



Comparative study between adenine and folic acid as optimal models to induce anemia of chronic kidney disease in male rats; an experimental study

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Abstract

Introduction: Anemia of chronic kidney disease (CKD) is a prevalent complication characterized by reduced erythropoiesis and altered iron metabolism.

Objectives: This study aims to compare the efficacy of adenine and folic acid in inducing anemia in male rats, providing insights into the underlying mechanisms and potential therapeutic targets. Through a systematic evaluation of hematological parameters and renal function, we assess the suitability of these agents as models for studying CKD-related anemia.

Materials and Methods: In this experimental study, 30 male albino Wistar rats were allocated into five groups, each consisting of six rats. Group I served as the healthy control group and received weekly intraperitoneal injections of normal saline. Group II, designated as the adenine model group, was administered a weekly intraperitoneal injection of adenine at a dosage of 250 mg/kg. Group III, also part of the adenine model group, received an intraperitoneal injection of adenine at the same dosage but every two weeks. Group IV represented the folic acid model group, receiving weekly intraperitoneal injections of folic acid at 250 mg/kg, while group V, also part of the folic acid model group, was treated with folic acid at the same dosage every two weeks. Following a 28-day induction period, all rats were sedated and euthanized to facilitate the collection of blood samples for the assessment of kidney function indicators, specifically neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C, across with hematological indicators such as transferrin, hepcidin, and hemoglobin.

Results: The results revealed that both folic acid and adenine significantly elevate NGAL, cystatin C, and hepcidin levels in rats, with folic acid showing superior efficacy, particularly at weekly doses. While both treatments effectively reduce hemoglobin and transferrin levels, they do not differ significantly in their overall therapeutic outcomes. Notably, folic acid produced a more pronounced reduction in transferrin compared to adenine.

Conclusion: The results demonstrated that both folic acid and adenine significantly affect biomarkers related to kidney function and iron metabolism in rats, with folic acid showing superior efficacy in increasing NGAL, cystatin C, and hepcidin levels. Although both treatments effectively reduced hemoglobin and transferrin levels, they did not differ significantly in their overall impact on hemoglobin reduction.



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Introduction

Renal anemia is a prevalent and significant complication of chronic kidney disease (CKD), affecting a substantial proportion of patients globally. This condition arises primarily due to inadequate erythropoietin production, shortened red blood cell lifespan, and disrupted iron metabolism, leading to decreased hemoglobin levels and increased morbidity and mortality among affected individuals (1-3). According to the 2012 KDIGO Clinical Practice recommendations, anemia is defined as a hemoglobin level below 130 g/L in males or 120 g/L in females aged 15 years or older. Renal anemia itself

is not independently associated with initial cognitive function or a decline in CKD, but it is significantly linked to a rapid decline in the estimated glomerular filtration rate (eGFR) (4,5). As the GFR falls below 60 mL/min/1.73 m², indicating a loss of more than 50% of renal function, clinical symptoms of CKD often begin to manifest. With the progression of CKD, the severity of anemia typically worsens, making it a common complication associated with this condition. Anemia in CKD is linked to increased mortality rates and a significantly heightened risk of cardiovascular morbidity, underscoring the critical need for effective management strategies to address both renal

Key point

In an experimental study on rats, we found that both folic acid and adenine significantly elevated neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C levels in rats, with folic acid demonstrating superior efficacy, particularly at weekly doses. Both treatments effectively reduced hemoglobin and transferrin levels without significant differences in their overall effectiveness regarding hemoglobin reduction. However, folic acid produced a more pronounced reduction in transferrin levels compared to adenine. Additionally, both compounds increased hepcidin levels, with folic acid again showing greater efficacy at weekly doses. These findings suggest important implications for optimizing dosing regimens in clinical settings to manage iron metabolism effectively.

function decline and its associated complications (6). Many patients with renal anemia experience cognitive decline, dyspnea, lightheadedness, headaches, anorexia, depression, and fatigue, all of which negatively impact their quality of life (7).

High doses of folic acid, such as 250 mg/d, commonly used to induce renal disease in animal models, pose significant health risks and are considered very dangerous (8). While folic acid is essential for various physiological functions, excessive intake can lead to adverse effects, including the masking of vitamin B12 deficiency symptoms, which may result in severe neurological complications (9). Previous studies have demonstrated that administering folic acid in amounts less than 200 mg/kg body weight can effectively induce symptoms of renal disease, thereby providing a valuable model for investigating the underlying mechanisms of acute kidney injury (AKI) and CKD (10). On the other hand, it has been shown that administering an oral dose of adenine at 600 mg/kg for 10 days in rats or 50 mg/kg for 28 days in mice leads to significant damage and impairment of renal tissue, alongside a discernible drop in red blood cell count. This model of adenine-induced renal injury is also instrumental for researchers, as it mimics the pathophysiological changes associated with CKD and AKI (11).

