secondary infections. These infections can amplify systemic inflammation, creating a feedback loop that intensifies organ dysfunction. Additionally, disruptions in endothelial function, activation of coagulation pathways, and altered nitric oxide signaling contribute to microvascular dysfunction in remote organs, exacerbating tissue hypoxia and injury (Lee S. A. et al., 2018). Together, these mechanisms explain why AKI is not merely a renal disorder but a systemic disease with the potential to precipitate multi-organ injury, even in patients without prior organ-specific comorbidities.

Other mechanisms contributing to distant organ dysfunction include endothelial injury, apoptosis, necrosis, and heightened oxidative stress, which together compromise microcirculatory perfusion and tissue homeostasis (Abdallah H. M. I., 2021; Rabb H. et al., 2016). Collectively, these interconnected pathophysiological processes demonstrate that AKI acts as a systemic inflammatory and metabolic insult rather than an isolated renal event. This systemic impact explains why patients with AKI and concurrent multiple organ dysfunction consistently exhibit higher morbidity, mortality, and prolonged recovery times compared to those with isolated AKI. Therefore, early recognition of renal injury, along with timely intervention to prevent or mitigate extrarenal complications, is essential to improving survival and long-term outcomes in patients with AKI (Lee S. A. et al., 2018).

1.2.4. Renal ischemia-reperfusion and liver effects

Renal ischemia-reperfusion (IR) injury occurs when blood supply to the kidneys is temporarily interrupted (ischemia) and subsequently restored (reperfusion). Although reperfusion is essential to restore oxygen and nutrient delivery to the affected tissue, it paradoxically exacerbates renal injury through multiple interconnected mechanisms (Zuk A. & Bonventre J. V., 2016). Initially, the sudden return of oxygen during reperfusion generates ROS, which induce oxidative stress, lipid peroxidation, protein oxidation, and DNA damage, ultimately impairing cellular integrity (Malek M., 2015). Moreover, ischemia-reperfusion activates inflammatory pathways, leading to the recruitment and activation of immune cells such as neutrophils and macrophages, which release cytokines, chemokines, and proteolytic enzymes that amplify tissue injury (Danobeitia J. S. et al., 2014). Concurrently, endothelial dysfunction occurs as a result of oxidative stress and

inflammation, causing increased vascular permeability, microvascular thrombosis, and impaired renal perfusion, which further compromises tissue oxygenation (Basile D. & Yoder M., 2014). In addition, mitochondrial dysfunction during ischemia and reperfusion impairs ATP production, disrupts ion homeostasis, and triggers apoptotic and necrotic cell death (Li C. et al., 2024). Taken together, these mechanisms contribute to tubular epithelial injury, glomerular dysfunction, and interstitial inflammation, driving the development and progression of AKI following ischemia-reperfusion events.

Importantly, AKI is increasingly recognized not merely as an isolated renal event but as a systemic condition with significant effects on distant organs, including the liver. The liver plays a central role in maintaining metabolic homeostasis, regulating immune responses, and detoxifying endogenous and exogenous compounds. Consequently, AKI disrupts these hepatic functions through complex pathophysiological mechanisms that involve oxidative stress, systemic inflammation, and immune dysregulation (Lee S. A. et al., 2018). Specifically, during AKI, the kidneys generate ROS as part of the inflammatory response, which can circulate and induce oxidative stress in the liver, leading to hepatocellular injury, mitochondrial dysfunction, and apoptosis (He J. et al., 2025). Furthermore, AKI activates systemic inflammatory pathways, resulting in the release of cytokines and other inflammatory mediators that reach the liver and activate hepatic macrophages, potentially progressing to acute liver failure (Lee S. A. et al., 2018). Additionally, the immune response in AKI can alter immune cell populations and function, compromising the liver's ability to respond to infections or other insults and increasing the risk of hepatic dysfunction (Sun S. et al., 2023).

In patients with cirrhosis, AKI can precipitate hepatorenal syndrome (HRS), a condition characterized by renal vasoconstriction and impaired renal perfusion. This syndrome exemplifies

the bidirectional relationship between the liver and kidneys, in which dysfunction in one organ exacerbates injury in the other (Golab F. et al., 2009). Experimental IR and nephrectomy models in rodents have consistently demonstrated increased hepatic ROS generation, lipid peroxidation, and neutrophil infiltration following renal injury (Golab F. et al., 2009; Shang Y. et al., 2016). Moreover, cytokines such as TNF- α , IL-6, and IL-1 β released during AKI propagate systemic inflammation, thereby exacerbating liver dysfunction and impairing its capacity to maintain redox balance and metabolic stability (Shang Y. et al., 2020).

Taken together, the liver's response to AKI involves both direct injury mechanisms—including oxidative stress and mitochondrial dysfunction—and indirect pathways mediated by systemic inflammatory mediators and leukocyte activation. Furthermore, the crosstalk between injured kidneys and the liver is thought to be mediated via damage-associated molecular patterns (DAMPs), cytokines, and altered hemodynamic factors. Ultimately, this systemic interplay contributes to what is now termed multi-organ dysfunction syndrome (MODS) in severe AKI, highlighting the importance of understanding kidney–liver interactions in critically ill patients (Grams M. E., & Rabb H., 2012; Husain-Syed F. et al., 2020).

1.3. Oxidative stress

The recognition of free radicals as biologically significant entities began in the mid-20th century. During the 1940s and 1950s, researchers started to uncover their potential role in physiology and pathology. A pivotal moment came when Gerschman et al. (1954) suggested that oxygen toxicity and ionizing radiation shared a common mechanism mediated by free radicals, marking one of the

earliest connections between free radical biology and disease processes (Gerschman R. et al., 1954).

Shortly thereafter, Denham Harman (1956) advanced the Free Radical Theory of Aging, proposing that free radicals cause cumulative molecular and cellular damage, thereby driving the aging process and predisposing to degenerative diseases (Harman D., 1956). This idea was revolutionary, as it shifted scientific thinking by linking oxidative chemistry directly to biological aging and pathology.

In the 1960s and 1970s, experimental discoveries strengthened this emerging field. McCord and Fridovich (1969) identified superoxide dismutase (SOD) as a key endogenous antioxidant enzyme capable of neutralizing superoxide radicals, thus providing the first biochemical evidence of evolved cellular defenses against free radical damage (McCord J. M., & Fridovich I., 1969). Around the same time, other antioxidant systems, such as catalase and glutathione peroxidase, were also described, establishing that living organisms had intricate protective mechanisms to maintain redox balance (McCord J. M. & Fridovich I., 1969).

Building on these foundations, Helmut Sies (1985) introduced the concept of "oxidative stress," formally defining it as an imbalance between the excessive generation of ROS and the capacity of antioxidant defenses (Sies H., 1986). This term provided a unifying framework for understanding how oxidative damage contributes not only to aging but also to a wide spectrum of diseases, including cancer, neurodegeneration, cardiovascular disorders, and kidney injury (Sies H., 1986). Oxidative stress arises in living organisms when there is a disruption in the delicate balance between the generation of ROS and the body's antioxidant defense systems. ROS encompass both free radicals, such as superoxide anion (O_2^-) and hydroxyl radical ($^{\circ}OH$), and non-radical oxidants like hydrogen peroxide (H_2O_2). Under normal physiological conditions, ROS are continuously

produced as natural byproducts of aerobic metabolism, particularly within the mitochondria during oxidative phosphorylation. At controlled levels, these molecules play important roles in intracellular signaling, host defense, and redox regulation (Tomsa A. M., et al., 2019).

However, when ROS are generated in excessive amounts or when antioxidant defenses including enzymatic systems like SOD, catalase, and glutathione peroxidase, as well as non-enzymatic antioxidants such as vitamins C and E are insufficient, oxidative stress develops. In this state, highly reactive molecules attack and oxidize essential cellular macromolecules. Lipid peroxidation compromises membrane integrity and fluidity, protein oxidation disrupts enzyme activity and structural stability, and oxidative DNA damage leads to mutations that compromise genomic stability (Sies H., 2020).

Over time, the cumulative effects of this molecular injury contribute to the onset and progression of diverse pathological conditions, including neurodegenerative diseases, cancer, cardiovascular disorders, diabetes, liver injury, and acute kidney injury. Thus, oxidative stress is not merely a byproduct of cellular metabolism; rather, it represents a central pathophysiological mechanism that connects redox imbalance to the onset and progression of numerous human diseases (Jones D. P., 2006; Sies H., 2020).

1.3.1. Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules, often referred to as free radicals, that are derived from molecular oxygen (O_2). Under physiological conditions, cells continuously generate ROS as natural byproducts of metabolic processes, most notably during mitochondrial oxidative phosphorylation, where oxygen is consumed to produce ATP. Importantly, ROS are not

inherently detrimental. Rather, they exhibit a dual role in biology: at physiological concentrations, they act as essential signaling molecules, whereas at elevated levels, they contribute to cellular and tissue damage (Sies H., 2020).

ROS encompass a diverse group of species that can be broadly classified into two categories. The first includes free radicals, which contain unpaired electrons and are therefore highly reactive, such as superoxide anion ($O_2^{\circ-}$) and hydroxyl radical ($^{\circ}OH$). The second category consists of non-radical derivatives, which lack unpaired electrons but remain reactive, including hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), and hypochlorous acid ($HOCl$). These reactive molecules arise from multiple cellular compartments: mitochondria during ATP production, NADPH oxidase (NOX) enzymes at the plasma membrane, peroxisomes during fatty acid metabolism, and the endoplasmic reticulum during protein folding. In addition, immune cells such as neutrophils and macrophages produce large amounts of ROS during the respiratory burst, an antimicrobial defense mechanism designed to eliminate invading pathogens (Garcia J. et al., 2010).

At low or moderate concentrations, ROS are indispensable regulators of physiological processes, including cellular proliferation, differentiation, apoptosis (programmed cell death), and innate immune responses. However, when their levels exceed the neutralizing capacity of endogenous antioxidant defenses, a condition termed oxidative stress arises. Under such circumstances, ROS can damage critical cellular macromolecules lipids, proteins, and nucleic acids thereby disrupting membrane integrity, enzyme function, and genomic stability. Persistent oxidative stress is a well-established contributor to the pathogenesis of numerous diseases, ranging from cardiovascular disorders and neurodegenerative conditions such as Alzheimer's and Parkinson's disease, to cancer, chronic kidney disease, and liver injury (Joshi R. et al., 2001).

1.3.2. Antioxidant defense

Antioxidant defense refers to the complex biological system that protects cells from damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive molecules are byproducts of normal cellular metabolism but can increase during stress, leading to oxidative stress (Sies H., 2020). The antioxidant defense system includes enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and non-enzymatic antioxidants such as glutathione (GSH), vitamins C and E, flavonoids and carotenoids (Asbaghi O. et al., 2021).

Glutathione (GSH) is a tripeptide antioxidant made of glutamate, cysteine and glycine. It's the most abundant intracellular non-enzymatic antioxidant in mammalian cells, and critical for maintaining redox homeostasis, detoxifying ROS, and modulating cell signaling and immune responses (Lu S. C., 2009; Townsend D.M. et al., 2003). In neutralizing reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), two molecules of reduced glutathione convert H_2O_2 into water via glutathione peroxidase (GPx), producing oxidized glutathione (GSSG), which is then recycled back to GSH by glutathione reductase (GR) using NADPH. The cellular GSH:GSSG ratio that is typically maintained at over 100:1 in healthy cells serves as a sensitive marker of oxidative stress (Forman H. J. et al., 2009; Jones D. P., 2006).

Ischemia-reperfusion, nephrotoxins, or sepsis can cause a surge in ROS/RNS. GSH levels rapidly decline due to overconsumption in ROS detoxification and impaired regeneration. Depletion leads to oxidative damage to lipids, proteins, and DNA, mitochondrial dysfunction, activation of pro-inflammatory and pro-apoptotic pathways, and disruption of redox-sensitive signaling (e.g., NF- κ B, Nrf2) (Ngo V. & Duennwald M. L., 2022; Zhang J. et al., 2016). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that, when activated under oxidative stress,

upregulates genes involved in GSH synthesis (like GCL and GS), promoting antioxidant defense. Experimental AKI models consistently demonstrate decreased renal GSH levels, elevated GSSG and a reduced GSH:GSSG ratio, and correlation with histological injury, renal dysfunction, and oxidative stress markers (Tomás-Simó P. et al., 2021). Therapeutic approaches like N-acetylcysteine (NAC, a cysteine precursor) or agents targeting Nrf2 pathways aim to restore GSH homeostasis and mitigate injury (Aldini G. et al., 2018; Baird L. & Yamamoto M., 2020).

1.3.3. AKI and oxidative stress

A central feature in the pathophysiology of AKI is oxidative stress, an imbalance between ROS production and the body's antioxidant defenses (Tomsa A. M. et al., 2019). In AKI, excess ROS are produced in large quantities, especially during ischemia-reperfusion. These ROS damage cellular lipids, proteins, and DNA, leading to lipid peroxidation of cell membranes, mitochondrial dysfunction, endothelial and tubular epithelial cell injury, activation of inflammatory pathways via cytokine release (e.g., TNF- α , IL-1 β), and antioxidant defenses (e.g., SOD, catalase, glutathione) become overwhelmed and inactivated (Wijerathne C. U. B. et al., 2020). This oxidative stress-mediated damage worsens tubular necrosis, microvascular dysfunction, and inflammation, which accelerates kidney dysfunction (Wang P. et al., 2014; Zhang J. et al., 2016).

