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Research Article

Impact of Acute and Chronic Stress on Novel Kidney Injury and CBC-Derived Inflammatory Biomarkers in Rats

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Abstract

Background: Stress is associated with structural and physiological changes in various organs, but its impact on kidney injury is not well-studied. ***Objective***: To investigate the physiological changes in the kidneys of acutely and chronically stressed rats. ***Methods***: Twenty-four rats were assigned to three groups (n=8). Negative control (NC): unstressed, acute stress (AS): exposed to cold-restraint stress for three hours, chronic stress (CS): exposed to chronic unpredictable stress (CUS) protocol for one month. The animals were sacrificed, and blood and kidney tissue were collected for assessment of kidney injury molecule-1 (KIM-1), cystatin C, serum creatinine, blood urea, interleukin-6 (IL-6), total antioxidant capacity (TAC), and complete blood count (CBC)-derived markers. ***Results***: A significant elevation of KIM-1 and cystatin C levels in AS and CS groups and serum creatinine in rats exposed to CS was observed with a significant reduction in the serum level of IL-6 in acute and chronically stressed rats. TAC was elevated non-significantly in the AS group; however, it was significantly elevated in the CS group. CBC-derived biomarkers like neutrophil/lymphocyte, platelet/lymphocyte, and monocyte/lymphocyte ratios showed a significant increase in favor of inflammatory responses. ***Conclusions***: Chronic and acute stress exert a detrimental effect on the kidney, with chronic stress resulting in more severe injuries. Chronic stress has a complex effect on antioxidants and inflammatory processes. This might be related to the adaptive physiological mechanism and/or alteration in the hematological parameters, which represents the stress-induced inflammatory process.

Keywords: Acute Stress, Chronic Stress, KIM-1, Kidney injury, novel biomarkers.

تأثير الإجهاد الحاد والمزمن على مؤشرات الكلى الجديدة والمؤشرات الحيوية الالتهابية المشتقة من CBC في الجرذان

الخلاصة

الخلفية: ارتبط الإجهاد بالتغيرات الهيكلية والفسيولوجية في الأعضاء المختلفة، لكن تأثيره على إصابة الكلى لم يتم دراسته جيدا. **الهدف**: التحقيق في التغيرات الفسيولوجية في الكلى للجرذان المجهدة بشكل حاد ومزمن. **الطرائق**: تم تعيين أربعة وعشرين جرذا إلى ثلاث مجموعات (ن = 8). التحكم السلبي (NC): الإجهاد الحاد البسيط (AS): التعرض لإجهاد ضبط النفس اثناء التعرض للبرد لمدة ثلاث ساعات، الإجهاد المزمن (CS): التعرض لبروتوكول الإجهاد المزمن غير المتوقع (CUS) لمدة شهر واحد. تم التضحية بالحيوانات، وتم جمع الدم وأنسجة الكلى لتقييم المؤشرات: جزيء إصابة الكلى -1 (KIM-1)، والسيستاتين C، والكرياتينين واليوريا في الدم، والإنترلوكين -6 (IL-6)، والقدرة الكلية المضادة للأكسدة (TAC)، وعلامات تعداد الدم الكامل (CBC). **النتائج**: لوحظ ارتفاع كبير في مستويات KIM-1 و cystatin C في مجموعات AS و CS والكرياتينين في الدم في الجرذان المعرضة ل CS مع انخفاض كبير في مستوى مصل IL-6 في الجرذان الحادة والمجهدة المزمنة. ارتفع TAC بشكل غير ملحوظ في مجموعة AS. ومع ذلك ، فقد ارتفعت بشكل كبير في مجموعة CS. أظهرت المؤشرات الحيوية المشتقة من CBC مثل العدلات / الخلايا الليمفاوية، والصفائح الدموية/ الخلايا الليمفاوية، ونسب الخلايا الوحيدة/الخلايا الليمفاوية زيادة كبيرة في تفضيل الاستجابات الالتهابية. **الاستنتاجات**: أثر كل من AS و CS بشدة على الكلى المريضة بشكل مزمن. الإجهاد المزمن له تأثير معقد على مضادات الأكسدة والعمليات الالتهابية. قد يكون هذا مرتبطا بالآلية الفسيولوجية التكيفية و / أو التغيير في المعلمات الدموية ، والتي تمثل العملية الالتهابية الناجمة عن الإجهاد.

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INTRODUCTION

The occurrence of kidney diseases has significantly increased in recent years. Many researchers have started to focus on the impact of psychological, social, and economic factors on the onset and progression of renal diseases [1]. These factors mostly targeted vulnerable populations, where high social and occupational burdens and poor socioeconomic conditions contribute to chronic stress [2]. Stress is

characterized by a reactive state that triggers both physiological and behavioral responses. These responses are often associated with a series of systemic changes, primarily driven by the overactivation of the adrenal glands. This leads to an elevated secretion of glucocorticoids from the adrenal cortex, forming the core of the stress response mechanism [3]. Both acute and chronic stress have been linked to the alteration of the physiological function of several tissues or organs; thus, it affects multiple organs and tissues in the body, including the digestive system, cardiovascular system, and hepatobiliary system [4,5]. It also has a detrimental effect on the kidneys [6,7]. Numerous studies indicate that stress profoundly affects kidney function through multiple mechanisms. An experimental study demonstrates that chronic stress in both prepubertal and adult rats causes irreversible glomerular damage and altered kidney morphology, implying that stress exerts immediate and long-term detrimental effects on the renal system [6]. One study highlights how maternal psychosocial stress during pregnancy reduces nephron endowment in offspring, thereby increasing the risk of chronic kidney disease (CKD) [8]. Furthermore, a study finds that job-related stress intensifies the connection between metabolic risk factors and renal dysfunction in adult men [9]. In another study, social, economic, and psychological stressors demonstrate a pivotal role in CKD prevalence and progression, particularly in high-risk groups [1]. Based on these findings, multiple mechanisms of stress influence kidney structure and function. Although emerging evidence suggests a link between stress and CKD, this area of research remains largely unexplored. Detecting stress-induced kidney injury at an early stage remains difficult. Conventional biomarkers like serum creatinine, blood urea nitrogen (BUN), and blood urea are frequently used but are not very reliable for initial damage; they are limited in their sensitivity for early identification and kidney injury diagnosis [10]. The newer identified biomarkers, such as kidney injury molecule-1 (KIM-1) [11], cystatin C, and neutrophil gelatinase-associated lipocalin (NGAL), have shown potential for early detection of renal injury [12,13]. Furthermore, recent investigations have highlighted the value of inflammatory markers derived from complete blood count (CBC) in the diagnosis of a range of clinical conditions, including renal dysfunction [14], malignancy [15], and metabolic syndrome [16], and their analysis has been recently applied in patients who underwent arthroplasty [17]. Ratios such as neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), monocyte/lymphocyte ratio (MLR), platelet/neutrophil ratio (PNR), red cell distribution width/platelet ratio (RPR), lymphocyte/monocyte ratio (LMR), systemic immune-inflammation index (SII), and red cell distribution width-coefficient of variation (RDW-CV) have demonstrated significant utility as early indicators for the diagnosis and management of disease-associated complications [18]. Despite the importance of these novel biomarkers and CBC-inflammatory markers, their roles and mechanisms in stress-related kidney damage are not fully explored. Therefore, the current study aimed to explore the impact of acute and chronic stress models on kidney injury in experimental animals, with a focus on the assessment of novel biomarkers for kidney injury such as KIM-1 and cystatin C, in addition to the conventional biomarkers. Additionally, the impact of acute and chronic stress on inflammatory response and hematological parameters, including novel CBC-derived inflammatory biomarkers, was investigated.

