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The Metabolic Mechanism Underlying the Enhancing Effects of Glycine and Tryptophan on Kidney Function: How to Reduce EGFR Inhibitory Effect on AAs

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ABSTRACT

Background: Chronic kidney disease (CKD) leads to a progressive decline in kidney function that eventually progresses to end-stage renal disease. It is critical to identify people at risk for renal disease and to intervene early to avoid kidney damage. Tryptophan hydroxylase (TPH-1) is an accurate biomarker for CKD early stage. The loss of tryptophan hydroxylase contributes to kidney injury. N-acetylcysteine, glycine, and tryptophan can arrest renal fibrosis development and progression through the anti-fibrotic effect of tryptophan hydroxylase and the protective effect of N-acetylcysteine.

Objectives: This study aims to assess the metabolic effect of N-acetyl-L-cysteine on kidney function as an EGFR inhibitor and its effect on AAs depletion. In addition, to evaluate the effect of glycine and tryptophan on kidney injury and renal fibrosis progression.

Results: Eight weeks of administration of pemetrexed led to a significant increase in serum, urea, creatinine, and uric acid. Administration of N-acetylcysteine (NAC with pemetrexed) resulted in a significant decrease in biochemical kidney function. Also, glycine and tryptophan directly affect renal function; increased expression of tryptophan hydroxylase with decrease expression of EGFR and transforming growth β (TGF- β), compared to N-acetylcysteine group.

Conclusion: N-acetylcysteine, glycine, and tryptophan can halt the depletion of AAs in the presence of urea and EGFR. Glycine and tryptophan both have a direct effect on renal function due to their uric acid-lowering properties.

INTRODUCTION

The increasing chronic kidney disease (CKD) prevalence threatens the health and overall quality of life. The biomarkers of the pathogenesis of CKD and the associated complications are essential for CDK diagnosis and treatment (Yiming Hao *et al.*, 2020). Pemetrexed is a methotrexate derivative. It is authorized for advanced non-small cell lung cancer and pleural mesothelioma treatment. Pemetrexed may result in a decrease in

creatinine clearance, AKI, and other types of renal impairment, which may lead to irreversible CKD (Stavroulopoulos, A. *et al.*, 2010).

After kidney damage, the loss of functioning nephrons stimulates compensatory development of the remaining ones, allowing functional adaptability. These compensatory processes, however, activate signaling pathways, resulting in pathological changes and chronic kidney disease (Mohamad Zaidan *et al.*, 2020).

In cell signaling, tyrosine kinases play a critical role. Dysregulation of their activity can lead to fibrotic disorders. Thus, pathogenic tyrosine kinase activation can promote fibrogenesis (Overstreet, J. M. *et al.*, 2017). In renal tissue, the epidermal growth factor receptor (EGFR) is expressed, primarily in the distal and collecting tubules epithelial cells, peritubular vessels, and glomeruli, with increased expression in affected kidneys, compared to normal kidneys (Skibba, M. *et al.*, 2016) (Yang, L. *et al.*, 2019). Because activation of these receptors has been linked to a variety of forms of chronic kidney disease, inhibiting target EGFR by interfering with its signaling pathway has been shown to have a protective impact against renal fibrosis.

A method for managing individuals with chronic renal disease is to use angiotensin-converting enzyme inhibitors (ACEI) to block the renin-angiotensin-aldosterone pathway. Currently, there is no effective treatment that can halt chronic renal disease development. In individuals with chronic kidney disease, antioxidants such as N-acetylcysteine (NAC) have been described as potential treatments and surrogate biomarkers for tubular damage or renal fibrosis. (Marcin Renke *et al.*, 2009).