During ischemia, anaerobic metabolism becomes the primary energy source, leading to an accumulation of hydrogen ions and a decrease in intracellular pH (Kalogeris T. et al., 2012). To counteract this acidification, the Na⁺/H⁺ exchanger is activated, which expels hydrogen ions in exchange for sodium ions. This process results in a significant influx of sodium into the cell. Meanwhile, ischemia causes a depletion of cellular ATP, which inactivates essential ATP-

dependent ion pumps, such as the Na⁺/K⁺-ATPase. This impairment disrupts the balance of sodium and potassium ions in the cell (Chen W. et al., 2022). The loss of ATP also affects the active removal of calcium ions from the cell and limits their reuptake into the endoplasmic reticulum (ER), leading to an overload of calcium inside the cell. These ionic imbalances and energy deficits contribute to the opening of the mitochondrial permeability transition (MPT) pore, which disrupts the mitochondrial membrane potential and further inhibits ATP synthesis (Halestrap A. P., 2009). In cardiac cells, these events are accompanied by the activation of intracellular proteases, contributing to structural and functional cellular injury (Kalogeris T. et al., 2012). Just like in cardiac cells, when kidney cells such as tubular epithelial cells, podocytes, or endothelial cells are exposed to stressors like ischemia-reperfusion injury, toxins, sepsis, or oxidative stress, intracellular proteases including calpains, caspases, and cathepsins become activated (Kalogeris T. et al., 2012). These proteases degrade key structural and functional proteins, leading to cytoskeletal breakdown, mitochondrial dysfunction, disruption of cell–cell and cell–matrix adhesions, and the promotion of apoptotic or necrotic cell death (Havasi A. & Borkan S. C., 2011). For example, calpains are calcium-dependent proteases that become activated by intracellular Ca²⁺ overload, commonly occurring during ischemic injury. They target and degrade cytoskeletal proteins such as spectrin and talin in renal tubular cells (Goll D. E. et al., 2003). Caspases are involved in programmed cell death pathways and become activated during both apoptotic and necrotic processes in AKI (Bonventre J. V. & Yang L., 2011). Additionally, cathepsins, typically confined within lysosomes, can leak into the cytosol under stress conditions, contributing to proteolysis and cell death (Chen N. et al., 2018). This cascade of protease activation is central to the pathogenesis of AKI and progression of AKI to chronic, closely mirroring the cellular injury mechanisms seen in ischemic cardiac injury (Kalogeris T. et al., 2012). It is one of the key reasons

why the kidney is particularly vulnerable to oxidative stress and ischemia (Sutton T. A. et al., 2002).

The transcription factor Nrf2 plays a pivotal role in regulating the antioxidant defense system. Nrf2 promotes the expression of antioxidant enzymes to preserve redox homeostasis in the body. Under normal conditions, Nrf2 is bound to the repressor protein Keap1 in the cytoplasm. However, during oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it activates the expression of genes responsible for antioxidant enzyme production (Nezu M. & Suzuki N., 2020; Schmidlin C. J. et al., 2019; Wijerathne C. U. B. et al., 2022). During AKI, particularly when triggered by ischemia-reperfusion, toxins, sepsis, or oxidative insults, oxidative stress levels increase dramatically. In response, the Nrf2–Keap1 signaling pathway is activated to counteract the damage (Nezu M. et al., 2017). Elevated ROS modify critical cysteine residues on Keap1, disrupting its ability to ubiquitinate and target Nrf2 for degradation. This allows Nrf2 to accumulate in the cytoplasm and translocate into the nucleus, where it initiates the transcription of antioxidant and cytoprotective genes (Baird L. & Yamamoto M., 2020). Within the nucleus, Nrf2 binds to antioxidant response elements (AREs) in the promoter regions of its target genes, promoting the transcription of various antioxidant and cytoprotective enzymes, including heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic/modifier subunits (GCLC/GCLM), superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Loboda A. et al., 2016). However, although Nrf2 is initially activated as a compensatory defense mechanism, in cases of severe or prolonged AKI, this antioxidant response may become insufficient or dysregulated. Studies have demonstrated that Nrf2 activation can be transient or impaired in sustained injury models (Nezu M. & Suzuki N., 2020). Furthermore, persistent mitochondrial dysfunction and excessive ROS production can overwhelm the cellular antioxidant defenses (Wei J.-P. et al., 2025).

Notably, in several experimental AKI models, pharmacological activation of Nrf2 has been shown to improve outcomes by reducing oxidative stress, inflammation, and cell death (Wei W. et al., 2020).

1.4. Lipid metabolism in liver

The liver plays a central role in both lipid synthesis and degradation, as well as in fatty acid oxidation. Within hepatocytes, fats can be stored as triglycerides within lipid droplets or utilized for the production of phospholipids (Postic C. & Girard J., 2008).

Fat accumulation depends on the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis or fatty acid oxidation). Lipogenesis is the metabolic process by which acetyl-CoA is converted into fatty acids. This primarily occurs in the liver and adipose tissue (Kersten S., 2001). Hepatic lipid accumulation originates from several key sources (Fabbrini E. et al., 2010). One of these is *de novo* lipogenesis (DNL), a process where the liver converts excess glucose into fatty acids. Under normal conditions, DNL contributes minimally to liver fat stores. However, in metabolic diseases like metabolic dysfunction–associated steatotic liver disease (MDSLD), it can become a significant source of lipid accumulation (Donnelly K. L. et al., 2005). Another major contributor is the uptake of free fatty acids (FFAs) from peripheral tissues. During states of insulin resistance, obesity, or fasting, adipose tissue releases non-esterified fatty acids (NEFAs) through lipolysis. These circulating NEFAs are then taken up by the liver (Matsuzaka T. & Shimano H., 2011). Dietary fat also contributes to hepatic lipid content. After absorption in the intestine, dietary fats are packaged into chylomicrons. The liver subsequently takes up chylomicron remnants, further adding to its lipid pool (Gaggini M. et al., 2013). Reduced fatty acid oxidation plays an

additional role. When mitochondrial β -oxidation is impaired the liver's ability to break down fatty acids is compromised, resulting in their accumulation (Sunny S. et al., 2011). Finally, impaired triglyceride export can exacerbate lipid buildup. If the liver's capacity to assemble and secrete very low-density lipoproteins (VLDL) is disrupted, triglycerides remain trapped within hepatocytes, contributing to steatosis (Yamaguchi K. et al., 2007).

Lipid breakdown involves two key processes: lipolysis and fatty acid oxidation. Lipolysis occurs in adipose tissue, where hormones like catecholamines (epinephrine, norepinephrine) stimulate the breakdown of stored triglycerides into FFAs and glycerol, especially during fasting, exercise, or stress (Duncan R. E. et al., 2007). In contrast, insulin suppresses lipolysis by inhibiting hormone-sensitive lipase activity (Large V. et al., 2004). Once released, FFAs are transported to the liver and other tissues for fatty acid oxidation, which takes place in mitochondria. This process is enhanced during energy-demanding states such as fasting or prolonged exercise and is regulated by factors like low insulin, increased CPT1 α activity, and activation of AMP-activated protein kinase (AMPK) (Hardie D. G., 2007). Efficient oxidation also depends on mitochondrial function and regulators like PPAR α and thyroid hormone (Kersten S., 2001; Mullur R. et al., 2014).

However, disruptions in hepatic lipid metabolism can lead to the abnormal accumulation of triglycerides within hepatocytes, a hallmark of steatosis. This condition arises when there is an imbalance between lipid acquisition, storage, and disposal (Rinella M. E., 2015). This state, referred to as hepatosteatosis, is a defining feature of both alcoholic fatty liver disease (AFLD) and metabolic dysfunction-associated steatotic liver disease (MDSLD) (Buzzetti E. et al., 2016; Rinella M. E., 2015).

While triglycerides are the primary fat stored in the liver, excess free (unesterified) cholesterol is toxic to liver cells. It disrupts mitochondrial and cellular membranes, triggers oxidative stress, and

promotes inflammation and cell death. Cholesterol crystals can also activate immune cells, leading to fibrosis. Thus, hepatic cholesterol accumulation not only worsens steatosis but also contributes to liver injury and disease progression (Ioannou G. N., 2016).

1.4.1. AMP-activated protein kinase (AMPK)

AMPK is a widely present heterotrimeric enzyme that functions as a key energy sensor and regulator of cellular metabolism (Kim J. et al., 2016). Structurally, AMPK is composed of three subunits: the catalytic α -subunit (with two isoforms, $\alpha 1$ and $\alpha 2$), the structural β -subunit (with isoforms $\beta 1$ and $\beta 2$), and the regulatory γ -subunit, which contains the AMP-binding sites (including $\gamma 1$, $\gamma 2$, and $\gamma 3$). Activation of AMPK is triggered by an increase in the intracellular AMP: ATP or ADP:ATP ratios under conditions of energy stress, such as ATP depletion. Binding of AMP or ADP to the γ -subunit induces a conformational change that promotes the phosphorylation of the catalytic α -subunit at threonine-172 (Thr172). This phosphorylation is mediated by upstream kinases, including liver kinase B1 (LKB1), calcium/calmodulin-dependent protein kinase kinase β (CaMKK β), and transforming growth factor- β -activated kinase 1 (TAK1), depending on the cell type and physiological stimulus (Rodríguez C. et al., 2020).

AMPK plays a central role in regulating cellular and systemic energy balance. Under physiological conditions, it is activated in response to situations where ATP consumption exceeds production, leading to a rise in the intracellular AMP: ATP and ADP:ATP ratios. This occurs during metabolic stresses such as exercise, fasting, hypoxia, ischemia, and nutrient deprivation (Hardie D. G. et al., 2012).

Once activated, AMPK functions as a metabolic master switch by promoting energy-generating (catabolic) pathways, including glucose uptake, glycolysis, and fatty acid oxidation, to restore ATP levels. At the same time, it suppresses energy-consuming (anabolic) processes such as the synthesis of fatty acids, triglycerides, cholesterol, and proteins, which would otherwise deplete energy reserves. In this way, AMPK ensures that the balance between ATP production and consumption is maintained under varying physiological and environmental influences, such as nutrient availability, oxygen levels and temperature stress. (Song X. et al., 2020). In this way, AMPK serves as a master energy sensor and metabolic regulator, ensuring that energy production and expenditure remain balanced under diverse physiological and environmental conditions.

1.4.2. Role of AMPK in the liver

In the liver, AMPK acts as a central regulator of lipid metabolism by integrating signals related to the cellular energy state and modulating key metabolic pathways accordingly (Hardie D. G., 2008). Upon activation, AMPK shifts hepatic metabolism toward energy-generating processes while inhibiting energy-consuming pathways, particularly those involved in lipid biosynthesis (Viollet B. et al., 2006). A key target of AMPK in hepatic lipid metabolism is acetyl-CoA carboxylase-1 (ACC1), the rate-limiting enzyme in fatty acid synthesis. AMPK phosphorylates and inactivates ACC1, leading to a reduction in malonyl-CoA levels. This decreases not only limits substrate availability for fatty acid synthesis but also relieves inhibition on carnitine palmitoyltransferase-1 (CPT1 α) that facilitates mitochondrial fatty acid β -oxidation. AMPK also negatively regulates the activity of lipogenic transcription factors, including sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP). By suppressing

these transcription factors, AMPK reduces the expression of genes that are involved in fatty acid and triglyceride synthesis. Additionally, AMPK promotes lipid catabolism by increasing the expression of peroxisome proliferator-activated receptor- α (PPAR α) target genes involved in fatty acid oxidation pathways. It can further contribute to hepatic lipid clearance by stimulating lipophagy, a selective form of autophagy responsible for degrading lipid droplets (Herzig S. & Shaw R. J., 2018). While AMPK activation is typically considered a response to severe energy stress, evidence suggests it is also engaged by physiological stimuli such as exercise and nutrient deprivation, as well as pathophysiological conditions including prolonged starvation, ischemia-reperfusion injury, and chronic alcohol consumption. These findings highlight the broader role of AMPK in maintaining hepatic energy and lipid homeostasis under diverse conditions (Viollet B. et al., 2006).

1.4.3. Role of AMPK in ischemia reperfusion injury

In AKI, changes in AMPK signaling can profoundly impact the metabolic state of the kidneys and other organs (Day E. A. et al., 2017). During ischemia, ATP levels drop, leading to an increased AMP/ATP ratio, which activates AMPK. Activated AMPK inhibits anabolic (ATP-consuming) pathways and stimulates catabolic (ATP-producing) pathways like glycolysis and fatty acid oxidation. This metabolic shift helps preserve cellular energy and viability during ischemic stress. Notably, AMPK activation has been shown to mitigate ischemia-reperfusion-induced kidney injury by promoting autophagy, reducing oxidative stress, and preserving mitochondrial integrity (Kandula N. et al., 2022). Reperfusion rapidly generates ROS, leading to oxidative damage. AMPK activation reduces ROS by promoting mitochondrial biogenesis and function (Zhao Y. et

al., 2017). Moreover, it enhances antioxidant defenses (e.g., via upregulation of superoxide dismutase and catalase) and reducing mitochondrial ROS production by limiting excessive fatty acid oxidation during reperfusion (Rodríguez C. et al., 2020).

A growing body of evidence highlights a critical link between AMPK inactivation and inflammation in AKI (Kandula N. et al., 2022). In AKI, AMPK activity is frequently suppressed, which exacerbates renal inflammation and tissue injury (Guo Y. et al., 2014). Under normal conditions, AMPK exerts anti-inflammatory effects by inhibiting key pro-inflammatory pathways, notably the nuclear factor-kappa B (NF- κ B) signaling cascade (Kim J. et al., 2016; Mo C. et al., 2014). When AMPK activity declines during AKI, such an inhibition is lost, resulting in increased NF- κ B activation, elevated production of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β , and heightened recruitment of immune cells to the damaged kidney (Guo Y. et al., 2014). In parallel, AMPK inactivation disrupts mitochondrial function and elevates oxidative stress, both of which further potentiate inflammatory responses (Herzig S., & Shaw R. J., 2018). Collectively, the loss of AMPK's regulatory control fosters a self-perpetuating cycle of inflammation, oxidative damage, and progressive renal dysfunction in the setting of AKI (Salminen A. et al., 2011; Viollet B. et al., 2006).