Studying the molecular mechanisms of renal anemia using animal models is essential for developing therapeutic interventions and identifying targets for novel pharmacological approaches.

Objectives

The objective of this study is to compare the efficacy of adenine and folic acid as models for inducing anemia in male rats with CKD. By evaluating hematological parameters and renal function, the study aims to determine which agent more effectively mimics the pathophysiological characteristics of anemia associated with CKD. This comparison will provide insights into the mechanisms underlying anemia in CKD and facilitate the development of targeted therapeutic interventions.

Materials and Methods**Study design**

This experimental study was conducted at the animal

facility and laboratory of the College of Pharmacy, Mustansiriyah University, Iraq, following the necessary approvals from the scientific and ethical council. The research spanned from November 8 to December 8, 2023, and involved an investigation into the effects of various treatments on male albino Wistar rats. The study adhered to ethical guidelines to ensure the humane treatment of animals throughout the experimental procedures.

Animals' preparation

Thirty male albino Wistar rats, aged between 10 to 12 weeks and weighing between 200 and 300 g, were procured from local markets for this study. Upon arrival, the animals were housed in well-ventilated cages that allowed for optimal airflow and were provided with unrestricted access to water and standard chow pellets to ensure adequate nutrition. The environmental conditions were carefully controlled, maintaining a temperature of 25 ± 5 °C, with natural light/dark cycles and humidity levels ranging from 30% to 40%. To facilitate adaptation to their new environment, the rats underwent a 14-day acclimatization period before the commencement of the experimental trials, ensuring that they were physiologically stable and minimizing stress-related variables that could affect the outcomes of the study.

Drugs' preparation

Adenine was prepared for administration by dissolving it in a sodium hydroxide (NaOH) solution, which was formulated according to a specific procedure (11). A 1M NaOH solution was created by dissolving 40 grams of sodium hydroxide pellets in 250 mL of distilled water and then diluting this mixture to a final volume of one liter (12). The adenine injections were delivered at a dosage of 250 mg/kg through two different models: either weekly or every two weeks (13), with the dosage calculated based on the average body weight of each group, resulting in each rat receiving approximately 60 mg of adenine. To ensure compatibility with the physiological pH range of the rats, adenine was dissolved in 10 mL of the 1M NaOH solution and subsequently adjusted with 7 drops of strong hydrochloric acid (HCl). Previous studies have established adenine's efficacy in inducing renal anemia associated with kidney failure across various experimental models (8). In parallel, folic acid was dissolved in a sodium bicarbonate (NaHCO_3) solution, prepared by dissolving 1 g of sodium bicarbonate in 10 mL of distilled water, followed by the addition of 1 g of folic acid to this mixture. The solution was stirred at 40 °C for 5 minutes to ensure complete dissolution (14). Folic acid injections were administered at a volume of 0.57 mL based on body weight to group IV once weekly, while the dosage for the folic acid group was adjusted to 0.6 mL every two weeks according to the average body weight of the rats, ensuring precise and effective dosing throughout the study.

Intervention

In this experimental study, 30 male albino Wistar rats were utilized and divided into five groups, each consisting of six rats, to assess the effects of different treatment regimens over four weeks. Group I served as the healthy control group, receiving 0.5 mL of normal saline via intraperitoneal injection once weekly for the duration of the study. Group II, the adenine model group, was administered 0.55 mL of adenine at a concentration of 250 mg/kg through intraperitoneal injection once weekly. Group III also belonged to the adenine model group but received 0.6 mL of adenine at the same concentration every two weeks. In group IV, designated as the folic acid model group, rats were injected with 0.58 mL of folic acid at a dosage of 250 mg/kg once weekly. Finally, group V, also part of the folic acid model group, received 0.58 mL of folic acid at the same concentration every two weeks. The drug doses were administered daily between 8:30 and 9:30 AM to minimize potential hormonal fluctuations that could interfere with the estrus cycle of the rats.

Serum collection and kidney harvesting

To collect serum samples, the rats were first rendered unconscious through intraperitoneal administration of 100 mg/kg of ketamine and 10 mg/kg of xylazine (15). Following sedation, blood was drawn directly from the right ventricle using a syringe to ensure accurate sampling. The collected blood was then transferred into gel tubes and subjected to centrifugation at 1000 rpm for 15 minutes to separate the serum from cellular components. Subsequently, the serum was carefully extracted using micropipettes and transferred into labeled Eppendorf tubes for storage at -20 °C until further analysis of serum indicators was conducted. The rat's abdomen was meticulously dissected using forceps and scissors to facilitate the removal of the kidneys, which were subsequently preserved in a 10% formalin solution for further analysis. The kidneys obtained from the rats were utilized to determine their relative index and to conduct histopathological assessments, providing valuable insights into the morphological changes associated with the experimental treatments (16). The serum obtained was preserved for subsequent biomarker analysis utilizing enzyme-linked immunosorbent assay (ELISA) kits, and kidney histopathological alterations were evaluated through hematoxylin and eosin staining.