Tryptophan hydroxylase is an accurate metabolic biomarker of early-stage kidney disease. TPH-1 deficiency exacerbates renal damage by activating NF- κ B and suppressing Nrf2 signaling pathways. As a result, targeting TPH-1 may provide a novel method for the treatment of chronic kidney disease. (Dan-Qian Chen *et al.*, 2019). Alterations in tryptophan act as a chemical fingerprint and can be utilized as biomarkers for detecting or monitoring biological processes (Kosmidis A.K. *et al.*, 2013) (Holmes E. *et al.*, 2008).

MATERIALS AND METHODS

Animal Preparation:

Thirty-two male Sprague-Dawley rats (10–12 weeks of age, 250–300 g) were utilized in this study. They were purchased from Helwan Experimental Animal Breeding Farm. The Laboratory Animal Care and Use Committee approved all protocols. The experimental rats were kept in a room with a temperature of $25 \pm 1^\circ \text{C}$ with a 12-hour light/dark cycle and provided regular laboratory food and water. To minimize the possibility of our results being influenced by the sex hormones variation during the menstrual cycle in female rats, only male rats were utilized.

Animal Treatment:

Rats were randomly categorized into four groups (eight per group). Group 1 received pemetrexed. Each rat was injected with ALIMTA (IV, 10 mg/kg), a sterile white-to-light yellow or green-yellow lyophilized powder, for 8 weeks. Group 2 received N-acetylcysteine (IP, 80 mg/kg) 30 minutes before pemetrexed for five weeks. Group 3 received Glycine (6 g) and L-Tryptophan (0.4 g) 30 minutes before pemetrexed for eight weeks. Group 4 was a control group, received normal saline by gavage.

Drugs:

N-acetylcysteine (NAC), glycine, and tryptophan were supplied from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). Before use, all drugs were dissolved in distilled water and were freshly prepared. After eight weeks, animals were anesthetized with isoflurane and underwent cardiac puncture for blood collection. To evaluate renal function,

blood creatinine, urea, and uric acid were quantified using colorimetric assays in a clinical laboratory utilizing an automated biochemical analyzer (Pointe Scientific, Canton, MI, or Sigma kits).

RNA Extraction, Reverse Transcription, Quantitative Analysis of Transcripts Using qRT-PCR:

RNA was isolated from serum according to the manufacturer's instructions using a RNeasy kit (Qiagen). Nanodrop analysis was used to assess RNA quality and amount. Reverse transcription was done at 25° C, 42° C, and 70° for 10 min, 1 h, and 10 min, respectively. qRT-PCR was performed using 1 µl of cDNA (50 ng), 5 µl of SYBR Green/0.2 µl of ROX qPCR Master Mix (2X, Fermentas, Germany), and 1 µl of forward and reverse primers adjusted to a total volume of 10 ml using nuclease-free water. GAPDH was used as a reference gene.

Relative quantification of EGFR, TPH1, and TGF was performed with the 2^{-ddCt} method and analyzed by ABI prism 7500. The Boxplot software and values were normalized to the quantity of GAPDH.

Table 1: primers sequences of EGFR. TGF. TPH1 and GAPDH.

EGFR	Forward Sequence GGAGGACCGTCGCTTGGT-
	Reverse Sequence AACACCGCAGCATGTCAAGA-
TGF beta	Forward Sequence TACCTGAACCCGTGTTGCTCTC
	Reverse Sequence GTTGCTGAGGTATCGCCAGGAA
Tryptophan hydroxylase	Forward Sequence TTCTGACCTGGACCATTGTGCC
	Reverse Sequence ACGGTAGACATTGTCTTTGAAGCC
GAPDH	Forward Sequence CAGTGCCAGCCTCGTCTCAT
	Reverse Sequence AGGGGCCATCCACAGTCT TC

Statistical Methods:

SPSS software version 25 was used for statistical analysis (IBM, Armonk, New York, United States). Quantitative data were presented as means and standard deviations or medians and IQRs. Comparisons between the study groups were done using one-way ANOVA or Kruskal-Wallis test. Post-hoc analyses were done using Bonferroni's method. All statistical tests were two-sided. P values less than 0.05 were considered significant.