In AKI, particularly following reperfusion, massive oxidative stress arises due to mitochondrial dysfunction, excessive ROS production, and infiltration of inflammatory cells (Zuk A. & Bonventre J. V., 2016). ROS can directly oxidize critical cysteine residues on AMPK itself or its upstream kinases such as LKB1 and CaMKK β , impairing their activity or interfering with AMPK phosphorylation at the essential Thr172 site (Herzig S. & Shaw R. J., 2018). Additionally, oxidative stress can activate protein phosphatases like PP2C and PP2A, which dephosphorylate and inactivate AMPK. These phosphatases are also regulated by ROS-sensitive signaling pathways

during AKI (Zaha V. G. & Young L. H., 2012). Paradoxically, although AMPK is typically activated in response to low ATP levels, the extensive mitochondrial injury and overwhelming ROS production in AKI can disrupt proper ATP/AMP ratio sensing and compromise the cellular energy-sensing machinery, including AMPK (Hinchey E. C. et al., 2018). Moreover, ROS-induced activation of the NF- κ B pathway and subsequent release of pro-inflammatory cytokines such as TNF- α and IL-1 β further exacerbate the situation (Andrade-Oliveira V. et al., 2019). These cytokines activate stress kinases like JNK and p38 MAPK, which can negatively regulate AMPK activity either directly or by enhancing the expression and activity of inhibitory phosphatases (Cuenda A. & Rousseau S., 2007).

1.5. Folate

Folate (vitamin B9) is an essential water-soluble B-complex vitamin that plays a fundamental role in human health. It occurs naturally in a wide variety of foods such as leafy green vegetables, legumes, citrus fruits, and liver and is also provided in the form of folic acid, the fully oxidized synthetic compound used in fortified foods and dietary supplements. From a biochemical perspective, all folates are derivatives of pteroylglutamic acid, and they are classified according to their reduction state and the nature of the one-carbon group attached (Bailey L. B. & Gregory J. F., 1999).

Structurally, folate is a conjugated molecule composed of three distinct but interconnected components that together define its chemical reactivity and biological functions. The first component is the pteridine ring system, a bicyclic heteroaromatic structure derived from pterin, which undergoes enzymatic reduction to form dihydrofolate and ultimately tetrahydrofolate

(THF), the biologically active form capable of carrying one-carbon units. Attached to the pteridine nucleus via a methylene bridge is the second component, para-aminobenzoic acid (PABA), which acts as a linker and provides the aromatic backbone connecting the pteridine moiety to the third component (Lucock M., 2000). The third region is a polyglutamate chain, composed of one or more glutamate residues joined through γ -peptide bonds. Naturally occurring folates in foods often contain multiple glutamate residues (polyglutamates), which enhance intracellular retention and binding affinity to folate-dependent enzymes. In contrast, synthetic folic acid typically exists in the monoglutamate form, which must be converted and polyglutamated within cells to participate efficiently in metabolic processes (Bekaert S. et al., 2008; Lucock M., 2000).

The central biological role of folate is as a carrier and donor of one-carbon units in a variety of oxidation states, a function that underpins its importance in cellular metabolism. These one-carbon transfers, primarily mediated through tetrahydrofolate (THF) derivatives, provide the chemical versatility necessary for multiple biochemical reactions. THF and its substituted forms serve as essential cofactors in pathways responsible for the synthesis, modification, and regulation of key biomolecules (Bailey L. B. et al., 2015).

One of the most critical functions of folate-dependent one-carbon metabolism is its involvement in de novo nucleotide biosynthesis. Specifically, folate derivatives such as 5,10-methylene-THF are required for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a reaction catalyzed by thymidylate synthase. This process supplies thymidine, a building block of DNA, and ensures proper DNA replication and repair. Folate is also indispensable in the synthesis of purine nucleotides, which are required for both DNA and RNA production. Impairments in these pathways due to folate deficiency can lead to faulty DNA

synthesis, resulting in genomic instability, megaloblastic anemia, and increased risk of developmental abnormalities (Bailey L. B., & Gregory J. F., 1999; Stover P. J., 2009).

Beyond nucleotide biosynthesis, folate-derived cofactors participate in the methylation cycle, where 5-methyltetrahydrofolate (5-MTHF) donates a methyl group for the remethylation of homocysteine to methionine. Methionine is then converted to S-adenosylmethionine (SAM), the universal methyl donor for methylation reactions throughout the body. This cycle regulates the methylation of DNA, histones, proteins, neurotransmitters, and phospholipids, thereby influencing gene expression, chromatin structure, cellular signaling, and membrane integrity. Proper methylation status is essential not only for normal cellular function but also for long-term epigenetic regulation and developmental programming (Crider K. S. et al., 2012; Lucock M., 2000).

Because of these diverse and indispensable roles, folate is especially critical for tissues with high rates of cell turnover and division. During embryonic and fetal development, when rapid DNA synthesis and cell proliferation are required for organogenesis, folate demand is greatly increased. Adequate maternal folate intake before and during pregnancy is crucial for preventing neural tube defects and supporting proper growth of the fetus. Similarly, in adults, folate is essential for maintaining hematopoietic function, reproductive health, and neurological integrity (Bailey L. B. et al., 2015; Lindzon G. & O'Connor D. L., 2007).

In clinical and nutritional contexts, folic acid is the most widely used form in fortified foods and supplements. It is more stable than natural food folates and has been successfully implemented in public health strategies to reduce the risk of neural tube defects during pregnancy. Supplementation also helps prevent and treat folate deficiency, which can lead to megaloblastic anemia and other health complications (Matherly L. H. & Hou Z., 2008).

Among the multiple folate derivatives, 5-methyltetrahydrofolate (5-MTHF) is the predominant form circulating in blood plasma and stored in tissues. It serves as the principal methyl group donor in the remethylation of homocysteine to methionine, a reaction catalyzed by methionine synthase in conjunction with vitamin B12 as a cofactor. This pathway is a cornerstone of one-carbon metabolism, linking folate status to methylation capacity and overall genomic stability (House J. et al., 2012; Woo C. W. H. et al., 2006). The natural stereoisomer L-5-MTHF is considered the biologically active form, often referred to as bioactive folate, and it has gained attention as a supplement alternative to folic acid due to its direct usability by cells without requiring enzymatic reduction (Lucock M., 2000).

1.5.1. Folate sources

The intestine is exposed to two primary sources of folate: dietary folate and folate produced by the gut microbiota. Among these, dietary folates are the most significant, as naturally occurring forms are abundant in leafy green vegetables, legumes, liver, and certain fruits. In these foods, folates are typically present as polyglutamated derivatives. Once ingested, they undergo intestinal hydrolysis and absorption, after which they are metabolized primarily to 5-MTHF, the major circulating form in plasma that supports one-carbon metabolism. By contrast, although the mammalian gut microbiota can produce small amounts of folate, this contribution is generally considered minor and insufficient to meet physiological needs. Consequently, dietary folate remains the dominant and essential source for maintaining systemic folate status (Crittenden R. G. et al., 2003; Lucock M., 2000). Together, these sources help sustain the body's folate pool, which is indispensable for several physiological processes, including nucleotide synthesis, methylation

reactions, and amino acid metabolism (Bailey L. B. et al., 2015; Tibbetts A. S. & Appling D. R., 2010).

Importantly, naturally occurring folates are predominantly present in foods as polyglutamated forms, in which multiple glutamate residues are attached to the folate molecule. Although these polyglutamates enhance folate retention and intracellular function, they are not efficiently absorbed in the human intestine. Therefore, before absorption, they must first be enzymatically cleaved to monoglutamate derivatives by the brush-border enzyme folylpolyglutamate hydrolase (also known as γ -glutamyl hydrolase or conjugase), which is located on the apical surface of enterocytes in the proximal small intestine. Once converted into the monoglutamate form, folates can be actively transported across the intestinal epithelium via the proton-coupled folate transporter (PCFT), which operates optimally in the mildly acidic environment of the proximal jejunum. After uptake, the absorbed folates undergo reduction and methylation within enterocytes, yielding primarily 5-MTHF. This circulating form ensures that folate enters the systemic circulation in a biologically active and transportable state, ready to participate in one-carbon metabolism (House J. et al., 2012; Rezk B. M. et al., 2003).

In contrast to natural folates, folic acid is a fully oxidized, monoglutamated form of folate that does not occur naturally in foods. Instead, it is added to fortified grain products (e.g., bread, cereals, flour, pasta) as well as to multivitamin and prenatal supplements. Folic acid is more chemically stable during storage and cooking compared to natural food folates and exhibits higher bioavailability, making it an effective source for supplementation and public health fortification programs (Pfeiffer C. M. et al., 2019).

Additionally, the gut microbiota particularly in the distal small intestine and colon, can produce folate. Certain gut bacteria possess genes encoding enzymes involved in the folate biosynthesis

pathway, converting guanosine triphosphate (GTP) to tetrahydrofolate (THF) derivatives. which can contribute to the body's overall folate supply (Engevik M. A. et al., 2019; Rossi M. et al., 2011). However, the degree to which microbial folate impacts an individual's folate status can vary based on factors such as diet and the composition of the gut microbiota (Kok D. E. et al., 2020).

The human gut microbiota is a diverse and dynamic microbial ecosystem, primarily dominated by the phyla Firmicutes and Bacteroidetes, with smaller contributions from *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*. These microorganisms play a critical role in host metabolism by fermenting and metabolizing dietary components, resulting in the production of a variety of metabolites, including short-chain fatty acids (SCFAs), long-chain fatty acids, lactic acid, and biogenic amines (Crittenden R. G. et al., 2003; Rossi M. et al., 2011).

Since mammals are unable to synthesize most vitamins endogenously, these essential micronutrients must be obtained from exogenous sources, namely the diet and, to a lesser extent, the gut microbiota (Crittenden R. G. et al., 2003). Notably, certain bacterial genera in the distal intestine such as *Bacteroides*, *Bifidobacterium* (Actinobacteria), and *Enterococcus* (Firmicutes) are capable of synthesizing a range of vitamins, including B1 (thiamine), B6 (pyridoxine), B12 (cobalamin), and B9 (folate). Although the contribution of microbial folate to systemic folate status in humans is still considered limited, it may support local intestinal metabolism and influence host folate homeostasis, particularly under conditions of low dietary intake (Engevik M. A. et al., 2019).

1.5.2. Bioavailability of folate

Bioavailability refers to the proportion of a nutrient that is digested, absorbed, and made available for use or storage in the body (Melse-Boonstra A., 2020). In the case of folate, bioavailability is highly variable and influenced by several factors, including the chemical form of folate (natural vs. synthetic), the food matrix, and the physiological condition of the individual. Thus, not all sources of folate contribute equally to meeting dietary requirements (Bailey L. B. et al., 2015).

Natural food folates, typically present as polyglutamated forms, require enzymatic hydrolysis before absorption. This processing step, along with losses during cooking and storage, contributes to their relatively modest bioavailability, which is estimated to range from 50% to 70% (Ohrvik V. E. & Witthoft C. M., 2011; Siatka T. et al., 2025). By contrast, synthetic folic acid the fully oxidized, monoglutamate form used in fortified foods and supplements is far more stable and does not require the same enzymatic cleavage, making it more efficiently absorbed. However, its bioavailability also depends on timing and context of intake: when consumed with meals, folic acid has an estimated bioavailability of 50–60%, while ingestion on an empty stomach increases this value to about 85% (Ohrvik V. E. & Witthoft C. M., 2011).

Because of these substantial differences, nutrient recommendations standardize intake using Dietary Folate Equivalents (DFE). By definition, 1 µg DFE equals 1 µg food folate, 0.6 µg folic acid from fortified food or supplements consumed with meals, and 0.5 µg folic acid when taken on an empty stomach (Gregory J. F., 2001; NIH Office of Dietary Supplements. Folate Fact Sheet for Health Professionals, 2023; Pfeiffer C. M. et al., 2019). This conversion system allows a more accurate assessment of total folate intake from diverse dietary and supplemental sources.

1.5.3. Dietary folate absorption

Folate absorption primarily occurs in the duodenum and jejunum, where an acidic microenvironment at the surface of intestinal epithelial cells facilitates uptake. Because the human body cannot synthesize folate endogenously, it must be obtained from external sources, with dietary folate and folic acid from fortified foods and supplements serving as the primary sources of folate for mammals (Siatka T. et al., 2025). This process has been extensively studied using a range of experimental models, including *in vivo* studies in humans and animals, as well as *ex vivo* and *in vitro* systems such as isolated intestinal segments, everted intestinal sacs, isolated intestinal tissues and cells, and membrane vesicles. These models have helped elucidate the mechanisms of folate transport, highlighting the importance of specialized carriers such as the proton-coupled folate transporter (PCFT), which operates optimally in an acidic milieu. Once absorbed, folates are transported into the enterocytes, where they undergo enzymatic reduction and methylation before entering the systemic circulation (Visentin M. et al., 2014). The pH at the transport interface is a critical determinant of the relative contributions of the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT) to overall folate uptake, particularly when both transporters are co-expressed within the same tissue and at the same site. At physiologic pH (7.4) RFC function is maximal, efficiently transporting folates across the membrane and PCFT activity is minimal to negligible, depending on the specific folate substrate. As pH decreases, RFC-mediated transport progressively declines while PCFT activity increases, due to its proton-coupled mechanism. This pH-dependent transporter switching ensures efficient folate absorption in the acidic microenvironment of the small intestine while also allowing for folate transport in systemic tissues where pH is neutral and RFC predominates (Zhao R. et al., 2009). Once inside the intestinal cells, folate is converted into its active form, 5-MTHF, which is then transported to various tissues

and organs throughout the body. Although the small intestine is the main site for folate absorption, there is minimal absorption in the colon. The primary source of folate for the body remains in the small intestine, where dietary folate and folic acid are absorbed and utilized to meet the body's needs for this essential vitamin (Kok D. E. et al., 2020).