METHODS

Laboratory kits and chemicals

Rat kidney injury molecule-1 (KIM-1), cystatin C, total antioxidant capacity (TAC), and interleukin-6 (IL-6) were measured by enzyme-linked immunosorbent assay (ELISA) kit from Bioassay Technology Laboratory, Shanghai, China. Serum creatinine and blood urea were measured using the Cobas c 311 analyzer, while complete blood counts (CBC) were tested by the Medonic M51 hematology analyzer. Urine test strips Cybow 10 (dipstick test) from Winterthur Medical AG, Germany, were utilized for semi-quantitative urinalysis.

Animal groups and ethical approval

Twenty-four healthy male Wistar albino rats with a weight of 150 ± 20 g were purchased from the animal house of the College of Pharmacy-University of Sulaimani. The room temperature was kept at 22 ± 3.0 with a relative humidity of 50%. The rats were housed in a separate area with a 12-hour light-dark cycle and free access to food and water for two weeks to be acclimated. The experiments were conducted in compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In addition, the experiment protocol was registered and formally approved by the Research Registration and Ethics Committee of the College of Pharmacy-University of Sulaimani (registration number PH146-24 on the date 28/11/2024). The experiments were conducted during the light and dark phases of the circadian cycle of the animals.

Study design and protocols

The 24 rats were assigned to three groups, each of 8 rats, as follows: Negative control (NC) group: a control group of unstressed rats was kept in their home cages until the time of scarification. Fasting is not allowed

during the modeling process to ensure that the body condition remains consistent with the other groups. Acute stress (AS) group: the rats were exposed to acute stress, which was induced by the cold-restraint stress method for three hours [19] and sacrificed after verifying the stress induction. Chronic stress (CS) group: the rats were exposed to chronic unpredicted stress (CUS) exposure [20,21] (Figure 1).

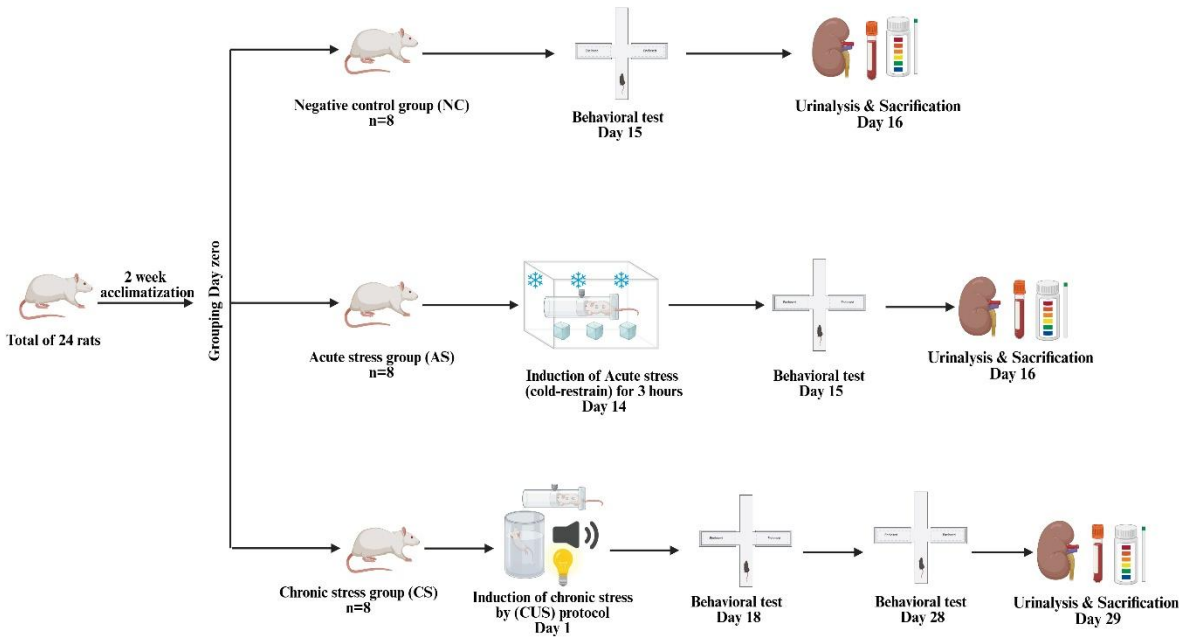

Figure 1: Flowchart of the study. NC: negative control, AS: acute stress, CS: chronic stress.

Stress protocols

A validated psychological stressor, the cold-restraint stress method [19], was adopted. Briefly, rats were starved for 24 hours and then immobilized using restrainers individually and placed in a cold room for 3 hours at 4 ± 0.3°C. To induce chronic stress in rats, a modified version of the chronic unpredictable stress protocol (CUS) was performed, which involves subjecting animals to stressors that are variable and unpredictable for several days or weeks at a time; this lowers the risk of adaptation. [20–24]. CUS protocol was performed for 4 weeks following the schedule and timing previously reported by Wu *et al*. (2004). To verify the success of stress induction, open field tests (OFT) and elevated plus mazes (EPM) were used.

Open field test

The open field test (OFT) apparatus consists of a wooden box with a square base measuring 80 cm in length and surrounded by 60 cm height for each side. The floor is marked into 16 equal squares. Observations included the number of squares each rat entered with its paws (crossing); instances of standing upright on the hind limbs against the walls (walling); and behaviors such as wiping, licking, or combing any body part (grooming). The number of times the rat urinated or defecated was also counted. Each rat was introduced at the center of the box, and the duration (in seconds) of the time spent in the margins and in the center, as well as the total time of movement and staying in one square, were measured over a 5-minute period. The floor was cleaned with ethyl alcohol and allowed to dry between each trial [25,26]. The testing took place in the morning, between 9:00 AM and 12:00 PM.