Enzyme-linked immunosorbent assay method

The readings for kidney function parameters, specifically neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C, as well as hematological parameters including hemoglobin, transferrin, and hepcidin, were conducted 28 days after the initiation of the experiment. Serum levels of these biomarkers were determined using the ELISA technique, following the manufacturer's guidelines to ensure accuracy and reliability in the measurement process (17).

Kidney histopathologic analysis

For kidney histopathological analysis, following four weeks of administration of folic acid and adenine, the animals were anesthetized and subsequently sacrificed to collect kidney tissue for detailed histological examination. The harvested kidney tissues were fixed in formalin, embedded in paraffin, sectioned into thin slices, and stained with hematoxylin and eosin (H&E) to visualize cellular structures and assess morphological changes. The stained sections were then analyzed using light microscopy, allowing for the evaluation of histopathological alterations associated with the treatments administered (18).

Statistical analysis

In the current research, data were reported as mean $\pm$ standard deviation (SD) to represent the central tendency and variability within the sample. Statistical analyses were conducted using version 27 of the Statistical Package for the Social Sciences (SPSS, IBM Corp., USA), facilitating comprehensive data evaluation. The significance of mean differences among groups was assessed using analysis of variance (ANOVA), followed by a post hoc least significant difference (LSD) test to identify specific group differences. Differences were deemed statistically significant based on *P* values less than 0.05.

Results

The control group exhibited significantly lower NGAL and cystatin C levels compared to all intervention groups. Among the adenine treatment groups, those receiving 250 mg/kg weekly showed markedly greater NGAL and cystatin C levels compared to those received every two weeks, indicating a pronounced impact on renal function. In contrast, the folic acid treatment groups revealed similarly elevated NGAL and cystatin C levels compared to the control, with those receiving folic acid weekly showing greater concentrations. The group treated with folic acid every two weeks presented intermediate values for both parameters (Table 1).

The analysis of kidney function parameters indicated that the administration of both adenine and folic acid, at both weekly and biweekly doses, significantly elevated NGAL levels in rats compared to the control group, which received no treatment. When comparing adenine and folic acid, it was found that the weekly administration of adenine did not exhibit a statistically significant difference from the weekly administration of folic acid; however, it did result in a notable increase in NGAL levels compared to both its biweekly dose and the biweekly dose of folic acid. Furthermore, the NGAL levels associated with both weekly and biweekly doses of folic acid were significantly higher than those observed with adenine administered every two weeks. Additionally, within the folic acid treatment comparisons, the weekly dosage significantly increased NGAL levels compared to the biweekly dosage. In terms of cystatin C levels, a significant increase was

Table 1. Frequency distribution of kidney function parameters of included rats in the intervention groups

Group	Parameter			
	NGAL (ng/L)		Cystatin C (mg/L)	
	Mean	SD	Mean	SD
Control	3.12	0.56	8.79	1.39
Adenine 250 mg/kg weekly	26.69	3.21	18.92	0.92
Adenine 250 mg/kg every 2 weeks	14.55	0.63	17.47	0.51
Folic acid 250 mg/kg weekly	28.20	0.71	25.58	1.47
Folic acid 250 mg/kg every 2 weeks	19.26	0.51	18.91	0.68

SD, Standard deviation; NGAL, Neutrophil gelatinase-associated lipocalin.

found when comparing the control group to both adenine and folic acid treatment groups. The adenine weekly group also demonstrated a significant increase in comparison to the adenine every two weeks group. On the other hand, both folic acid treatment groups showed a significant increase against both adenine groups as well (Table 2).

The data presented in Figure 1 indicate a significant elevation in the mean serum levels of NGAL and cystatin C in response to both adenine and folic acid treatments across all dosages when compared to the control group. Notably, the weekly administration of folic acid resulted in the highest concentrations of NGAL and cystatin C, suggesting a pronounced effect on renal function markers. In descending order of concentration, the weekly adenine treatment, biweekly folic acid, and biweekly adenine treatments exhibited progressively lower levels of these biomarkers (Figure 1).

In the analysis of hematological parameters among the intervention groups, significant differences were observed in hemoglobin, transferrin, and hepcidin levels when compared to the control group. The control

group exhibited the highest hemoglobin and transferrin concentrations, while both adenine treatment groups showed markedly lower levels of hemoglobin and transferrin, indicating a detrimental effect on these parameters. Notably, the adenine group receiving weekly doses demonstrated a substantial increase in hepcidin levels, suggesting altered iron metabolism. Similarly, the folic acid treatment groups also presented reduced hemoglobin and transferrin levels compared to controls, with the weekly administration resulting in the lowest values for both parameters. Conversely, the folic acid groups exhibited significantly elevated hepcidin levels, particularly in those receiving treatment weekly. These findings highlight the pronounced impact of both adenine and folic acid interventions on hematological parameters, emphasizing their roles in influencing iron homeostasis and erythropoiesis in the experimental model (Table 3).