1.5.4. Role of folate in health

Folate operates as a cofactor and coenzyme in one-carbon metabolism, a collection of biochemical reactions that transfer single-carbon units (methyl, methylene, formyl, formimino, and methenyl groups) crucial for cellular function (He Q. & Li J., 2023). Folate participates as tetrahydrofolate (THF) and its derivatives, which act as one-carbon donors or acceptors in a variety of reactions catalyzed by methyltransferases and other enzymes, including 1) DNA synthesis and repair, 5,10-methylene-THF donates a methylene group to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) via thymidylate synthase essential for DNA replication. Folate also contributes to purine synthesis; 2) amino acid metabolism, converts homocysteine to methionine via methionine synthase, using 5-methyl-THF as a methyl donor and vitamin B12 as a cofactor. Methionine is then converted to S-adenosylmethionine (SAM), the universal methyl donor for over 100 methylation reactions; 3) methylation reactions, Folate indirectly regulates DNA, RNA, histone, protein, lipid, and neurotransmitter methylation through its role in SAM synthesis. DNA methylation modulates gene expression, cell differentiation, and genome stability. Alterations in folate-dependent methylation are implicated in cancer and developmental disorders; 4) formylation reactions, 10-formyl-THF donates formyl groups in purine nucleotide biosynthesis, necessary for ATP and GTP formation (He Q. & Li J., 2023; Sobral A. F. et al., 2024). Collectively,

these processes position folate as an essential nutrient for maintaining genomic integrity, regulating epigenetic modifications, supporting cellular proliferation, and enabling normal growth and development. Impaired folate metabolism has been associated with a range of health consequences, including anemia, birth defects, cardiovascular disease, and cancer. Given its critical functions and the inability of mammals to synthesize folate endogenously, an adequate dietary intake through food and supplements remains vital to support health across the lifespan.

1.5.5. Folate deficiency

Although folate deficiency is relatively uncommon in the general population, particularly in countries with mandatory folic acid fortification programs. However, it remains a significant concern in specific clinical populations. Folate deficiency can still occur in individuals with increased metabolic demands, poor dietary intake, malabsorption syndromes, or chronic diseases. Notably, patients with conditions such as metabolic dysfunction–associated steatotic liver disease (MDSLD), chronic kidney disease, and certain gastrointestinal disorders are at increased risk of folate insufficiency (Bailey L.B. et al., 2015; NIH Office of Dietary Supplements. Folate Fact Sheet for Health Professionals, 2023). In these populations, impaired folate metabolism or increased oxidative stress may exacerbate folate depletion, contributing to disease progression through mechanisms like disrupted DNA synthesis, altered methylation patterns, and compromised antioxidant defenses (Sobral A. F. et al., 2024). Given its essential roles in nucleotide biosynthesis, methylation, and redox homeostasis, maintaining adequate folate status is critical for both disease prevention and management.

AKI, in particular, can contribute to low folate status through several interrelated mechanisms. Impaired renal function during AKI leads to reduced tubular reabsorption and increased urinary loss of folate (Bottiglieri T., 2000; Palevsky P. M. et al., 2013). Furthermore, the systemic inflammation and oxidative stress associated with AKI can impair intestinal absorption and accelerate folate degradation (Aparicio-Trejo O. E. et al., 2020). Supporting this, research has shown that during ischemia-reperfusion, there is a decrease in the mRNA and protein levels of folate receptor 1 (FOLR1) and reduced folate carrier (RFC) in kidney tissue of both male and female rats (Yang C. et al., 2021). This reduction is linked to a decline in Sp1/DNA binding activity, which leads to decreased FOLR1 expression in the kidney. Sp1, a transcription factor that regulates FOLR1 expression, plays a crucial role in this process. Silencing Sp1 has been found to significantly lower intracellular folate levels and increase IL-6 mRNA expression. These findings suggest that Sp1 contributes to the downregulation of FOLR1 expression, resulting in reduced folate reabsorption in tubular cells during ischemia-reperfusion injury (Yang C. et al., 2021).

1.5.6. Folate as an antioxidant

Folates, particularly in their reduced forms, exhibit notable antioxidant properties. They efficiently scavenge several types of free radicals, including DPPH radicals, and play an important role in protecting lipids from peroxidation (Hwang S.-Y. et al., 2011; Qin L. et al., 2014; Sarna L. K. et al., 2012). Folate operates as an effective *in vitro* scavenger of free radicals, capable of neutralizing thiol radicals and repairing thiol groups, which are central to cellular redox balance and antioxidant defense. Structurally, folic acid consists of purine- and pyrazine-type rings with two amino acid side chains on the pyrazine ring. Its lactim form contains a hydroxyl group on the purine-type ring,

which is particularly important in mitigating oxidative damage by reacting with oxidizing free radicals (Joshi R. et al., 2001).

Additionally, folic acid and its components, including pyrazine and pterin, can be readily reduced by hydrated electrons and acetone ketyl radicals, producing hydroderivatives within the pyrazine ring structure (Xu Y. et al., 2010). Although folate is water-soluble, it contributes to the inhibition of lipid peroxidation and supports cellular antioxidant defenses, further highlighting its multifunctional biological significance (He Q. & Li J., 2023; Lucock M., 2000).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a crucial regulator of oxidative stress (OS) and is widely distributed in oxygen-consuming organs such as muscle tissue, the heart, brain, liver, kidney, and blood vessels. Upon activation, Nrf2 promotes the expression of antioxidant enzymes, phase II detoxification enzymes, and anti-inflammatory factors (Cao P. et al., 2021). Under normal physiological conditions, Nrf2 is sequestered in the cytoplasm by its adapter protein, Kelch-like ECH-associated protein 1 (Keap1), and is continuously targeted for degradation via the ubiquitin-proteasome system, maintaining its activity at low basal levels. However, in the presence of excessive oxidative stress, Nrf2 dissociates from Keap1, becomes activated, and translocates into the nucleus, where it binds to antioxidant response elements (AREs) within the DNA. This interaction triggers the transcription of downstream antioxidant genes, including heme oxygenase-1 (HO-1), superoxide dismutase and glutathione synthesizing enzymes, contributing to cellular protection against oxidative damage (Baird L. & Yamamoto M., 2020). Keap1 contains several reactive cysteine residues, notably Cys151, Cys273, and Cys288, which act as sensors for oxidative and electrophilic stress. Under conditions of oxidative stress, these cysteine residues undergo modifications, leading to conformational changes in Keap1. These alterations disrupt the Keap1-Nrf2 interaction, inhibiting Nrf2 ubiquitination and allowing newly synthesized Nrf2 to

accumulate and translocate into the nucleus (Ngo V. & Duennwald M. L., 2022). Our previous study demonstrated that the injection of a low dose of the biologically active form of folic acid (5-MTHF) could reduce oxidative stress and enhance kidney function in rats with ischemia-reperfusion-induced AKI. The antioxidant effects of folic acid are partly mediated by the activation of Nrf2, which leads to the increased expression of antioxidant enzymes and the restoration of glutathione levels in the kidney (Wijerathne C. U. B. et al., 2022).

CHAPTER II: RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1. Rationale

AKI, particularly when caused by IR, leads to systemic oxidative stress that can damage distant organs, including the liver. Liver is a vital organ for detoxification, nutrient metabolism and storage. The oxidative burden can disrupt hepatic lipid metabolism, contributing to hepatic steatosis and liver injury. A key regulatory pathway in this process is the AMPK pathway, which maintains lipid homeostasis but may be suppressed under oxidative stress conditions.

Our previous studies indicate that IR injury can downregulate Sp1-mediated expression of the folate transporter in tubular cells, potentially reducing folate reabsorption and leading to low folate levels. Earlier findings also suggest that low-dose folic acid improves kidney function by activating Nrf2 and restoring antioxidant defenses, highlighting the potential of micronutrient supplementation to enhance clinical outcomes in AKI. Therefore, the biologically active form of folic acid, 5-MTHF, possesses antioxidant properties and may counteract oxidative injury while modulating metabolic signaling pathways such as AMPK. However, its effects on liver function and lipid metabolism following AKI remain largely unexplored.

2.2. Hypothesis

IR injury triggers the overproduction of ROS, leading to oxidative stress that extends beyond the initially affected organ. In this study, we hypothesized that IR-induced AKI initiates systemic inflammatory and oxidative stress responses that impair liver function by disrupting redox balance, depleting antioxidant defenses, and promoting lipid peroxidation and cellular damage. These processes may compromise hepatocellular integrity, disrupt hepatic lipid metabolism, and promote triglyceride accumulation within hepatocytes (hepatic steatosis), ultimately contributing to liver

injury. This distant organ crosstalk is likely mediated by elevated circulating pro-inflammatory cytokines and oxidative byproducts, which impair mitochondrial function and alter lipid regulatory pathways in the liver of affected rats. Furthermore, folic acid may also exert a protective effect on the liver against oxidative stress associated with AKI. Through enhancement of cellular antioxidant defenses and reduction of reactive oxygen species, folic acid supplementation may help preserve hepatic cellular integrity and function in conditions where oxidative stress is elevated due to kidney injury.

2.3. Objectives

General objective: This study aimed to determine whether 5-MTHF supplementation mitigates oxidative stress and improves hepatic lipid metabolism following AKI. By evaluating oxidative stress markers, antioxidant enzyme activity, and hepatic lipid content, the study seeks to clarify the protective role of folate against systemic oxidative injury and AKI-associated hepatic steatosis.

Specific objective: This study aimed to elucidate the molecular link between oxidative stress and the AMPK signaling pathway in regulating hepatic lipid homeostasis following AKI-induced systemic injury. Specifically, it examined how ischemia-reperfusion-induced oxidative stress affects AMPK phosphorylation and activity, and how these changes influence downstream targets involved in lipid synthesis and oxidation, including ACC1, SREBP-1c, PPAR α , and CPT1 α . Furthermore, it investigated whether folate treatment modulates this signaling axis to restore hepatic lipid balance.

Generally, this study aimed to (1) assess the effect of folate on oxidative stress and hepatic lipid metabolism in AKI, and (2) explore the interaction between oxidative stress and the AMPK

signaling pathway in this context. The findings may uncover new therapeutic strategies to prevent distant organ injury associated with AKI.

CHAPTER III: MATERIALS AND METHODS

3.1. Animal model - kidney ischemia-reperfusion

Male Sprague–Dawley rats (6 weeks old, weighing 250–300 g) were obtained from the University of Manitoba Central Animal Care Services (Winnipeg, MB, Canada). All animal procedures adhered to the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and were approved by the University of Manitoba Protocol Management and Review Committee. Animals were housed under controlled environmental conditions (22 ± 2 °C, 12 h light/dark cycle, 50–60% relative humidity) with ad libitum access to standard chow and water.

Rats were randomly assigned into three experimental groups (n = 8 per group):

Group 1. Ischemia–Reperfusion (IR): Rats were anesthetized, and the left renal pedicle was occluded for 45 minutes using a non-traumatic vascular clamp to induce ischemia. Reperfusion was achieved by removal of the clamp, restoring blood flow to the kidney. Following reperfusion, a right nephrectomy was performed to mimic reduced renal reserve, as previously described (Shang et al., 2016; Wang et al., 2014).

Group 2. Ischemia–Reperfusion with 5-MTHF Treatment (IR + 5-MTHF): Rats underwent the same IR procedure as described above. In addition, they received intraperitoneal injections of 5-methyltetrahydrofolate (5-MTHF; 3 µg/kg body weight; Sigma-Aldrich, Oakville, ON, Canada) administered 30 minutes prior to ischemia and again 3 hours after reperfusion. A stock solution of 5-MTHF (2 mg/mL) was freshly prepared in saline and diluted to the required concentration before injection.

Group 3. Sham-Operated Control (Sham): Rats underwent identical surgical procedures, including exposure of the renal pedicle, but without clamping to induce ischemia. Instead of 5-MTHF, they received equivalent volumes of saline intraperitoneally.

At 24 hours following reperfusion (or sham surgery), all animals were euthanized under deep anesthesia. Blood samples were collected into lithium heparin tubes, and plasma was separated by centrifugation at 3000×g for 20 minutes at 4 °C. Liver tissues were excised, washed in ice-cold saline, blotted dry, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. Plasma and tissue samples were subsequently used for biochemical and histological assessments. Kidney and liver functions were evaluated by measuring plasma creatinine and aspartate aminotransferase (AST) levels, respectively, using the Cobas C111 Analyzer (Roche, Laval, QC, Canada).

3.2. Cell culture

The human hepatoma cell line HepG2 (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; VWR, Radnor, PA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA). Cultures were grown under standard conditions in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were routinely passaged at 70–80% confluence and seeded at a density of 1×10^5 cells/mL into 6-well plates, 60 mm, or 100 mm tissue culture dishes, depending on the experimental design. After overnight attachment, cells were treated with 5-methyltetrahydrofolate (5-MTHF; 2 µg/mL) and/or hydrogen peroxide (H₂O₂; 200 µM) for 24 hours. Here, H₂O₂ served as an inducer of oxidative stress to mimic redox imbalance in hepatocytes. Untreated cells maintained in complete medium were used as negative controls, while H₂O₂-treated cells without 5-MTHF supplementation served as positive oxidative stress controls.

All experiments were performed in triplicate biological replicates, each with three technical replicates to ensure reproducibility.