RESULTS

Creatinine showed an overall significant difference (P-value <0.001) between groups. Pairwise analysis revealed that group I had higher creatinine than groups II, III, and IV. Also, group II showed higher creatinine compared to groups III & IV. No significant difference was observed between groups II & IV (Table 2 & Fig. 1).

Table (2) Creatinine in the studied groups

	Group I	Group II	Group III	Group IV	P-value
Creatinine	3.49 ±0.49	1.8 ±0.19	1.33 ±0.29	1.07 ±0.12	<0.001
Post-hoc	2,3,4	1,3,4	1,2	1,2	

Data was presented as mean ±SD. One-way ANOVA was used. All post hoc comparisons were Bonferroni adjusted 1; sig. diff. from G I 2; sig. diff. from G II 3; sig. diff. from G III 4; sig. diff. from G IV

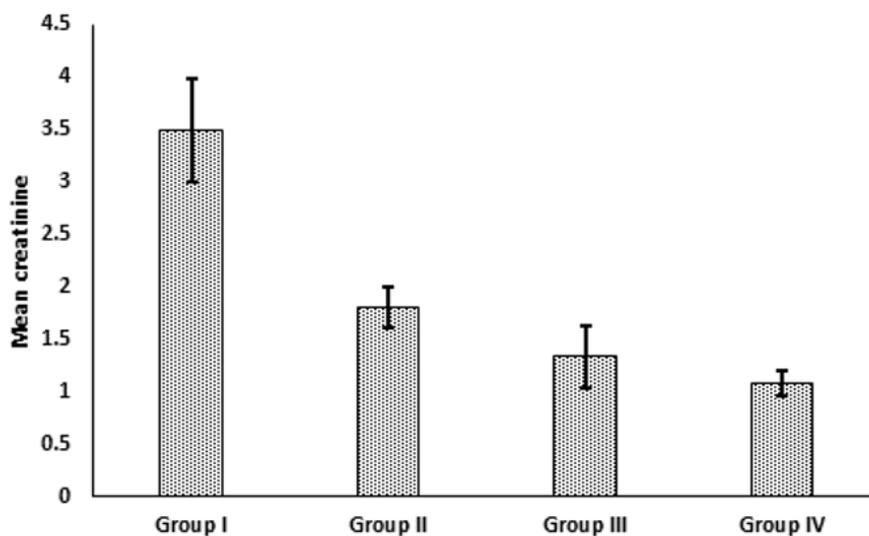


Fig. 1: Creatinine in different study groups

Urea showed an overall significant difference (P-value <0.001) between groups. Pairwise analysis revealed that group I had higher urea than groups II, III, and IV. Also, group II showed higher urea compared to groups III & IV. No significant difference was observed between groups II & IV (Table 3& Fig. 2).

Table 3: Urea in the studied groups

	Group I	Group II	Group III	Group IV	P-value
Urea	94 ±12	56 ±4	41 ±5	38 ±2	<0.001
Post-hoc	2,3,4	1,3,4	1,2	1,2	

Data was presented as mean ±SD. One-way ANOVA was used. All post hoc comparisons were Bonferroni adjusted

1; sig. diff. from G I 2; sig. diff. from G II 3; sig. diff. from G III 4; sig. diff. from G IV