The study results demonstrated that the administration of folic acid and adenine led to a significant reduction in hemoglobin levels compared to a control group that received no treatment. However, when comparing

Table 2. Comparison of the frequency distribution of kidney function parameters (NGAL and cystatin C) of included rats between intervention groups using ANOVA and post hoc LSD

First group	Second group	Mean Difference	P value
NGAL (ng/L)	Adenine weekly	23.57	<0.001
	Adenine every 2 weeks	11.43	<0.001
	Folic acid weekly	25.08	<0.001
	Folic acid every 2 weeks	16.14	<0.001
	Adenine every 2 weeks	12.13	<0.001
	Folic acid weekly	1.50	0.102
	Folic acid every 2 weeks	7.43	<0.001
	Folic acid weekly	13.64	<0.001
	Folic acid every 2 weeks	4.70	<0.001
	Folic acid every 2 weeks	8.94	<0.001
Cystatin C (mg/L)	Adenine weekly	10.13	<0.001
	Adenine every 2 weeks	8.68	<0.001
	Folic acid weekly	16.79	<0.001
	Folic acid every 2 weeks	10.12	<0.001
	Adenine every 2 weeks	1.44	0.027
	Folic acid weekly	6.66	<0.001
	Folic acid every 2 weeks	0.09	0.988
	Folic acid weekly	8.11	<0.001
	Folic acid every 2 weeks	1.43	0.028
	Folic acid every 2 weeks	6.67	<0.001

NGAL, Neutrophil gelatinase-associated lipocalin.

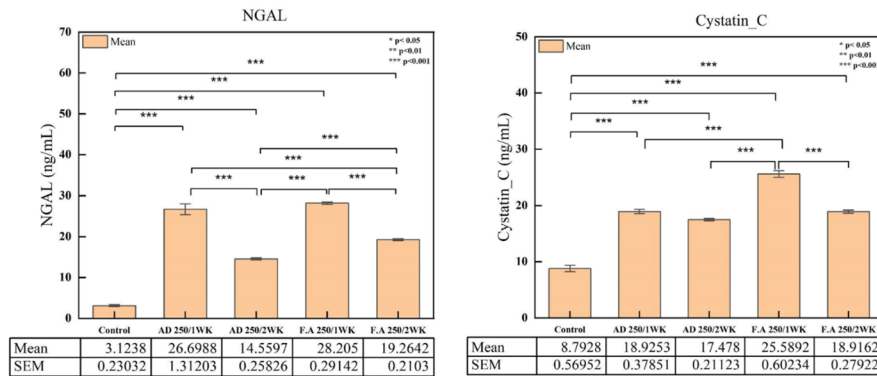


Figure 1. Comparison of NGAL and cystatin C levels between different intervention groups. NGAL, Neutrophil gelatinase-associated lipocalin; AD, Adenine; F.A, Folic acid. *** $P < 0.001$.

the effects of these two drugs, the analysis revealed no significant difference in their efficacy regarding hemoglobin reduction. Regarding transferrin, the administration of folic acid and adenine significantly reduced the mean levels of transferrin compared to a control group that did not receive treatment. Notably, the reduction in transferrin levels was more pronounced with folic acid than with adenine, indicating a superior efficacy of folic acid in this context. Furthermore, while there was no significant difference observed among the various dosages of folic acid administered, the results indicated that a once-weekly dose of adenine produced a significantly greater reduction in transferrin levels compared to a biweekly dosage. In terms of hepcidin, the administration of folic acid and adenine significantly increased mean levels of hepcidin compared to the control group that did not receive treatment. When comparing the two treatments, a once-weekly dose of folic acid resulted in a significantly greater increase in hepcidin levels than both dosages of adenine. Additionally, a once-weekly dose of adenine also demonstrated a significant increase in hepcidin compared to its biweekly dosage. However, there was no significant difference observed between the once-weekly dosages of adenine and the biweekly dosages of folic acid (Table 4).

As illustrated in Figure 2, the hemoglobin concentration in both the folic acid and adenine treatment groups was significantly lower than that of the control group, with no notable difference between the two treatment groups. Similarly, transferrin levels were lower in both groups

compared to the control; however, the reduction was more pronounced in the folic acid group. In terms of hepcidin concentration, both the folic acid and adenine groups exhibited significantly higher mean levels compared to the control group, with a tendency for higher levels in the folic acid group (Figure 2).