To evaluate cell viability, a water-soluble tetrazolium salt (WST-1; Sigma-Aldrich, Taufkirchen, Germany) assay was performed according to the manufacturer's protocol. Briefly, HepG2 cells were seeded into 96-well plates (1×10^4 cells/well) and incubated overnight to allow adherence. The following day, cells were exposed to increasing concentrations of H_2O_2 (0–500 μM) for 24 hours to generate a dose–response curve and determine the concentration that induced ~50% reduction in viability (IC_{50}). After treatment, cells were gently washed twice with phosphate-buffered saline (PBS; pH 7.4) to remove residual compounds. Subsequently, 100 μL of fresh culture medium containing 10% WST-1 reagent was added to each well, and cells were incubated for 1 hour at 37 °C under standard culture conditions.

Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA) with a reference wavelength of 630 nm to correct for background signal. The extent of formazan dye formation, which directly reflects the metabolic activity of viable cells, was quantified. Cell viability was expressed as a percentage relative to the untreated control group (0 μM H_2O_2), which was set to 100%. Data from independent experiments were pooled, and results were reported as mean $\pm$ standard error of the mean (SEM).

3.3. Biochemical analysis

Malondialdehyde (MDA), a well-established biomarker of lipid peroxidation and oxidative stress, was quantified in both liver and plasma samples using the thiobarbituric acid reactive substances (TBARS) assay as described previously (Au-Yeung K. K. W. et al., 2023; Shang Y. et al., 2016).

Briefly, liver tissue or cultured cell pellets were homogenized in phosphate-buffered saline (PBS; 1:10, w/v) kept on ice to minimize oxidation during processing. The homogenates were incubated with 10% phosphotungstic acid for 10 minutes to precipitate proteins and other interfering compounds, followed by centrifugation at $1,000 \times g$ for 10 minutes at 4 °C. The resulting supernatants were collected and incubated with 0.67% thiobarbituric acid (TBA) at 95 °C for 1 hour in a water bath to allow the formation of a pink MDA–TBA adduct. Samples were then cooled to room temperature, and absorbance was measured at 532 nm using a spectrophotometer (Molecular Devices, San Jose, CA, USA). MDA concentrations were determined against a standard curve prepared with 1,1,3,3-tetraethoxypropane and normalized to total protein content. To further assess redox balance, reduced (GSH) and oxidized (GSSG) glutathione levels were measured in kidney tissue using a spectrophotometric assay (Folch J. et al., 1957). Briefly, samples were homogenized in ice-cold 5% sulfosalicylic acid to prevent auto-oxidation of glutathione. The homogenates were centrifuged, and supernatants were analyzed for GSH and GSSG content using DTNB [5,5'-dithiobis (2-nitrobenzoic acid)], which reacts with free thiol groups to form a chromophore detectable at 412 nm. The GSH/GSSG ratio was then calculated as an indicator of oxidative stress status.

Hepatic lipids were extracted from liver tissue and cultured HepG2 cells using the classical Folch method (Folch J. et al., 1957). In brief, samples were homogenized in a solvent mixture of chloroform:methanol:distilled water (4:2:3, v/v/v) under nitrogen gas to minimize oxidation. Following centrifugation, the lipid-soluble fraction (lower organic phase) was carefully separated, dried under a gentle stream of nitrogen, and reconstituted in absolute ethanol for downstream analyses (Folch J. et al., 1957).

For intracellular triglyceride quantification, HepG2 cells were first harvested in cold PBS and lysed by sonication (3×10 -second pulses on ice, with 30-second intervals) as previously described (Madduma Hewage et al., 2022). The lysates were then subjected to colorimetric enzymatic assays to determine triglyceride (TG) and total cholesterol (TC) levels, using commercially available kits (Sekisui Diagnostics, Burlington, MA, USA). Absorbance was measured according to the manufacturer's instructions, and results were normalized to cellular protein concentration.

3.4. Quantitative real-time RT-PCR

Total RNA was extracted from liver tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Briefly, approximately 50–100 mg of frozen liver tissue was homogenized in 1 mL of TRIzol, followed by phase separation with chloroform and centrifugation at $12,000 \times g$ for 15 minutes at 4 °C. The aqueous phase was collected, and RNA was precipitated with isopropanol. After washing with 75% ethanol, RNA pellets were air-dried and resuspended in RNase-free water. RNA purity and concentration were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was verified by agarose gel electrophoresis.

For complementary DNA (cDNA) synthesis, 1 µg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the protocol described by Madduma Hewage et al. (Madduma Hewage S. et al., 2022). The synthesized cDNA was stored at –20 °C until further use.

The relative messenger RNA (mRNA) expression levels of peroxisome proliferator-activated receptor-alpha (PPAR α), acyl-CoA oxidase 1 (ACOX1), carnitine palmitoyltransferase-I-alpha

(CPT1 α), sterol regulatory element-binding protein-1c (SREBP-1c), and the internal control β -actin were quantified by quantitative real-time polymerase chain reaction (qPCR). Reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a 20 μ L total reaction volume containing 100 ng of cDNA, 1 $\times$ iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 300 nM of each forward and reverse primer, and RNase-free water.

Amplification conditions consisted of an initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing/extension at 60 °C for 30 seconds. Melt curve analysis was performed to confirm amplification specificity.

Amplification was conducted using the comparative CT method, with gene expression levels normalized to the housekeeping gene β -actin (Amplification and Data Analysis). Primer sequences for all target and reference genes are listed in Table 1.

Table 1. Gene primer sequences of rats and humans used for real-time PCR

Target Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	Size (bp)	Accession Number
<i>SREBP-1c</i>	GGCCCTGTGTGTACTGGTCT	AGCATCAGAGGGAGTGAGGA	88	NM_001276708.1
<i>PPARα</i>	TCACACAATGCAATCCGTTT	GGCCTTGACCTTGTTTCATGT	177	XM_030213757.1
<i>ACCI</i>	TGAGGAGGACCGCATTTATC	AAGCTTCCTTCGTGACCAGA	221	NM_022193.2
<i>CPTIα</i>	CAGCTCGCACATTACAAGGA	TGCACAAAGTTGCAGGACTC	128	XM_039102321.1
<i>β-actin</i>	ACAACCTTCTTGCAGCTCCTC	GACCCATACCCACCATCACA	198	NM_031144.3

3.5. Western immunoblotting analysis

Total proteins were extracted from rat liver tissue or cultured cells using a lysis buffer containing protease and phosphatase inhibitors to prevent protein degradation and dephosphorylation. Protein concentrations were determined to ensure equal loading across samples. Subsequently, equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (Madduma Hewage S. et al., 2022; Shang Y. et al., 2016).

Following electrophoresis, the resolved proteins were transferred onto nitrocellulose membranes for immunodetection. To investigate the activation of AMPK, membranes were incubated with a rabbit anti-phospho-AMPK- α monoclonal antibody (1:1000) or with a rabbit anti-AMPK- α monoclonal antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA) to detect total AMPK. To confirm equal protein loading across all lanes, membranes were simultaneously probed with a rabbit anti- β -actin monoclonal antibody (1:2000) (Cell Signaling Technology, Danvers, MA, USA) as a loading control.

After primary antibody incubation, membranes were washed and incubated with an HRP-conjugated anti-rabbit IgG secondary antibody (1:2000) (Cell Signaling Technology, Danvers, MA, USA). Protein bands were subsequently visualized using an Enhanced Chemiluminescence (ECL) detection system (Millipore Ltd., Burlington, MA, USA).

Finally, densitometric analysis of the protein bands was performed using Quantity One software (version 4.6.8, Bio-Rad, Hercules, CA, USA). Band intensities for phosphorylated AMPK were normalized to total AMPK levels, and total AMPK was normalized to β -actin to account for loading differences, enabling quantitative comparison across experimental groups.

3.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 for Windows (version 8.0.2). Data are presented as mean $\pm$ standard error of the mean (SEM) unless otherwise stated. Comparisons between two groups were conducted using an unpaired two-tailed t-test, while comparisons among three or more groups were performed using one-way analysis of variance (ANOVA), followed by Newman–Keuls post-hoc test to identify specific group differences when appropriate. Normality and homogeneity of variance assumptions were verified before applying parametric tests. A p-value ≤ 0.05 was considered statistically significant. Graphical representations of the data were generated using Prism software, and all statistical tests were two-sided.

CHAPTER IV: RESULTS

4.1. Effect of ischemia-reperfusion and folic acid on kidney and liver function

Renal ischemia for 45 minutes followed by 24 hours of reperfusion resulted in a marked elevation of plasma creatinine and aspartate aminotransferase (AST) levels, consistent with significant impairment of both renal and hepatic function (Figure 1). Preincubation of cultured cells with 5-methyltetrahydrofolate (5-MTHF; 1 and 2 $\mu\text{g/mL}$) produced notable protective effects against IR-induced cellular injury. The higher concentration (2 $\mu\text{g/mL}$) elicited a more pronounced protective response compared to the lower dose, indicating a clear dose-dependent mechanism, where increased levels of 5-MTHF enhanced the cytoprotective outcome. Consistent with these *in vitro* findings, *in vivo* experiments demonstrated that systemic administration of 5-MTHF (2 $\mu\text{g/mL}$) significantly mitigated IR-induced renal and hepatic dysfunction, as evidenced by a marked reduction in plasma creatinine and aspartate aminotransferase (AST) levels in rats measured 24 hours after reperfusion. These results suggest that 5-MTHF confers dose-dependent protection against oxidative and metabolic stress associated with IR injury, both at the cellular and systemic levels. (Figure 1). These findings suggest that 5-MTHF exerts renoprotective and hepatoprotective effects in the context of IR injury, likely through its antioxidant capacity, ability to preserve redox balance, and potential to mitigate oxidative stress-mediated cellular damage.

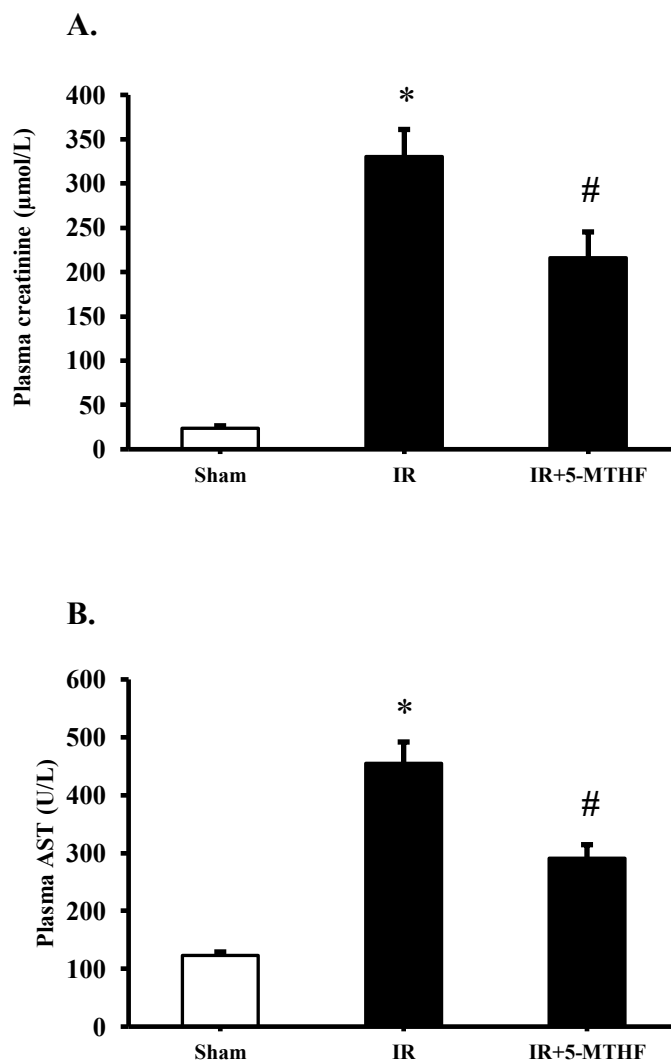


Figure 1. Effect of ischemia-reperfusion (IR) on kidney and liver function

A. Plasma creatinine levels were elevated, indicating impaired kidney function; 5-MTHF treatment partially ameliorated this effect. **B.** Plasma AST levels were elevated, indicating impaired liver function; 5-MTHF treatment restored liver function. Results are expressed as mean $\pm$ SD ($n = 6-8$). $P \leq 0.05$

4.2. Effect of folic acid on ischemia-reperfusion induced oxidative stress

Key biochemical indicators of oxidative stress include depletion of reduced glutathione (GSH), a decreased GSH-to-oxidized glutathione (GSSG) ratio, and accumulation of lipid peroxidation products such as malondialdehyde (MDA). To assess whether AKI induced by IR impacts hepatic redox status, we measured GSH, GSSG, the GSH/GSSG ratio, and MDA levels in liver tissue.

IR resulted in a marked decline in hepatic GSH levels and a significant reduction in the GSH/GSSG ratio, demonstrating impaired antioxidant defense capacity (Figure 2).

Strikingly, administration of 5-MTHF restored both GSH levels and the GSH/GSSG ratio in IR-injured rats, suggesting effective preservation of hepatic redox balance (Figure 2). In parallel, IR induced a significant elevation of hepatic MDA levels, reflecting enhanced lipid peroxidation and membrane damage, whereas treatment with 5-MTHF markedly suppressed this increase (Figure 2). These *in vivo* findings were further corroborated *in vitro*, where incubation of cells with 5-MTHF significantly counteracted H₂O₂ induced oxidative stress reinforcing the antioxidant role of 5-MTHF (Figure 3).