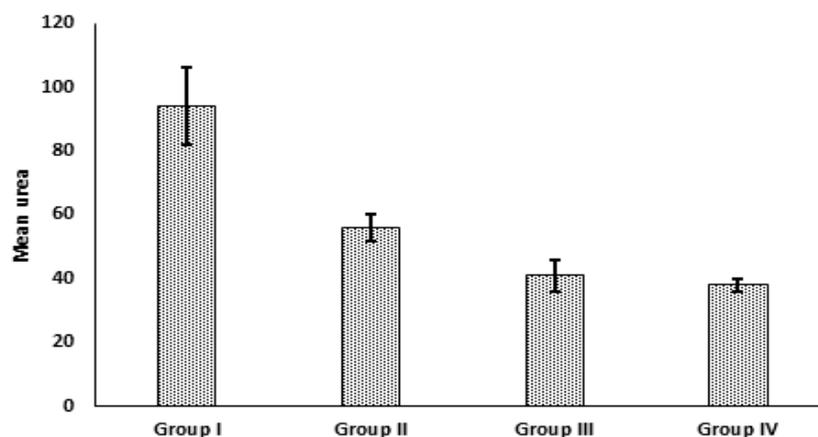


Fig. 2: Urea in different study groups.

Uric acid showed an overall significant difference (P-value <0.001) between groups. Pairwise analysis revealed that group I had higher uric acid than groups II, III, and IV. Also, group II showed higher uric acid compared to groups III & IV. Moreover, group II was significantly higher compared to group IV (Table 4).

Table 4: Uric acid in the studied groups

	Group I	Group II	Group III	Group IV	P-value
Uric acid	6.7 ±0.6	4.4 ±0.7	3.5 ±0.2	1 ±0.2	<0.001
Post-hoc	2,3,4	1,3,4	1,2,4	1,2,3	

Data was presented as mean ±SD. One-way ANOVA was used. All post hoc comparisons were Bonferroni adjusted

1; sig. diff. from G I 2; sig. diff. from G II 3; sig. diff. from G III 4; sig. diff. from G IV

TGF gene showed an overall significant difference (P-value <0.001) between groups. Pairwise analysis revealed group I had significantly higher TGF than groups III, IV. There were no other significant differences between groups (Table 5).

Table 5: TGF gene in the studied groups

	Group I	Group II	Group III	Group IV	P-value
TGF gene	5.099 (4.787 - 5.495)	1.2285 (1.033 - 2.359)	1.2245 (0.589 - 1.49)	0.194 (0.181 - 0.389)	<0.001
Post-hoc	3,4	-	1	1	

Data were presented as median (IQR). Kruskal-Wallis test was used. All post hoc comparisons were Bonferroni adjusted

1; sig. diff. from G I 2; sig. diff. from G II 3; sig. diff. from G III 4; sig. diff. from G IV

Epidermal growth factor receptor (EGFR) showed an overall significant difference (P-value <0.001) between groups. Pairwise analysis revealed that group I had significantly higher EGFR than groups III, IV. Also, group II had higher EGFR than group IV. There were no other significant differences between groups (Table 6 & Fig. 3).

Table (6) EGFR in the studied groups

	Group I	Group II	Group III	Group IV	P-value
EGFR	5.23 (4.452 - 5.981)	2.164 (1.59 - 3.521)	1.581 (1.179 - 2.169)	0.176 (0.166 - 0.71)	<0.001
Post-hoc	3,4	4	1	1,2	

Data were presented as median (IQR). Kruskal-Wallis test was used. All post hoc comparisons were Bonferroni adjusted

1; sig. diff. from G I 2; sig. diff. from G II 3; sig. diff. from G III 4; sig. diff. from G

EGFR; Epidermal growth factor receptor.