Elevated plus maze

The elevated plus maze (EPM) test is a commonly used behavioral method for evaluating anxiety-related responses. The apparatus consisted of four arms measuring 50 cm in length and 10 cm in width crossing at a right angle; two opposing arms are enclosed by walls 40 cm in height, while the other two arms remain open, except at the central junction where all arms meet. The maze is positioned 50 cm above ground. Each rat was placed at the center of the maze and observed for five minutes, with behavior recorded using a mobile phone camera. The time the animal spent in each arm, the total time of movement and immobilization, frequency of urination and defecation, as well as the occurrence of specific behaviors such as grooming, walling, and head dipping, were recorded manually. The test was performed in the morning, from 9:00 AM to 12:00 PM [26].

Outcome measures

The study assessed KIM-1 and cystatin C in serum and kidney tissue homogenate and traditional markers of kidney function, such as serum creatinine and blood urea, in addition to urinalysis using dipstick tests. IL-6, TAC, and CBC were measured, and CBC-derived inflammatory biomarkers, including neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), platelet/lymphocyte ratio (PLR), platelet/neutrophil ratio (PNR), red cell distribution width/platelet ratio (RPR), lymphocyte/monocyte ratio (LMR), systemic immune-inflammation index (SII), red cell distribution width-coefficient of variation (RDW-CV), were calculated as previously described [15,17].

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism software (version 10.4.1, LLA, CA, USA). Shapiro-Wilk test was used to test the distribution of the variables. For normally distributed data, parametric analysis was performed using a one-way ANOVA test followed by Tukey's multiple comparison for comparison between different groups. While the Kruskal-Walli's test followed by Dunn's multiple comparisons test was used for non-parametric values. A p-value of <0.05 was considered statistically significant.

RESULTS

To verify the successful induction of acute and chronic stress, behavioral evaluation was performed through analysis of locomotor and anxiety-associated behaviors using OFT and EPM tests. In OFT, the rats exposed to acute and chronic stressors demonstrated a significant behavioral alteration ($p<0.05$), as shown in Figure 2 (A-I).

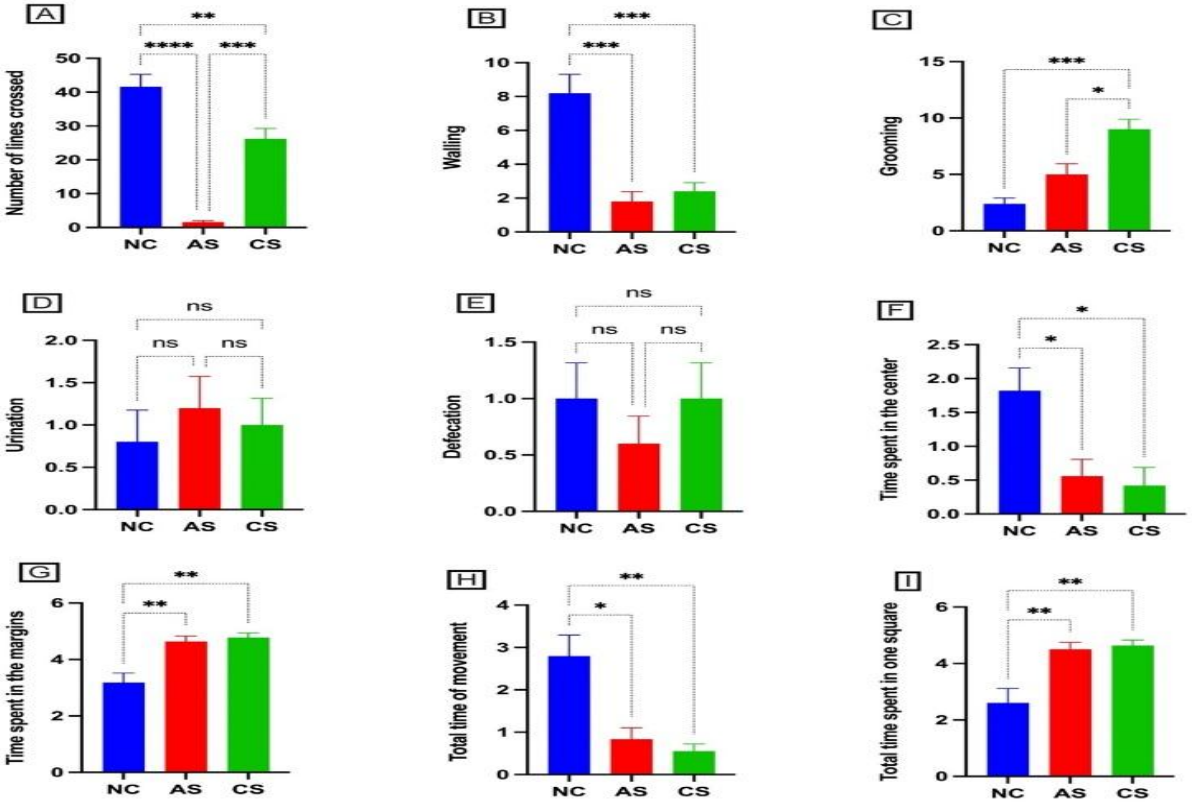

Figure 2 (A-I): Verification of stress using Open Field Test (OFT). **A**) Number of lines crossed; **B**) Walling; **C**) Grooming; **D**) Urination; **E**) Defecation; **F**) Time spent in the center; **G**) Time spent in the margins; **H**) Total time of movement; and **I**) Total time spent in one square. One-way ANOVA confirmed by Tukey's multiple comparisons was used. *p< 0.01, **p<0.005, ***p<0.0002, ****p<0.0001. ns: statistically non-significant. NC: negative control, AS: acute stress, CS: chronic stress.

Urination and defecation were not significantly altered (p>0.05). Collectively, these changes confirm the successful induction of stress. Consistent with the OFT analysis, behavioral outcomes in the EPM provided additional verification for the successful induction of stress by acute and chronic stress, as shown in Figure 3 (A-I). The change in the body weight of all the animals before and after the experiment is shown in Figure 4 A. There was a significant difference in the rate of weight gain of the rats exposed to one month of stress in the CS group (p<0.0001) compared to the unstressed animals in the NC group and animals exposed to acute stress (p>0.05). Minimum weight gain was observed in

the CS group. The body weight of rats in CS was monitored every week for four weeks; no significant difference was observed in body weight across the experimental period (p>0.05). However, a significant reduction in kidney index was observed (p<0.05) in the CS group on the last day of the experiment compared to the NC and AS groups (Figure 4 B and C). The effect of AS and CS on KIM-1 in both serum and kidney tissue homogenate is shown in Figure 5 (A and B). There was a significant elevation of tissue homogenate KIM-1 level in AS- and CS-stressed rats (p<0.05). Elevation of serum KIM-1 level was detected non-significantly (p>0.05). Moreover, the impact of AS and CS on glomerular function biomarker cystatin C resulted in a significant elevation of its level in serum (p<0.05). However, in tissue homogenate, cystatin C was significantly elevated only in the CS group (p<0.05) as shown in Figure 6 (A and B).