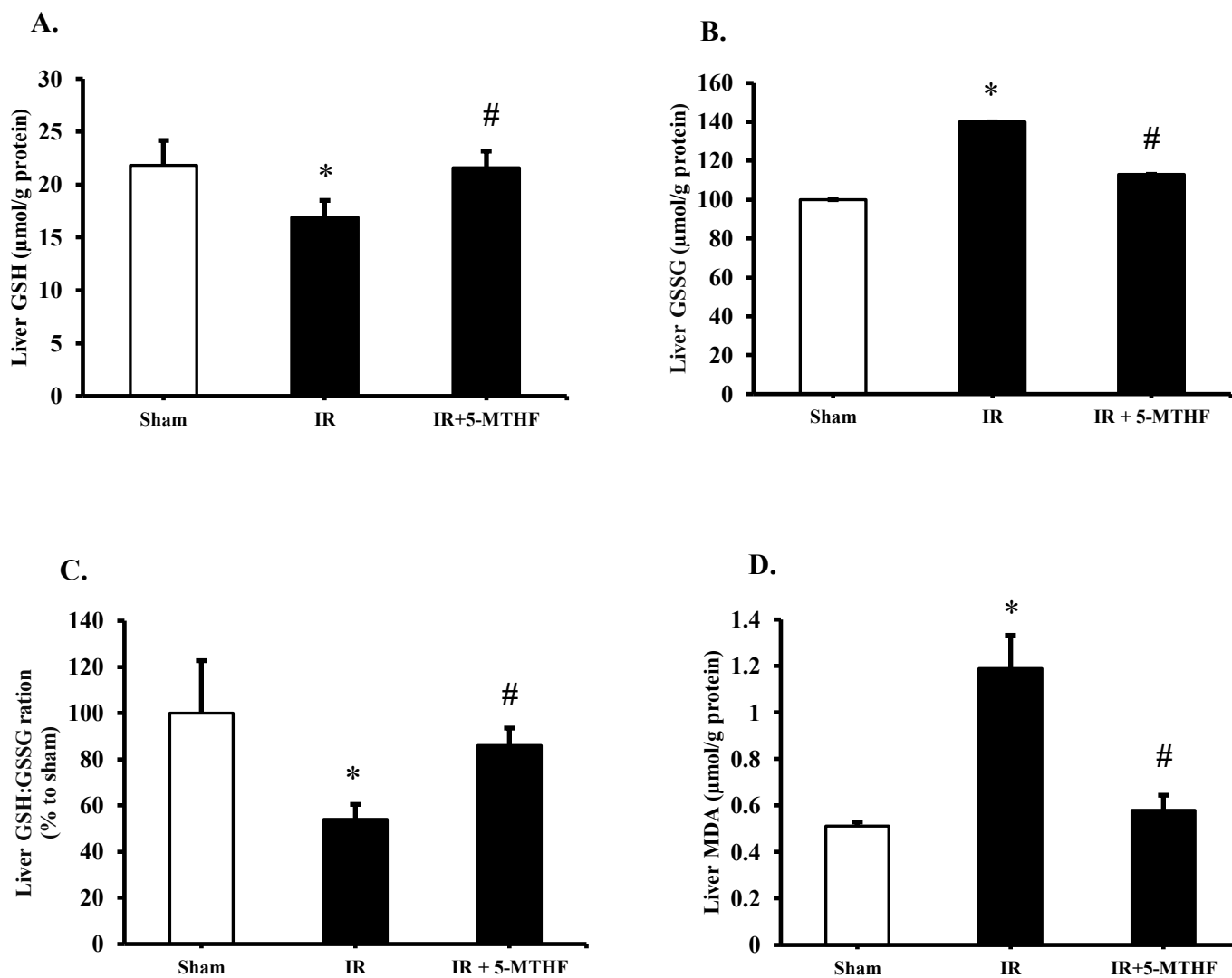


Figure 2. Hepatic oxidative stress biomarkers in rats subjected to IR injury and folate treatment

A. IR reduced hepatic GSH levels, while 5-MTHF treatment effectively restored them. **B.** IR elevated hepatic GSSG levels, whereas 5-MTHF treatment normalized them. **C.** IR decreased the hepatic GSH: GSSG ratio; 5-MTHF treatment effectively restored it. **D.** IR increased hepatic MDA levels, while 5-MTHF treatment effectively normalized them. Results are expressed as mean $\pm$ SD ($n = 6-8$). $P \leq 0.05$

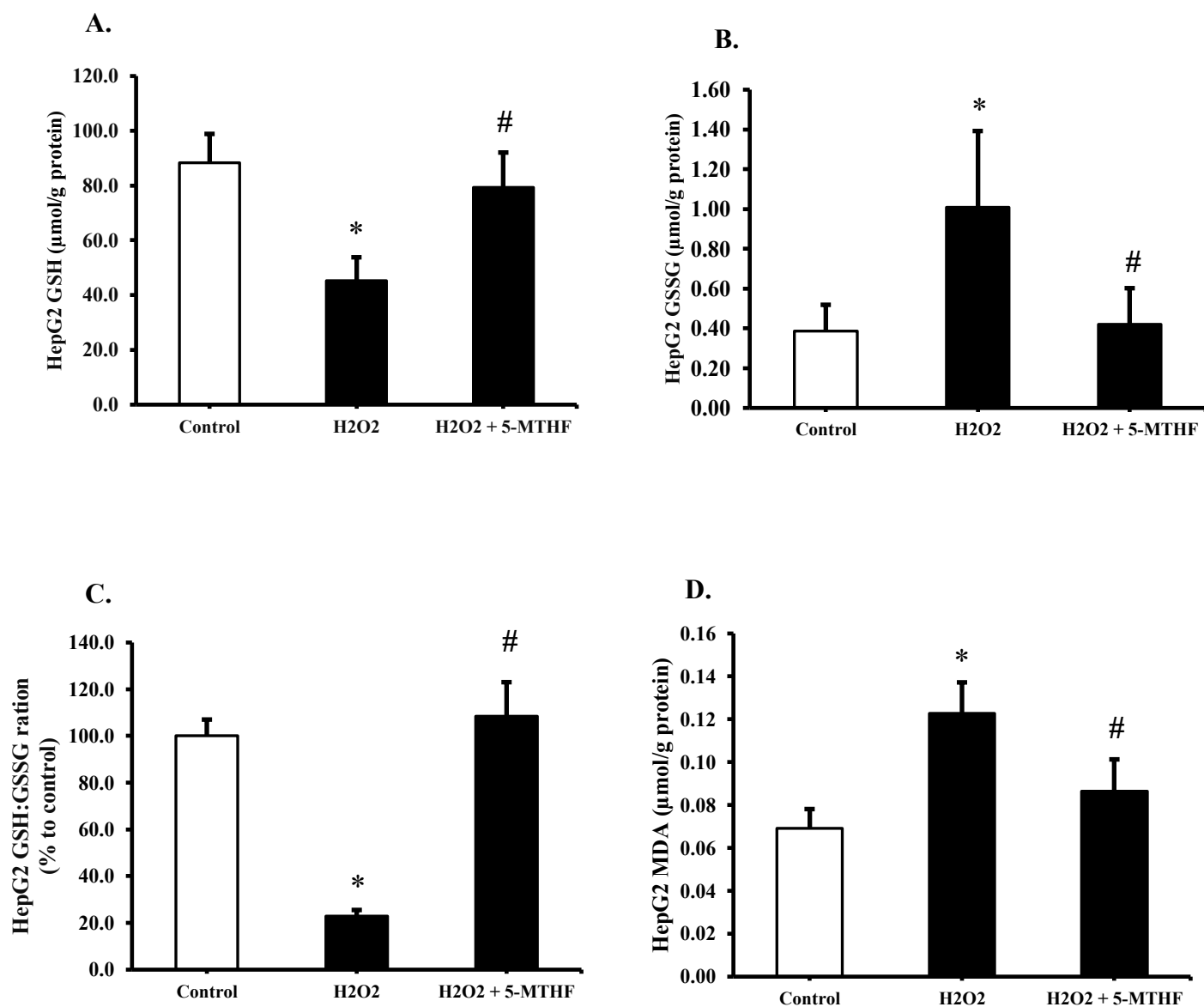


Figure 3. Oxidative stress biomarkers in HepG2 cells subjected to H₂O₂ and folate treatment

A. H₂O₂ treatment significantly reduced GSH levels, whereas 5-MTHF treatment effectively restored them. **B.** H₂O₂ exposure increased GSSG levels in HepG2 cells, while 5-MTHF treatment normalized them. **C.** H₂O₂ decreased the hepatic GSH:GSSG ratio, and 5-MTHF treatment effectively restored it. **D.** H₂O₂ elevated hepatic MDA levels, which were normalized by 5-MTHF treatment. Results are expressed as mean ± SD (n = 6). $P \leq 0.05$

4.3. Effect of folic acid against IR- and H₂O₂-induced lipid dysregulation

IR injury resulted in a marked increase in hepatic triglyceride and cholesterol accumulation, as evidenced by enhanced lipid deposition in liver tissue (Figure 4). This dysregulation of lipid metabolism is consistent with impaired mitochondrial function and redox imbalance, processes known to drive hepatic steatosis under oxidative stress conditions. Remarkably, treatment with 5-MTHF significantly attenuated this lipid accumulation, restoring hepatic lipid levels closer to baseline values (Figure 4).

Complementary in vitro experiments supported these findings: exposure of HepG2 cells to H₂O₂ induced intracellular triglyceride and cholesterol buildup, mimicking oxidative stress driven lipid dysregulation. Notably, co-treatment with 5-MTHF effectively reduced intracellular lipid content, underscoring its ability to protect against oxidative stress-mediated disturbances in lipid homeostasis (Figure 5). Together, these results suggest that 5-MTHF not only mitigates IR-induced oxidative stress but also prevents secondary metabolic consequences such as lipid accumulation in the liver.

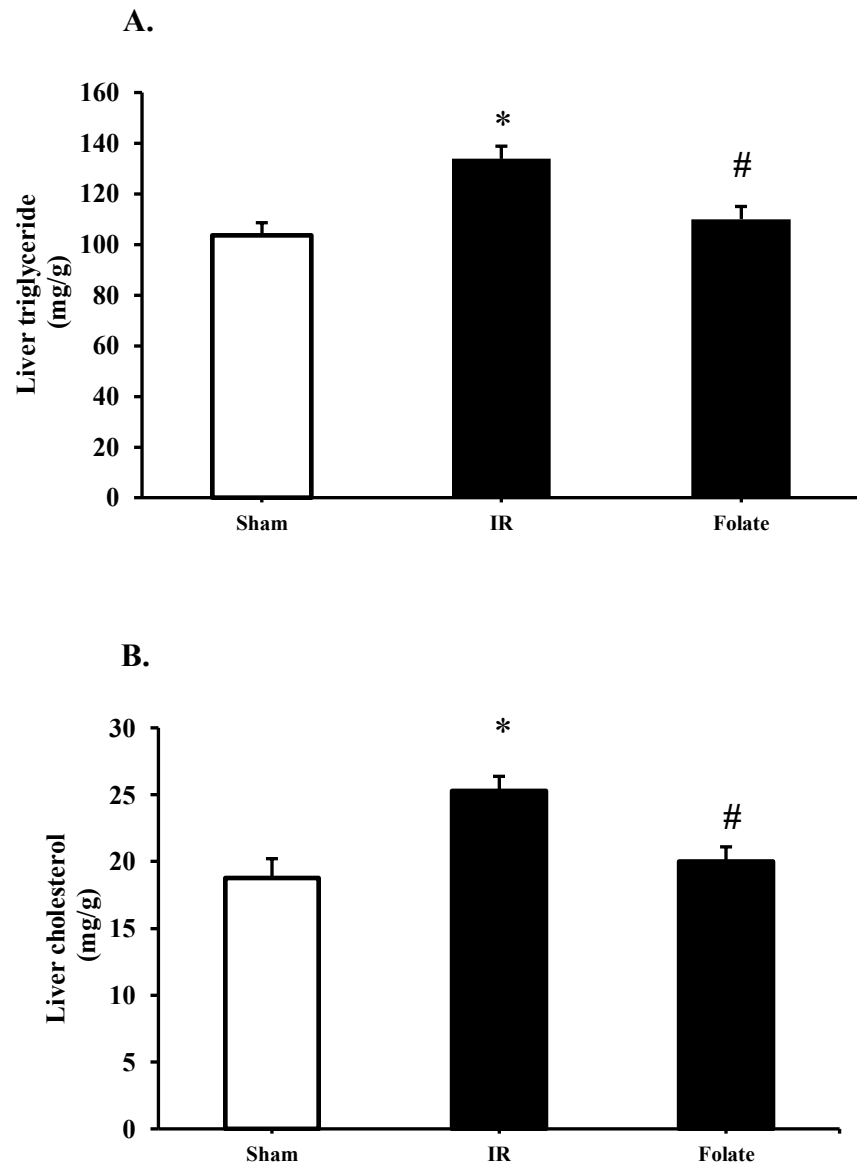


Figure 4. Effect of IR on hepatic lipid accumulation

A. The IR group exhibited elevated hepatic triglyceride levels, which were reduced to normal by 5-MTHF treatment. **B.** Hepatic cholesterol levels were elevated but were restored to normal with 5-MTHF treatment. Results are expressed as mean $\pm$ SD ($n = 6-8$). $P \leq 0.05$

