ORIGINAL ARTICLE

CHRONIC UNPREDICTABLE STRESS INDUCES RENAL DAMAGE IN RATS BY OXIDATIVE STRESS PROVOKED APOPTOSIS AND ALTERING THE FUNCTION OF Na$^+$/K$^+$-ATPase

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Background: Recently, some shocking epidemics of chronic kidney disease of undetermined origin have been reported. In this regard, numerous lines of evidence suggest that socioeconomic and environmental stressors have a key role, an effect that needs further confirmation. The present study was undertaken to explore the effect of Chronic Unpredictable Stress (CUS) on kidney function and structure in rats. **Methods:** It was a randomized control trial. Rats were divided into control and experimental groups (n=8 each). The experimental group (CUS group) consisted of rats which were exposed to a set of mild stressors for 21 days. After that, biochemical and molecular studies were conducted to measure kidney function tests, renal oxidative stress, inflammatory response, components of intrinsic apoptosis, as well as function of Na$^+$/K$^+$-ATPase. In addition, renal histopathological study was conducted. **Results:** Chronic Unpredictable Stress resulted in sever renal damage as indicated by enhanced serum urea and reduced creatinine clearance (Ccr), also evident by the severe glomerular and tubular damage and neutrophils infiltration. Concomitantly, CUS exaggerated oxidative stress and lipid peroxidation by inhibiting activities of endogenous antioxidant enzymes and activating renal inflammatory response. CUS stress resulted in inhibiting activities of renal Na$^+$/K$^+$-ATPase and induced Na$^+$ retention. CUS activated intrinsic apoptotic pathway as evident by decrease renal levels of Bcl-2 and enhanced levels of caspase 3 and *m*RNA levels of p53 and Bax. **Conclusion:** Chronic Unpredictable Stress causes renal damage by exaggerating oxidative stress, inhibiting Na$^+$/K$^+$-ATPase pump activity and activation of inflammation and apoptosis.
Keywords: Chronic unpredictable stress, Oxidative stress, Apoptosis, Na$^+$/K$^+$-ATPase

INTRODUCTION

Oxidative stress is defined as the loss of balance between the production of reactive oxygen species (ROS) and protective antioxidant systems. During generation of high ROS levels, both enzymatic and non-enzymatic antioxidant defence systems become overwhelmed leading to oxidative stress-induced tissue injury. Reactive oxygen species can damage cells by inducing lipid peroxidation, protein oxidation, and nucleic acid damage, which lead to structural and functional impairments.[1,2]

In daily life, exposure to adverse environmental changes, or stressful situations, can provoke behavioural, neuro-humoral, immunological and signalling responses leading to increased production of ROS.[3] Experimentally, oxidative stress has been recognized as a substantial mechanism involved in the cytotoxicity and tissue damage induced by chronic stress situations in different tissues like brain and heart.[4–7]

In the kidney, experimental evidences have shown that ROS and associated oxidative stress response mediate numerous mechanisms leadings to nephropathies and variety of experimental and clinical renal diseases,[8] including acute renal failure,[9] obstructive nephropathies,[10] glomerular damage,[11] and chronic renal failure with associated inflammation[12]. Indeed, increased levels of ROS as well as malondialdehyde and F2-isoprostanes, two products of lipid peroxidation, have been reported in various clinical settings associated with renal damage.[13]

During the last decades, some alarming epidemics of chronic kidney disease of undetermined origin have been reported. One example is the increased chronic kidney disease in many countries on the Pacific Ocean's coast, where many people are exposed to heat stress.[14–16] Also, the increased incidence of nephropathies among Japanese after earthquakes.[17] It has been previously reported that co-morbidities, such as diabetes, hypertension, or obesity, do not entirely explain the rapid initiation of chronic kidney disease and its progression to end-stage renal disease among high-risk population. Indeed, other factors such as social, economic and environment stresses have been found to be as important elements in the course from chronic to end-stage renal disease.[18,19]

Despite the broad agreement concerning the relationship between undergoing stressful experiences and the commencement and progression of kidney disease, this link has not been pursued extensively, and needs further attention and investigation.[19] This study was designed to experimentally investigate the effect of Chronic Unpredictable Stress (CUS) on kidney function in rats with emphasis on renal oxidative stress, apoptosis and function of renal Na$^+$/K$^+$-ATPase.