In this study, the histopathological examination of the renal cortex and medulla revealed distinct alterations across different treatment groups. The control group exhibited a normal appearance and cytoarchitecture of the renal tissue, as illustrated in Figure 3A. Conversely, the adenine group receiving 250 mg/kg weekly presented significant histological abnormalities, including focal necrosis and severe damage to the renal tubules, accompanied by a noticeable accumulation of mononuclear leukocytes in certain areas (Figure 3B). In contrast, the adenine group treated bi-weekly displayed milder changes characterized by slight expansion of collecting tubules, mild degeneration and karyorrhexis of lining cells, minor necrosis, capillary congestion in glomeruli, and mild thickening of the parietal glomerular capsule (Figure 3C). The folic acid group administered 250 mg/kg weekly showed moderate to severe glomerulonephritis with significant dilation of collecting tubules and pronounced focal necrosis alongside mononuclear leukocyte accumulation (Figure 3D). Finally, the bi-weekly folic acid group exhibited moderate tubular dilatation with intact lining cells, normal glomeruli, and minor vascular degeneration of interstitial cells, as depicted in Figure 3E.

The rats in the group that received folic acid at a dosage

Table 3. Frequency distribution of hematological parameters of included rats in the intervention groups

Group	Parameter					
	Hemoglobin (mg/dL)		Transferrin (mg/dL)		Hepcidin (nmol/L)	
	Mean	SD	Mean	SD	Mean	SD
Control	15.53	0.90	11.52	0.57	4.55	1.30
Adenine 250 mg/kg weekly	12.38	0.91	5.66	0.70	146.76	31.29
Adenine 250 mg/kg every 2 weeks	12.75	1.09	8.54	0.29	89.63	10.32
Folic acid 250 mg/kg weekly	11.63	1.00	4.57	0.45	249.92	20.47
Folic acid 250 mg/kg every 2 weeks	12.51	0.44	4.95	0.55	132.09	11.89

SD, Standard deviation.

Table 4. Comparison of the frequency distribution of hematological parameters (hemoglobin, transferrin, and hepcidin) of included rats between intervention groups using ANOVA and post hoc LSD

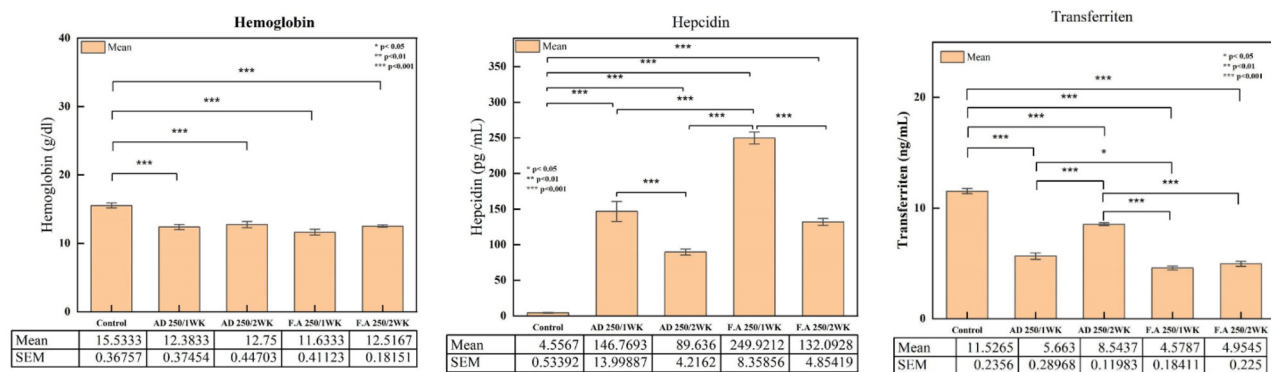
First group	Second group	Mean difference	P value
Hemoglobin (mg/dL)	Adenine weekly	3.15	<0.001
	Adenine every 2 weeks	2.78	<0.001
	Folic acid weekly	3.90	<0.001
	Folic acid every 2 weeks	3.01	<0.001
	Adenine weekly	0.36	0.488
	Folic acid weekly	0.75	0.162
	Folic acid every 2 weeks	0.13	0.800
	Folic acid weekly	1.11	0.062
	Folic acid every 2 weeks	0.23	0.655
	Folic acid every 2 weeks	0.88	0.102
Transferrin (mg/dL)	Adenine weekly	5.86	<0.001
	Adenine every 2 weeks	2.98	<0.001
	Folic acid weekly	6.94	<0.001
	Folic acid every 2 weeks	6.57	<0.001
	Adenine weekly	2.88	<0.001
	Folic acid weekly	1.08	0.002
	Folic acid every 2 weeks	0.70	0.030
	Folic acid weekly	3.96	<0.001
	Folic acid every 2 weeks	3.58	<0.001
	Folic acid every 2 weeks	0.37	0.235
Hepcidin (nmol/L)	Adenine weekly	142.21	<0.001
	Adenine every 2 weeks	85.07	<0.001
	Folic acid weekly	245.36	<0.001
	Folic acid every 2 weeks	127.53	<0.001
	Adenine weekly	57.13	<0.001
	Folic acid weekly	103.15	<0.001
	Folic acid every 2 weeks	14.67	0.198
	Folic acid weekly	160.28	<0.001
	Folic acid every 2 weeks	42.45	<0.001
	Folic acid every 2 weeks	117.82	<0.001