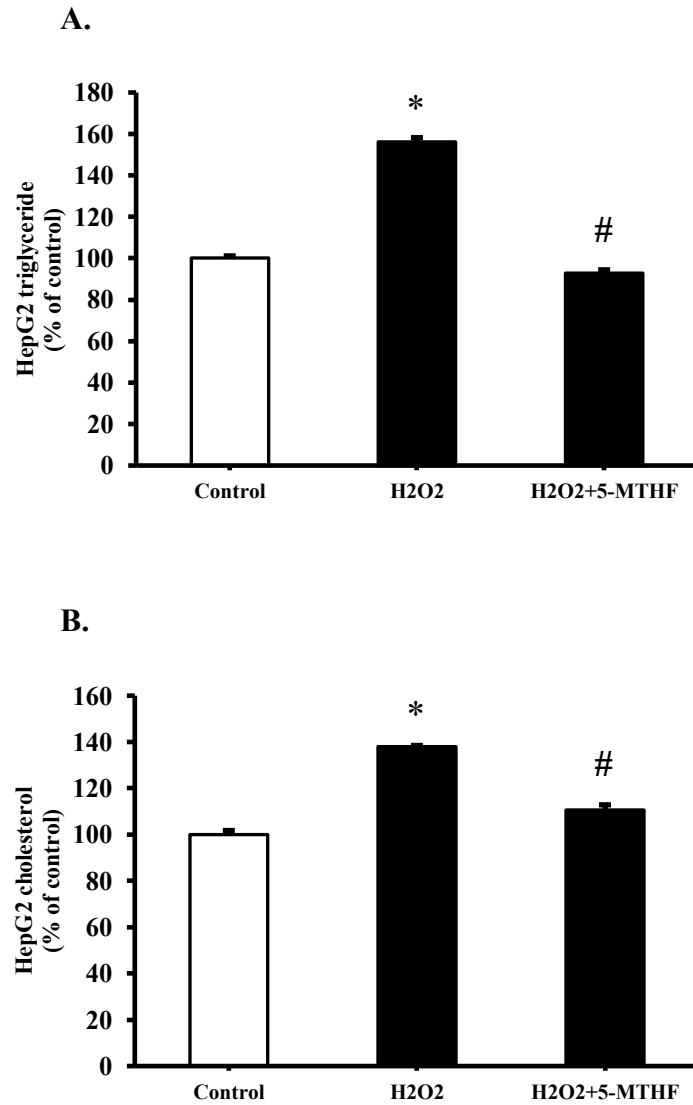


Figure 5. Effect of H₂O₂ on lipid accumulation in HepG2 cells

A. The H₂O₂ group showed an accumulation of triglycerides, which was reduced to baseline by 5-MTHF treatment. **B.** Hepatic cholesterol content increased but was corrected after 5-MTHF administration. Results are expressed as mean $\pm$ SD (n = 6-8). $P \leq 0.05$

4.4. Folic acid upregulated AMPK pathway and affected lipid metabolism in the liver

AMPK serves as a central regulator of lipid metabolism by integrating signals related to the cellular energy state and thereby modulating key metabolic pathways. Therefore, it is considered a therapeutic target for IR injury (Hardie, 2008). In this study, AMPK activation was evaluated by measuring phosphorylated AMPK (pAMPK) relative to total AMPK protein levels in the liver. The results demonstrated that renal IR significantly reduced pAMPK levels, which were subsequently increased following folate administration. (Figure 6).

To investigate whether the downregulation of AMPK affected lipid metabolism, the expression of genes involved in lipogenesis and fatty acid oxidation was examined. The results showed that renal IR significantly increased the mRNA expression of ACC1 and SREBP-1c in the liver, while folate administration attenuated these elevations (Figure 6). Additionally, the expression of genes regulating fatty acid oxidation, including PPAR α and CPT1 α , was markedly reduced by renal IR, but folate treatment restored their levels (Figure 7). Collectively, these findings suggested that renal IR suppressed AMPK phosphorylation, thereby contributing to dysregulated lipid metabolism and hepatic lipid accumulation. Folic acid administration could ameliorate these alterations.

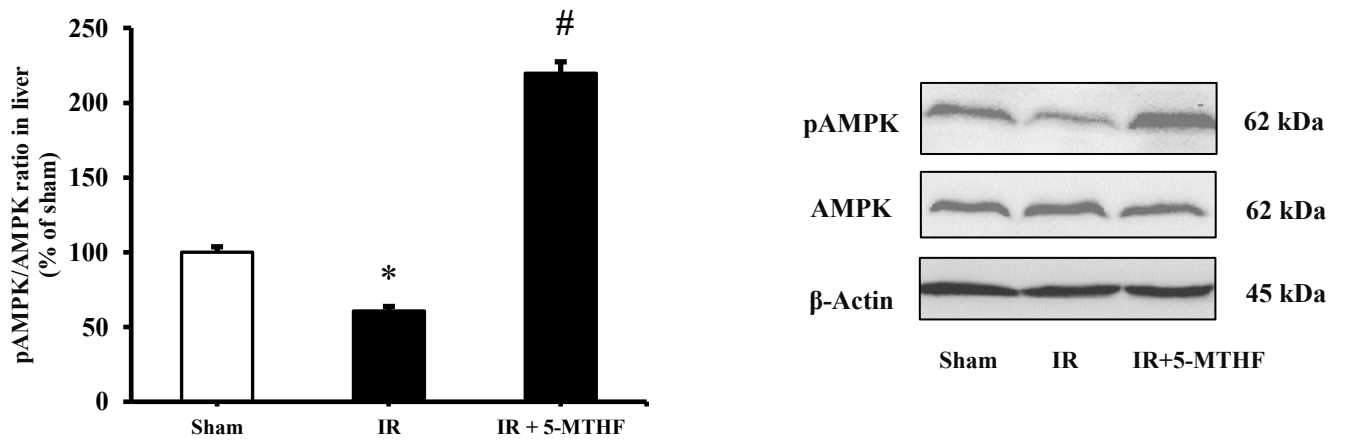


Figure 6. Effects of IR induction and folate on pAMPK activation in hepatic cells

IR induction decreased the pAMPK/AMPK ratio. Folate treatment elevated pAMPK activation.

Results are expressed as mean $\pm$ SD (n = 6-8). $P \leq 0.05$

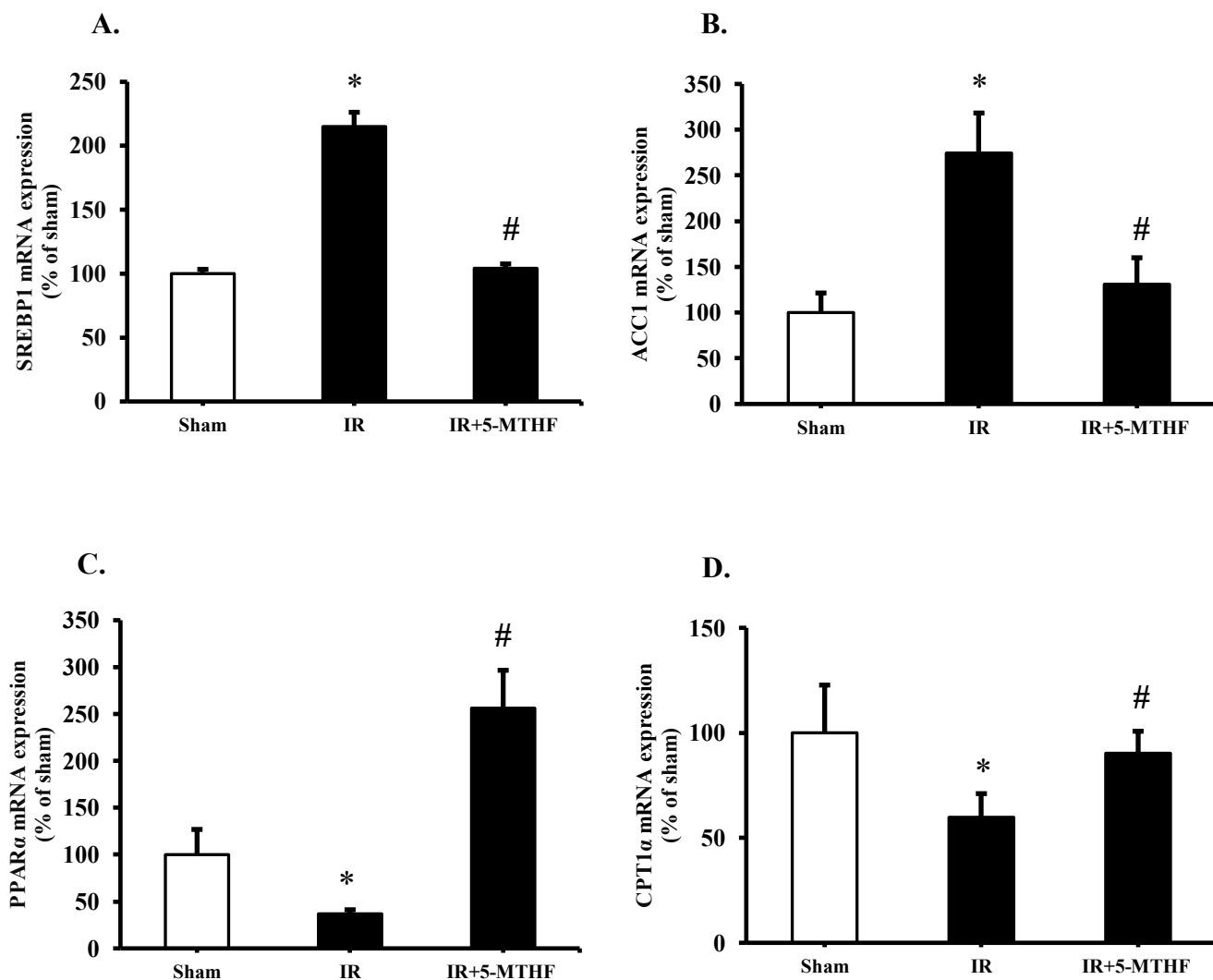


Figure 7. Hepatic gene expression related to lipogenesis and fatty acid oxidation in rats

A. IR increased hepatic SREBP1 mRNA expression, while 5-MTHF treatment effectively restored it. **B.** IR elevated hepatic ACC1 mRNA expression, whereas 5-MTHF treatment normalized it. **C.** IR decreased hepatic PPARα mRNA expression; 5-MTHF treatment effectively restored it. **D.** IR reduced hepatic CPT1α mRNA expression, while 5-MTHF treatment effectively normalized it. Results are expressed as mean $\pm$ SD (n = 6-8). $P \leq 0.05$

4.5. Effect of H₂O₂ and folic acid on AMPK phosphorylation and lipid metabolism-related gene expression in hepatocytes

Our previous studies indicated that after treating cells with various concentrations of H₂O₂, no significant toxicity was observed up to 200 µM H₂O₂. Additionally, the dosage of 5-MTHF used in the present study was determined based on the findings from our earlier research (Au-Yeung K. K. W. et al., 2023; Wijerathne C. U. B. et al., 2022). To further investigate the effects of H₂O₂ and 5-MTHF on lipid metabolism in HepG2 cells, the phosphorylation status of AMPK was assessed. H₂O₂ treatment significantly reduced phosphorylated AMPK protein levels, an effect that was reversed by 5-MTHF administration (Figure 8). Moreover, H₂O₂ significantly upregulated the expression of the lipogenic genes ACC1 and SREBP-1c, while 5-MTHF treatment attenuated these increases (Figure 9). In contrast, the mRNA expression of fatty acid oxidation-related enzymes, PPARα and CPT1α, was markedly decreased following H₂O₂ exposure and subsequently increased with 5-MTHF treatment (Figure 9). These findings suggest that oxidative stress disrupts AMPK phosphorylation and alters the expression of key lipid metabolism-related genes, effects that can be ameliorated by 5-MTHF.

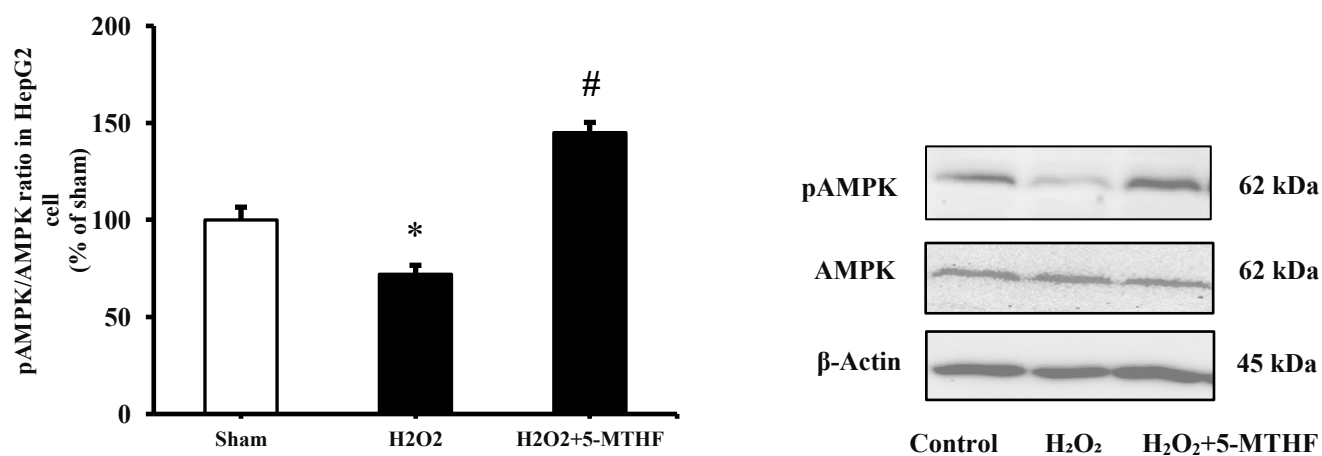


Figure 8. Regulation of AMPK and pAMPK in HepG2 cells following H₂O₂ exposure and folate treatment

H₂O₂ exposure markedly decreased the pAMPK/AMPK ratio. In contrast, folate treatment increased the pAMPK/AMPK ratio compared to the H₂O₂ group. Results are expressed as mean $\pm$ SD (n = 6-8). $P \leq 0.05$