MATERIAL AND METHODS

Sixteen adult male Wistar rats (150–230 g) bred in the Central Animal House, College of Medicine at King Khalid University, were used in this study. The rats were housed at a controlled ambient temperature of 25±2 °C and 50±10% relative humidity, with 12-h light/12-h dark cycles, and were fed standard rat chow and water *ad libitum*. Experiments were performed with the approval of the Medical Research Ethics Committee of the College of Medicine, King Khalid University, and all procedures were conducted in accordance with National Institutes of Health's Guide for the Care and Use of Laboratory Animals.[20]

After one week adaptation period, the rats were randomly divided into two groups (n=8 each). The first was the Control Unstressed Group. The second was the Chronic Unpredictable Stress (CUS) Group, where rats were exposed to a set of recognized chronic unpredictable mild stressors including swimming in 4 °C cold water for 5 min, tail clamp for 1 min, cage tilting and damp sawdust for 24 h, exposure to an experimental room heat at 50 °C for 5 min, noises for 1 hour (alternative periods of 60 dB (noise for 10 min and 10 min of silence), food deprivation for 48 hours, water deprivation for 24 hours, unpredictable shocks (15 mA, one shock/5 s, 10 s duration), and restricted movement for 4 hours. The whole stress protocol lasted for 3 weeks, with one random stressor been applied each day.

Twelve hours after day 21, the rats were housed in individual metabolic cages to collect a 24-h urine specimen to measure urine volume and urinary creatinine. Then, blood samples were withdrawn, centrifuged at 5,000 rpm for 10 min to collect serum used later to measure levels of urea, Na^+ and K^+. The rats were then humanely killed by cervical dislocation under terminal anaesthesia (sodium pentobarbital, 65 mg/Kg, intraperitoneally), after which both kidneys were removed. Parts of the first kidney were used for histological evaluation and other parts were directly frozen in RNA preservative solution and used later for reverse transcriptase-polymerase chain reaction (RT-PCR) studies. Parts of other kidney were frozen and homogenized later in appropriate buffers as per kits instructions to prepare homogenates which stored at -70 °C and used later for biochemical analysis.

Serum and urine creatinine concentrations were determined by colorimetric assay kits (Cat. No. 700460 and 500701, respectively, Cayman Co., Ann Arbor, MI, USA). Blood urea levels were determined by commercially available kit (Human CO, Germany). Na^+ and K^+ levels were determined using an Olympus Autoanalyser (Olympus Instruments, Tokyo, Japan). Creatinine clearance (Ccr) was calculated using the following equation:

Ccr (ml/min/Kg)= [urinary Cr (mg/dL)×urinary volume (ml/min)]/[serum Cr (mg/dl)]×[1000/body weight (g)]×[1/1440 (min)]

Lipid peroxidation marker, Malondialdehyde (MDA), was measured as levels of Thiobarbituric acid reactive substances (TBARS) using a commercial assay kit (Cat. No. NWK-MDA01, NWLSS, USA). Superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) activity were measured using commercial kits (Cat. No. 706002 and 703102, respectively, Cayman Co., Ann Arbor, MI, USA). Caspase 3 activity and levels of Bcl-2 were measured using special ELISA kits purchased from STZ ELISA company, USA (Cat. No. R5814 and R6813, respectively). Levels of TNF-α were measured using ELISA kit purchased form Abcam, Cambridge, MA, USA (Cat. No. ab46070). Levels of IL-6 were measured using ELISA kit supplied by RayBio, MO, USA (Cat. No. ELR-IL6-001). Na^+/K^+-ATPase in renal homogenates were assayed by measuring the amount of inorganic phosphate (Pi) liberated from ATP in a buffer containing 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 40 mM Tris/HCl (pH 7.4) and 3 mM Na 2ATP. Enzyme activity was calculated as the difference between the activities assayed in the absence and presence of 2 mM ouabain, and Sch 28080 (0.2 mM) was added to both samples to block ouabain sensitive H^+/K^+-ATPase, which could interfere with the assay. Enzyme activity was expressed in micromoles of Pi liberated per hour per milligram of protein.

Specimens from kidney tissues were fixed in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol (70–100%), and prepared using standard procedures for Haematoxylin and Eosin staining.