of 250 mg/kg/wk showed damage and inflammation in the endothelial cells of the renal tubules, with a severity grade of 2 (31%-50%). Additionally, there was fibrosis and thickening of Bowman's capsule at the glomerular level, with a severity grade of 3 (51%-70%). Lastly, there was an expansion of tubular cells, with a severity grade of 4 (more than 70%). The group of rats that received a dosage of 250 mg/kg/wk experienced disruption and swelling of the endothelial cells in the renal tubules, with a severity grade of 1 (10-30%). Additionally, there was fibrosis and

thickening of Bowman's capsule at the glomerular level, also with a severity grade of 1 (10%-30%). Finally, there was dilatation of tubular cells with a severity grade of 2 (51%-70%) (Table 5).

Discussion

The results indicated that both folic acid and adenine notably raised the levels of NGAL and cystatin C in rats, with folic acid proving more effective, especially at weekly doses. Both treatments successfully lowered hemoglobin

**Figure 2.** Comparison of hemoglobin, transferrin, and hepcidin levels between different intervention groups. AD, Adenine; F.A, Folic acid. *** $P < 0.001$.

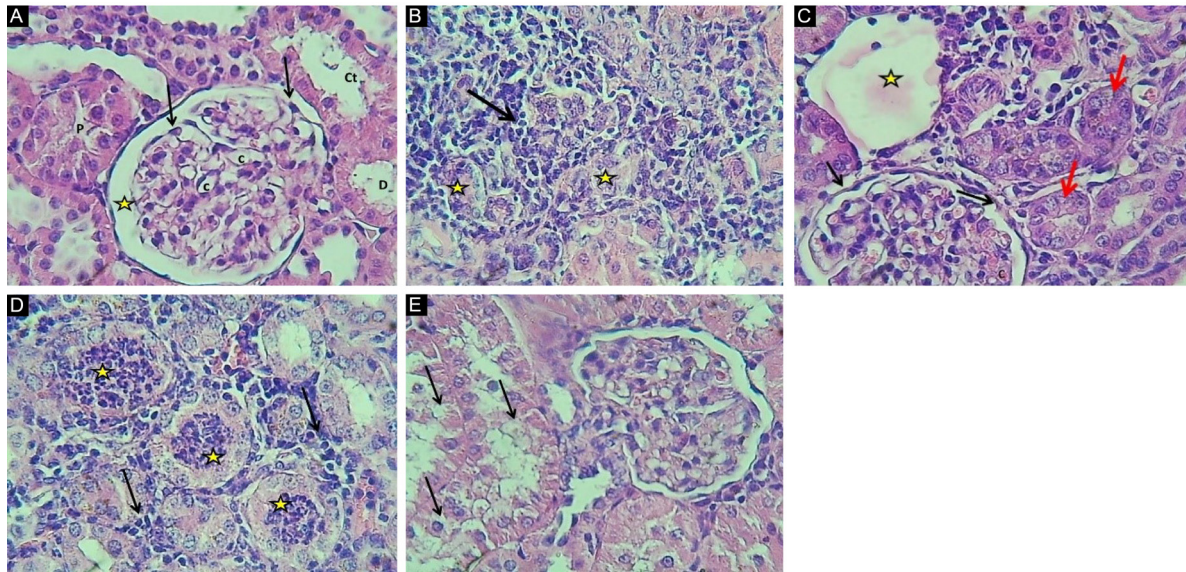


Figure 3. Cross-section of renal tissue in the control group and four different models represent histopathological changes in kidney tissue. **A:** Section of renal cortex (Control group/week) shows normal proximal tubule (P), distal tubule (D), collecting tubule (Ct), bowman space (Asterisk), podocytes (arrow) & glomerular capillary tuft (c). H&E stain, 400×. **B:** Section of renal cortex (Adenine group/ week) shows focal necrosis of renal tubules (Asterisk) with focal aggregation of mononuclear leukocytes (Arrow). H&E stain, 400×. **C:** Section of renal cortex (Adenine group-2 week) shows: mild tubular dilation at urinary pole (Asterisk), mild degeneration with karyorrhexis of lining cells and little necrosis of renal tubules (red arrow), glomerular congestion (C) with thickening of parietal glomerular capsule (black arrows). H&E stain, 400×. **D:** Section of renal cortex (Folic acid group -once/week) shows sever necrosis of lining cells renal tubules with marked luminal inflammatory cells exudation (asterisks) & interstitial infiltration of mean mononuclear cells (arrows). H&E stain, 400×. **E:** Section of renal cortex (Folic acid group-2 weeks) shows mild vascular degeneration of lining cells of renal tubules mostly collecting tubules (Arrows) with normal glomerulus. H&E stain, 400×.