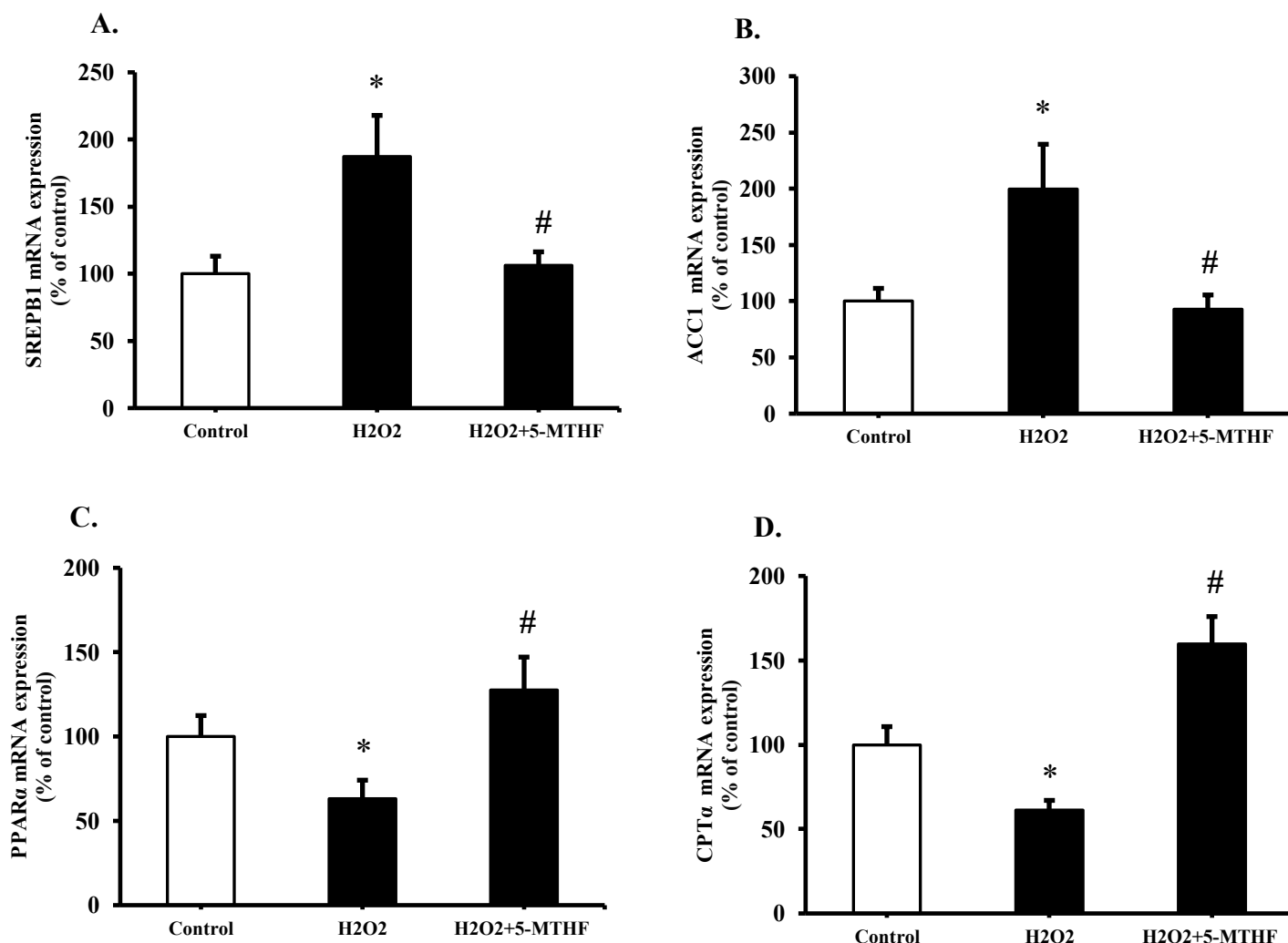


Figure 9. Regulation of lipogenic and fatty acid oxidation genes in HepG2 cells following H₂O₂ exposure and folate treatment

A. H₂O₂ increased SREBP1 mRNA expression, which was effectively restored by 5-MTHF treatment. **B.** H₂O₂ elevated ACC1 mRNA expression, whereas 5-MTHF treatment normalized it. **C.** H₂O₂ suppressed PPARα mRNA expression, which was restored by 5-MTHF treatment. **D.** H₂O₂ reduced CPT1α mRNA expression, while 5-MTHF treatment normalized it. Results are expressed as mean ± SD (n = 6-8). $P \leq 0.05$

CHAPTER V: DISCUSSION

The adverse effect of AKI can extend beyond the kidneys and precipitate dysfunction in distant organs, including the brain, lungs, heart, liver, and intestinal tract. This interorgan crosstalk, often referred to as the kidney - organ axis, underscores the systemic dysfunction of AKI (Husain-Syed F. et al., 2020). The harmful interaction between injured kidneys and the peripheral organs can amplify inflammatory responses, oxidative stress, and metabolic imbalances, thereby creating a vicious cycle of progressive organ damage. Over time, this cascade not only worsens the severity of AKI but also contributes to the development of multi-organ dysfunction syndrome (MODS), significantly increasing morbidity and mortality (Grams M. E. & Rabb H., 2012).

5.1. 5-MTHF improves kidney and liver function in AKI rats

Our previous research demonstrated that kidney IR injury was associated with significantly reduced plasma folate concentration (Yang C. et al., 2021). Moreover, plasma folate levels were inversely correlated with creatinine levels, suggesting that deterioration in kidney function was accompanied by a decline in circulating folate (Yang C. et al., 2021). In the present study, rats subjected to renal IR exhibited both kidney and liver injuries. This was indicated by elevated plasma creatinine, a marker of renal dysfunction, and increased ALT activity, a well-established indicator of hepatocellular damage. Given that folate is an essential micronutrient required for one-carbon metabolism, nucleotide synthesis, methylation reactions, and antioxidant defense, maintaining adequate folate status is a critical component of managing AKI. In this context, the current findings are particularly noteworthy, as supplementation with 5-MTHF, the biologically active form of folate, significantly improved both renal and hepatic function in rats subjected to

IR-induced AKI. These results highlight the therapeutic potential of folate supplementation in attenuating multi-organ dysfunction associated with AKI.

5.2. 5-MTHF attenuates ischemia-reperfusion induced oxidative stress

Oxidative stress is a major contributor to the adverse outcomes associated with AKI, not only impairing renal function but also promoting secondary dysfunction in other organs through systemic oxidative stress and metabolic disturbances (Husain-Syed F. et al., 2020; Pavlaku P. M. et al., 2017). In AKI, excessive production of ROS occurs in large quantities, particularly during IR. These ROS attack cellular macromolecules including lipids, proteins, and DNA, resulting in lipid peroxidation of cell membranes, mitochondrial dysfunction, and injury to both endothelial and tubular epithelial cells (Tomsa A. M. et al., 2019). In addition, ROS activate inflammatory pathways through the release of cytokines such as TNF- α and IL-1 β , while simultaneously overwhelming and inactivating endogenous antioxidant defenses, including superoxide dismutase, catalase, and glutathione (Wijerathne C. U. B. et al., 2020). This imbalance between ROS generation and antioxidant capacity drives oxidative stress mediated damage, which exacerbates tubular necrosis, promotes microvascular dysfunction, and amplifies inflammation, thereby accelerating the progression of kidney dysfunction (Zhang J. et al., 2016).

Accumulating evidence indicates that antioxidants can mitigate renal injury, particularly during the early stages of AKI, by limiting oxidative damage and preserving cellular homeostasis (Dennis J. & Witting P., 2017). In parallel, micronutrient deficiencies are frequently observed in patients with kidney disease, with folate deficiency being especially prominent (Yang C. et al., 2021). Folate plays a central role in redox balance, one-carbon metabolism, and nucleotide synthesis.

Inadequate folate availability may exacerbate oxidative stress and contribute to poor outcomes in AKI (Ostermann M. et al., 2020). In the present study, we demonstrated that administration of low-dose 5-MTHF attenuated oxidative stress and significantly improved kidney and liver function in rats subjected to IR-induced AKI. IR injury was associated with reduced hepatic GSH levels, increased MDA levels, and an elevated GSH-to-GSSG ratio in IR-injured rats. Treatment with 5-MTHF partially restored GSH level while reducing GSSG and MDA accumulation. These results indicate that the restoration of glutathione homeostasis may contribute, at least in part, to the antioxidant and hepatoprotective effects of folic acid against IR-induced liver injury.

HepG2 cells, which retain many key characteristics of hepatocytes, have been widely used as a valuable in vitro model for studying liver metabolism, including mechanisms regulating oxidative stress and cellular responses to metabolic challenges (Hou X. et al., 2008). In the present study, exposing HepG2 cells to H₂O₂ caused oxidative stress, as indicated by a decrease in GSH levels and an increase in MDA levels, along with a decrease in the GSH-to-GSSG ratio. The rise in MDA reflects enhanced lipid peroxidation, while changes in GSH and GSSG indicate disruption of the cellular redox balance. Treatment with 5-MTHF partially restored GSH levels, reduced GSSG accumulation, and mitigated MDA production, suggesting that reestablishing glutathione homeostasis and limiting lipid peroxidation are key mechanisms by which folic acid exerts its antioxidant and hepatoprotective effects against oxidative stress–induced cellular injury.

5.3. 5-MTHF attenuates AKI-induced AMPK inhibition and prevents hepatic lipid accumulation

AKI profoundly affects the metabolic state of the kidneys and other organs, in part through dysregulation of the AMPK signaling pathway (Day E. A. et al., 2017; Kandula N. et al., 2022). Under normal physiological conditions, AMPK functions as a central energy sensor that maintains cellular and hepatic lipid homeostasis by inhibiting ATP-consuming anabolic pathways, including lipogenesis, while stimulating ATP-producing catabolic pathways such as glycolysis and fatty acid oxidation (Hardie D. G., 2008). During ischemia, ATP levels drop and the AMP/ATP ratio rises, activating AMPK to preserve cellular energy and cell viability. AMPK activation has been shown to mitigate IR-induced kidney injury by promoting autophagy, enhancing mitochondrial integrity and biogenesis, reducing ROS production, and upregulating antioxidant defenses such as superoxide dismutase and catalase (Rodríguez C. et al., 2020).

However, in AKI, a significant reduction in AMPK phosphorylation disrupts these protective mechanisms, leading to metabolic dysregulation beyond the kidney (Guo Y. et al., 2014). In the liver, this manifests as unchecked lipogenesis, impaired fatty acid oxidation, and progressive hepatic lipid accumulation (Au-Yeung K. K. W. et al., 2023). Excess lipid deposition exacerbates oxidative stress and triggers inflammatory responses, compromising hepatocellular structure and critical liver functions including metabolism, protein synthesis, and detoxification (Kandula N. et al., 2022). In the present study, folic acid supplementation effectively restored AMPK phosphorylation and reactivated downstream lipid regulatory pathways, enhancing fatty acid oxidation, suppressing lipid synthesis, reducing hepatic lipid accumulation, and preserving liver function. These findings highlight the central role of AMPK in coordinating multi-organ metabolic responses during AKI. Our results suggest that targeted modulation of AMPK signaling,

potentially via folate supplementation, may provide a therapeutic strategy to prevent AKI-induced hepatic and systemic dysfunction.

CHAPTER VI: CONCLUSIONS AND FUTURE PERSPECTIVES

6.1. Conclusion

In conclusion this study demonstrated that renal IR in rats induced both kidney and liver damage, accompanied by a reduction in plasma folate levels and heightened oxidative stress. These findings underscore the systemic consequences of AKI beyond the kidney itself. Supplementation with 5-MTHF effectively improved renal and hepatic function, likely through its antioxidant property and its ability to restore glutathione homeostasis, thereby mitigating oxidative damage. Mechanistically, AKI promoted hepatic lipid accumulation through dysregulation of the AMPK signaling pathway, resulting in enhanced lipogenesis, impaired lipid clearance, and progressive fatty liver development. This hepatic lipid accumulation further amplified oxidative stress and inflammatory responses, exacerbating liver injury. Folic acid supplementation restored AMPK phosphorylation, reactivated downstream lipid regulatory pathways, promoted fatty acid oxidation, and reduced hepatic lipid deposition, ultimately preserving liver function. These findings highlight a mechanistic link between AKI-induced metabolic disturbances and secondary liver injury, and suggest that targeting AMPK signaling and restoring folate status may represent a promising therapeutic strategy to prevent multi-organ complications following AKI.

6.2. Future studies

Despite growing evidence that AKI exerts systemic effects beyond the kidney, the long-term impact of AKI and folate treatment on hepatic lipid metabolism remains poorly understood. AKI-induced alterations in renal function can trigger widespread metabolic disturbances, potentially promoting hepatic lipid accumulation, oxidative stress, and inflammatory responses, which may contribute to the development of liver dysfunction and metabolic disorders. While folate

supplementation has been suggested to exert hepatoprotective and antioxidant effects, the timing, dosage, and efficacy of such interventions in the context of AKI have not been systematically examined. Further research is needed to investigate the effects of AKI and folate treatment at critical time points, such as 48- and 72-hours post-injury. Research should also examine longer-term follow-up (e.g., weeks or months post-AKI). This will help elucidate the cellular and molecular mechanisms driving hepatic lipid accumulation. It is also important to determine whether these changes predispose individuals to long-term metabolic or inflammatory liver disorders. Additionally, sex-specific differences in response to AKI and folate treatment may significantly influence these outcomes, underscoring the importance of including both male and female subjects in experimental designs. A comprehensive understanding of these mechanisms could provide critical insights into multi-organ interactions following AKI, inform targeted therapeutic strategies, and ultimately improve clinical outcomes such as recovery, morbidity, and survival in the affected population.

Chapter VII: REFERENCES

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