*m*RNA levels of Bax, p53, Na^+/K^+-ATPase α subunit, and the housekeeping gene (β actin) were measured by RT-PCR, using established published sequences of PCR primers.[21] Total RNA was extracted from the frozen kidney sections (30 mg) using an RNeasy Mini Kit (Qiagen Pty. Ltd., Victoria, Australia) according to manufacturer's instructions. RNA purity was estimated by the 260/280 ηm absorbance ratio. Single-strand cDNA synthesis was performed with Superscript II reverse transcriptase (RT) and Oligo (dT)12–16 (Invitrogen), and the tested genes were amplified according to manufacturer's instructions. A control reaction without reverse transcriptase was included for every sample of RNA isolated to verify the absence of contamination. After running the PCR products (10 μl) on 2% agarose gels containing 100 ηg/ml ethidium bromide, gels were photographed, and bands intensities were normalized to those of the corresponding β actin band intensity using the 'my Image Analysis' software, Thermo Scientific, USA.

Statistical analysis was done using Graphpad Prism statistical software package (GraphPad Software Inc.). Student's *t*-test was used, results were expressed as Mean±SD and $p < 0.05$ was considered significant.

RESULTS

CUS exposed rats had significant higher urea, creatinine and Na^+ in their serum with a parallel significant decrease in urine creatinine levels, compared to control rats. Urine volume collected over 24-hour period significantly reduced in CUS rats. Consequently, a significantly reduced creatinine clearance (Ccr) levels were observed in CUS rats. (Table-1).

Table-1: Biochemical parameters in serum and urine of control and chronic unpredictable stressed rats (Mean±SD)

Parameter	Control	CUS
Serum		
Urea (mg/dl)	34.7±2.5	67.4±4.0*
Creatinine (mg/dl)	0.57±0.06	1.93±0.14*
Na^+ (mmol/l)	137.2±3.9	202.9±3.2*
K^+ (mmol/l)	4.1±0.3	3.9±0.4
Urine		
Volume (ml)	4.9±0.6	2.1±0.4*
Creatinine (mg/dl)	56.7±5.1	18.9±0.9*
Clearance		
Creatinine (Ccr) (ml/min/Kg)	1.35±0.34	0.05±0.009*

*$p<0.05$

CUS caused significant increases in Malondialdehyde (MDA) levels, and significant decreases in the activities of Superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the renal homogenates as compared to that of control unstressed rats (Figure-1).
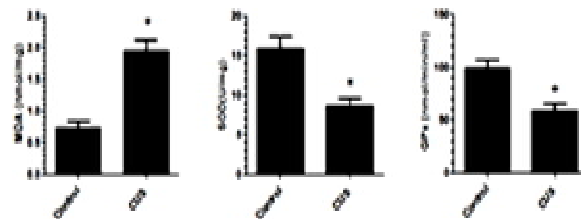

Figure-1: Levels of malondialdehyde and activities of superoxide dismutase and glutathione peroxidase in the kidney homogenates of the control and chronic unpredictable stressed rats. (*$p<0.05$)

Concomitantly, renal homogenates of CUS rats showed significantly higher levels of the measured inflammatory markers, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) when compared to control rats as shown in Figure-2.
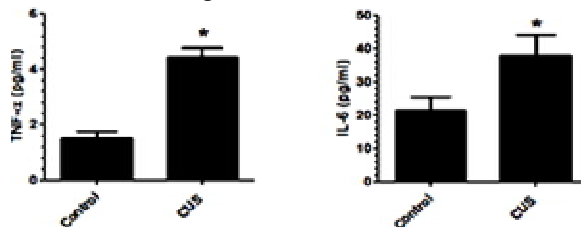

Figure-2: Levels of TNF-α and IL-6 in kidney homogenates of control and chronic unpredictable stressed rats. (*$p<0.05$)

The effect of CUS on renal tissue structure is shown in Figure-3 which shows photomicrographs of sections stained with haematoxylin and eosin (H&E). Control unstressed rats showed normal kidney architectures with well-preserved nephron structures where dense rounded renal corpuscles, comprising the glomeruli surrounded by Bowman's capsules, were dominant. Proximal convoluted tubules were lined with brush border and all tubules were lined by simple cuboidal epithelium. On the other hand, sections taken from CUS rats showed severe glomerular and tubular degeneration with severe neutrophils infiltration and dark pink cytoplasm, as indicators of the cell death. Most glomeruli were shrunken or ruptured. In some sections, few of the glomeruli epithelia and vessels were completely absent.
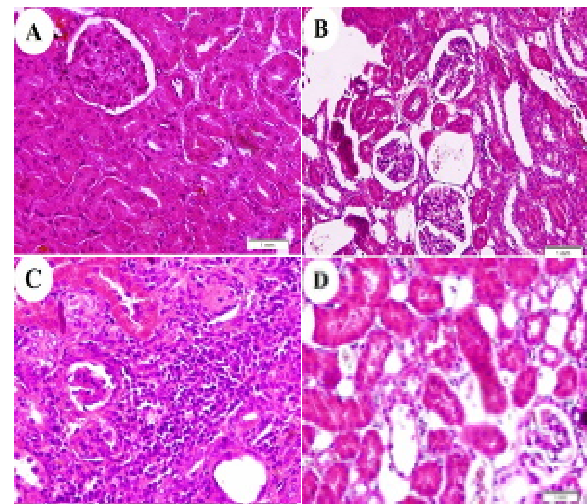