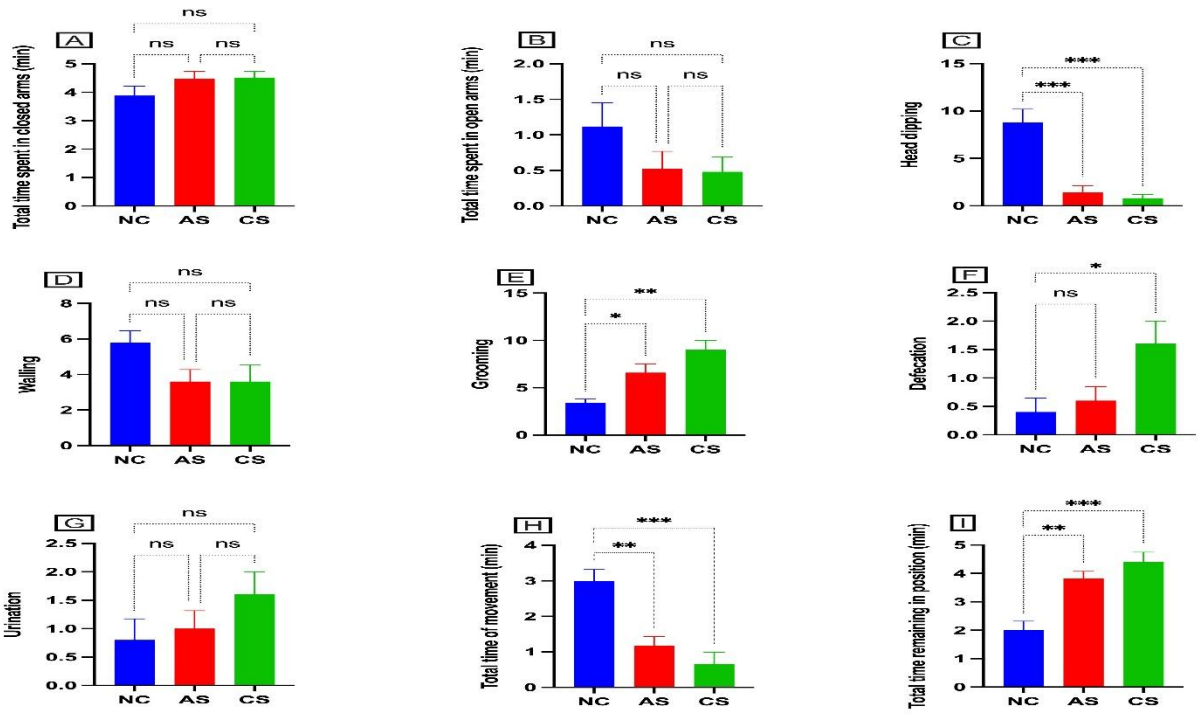

Figure 3 (A-I): Verification of stress using Elevated Plus Maze (EPM) Test. **A**) Total time spent in closed arms; **B**) Total time spent in open arms; **C**) Head dipping; **D**) Walling; **E**) Grooming; **F**) Defecation; **G**) Urination; **H**) Total time of movement; and **I**) Total time remaining in position. One-way ANOVA confirmed by Tukey's multiple comparisons was used. *p<0.05, **p<0.0001, ***p<0.0003. ns: statistically non-significant. NC: negative control, AS: acute stress, CS: chronic stress.

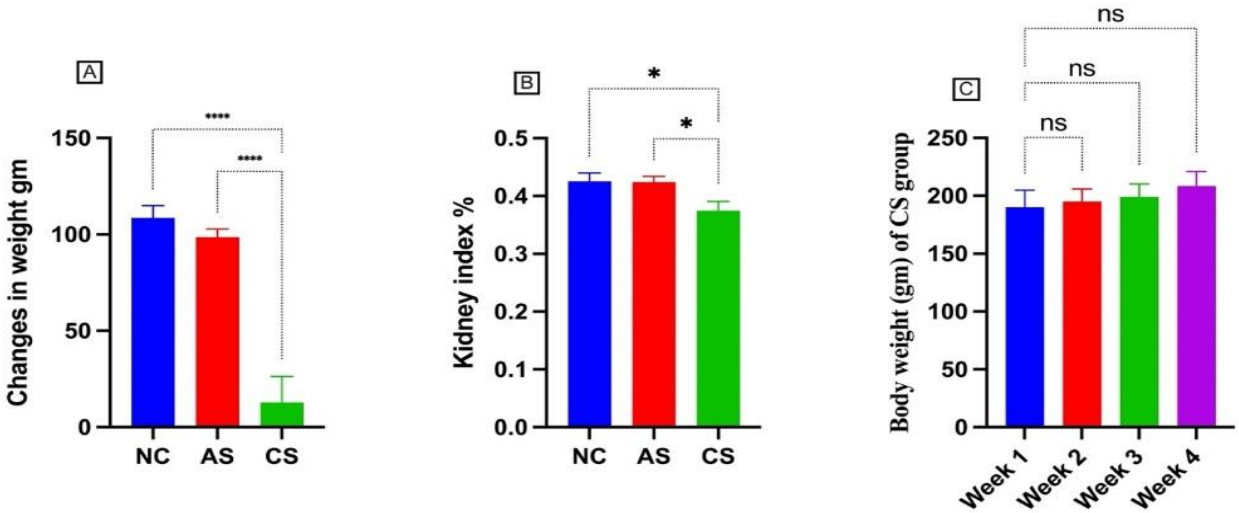

Figure 4 (A-C): Effect of stress on the body weight. **A**) Changes in the body weight of the animals before and after the experiment; **B**) Kidney index; and **C**) Body weight of the animals exposed to chronic stress during the experiment period. One-way ANOVA confirmed by Tukey's multiple comparisons was used at p<0.05 for statistical significance. ns: indicates statistically non-significant. NC: negative control, AS: acute stress, CS: chronic stress. *p<0.05, ****p<0.0001.