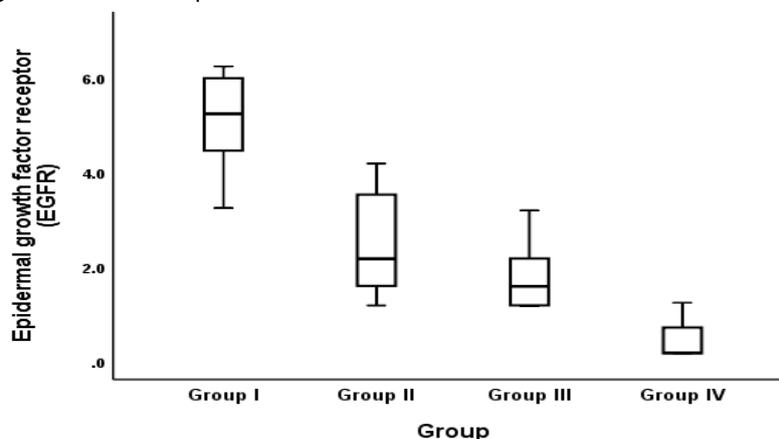


Fig.3: EGFR in different study groups

Tryptophan hydroxylase-1 (TPH-1) showed an overall significant difference (P-value <0.001) between groups. Pairwise analysis revealed that group I had significantly lower TPH than groups II, III, and IV. There were no other significant differences between groups (Table 7 & Fig. 4).

Table (7) TPH-1 in the studied groups

	Group I	Group II	Group III	Group IV	P-value
TPH-1	0.168 (0.164 - 0.477)	3.953 (3.234 - 4.709)	4.233 (3.444 - 4.717)	5.453 (4.969 - 6.239)	<0.001
	2,3,4	1	1	1	

Data were presented as median (IQR). Kruskal-Wallis test was used. All post hoc comparisons were Bonferroni adjusted

1; sig. diff. from G I 2; sig. diff. from G II 3; sig. diff. from G III 4; sig. diff. from TPH-1; Tryptophan hydroxylase-1

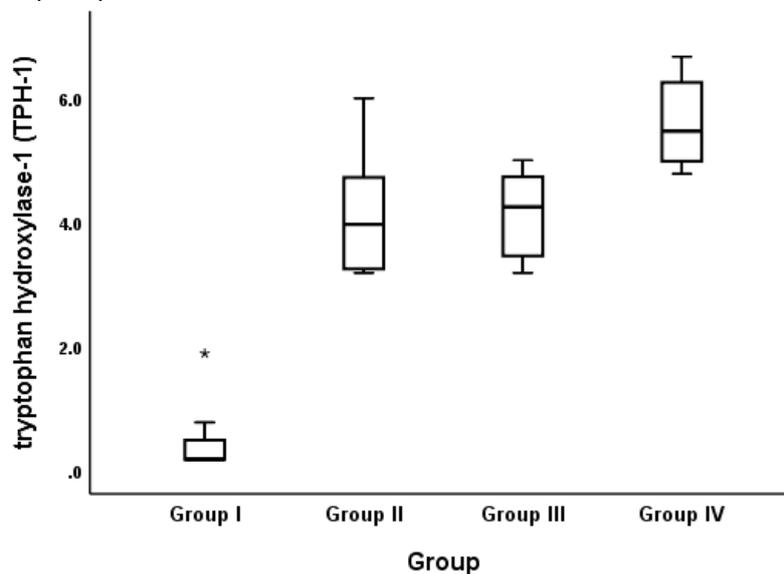


Fig.4: TPH-1 in different study groups

DISCUSSION

Chronic kidney disease (CKD) is a prominent cause of noncommunicable disease morbidity and death, affecting 10% of the world population, chronic kidney disease (CKD) is the main cause of cardiovascular illness, early death, and end-stage renal disease. As a result, avoiding the development of CKD is critical (Jha V *et al.*, 2013)

N-acetylcysteine (NAC) is a glutathione regenerator. NAC has been studied and is now being utilized to help avoid iatrogenic acute kidney damage and help reduce chronic kidney disease progress (Johnny W. *et al.*, 2021).

According to the current study, N-acetylcysteine with pemetrexed showed a significant decline ($P < 0.001$) in serum creatinine, urea, uric acid, TGF- β 1, and epidermal growth factor receptor (EGFR), and an increase in TPH expression, compared to the pemetrexed group.