Figure-3: Light photomicrographs of renal tissues from (A) Control rats, and (B to D) chronic unpredictable stressed rats. (H&E ×400)

The apoptotic pathway, that was producing renal glomeruli and tubular cells damage, was activated during CUS, as depicted in Table-2 showing the levels of caspase 3 and Bcl-2 in renal homogenates from control and CUS rats, and Figure-4 showing the *m*RNA levels of p53 and Bax. Relative expression of those genes were expressed as compared to the housekeeping gene, β-actin. Levels of Bcl-2 were significantly reduced while activities of activated caspase 3 were significantly elevated in CUS rats, compared to control unstressed rats. CUS rats additionally have significantly elevated expression levels of both p53 and Bax.

Table-2: Levels of caspase 3 and Bcl-2 in the renal homogenates of control and chronic unpredictable stressed rats. Mean±SD

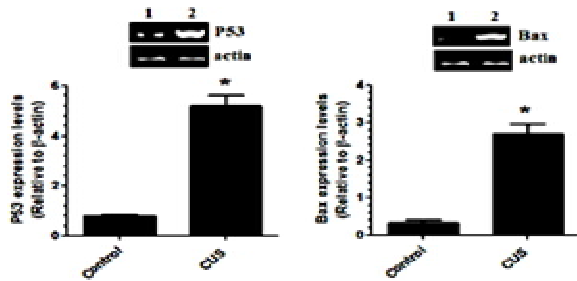| Parameter	Control	CUS
Caspase-3 (ηg/mg)	0.16±0.04	5.1±0.2*
Bcl-2 (ηg/mg)	7.8±1.2	2.9±0.6*

*$p<0.05$

Figure-4: Semi-quantitative reverse transcription PCR products and relative expression of renal tissue *m*RNA of p53 and Bax in reference to β-actin *m*RNA. (*p<0.05)

Activity and *m*RNA expression levels of Na$^+$/K$^+$-ATPase (α-subunit) were measured in renal homogenates of both control and CUS rats as shown in Figure-5. As compared to control unstressed rats, CUS significantly inhibited the biochemically measured enzyme activity, and resulted in a significant increase in the Na$^+$/K$^+$-ATPase α-subunit *m*RNA levels.
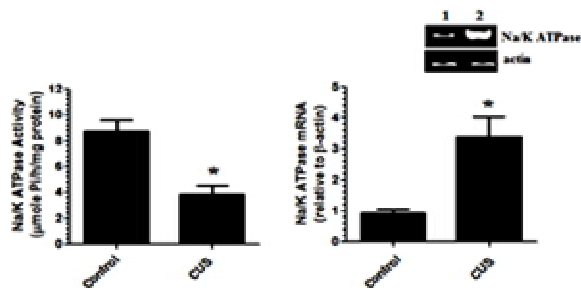

Figure-5: (Left) Na$^+$/K$^+$-ATPase Activity and (Right) Semiquantitative RT-PCR product and relative expression of renal tissue Na$^+$/K$^+$-ATPase α-1 subunit *m*RNA, in reference to β-actin *m*RNA. (*p<0.05)

DISCUSSION

In current study, chronic unpredictable stress (CUS) resulted in significant increase in levels of urea and creatinine with a parallel decrease in creatinine clearance in rats, all of which are indicators of renal dysfunction. Concomitantly, there were significant decreases in the activities of major antioxidant systems (SOD and GPx), and enhanced lipid peroxidation (MDA) in the renal homogenates of rats, indicating oxidative stress as a major pathway in CUS-induced renal damage. Histological findings showed severe glomerular and tubular damage. Previous research has shown that the glomerulus is considerably more sensitive to oxidative injuries than other nephron segments, and ROS can rapidly alter the structure and function of the glomerulus because of its direct apoptotic effect on mesangial and endothelial cells.[22] Besides the direct damaging effect of ROS on these cells, some authors have suggested oxidation of LDL in mesangial cells by ROS, or by infiltrating macrophages

increased glomerular damage, and consequently, activating the apoptosis pathway of endothelial and mesangial cells[23], an effect that needs further investigation.