and transferrin levels, showing no major differences in their overall effects on hemoglobin reduction; however, folic acid was more effective in decreasing transferrin levels. Furthermore, both substances significantly increased hepcidin levels, with folic acid again exhibiting greater effectiveness than adenine. These findings underscore the significance of dosing strategies in enhancing therapeutic approaches for managing iron metabolism and associated disorders.

Elevated NGAL, a member of the lipocalin family, is crucial for the stability of nephrons. AKI and abrupt

renal disease are often linked to elevated levels. NGAL, a systemic protein, is released from the injured proximal tubule, entering the urine and bloodstream. It is passed through the glomerulus and absorbed by the proximal tubular epithelia through endocytosis (19). The study conducted by Xiang et al revealed significant relationships between NGAL levels and iron status in patients with CKD, indicating that NGAL levels were markedly elevated in those with anemia compared to their non-anemic counterparts (20). The study by Devireddy et al documented that the gene undergoing maximal transcriptional induction after

Table 5. Histopathological changes in kidney tissue by the semi-quantitative scoring model of the ordinal scale of scores

Tissue type	Pathological changes	Control group	Adenine Once weekly	Adenine Twice weekly	Folic acid Once weekly	Folic acid Twice weekly
Endothelial	Loss	0	0	-	0	0
	Disruption	-	+	+	++	0
	Swelling	0	+	0	++	0
	No changes	+	No	-	+	0
Glomerular	Sclerosis	0	+	-	++	-
	Retraction of tuft	0	-	-	++	-
	Thickening of Bowman's capsule	0	+	-	+++	++
	No changes	+	0	-	0	+
Tubular	Inflammation with necrosis more than 60% with cast	0	++	-	+++	-
	Inflammation with necrosis, up to 60% with cast	0	+	-	++	-
	Inflammation with necrosis less than 20% without cast	0	+	-	++	-
	Necrosis more than 60%	0	-	-	-	-
	Dilation	0	+++	+	++++	++

cytokine withdrawal is 24p3, which encodes a secreted lipocalin leading to an increase in NGAL (21). Recently, Ali et al found that an increase in NGAL serum levels correlates with a decrease in iron levels (22). Additionally, adenine markedly elevated the production of NGAL, a hallmark of tubular damage, by activated mitogen protein kinase (MAPK) and phosphorylated nuclear factor kappa-light-chain enhancer of activated B (NF- κ B) cells, both as examples of kidney pro-inflammatory signaling mediators (23). NGAL upregulation, influenced by increased iron demand during anemia, has been shown to inhibit erythrocyte maturation and induce apoptosis. NGAL may control the hematopoietic system during renal anemia by suppressing erythropoiesis and activating apoptosis in the medulla, thus preventing erythroid progenitor cell development and disrupting iron absorption, resulting in renal anemia (24). Previous studies have indicated that the administration of folic acid in a single dose to rats is linked to kidney injury, acute tubular necrosis, epithelial regeneration, and renal cortical scarring (8,10,25). This finding suggests a potential link between NGAL and iron storage, highlighting the role of NGAL as a biomarker that may reflect both renal function and iron metabolism in CKD patients. The correlation between increased NGAL levels and altered iron status underscores the importance of monitoring these parameters in clinical practice, as they may provide insights into the pathophysiology of renal anemia and inform therapeutic strategies to improve patient outcomes in CKD management.

Cystatin C is a crucial protein synthesized by nucleated cells and predominantly excreted by the kidneys, making it an important biomarker for assessing renal function. Its serum levels reflect GFR more accurately than traditional markers like creatinine, particularly in the early stages of renal impairment, where timely diagnosis is critical for effective management. Elevated levels of Cystatin C indicate reduced kidney function and have been associated with various clinical conditions, including CKD (26,27). Our results found that both folic acid and adenine significantly increased the levels of cystatin C, with folic acid being more effective; in line with our study, Rattanasinganchan et al demonstrated that after a 28-day period, serum levels of cystatin C significantly increased in rats following a single dose injection of folic acid, indicating a potential impact on renal function (14). On the other hand, in a study by Zhu et al corticosteroids have been shown to increase cystatin C levels by promoting its production in tissues without affecting renal function, suggesting that factors other than renal impairment can influence cystatin C concentrations (28). Furthermore, the association between elevated cystatin C levels and conditions such as hyperuricemia has been documented, highlighting the multifactorial nature of cystatin C elevation (29). Overall, these findings suggest that while cystatin C is a valuable marker for renal function, its levels can be affected by various non-renal factors, including

dietary components like folic acid. This underscores the importance of considering these influences when interpreting cystatin C levels in both experimental and clinical settings.