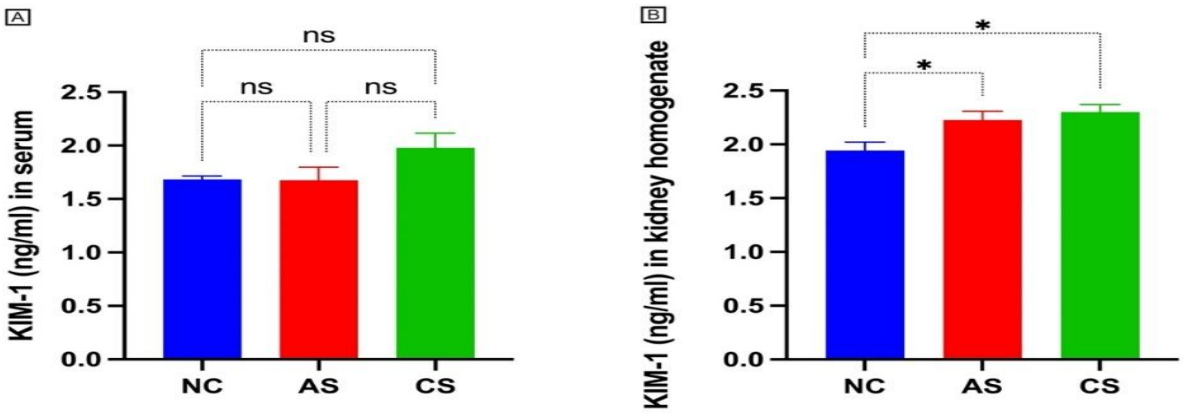

Figure 5 (A and B): Effect of Acute and Chronic Stress on Kidney Injury Molecule-1 in **A**) Serum and **B**) Kidney tissue homogenate. One-way ANOVA followed by Tukey's multiple comparisons was used. *p<0.05, is statistically significant. ns: indicates statistically non-significant. NC: negative control, AS: acute stress, CS: chronic stress. KIM-1: Kidney Injury Molecule-1.

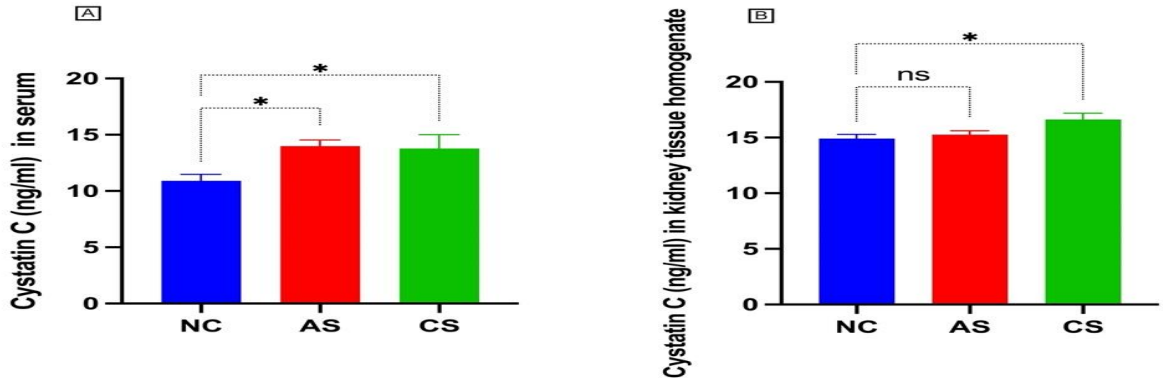

Figure 6 (A and B): Impact of acute and chronic stress on cystatin C in **A**) Serum and **B**) Kidney tissue homogenate. One-way ANOVA followed by Tukey's multiple comparisons was used. *p<0.05, is statistically significant. ns: statistically non-significant. NC: negative control, AS: acute stress, CS: chronic stress.

Additionally, the impacts of AS and CS on the semi-quantitative urinalysis dipstick test are shown in Figure 7A. Analysis of hematuria recorded a higher score on the dipstick scale in rats exposed to CUS for one month compared to the NC group in a non-significant manner (p<0.05). Additionally, analysis of protein urea and leukocytes on dipstick demonstrated higher scores in the CS-exposed group in a significant manner (p<0.05) as shown in Figures 7B and 7C. Serum samples from each rat were also analyzed to assess conventional kidney function biomarkers, including serum creatinine and blood urea. A significant elevation in serum creatinine was observed in rats exposed to CS for one month, while blood urea elevated non-significantly in the CS group. Non-significant changes have been observed in the AS group, as shown in Figure 7 (D and E). The effects of AS and CS on the pro-inflammatory biomarker IL-6 were investigated. Figure 8A shows a significant reduction in the serum level of IL-6 in animals subjected to both AS and CS (p<0.05). Additionally, TAC was elevated non-significantly in the AS group (Figure 8B); however, when animals were subjected to CUS for one month, a significant elevation of TAC was observed (p<0.05). Analysis of CBC-derived inflammatory biomarkers is demonstrated in Figure 9 (A-H). A significant change in most of the CBC-derived inflammatory biomarkers (p<0.05) such as NLR (neutrophil/lymphocyte ratio), PLR (platelet/lymphocyte ratio), MLR (monocyte/lymphocyte ratio), PNR (platelet/neutrophil ratio), RPR (red cell distribution width/platelet ratio), LMR (lymphocyte/monocyte ratio), SII (systemic immune-inflammation index), and RDW-CV (red cell distribution width-coefficient of variation) in favor of increasing inflammatory responses.

DISCUSSION

The main findings of the present study demonstrated that exposure to stress induces a significant alteration in renal function and physiology of the kidney function as evidenced by a) Significant elevation of the level of novel biomarkers of kidney injury and glomerular function including KIM-1 and cystatin C in the tissue homogenate and serum with significant elevation of serum creatinine in rats subjected to CUS; b) Detection

of hematuria, protein urea and presence of pus and leukocyte in urine; c) Alteration of pro-inflammatory biomarker IL-6 and total antioxidant capacity; d) A significant elevation of most CBC-derived inflammatory biomarkers including NLR, MLR and SII, and decline in the level of PNR, RPR, RDW-CV because of active hematological and immune response.