Prior studies evaluated tyrosine kinase inhibitors effect on N-acetyl glutamate synthase, which is a urea cycle key enzyme (Li, J. *et al.*, 2019) (Pham-Danis C. *et al.*, 2019). The current study showed that pemetrexed might interfere with glutamate incorporation in ERK1/2 stimulatory signals by halting glutamate receptors activation by Src tyrosine kinases, which may direct glutamate towards the elevation of urea formation in the blood.

Pemetrexed blocks thymidylate synthase (TS), decreasing the available thymidine for DNA synthesis. Additionally, pemetrexed inhibits dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT), two enzymes important for the de novo production of thymidine and purine nucleotides. After entering the cell via the reduced folate carrier, pemetrexed is polyglutamated. Glutamation increases pemetrexed cellular retention and intracellular half-life while simultaneously increasing the inhibitory activity of the polyglutamated metabolites by more than 60-fold (Paz-Ares L. *et al.*, 2003). Thus, an increase in uric acid levels may develop as a result of excessive nucleic acid breakdown from dead cells following a high dosage of the medication (Pui C.H. *et al.*, 1997).

N-acetyl-l-cysteine use as an EGFR inhibitor prevented the AAs depletion caused by urea. This finding shows the inhibitory effect of urea over the EGF/EGFR system (Rasoul Kowsar *et al.*, 2020). Urea signaling is sensitive to NAC supplementation (Zhao H. *et al.*, 2003).

After eight weeks of continuous ingestion of combined glycine and tryptophan with pemetrexed, serum uric acid significantly decreased, renal function improved, expression of TGF- β 1 decreased, and the expression of TPH-1 increased, according to the current study. The combination of glycine and tryptophan has a uric acid-lowering effect, which influences renal function and significantly suppresses the elevation in uric acid levels.

Afferent arteriopathy, inflammation, and stimulation of the renin-angiotensin system are all possible processes underlying kidney injury induced by uric acid buildup. The consequences of high uric acid are believed to be produced by uric acid's direct toxicity to the kidney (Kang DH, Chen W, 2011). Shunji Oshima *et al.* 2021) are in line with our results.

The excretion of urates into the urine leads to decreased serum uric acid concentration (Oshima S. *et al.*, 2019). The AA may impair renal function; a single combination dosage of glycine and tryptophan transiently improves eGFR, indicating that eGFR, as a measure of renal function, may be increased via continuous supplementation with combined glycine and tryptophan. Glycine is metabolized to produce various compounds, including glutathione, nucleic acid bases, heme, creatine, and bile. The conversion of glycine to creatinine via creatine may affect the blood creatinine level.

The EGFR may impede the transfer of AAs in the pemetrexed group. It is suggested that the greater depletion of AAs in renal injury is due to urea inhibitory effect on the EGF/EGFR pathway. (Garofalo, A. *et al.*, 2010) (Amaral D. N. *et al.*, 2019) (Jiang N. *et al.*, 2016).

Urea effects on AAs via the EGFR system were investigated using NAC. It has been demonstrated that NAC inhibits EGFR activation. NAC is a sensitive regulator of urea signaling (Zhao *et al.*, 2003).

The potential relation between altered levels of epidermal growth factor and elevated uric acid with reduced eGFR might be important in preventing, diagnosing, and managing impaired kidney function (Wu J. *et al.*, 2018).

TGF-1 is a fibrogenic cytokine that may promote the synthesis of ECM, change its breakdown, and is implicated in the primary pathogenic processes of chronic kidney disease. The stimulation of the EGFR signaling pathway was essential for boosting TGF-1 production. (Chen *et al.*, 2011) demonstrated that inhibiting EGFR can reduce TGF- β 1 /2/p38 expression (Lu T. C. *et al.*, 2009) (N Liu *et al.*, 2012). The EGFR inhibitor can slow the course of kidney damage and fibrosis in the kidney. This impact might be mediated at least by an antioxidant effect, which targets the STAT3 activation pathway, the ERK1/2 pathway, and the profibrogenic marker TGF-1. NAC treatment can delay the progression of fibrosis (Chen *et al.*, 2011).