The results of the current study indicate that both folic acid and adenine significantly decreased hemoglobin levels in rats; this finding aligns with previous studies exploring the hematological effects of these substances. Previous studies have established that folic acid is essential for erythropoiesis, but excessive intake can lead to imbalances, potentially resulting in lower hemoglobin levels under certain conditions, such as when combined with other dietary factors or health issues (30). In CKD models, low-hemoglobin levels and reduced serum ferritin levels indicate anemia, often linked to erythropoietin deficiency and impaired iron homeostasis (31). The observed decrease in hemoglobin levels following administration of both compounds suggests a complex interaction that warrants further investigation. While folic acid is typically recognized for its supportive role in red blood cell formation, its potential adverse effects when administered in excess or in specific contexts must be considered. Furthermore, the impact of adenine on erythropoiesis highlights the need for a nuanced understanding of how various compounds influence hematological health. In conclusion, these findings underscore the importance of further research to elucidate the mechanisms by which folic acid and adenine affect hemoglobin levels and to explore the implications for dietary recommendations and clinical practices regarding their use.

The results indicated that despite that both folic acid and adenine significantly reduced transferrin levels compared to control group, the folic acid was more effective in decreasing transferrin levels. Previous research has consistently highlighted the role of folic acid in influencing iron metabolism. For instance, a systematic review indicated that supplementation with vitamins B6, B12, and folic acid led to a significant reduction in homocysteine levels, which are often elevated in conditions associated with iron metabolism disorders. While this review did not focus solely on transferrin, it underscores the importance of folic acid in managing related biochemical markers (32). Moreover, a study investigating the effects of combined therapy (erythropoietin, iron, folate, and vitamin B12) on transfusion requirements in extremely low birth weight infants found that folate levels increased significantly alongside other markers⁵. This suggests that folic acid supplementation can have a beneficial impact on iron status and transferrin levels, aligning with the current findings (33). Overall, the current study reinforces the significance of folic acid as a potent agent for reducing transferrin levels compared to adenine. These findings are consistent with previous studies emphasizing the role of folic acid in iron metabolism and erythropoiesis. Future research should further explore the mechanisms through which adenine affects transferrin levels and consider

the implications of these findings for clinical practices aimed at managing anemia and optimizing nutritional interventions.

The results demonstrated that both folic acid and adenine significantly increased hepcidin levels; however, folic acid exhibited greater effectiveness than adenine. This result is consistent with previous studies that have explored the role of these compounds in iron metabolism. Research has established that hepcidin is a crucial regulator of iron homeostasis, influenced by various factors including dietary components. For instance, adenine has been recognized for its ability to induce hepcidin expression through the BMP/SMAD signaling pathway, demonstrating its potential as a therapeutic agent in conditions like hereditary hemochromatosis (34). This finding also aligns with findings from other studies that highlight the importance of folate in managing anemia and iron deficiency (35). Overall, these results underscore the significance of dietary interventions in modulating hepcidin levels and suggest that folic acid may be a more effective option than adenine for improving iron status in clinical settings.

Conclusion

In conclusion, the results of this study indicate that both folic acid and adenine significantly influence various biomarkers related to kidney function and iron metabolism in rats, particularly NGAL, cystatin C, transferrin, and hepcidin levels. Folic acid administration, especially at a weekly dosage, consistently resulted in higher NGAL, cystatin C, and hepcidin levels compared to adenine, suggesting a superior efficacy of folic acid in these areas. While both treatments effectively reduced hemoglobin and transferrin levels, they did not differ significantly in their overall therapeutic outcomes regarding hemoglobin reduction. These findings highlight the importance of dosing regimens in optimizing treatment strategies and suggest potential equivalence in the clinical applications of folic acid and adenine for conditions associated with elevated hemoglobin levels and altered iron metabolism and suggest potential equivalence in the clinical applications of folic acid and adenine for conditions associated with elevated hemoglobin levels and altered iron metabolism.

Authors' contribution

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Conflicts of interest

The authors declare no conflict of interest.

Ethical issues

The research protocol of this study followed the guidelines of animal studies and was approved by the Ethics Committee of the Mustansiriyah University College of Pharmacy (Ethical code #99). We conducted the guidelines related to animal experiments, approved by the United States National Institutes of Health (NIH, 1978). Besides, the authors have ultimately observed ethical issues (including plagiarism, data fabrication, and double publication).

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