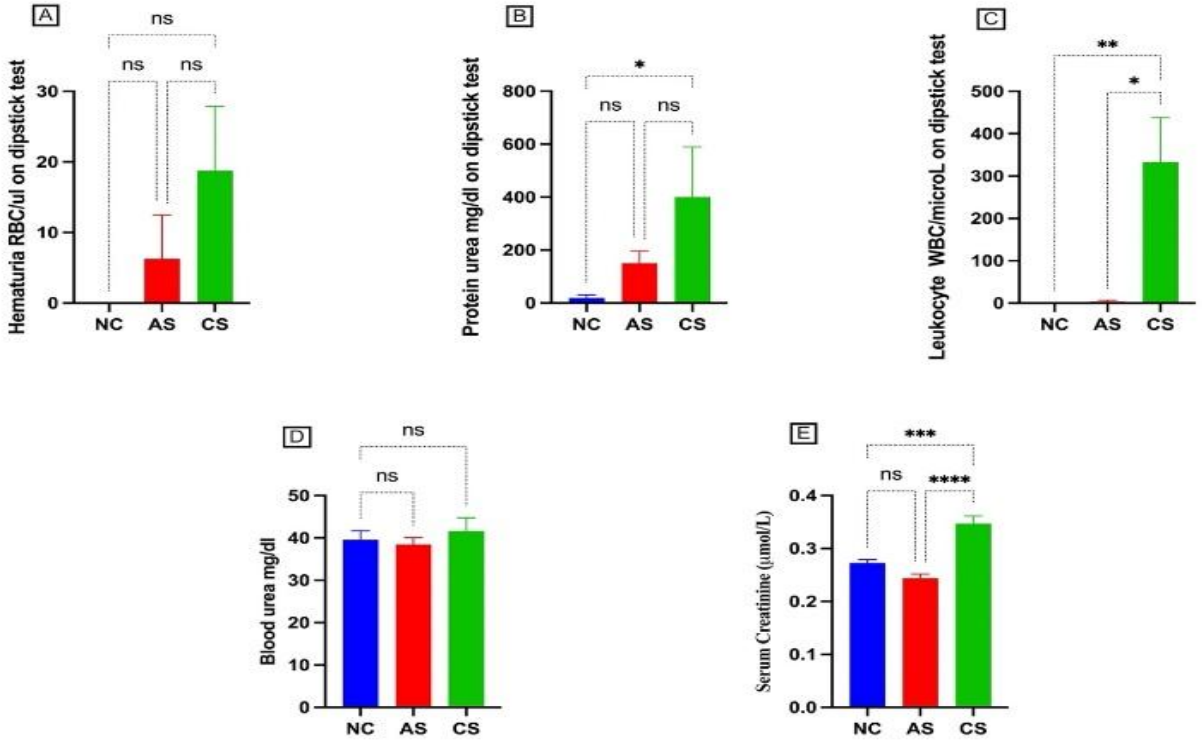

Figure 7 (A and E): Impact of acute and chronic stress on semi-quantitative urinalysis parameters: **A**) Hematuria; **B**) Protein urea, and C. Leukocyte in urine measured 24 hours post stress confirmation using dipstick test and classical kidney function markers; **D**) Blood urea and **E**) Serum creatinine. Kruskal-Wallis followed by Dunn's multiple comparison test was used to identify differences between the groups in Figures A, B, and C. While one-way ANOVA followed by Tukey's multiple comparison test was used to identify differences between the groups in D and E. *p<0.03, **p=0.008, ***p= 0.0002, ****p< 0.0001. p-value < 0.05 is considered statistically significant. NC: negative control, AS: acute stress, CS: chronic stress. ns: non-significant.

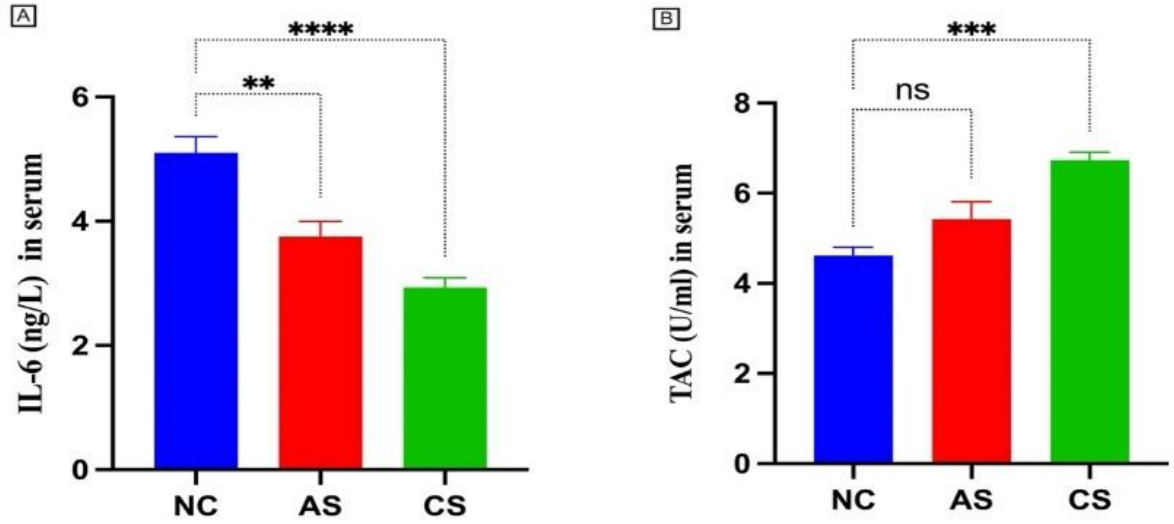

Figure 8 (A and B): Effect of acute and chronic stress on inflammatory and total antioxidant capacity. **A**) Interleukin-6 and **B**) Total antioxidant capacity. One-way ANOVA followed by Tukey's multiple comparison test was used to identify differences between the groups. ns: non-significant, **p=0.001, ***p=0.0002, ****p<0.0001. p-value < 0.05 is considered statistically significant. NC: negative control, AS: acute stress, CS: chronic stress, IL-6: Interleukin-6, TAC: total antioxidant capacity.