This study showed enhanced CUS-induced inflammatory response in the kidney homogenates, as indicated by significant raises in both tumour necrosis factor-α (TNF-α) and IL-6. It has been previously shown that inflammation could be produced by the resident renal cells, such as glomerular mesangial and endothelial cells, proximal tubular epithelial cells, and interstitial fibroblasts.[24] ROS may induce gene expression of the inflammatory mediators in the glomerulus and tubular epithelial cells, resulting in the recruitment of leukocytes.[24] Indeed, ROS have been shown to stimulate the nuclear factor kappa B (NF-κB) which is one of the most important regulators of pro-inflammatory gene expression and leukocyte infiltration in mesangial and tubular cells.[25] In support, increased circulating levels of TNF-α and interleukin-1 beta (IL-1β) have been reported in a rat model of anhedonia induced by CUS.[26] Higher levels of pro-inflammatory cytokines, including TNF-α and IL-6, and higher ratio of pro-inflammatory/anti-inflammatory cytokines have been found in healthy individuals under psychological stress.[27]

On the other hand, numerous studies reported involvement of high levels of ROS in apoptotic cell death after stressful conditions. In the current study, a multitude of pathways, including the intrinsic (Bcl-2 family, cytochrome c and caspase 3), and regulatory (p53 and NF-κB) factors, are activated during CUS induced kidney damage. CUS significantly upregulates *m*RNA levels of p53 and Bax, and enhances levels of renal caspase 3 with simultaneous decreased levels of renal Bcl-2. It has been previously shown that ROS not only contribute to injury of macromolecules such as lipids, proteins, and DNA but also to transduction of apoptotic signals.[28] It has been reported that high levels of ROS disrupt the cell's inner and outer mitochondrial membranes, relocate Bax, induce the release of cytochrome c and activate caspases 3 and 9 resulting in cell apoptosis.[28] ROS was shown to provoke p53 levels which also initiate apoptosis,[29] this supports our findings. Apoptosis through activation of these pathways was shown as the major mechanism of early glomerular and tubular cell death in ischemic acute renal injury.[29] The initiated acute inflammatory response could contribute to the death of renal cells as a combination of both necrosis and apoptosis.[30]

Impairment of renal Na$^+$/K$^+$-ATPase expression and/or activity play an essential role in the pathology and progress of kidney and other associated diseases.[31] In the current study, and unexpectedly with the presence of damaged tubules, we found decreased Na$^+$/K$^+$-ATPase activity in the renal homogenates of CUS,

which was associated with enhanced serum Na^+ levels. Decreases in Na^+/K^+-ATPase activity can be mediated by changes in the activity of pre-existing Na^+/K^+ pumps, recruitment/translocation of Na^+/K^+ pumps from intracellular pools to the basolateral membrane, or by decreased transcription/translation, all of which decrease the number of active Na^+/K^+ pumps on the cell plasma membrane.[32]

In this study, CUS resulted in significant increase in the expression of Na^+/K^+-ATPase *m*RNA which could be speculated as a compensatory mechanism due to inhibition of Na^+/K^+-ATPase activity. Enzyme glycation has been recognized as one of the mechanisms that contribute to decreased activity of Na^+/K^+-ATPase in diabetes.[33] Therefore, it could propose a significant role of ROS in this enzyme inhibition.[22]

Na^+/K^+-ATPase activity is differentially regulated in a hormone- and tissue-specific fashion.[34] For instance, dopamine secretion is known to be induced during a stressful situation. In renal tissue, dopamine inhibits Na^+/K^+-ATPase activity by either increasing ROS generation and/or through PKC-dependent phosphorylation of the Na^+/K^+-ATPase subunits and activation of phosphoinositide 3-kinase, thus inducing enzyme endocytosis.[34–37] Expected increase in dopamine levels could inhibit Na^+/K^+-ATPase activity.

CONCLUSION

Chronic unpredictable stress produces renal damage via exaggerating oxidative stress, escalating inflammation and apoptosis, and inhibiting Na^+/K^+-ATPase pump activity. Further investigation to explain the effect of CUS on enzyme regulation is required.

ACKNOWLEDGMENTS

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