Renal fibrosis is caused by the interaction of cellular and molecular processes triggered by glomerular damage and involving podocytes and numerous pro-fibrotic agents (Tiago Giuliani Lopes *et al.*, 2019).

Connective tissue growth factor (CTGF) functions as a mediator of TGF- β activity on mesenchymal cells. Both are co-expressed in various kinds of glomerular damage (Ito *et al.*, 2010). Multiple processes contribute to renal fibrosis, including cellular events (monocyte and T cell infiltration), the activity of critical signaling molecules (NF- κ B, TGF- β /Smad), cell death, and an excess of matrix-degrading enzymes (Liu Y. 2006).

For six weeks, a powder combination of glycine and tryptophan (3g and 0.2g, respectively) was eaten at bedtime once daily, and serum uric acid levels were considerably reduced (Oshima S *et al.*, 2019). Up to 90 g glycine or 5.0 g L-tryptophan per day was consumed without severe adverse effects (Pérez-Torres *et al.*, 2017) (Hiratsuka C *et al.*, 2013). The combination of two AA is safe, considering that they were continually ingested for six weeks with no change in physiological parameters (Ter Wee PM *et al.*, 1986).

TPH-1 deficiency promotes kidney damage and fibrosis via activating NF- κ B and blocking Nrf2 pathways. Thus, we propose that activation of TPH-1 be used as a target in the therapy of chronic kidney disease.

TPH-1 expression was decreased following TGF- β 1 and LPS-induced kidney damage. TPH-1 deficiency decreased epithelial cell marker expression and elevated pro-fibrotic marker expression, resulting in extracellular matrix buildup and kidney fibrosis (Wang, Y. F. *et al.*, 2016).

Wei Q *et al.*, 2014 revealed the role of tryptophan and amino acids as potential biomarkers and their biologically significant role, both within normal renal homeostasis and diseased kidney. Additionally, glycine at dosages up to 90 g per day for several weeks shows no significant side effects (Pérez-Torres I *et al.*, 2017) (Friedman M., 1947).

Glycine increased uric acid excretion and urate clearance. Continuous glycine and tryptophan feeding resulted in a rise in urine pH (Glycine acts as a buffer and is thus utilized as an antacid). Uric acid solubility in water increases as pH levels increase, and uric acid excretion is greater in alkaline urine than in acidic urine. Elevated urine pH caused by glycine and tryptophan increases uric acid solubility, thereby increasing urinary uric acid excretion (Kanbara A. *et al.*, 2012).

CONCLUSION

Early detection and precise monitoring of chronic kidney disease (CKD) can enhance care and delay development to end-stage renal disease. Depletion or appearance of AAs (tryptophan) could change in the presence of urea and EGFR.

List of Abbreviations

chronic kidney disease (CKD)

N-acetyl-l-cysteine NAC

Tryptophan hydroxylase (TPH-1)

Transforming growth factor β (TGF- β)

Acute Kidney injury AKI

ANGIOTENSIN-converting enzyme inhibitors (ACEI)

nuclear factor- κ B

nuclear factor erythroid 2-related factor 2 Nrf2

GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

EGFR, epidermal growth factor receptor

Thymidylate synthase (TS)

Dihydrofolate reductase (DHFR)

Glycinamide ribonucleotide formyltransferase (GARFT),

Connective tissue growth factor (CTGF)

5-MTP 5-methoxytryptophan

LPS lipopolysaccharide

eGFR estimated glomerular filtration rate

AA amino acid

* **Conflicts of Interest**/Competing interests no conflict

* **Ethics Approval**

* Consent to Participate (Ethics) not applicable

* Consent to Publish (Ethics) -not applicable

* Authors' contributions -no contribution

* Availability of data and material from molecular biology and biotechnology unit at Faculty of Medicine, Banha University

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