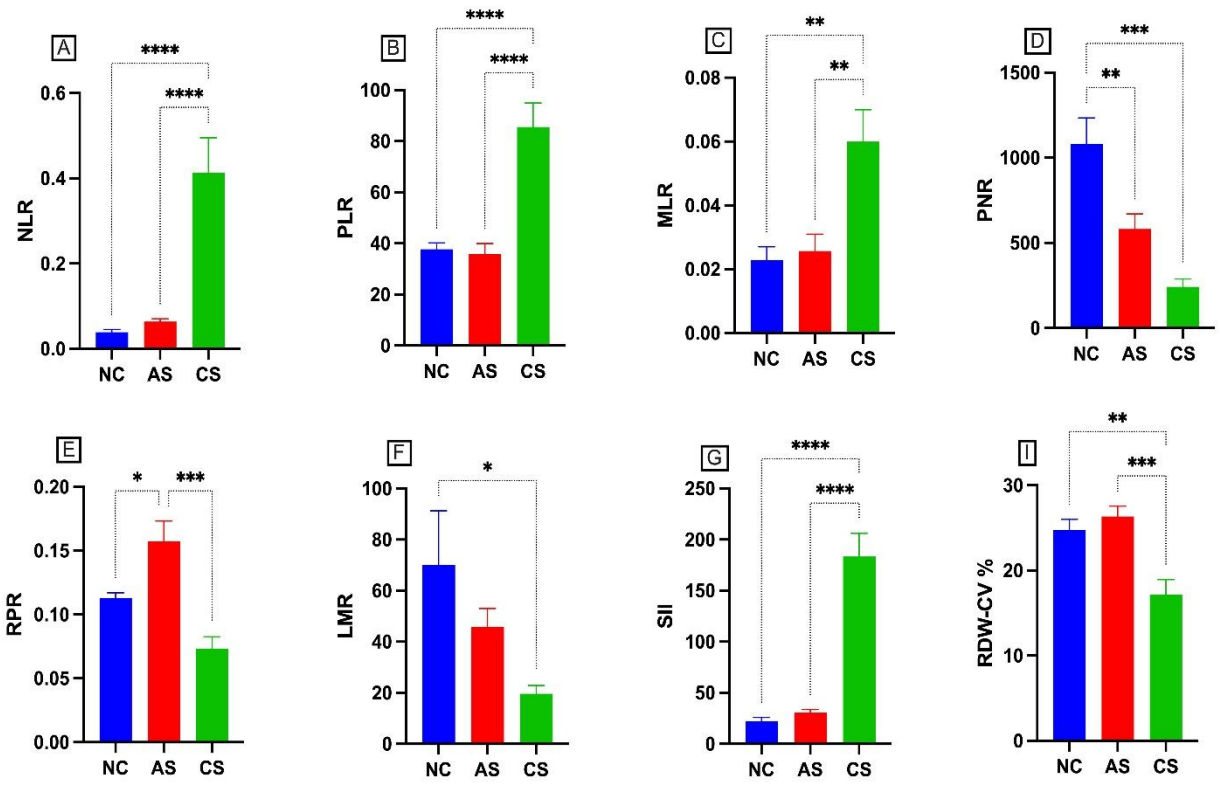

Figure 9 (A-H): Effect of acute and chronic stress on complete blood count (CBC)-derived Inflammatory biomarkers. **A**) NLR; **B**) PLR; **C**) MLR, **D**) PNR, **E**) RPR, **F**) LMR, **G**) SII, **H**) RDW-CV. One-way ANOVA followed by Tukey's multiple comparison test was used to identify differences between the groups. ns: non-significant. *p<0.05, **p=0.005, ***p=0.0002, ****p<0.0001. p-value < 0.05 is considered statistically significant. NC: negative control, AS: acute stress, CS: chronic stress. NLR: neutrophil/lymphocyte ratio, PLR: platelet/lymphocyte ratio, MLR: monocyte/lymphocyte ratio, PNR: platelet/neutrophil ratio, RPR: red cell distribution width/platelet ratio, LMR: lymphocyte/monocyte ratio, SII: systemic immune-inflammation index; RDW-CV: red cell distribution width-coefficient of variation.

In this *in vivo* study, both acute stress exposure protocol and chronic unpredictable stress (CUS) as a model of chronic stress induction are applied [27-29]. Behavioral assessments, including the OFT and the EPM [25,26], were utilized to confirm stress induction. Assessment of locomotion in the OFT is commonly applied to evaluate behavioral alterations induced by stress. In this study, rats subjected to AS and CUS exhibited motor deficits, as indicated by reduced crossing, walling behaviors, and total time of movement. Additionally, increased grooming, time spent in the margins, and time spent in one square of the apparatus have been recorded, which adds another piece of confirmatory evidence for the induction of stress. This finding is consistent with the other studies that follow the same locomotor evaluation for stress confirmation [26]. Anxiety is a psychological and behavioral response triggered by exposure to stress in both animals and humans, typically marked by fear and irritability [30]. The EPM is a well-established behavioral model used to evaluate anxiety-related responses. This test relies on rodents' discomfort in bright and open areas. In the present study, stressed groups demonstrated more pronounced anxiety-like behaviors, as evidenced by reduced head-dipping behavior, total time of movement, and spending more time in the enclosed arms compared to the control group. These alterations in the rats' behaviors suggest anxiety-related suppression of exploratory behavior and hypoactivity, which indicates stress induction. Cumulative behavioral changes provide evidence of stress response, especially in the CS group, which is in line with the previous studies that relied on the assessment of these behavioral tests in confirmation of stress induction [25,26]. In the current study, the rate of weight gain in the animals exposed to CS for one month was significantly slower than the unstressed animals in the NC group and the AS group exposed to acute stress. Thus, a minimum increase in weight gain was observed in the CS group across four weeks of stress exposure. However, the kidney index was significantly decreased in the CS group as observed on the last day of stress exposure compared to the NC and AS groups; this indicates a potential decrease in kidney weight relative to body weight. Possible reasons for this finding are associated with CUS protocol, which includes periods of fasting and food deprivation. The other possible reasons might be related to chronic stress, which has the potential to disturb the circadian rhythm, which is vital for managing body weight. The circadian rhythm affects metabolic functions, and any disturbances can result in fluctuations in weight [31]. In a research study, rats exposed to chronic

unpredictable mild stress exhibited a notable decrease in weight, highlighting the significance of the circadian rhythm in weight regulation influenced by stress [31]. In another in vivo study, chronic restraint stress reduced body weight and food intake in mice by altering hypothalamic gene expression related to energy metabolism. Also, stress has the potential to change carbohydrate metabolism, as evidenced by research demonstrating that stressed rats displayed high gluconeogenesis and glycogenolysis, resulting in chronic hyperglycemia. These metabolic alterations can impede weight gain by reducing liver glycogen concentration increase [32]. The significant elevation of KIM-1 in kidney tissue homogenate was observed in rats subjected to AS and CUS alongside a significant elevation of cystatin C level in serum, while in tissue homogenate cystatin C was significantly elevated only in the CS group. The concurrent elevation of KIM-1 in kidney tissue homogenates and cystatin C in both serum and renal tissue suggests the presence of early and persistent tubular injury induced by chronic stress exposure. KIM-1, a highly sensitive indicator of proximal tubular damage that is expressed following renal insult, confirms direct cellular injury within the kidney [13]. Similarly, the increase in cystatin C, a glomerular function biomarker capable of detecting even minor reductions in glomerular filtration rate (GFR), implies early functional impairment followed by detectable changes in creatinine as a conventional renal marker. To the best of our knowledge, there is no other previous study to compare with this novel finding in the current study. Although a mild alteration in some conventional biochemical markers was demonstrated by Khanthiyong *et al*. in chronic stress-induced rats [33], in another in vivo study, animals exposed to immobilization-induced stress demonstrated a diminished glomerular volume density in the kidneys [7]. In the current study, blood urea and creatinine as conventional biomarkers have been analyzed to demonstrate the impact of acute and chronic stress on these parameters. Significant elevation in serum creatinine has been observed in the chronic stress model, adding further evidence for kidney injury and reduced renal function by the chronic stressors. This finding is inconsistent with the study conducted by Khanthiyong *et al*., which stated that chronic stress resulted in a reduction of the functionally biochemical parameters, including creatinine [33]. Additionally, in another *in vivo* study, it has been shown that exposure to chronic stress before puberty significantly reduces nephron count in rats without producing changes in serum creatinine levels [7]. Serum creatinine and urinary output are significantly limited in accurately identifying the presence and severity of AKI. Serum creatinine lacks sensitivity and generally does not rise until nearly 50% of the GFR has been compromised [34]. This suggests that the significant elevation of creatinine in the current study reflects a remarkable kidney deterioration after subjecting the rats to CUS for 28 days. However, non-significant changes have been observed in rats subjected to acute stress. Furthermore, blood urea is not significantly altered in the current study; this is not in line with the previous study, which demonstrated a significant elevation of its serum level [33]. This inconsistency might be related to the adaptive response in CUS. Moreover, urinalysis using dipstick tests was performed 24 hours post-stress confirmation to evaluate the presence of hematuria, proteinuria, and pyuria. The dipstick method was selected for its cost-effectiveness and high sensitivity, particularly in detecting hematuria [35]. A significant elevation in protein urea and leukocytes (WBC) in dipstick was observed in the urine of rats subjected to CUS. However, hematuria was elevated in the same group non-significantly in comparison to the AS and NC groups. Assessment of hematuria, proteinuria, and leukocytes was utilized in the detection of kidney injury both visually through representative images and quantitatively using scoring methods consistent with those reported in previous studies [14,36]. Furthermore, the impact of AS and CS on the inflammation and antioxidant status of the experimental animals was demonstrated in this study. A significant drop in the serum level of IL-6 was observed in the rats subjected to AS and CS. This finding is typically inconsistent with the other studies that found the marked elevation of pro-inflammatory proteins or cytokines, including IL-6, IL-1β, nuclear factor kappa B (NF-κB), and tumor necrosis factor-α (TNF-α), following exposure to chronic stressors [37]. Additionally, rats exposed to acute stress through forced swimming or restraint stress showed elevated levels of TNF-α, IL-10, IL-6, and IL-4 compared to those in the negative control group that were not subjected to any stressor [38]. The finding in the current study might be related to the alteration in the hematological and immunological parameters in the rats subjected to AS and CS, as evidenced by a significant reduction in WBC counts and lymphocytes and an increase in neutrophils in CBC parameters in addition to the reduction in some CBC-derived inflammatory biomarkers, including PLR, PNR, and LMR. On the other hand, chronic stress stimulates the HPA axis and the sympathetic nervous system (SNS), resulting in the release of glucocorticoids. Although glucocorticoids are recognized for their capacity to attenuate inflammatory responses, chronic exposure may induce a disruption in cytokine production, which could potentially result in immune suppression and suppress the expression of IL-6, consequently diminishing levels of IL-6 [39,40]. In the other part of this study, the impact of AS and CS on hematological profiles, including CBC-derived inflammatory biomarkers, has been examined. Indeed, these inflammatory biomarkers are useful in predicting the progress of many diseases, such as metabolic syndrome

in obese adults and the severity of chronic kidney diseases, as demonstrated in the previous studies [16,41], For instance, NLR was utilized in predicting postoperative AKI after hepatobiliary and gastrointestinal surgery [42]. In our study a significant increase in the ratio of NLR, MLR, PLR, and SII and a decline in the level of PNR, RPR, LMR, and RDW-CV of CBC-derived inflammatory biomarkers were observed. Additionally, significant reductions in WBCs and lymphocytes were obtained, with significant neutrophilia and increase in monocyte percent. These findings were not parallel with the results of the previous study, which demonstrated that CUS induces early alterations in blood and biochemical parameters in C57BL/6 mice after 10 days of exposure, such as a reduction in monocytes and a non-significant increase in neutrophils, which suggests stress-related immune alterations [43]. This inconsistency might be related to the duration of stress exposure and the experimental models applied in the study. The other finding of this study is a significant elevation of TAC ($p<0.05$) in the CS animal group while non-significant in the AS group in comparison to the NC group. Since the elevation of TAC refers to the combined effects of all antioxidant systems, therefore, in acute stress, the elevation of TAC level in animals in the AS group can be explained by the induction of the Nrf2 pathway, as exposure to acute stressors upregulates antioxidant genes like Nrf2 and Hmox1 [44]. Additionally, non-enzymatic antioxidants, including glutathione (GSH), vitamin E, and lactate, also contribute significantly by neutralizing ROS [45]. Elevated GSH levels in organs like the liver and kidney, along with increased plasma lactate during stress, further strengthen antioxidant defenses. On the other hand, the TAC level in animals of the CS group is significantly elevated, which is in line with the previous findings that state that CS has a complex effect on the antioxidant system; therefore, it differentially affects this system. For instance, chronic psychological stress results in activation of superoxide dismutase (SOD) enzymes, particularly within the hippocampus and prefrontal cortex regions of the brain, which convert superoxide radicals into hydrogen peroxide, which is less detrimental and can subsequently be detoxified by catalase and various other enzymatic systems [46]. This phenomenon represents an adaptive physiological mechanism aimed at alleviating oxidative damage through alteration of the synthesis and function of several protective proteins, including enzymes, transcription factors, and chaperones, to enhance the defense mechanisms against the oxidative damage [47]. Additionally, in the prefrontal cortex, chronic stress alters catecholaminergic turnover, including norepinephrine, which correlates with activation of antioxidant enzyme activity. This implies a connection between neurotransmitter metabolism and the management of oxidative stress [48].

Study limitations

Despite the important findings, several limitations should be considered. First, behavioral tests such as OFT and EPM, while reliable, are influenced by environmental factors. Secondly, while the study incorporates conventional renal biomarkers with novel ones, more novel markers such as neutrophil gelatinase-associated lipocalin (NGAL) would provide more confirmatory outcomes. Although certain limitations exist, this study is distinguished by several strong points that support the validity and applicability of its outcomes. Firstly, the employment of a well-established experimental model facilitated a controlled examination of the targeted physiological responses, thereby reducing variability and improving reproducibility. Secondly, the combined use of behavioral and biochemical assessments enabled an interpretation of the underlying biological mechanisms. Thirdly, the parallel evaluation of both traditional and novel biomarkers enriched the data set. Lastly, the study's methodology, characterized by the inclusion of proper control groups and adherence to standardized protocols, ensured the generation of reliable and effective findings.

Conclusion

Both chronic and acute stress exert a detrimental effect on the kidney, with chronic stress resulting in more severe injurious effects, as evidenced by the significant increase in conventional and new kidney injury novel biomarkers KIM-1 and cystatin C. Chronic stress had a complex effect on the antioxidant system; it increased total antioxidant capacity, which might be related to the adaptive physiological mechanism. Additionally, the pro-inflammatory biomarker IL-6 was decreased; this could relate to the alteration in the hematological and immunological parameters in the animals subjected to AS and CS. A significant alteration in the ratios of CBC-derived inflammatory biomarkers was observed as an additional indicator for the stress-induced inflammatory process. Further study is recommended to observe the impact of stress on additional kidney injury-specific biomarkers while focusing on molecular mechanisms and the signaling pathways involved in stress-induced kidney damage.

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Conflict of interests

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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