The Effect of High Salt Diet in Renal Fibrosis Through CHOP Protein Stimulated Apoptosis in Rat Model

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Abstract

I. Background: Prolonged excessive salt intake is an important risk factor for development of renal fibrosis. In the onset of renal tubular destruction, KIM-1 appears in urine. CHOP is an important apoptosis stimulator protein. The aim of present study was to investigate the effect of high salt diet in development of renal fibrosis through apoptosis.

II. Methods and results: The 25 male Wistar rats were divided randomly into five groups and treated with 0%, 0.5%, 1%, 1.2%, 1.5% of NaCl dissolved in distilled water for 8 weeks. For confirmation of renal tubular destruction, the urinary KIM-1 was measured. The slides of renal tissue were prepared and stained with Hematoxylin and Eosin and Masson´s Trichrome for fibrosis detection. To investigate the role of CHOP protein in development of renal tubulointerstitial destruction, the relative gene expression of CHOP in renal tissue was analyzed using qRT-PCR method.There was no significant *differences* in urea, creatinine and total protein concentration of rats received different concentrations of NaCl compared to the control group. Urinary KIM-1 and mRNA level of CHOP was found to be increased significantly in rats treated with 1.5% NaCl compared to the control group. Mild renal fibrosis was observed in the same group too.

III. Conclusion: Excessive salt intake can lead to fibrosis through increasing the expression of apoptotic CHOP gene in renal tissue. KIM-1 can be detectable in urine long before the development of renal fibrosis.

Introduction

Chronic kidney disease (CKD) is a global threat to public health and if not treated in time, it will lead to renal failure (RF). In the early stages of CKD, there are usually no clinical symptoms and the disease does not appear unless there is a significant reduction in renal function. The total number of adults affected with CKD is 220 million men and 270 million women worldwide[1, 2]. CKD is defined as tubulointerstitial destruction with a glomerular filtration rate (GFR) of less than 60 ml / min / 1.73 m^2 for at least 3 months [3, 4].Prolonged excessive salt intake has been identified as a risk factor for the development of renal fibrosis and CKD[5]. Excessive salt intake increases the osmotic pressure inside the renal tubulointerstitial cells. High osmotic pressure inside the nucleus; destroys the chromatin structure, which will alter the expression of genes; like reducing the expression of genes involved in DNA repair. Additionally, high level of unfolded proteins in the cytoplasm of renal tubulointerstitial cells results in osmotic stress in the endoplasmic reticulum (ER) [6]. In other words, high osmotic pressure, causes DNA damage, disruption of DNA repair systems and ER osmotic stress. Due to destruction of DNA structure, the cell remains in the G_2 phase of the cell cycle and cannot enter mitosis [7-9].

It has been demonstrated that, inhibition of ER osmotic stress in the salt-sensitive rats, prevented from developing renal tissue fibrosis. It means that; prolonged excessive salt intake may cause inflammation and fibrosis in kidneys through ER osmotic stress [10]. Some in vitro studies indicated that ER osmotic stress increased the expression of the pre-apoptotic molecule CHOP (C/EBP Homologous Protein GADD 153), which promoted apoptosis through inhibiting the anti-apoptotic molecule BCL-2. It seems that,

CHOP is one of the key proteins in stimulating apoptosis [11-13]. Recent in vivo studies confirmed that rats with a defect in the CHOP gene did not develop inflammation and fibrosis in kidneys [7, 11].

CHOP is a 29 kDa protein consisting of 169 amino acids in humans and 168 amino acids in rodents. BCL-2 is an important inhibitor protein in apoptosis pathway. CHOP binds to BCL-2 and in this way; stimulates apoptosis [14, 15]. In apoptotic tissue, the inflammatory process will lead to fibrosis [7, 12, 16] .

Kidney Injury Molecule 1 (KIM-1) is a 90 kDa transmembrane protein found in the membrane of renal tubular cells. The outer domain of KIM-1 separates from the membrane and enters the lumen of the renal tubules during apoptosis [17]. Urinary KIM-1 is identified as a diagnostic marker for renal tissue destruction[1].

In an attempt to gain further insights into the effects of prolonged excessive salt intake on renal function; this study was carried out in rat model. We hypothesized that high salt diet could a) increase relative gene expression of CHOP in renal tissue , b) increase urinary KIM-1, c) develop renal tissue fibrosis. Our study focused on the association between renal tissue fibrosis and urinary diagnostic markers and could be applied as a strategy to prevent from developing progressive CKD and RF.

Material And Methods

Research design

8-Weeks old male Wistar rats, body weight of 200-250g, were purchased from Pasteur institute of Iran (IPI). Animals were housed in 12-Hour light/dark period in a stable temperature (21-23°C) and 55%±10% relative humidity. Rats were nourished by standard chow and water ad libitum. 25 animals were divided into 5 groups randomly each as given below. NaCl ACS reagent was dissolved in distilled water and provided for animals as drinking water: Group 1: distilled water as drinking waterGroup 2: 0.5% w/v NaCl in distilled water as drinking waterGroup 3: 1% w/v NaCl in distilled water as drinking waterGroup 4: 1.2% w/v NaCl in distilled water as drinking water Group5: 1.5% w/v NaCl in distilled water as drinking water Treatment was continued for 8 weeks. At the end of the treatment; 24 hour urine with the aid of metabolic cage was collected. The collected urine stored at -80 °C for measuring urinary KIM-1, creatinine, urea and total protein concentration. Animals were anesthetized by diethyl ether and then; kidney samples were separated. The part of kidney samples were immediately frozen in liquid nitrogen and transferred into -80˚C freezer for qRT-PCR assay; and the other part was fixed with 10% formalin for histopathology examination. The blood samples were taken from the cardiac puncture; serum was separated and stored at -20˚C for measuring creatinine and urea concentration.

Urea, creatinine, total protein and KIM-1 assay in serum and urine

Creatinine concentration was measured in urine and serum using Pars Azmon kit, based on the Jaffa colorimetric method. Urea concentration was tested in urine and serum with the aid of Pars Azmon kit, based on the Kinetic Urease method. Total protein concentration in urine was determined using Grainer kit, based on the Biuret colorimetric method. KIM-1 concentration in urine was measured by Crystalday ELIZA kit.

RNA extraction and qRT-PCR analysis

Total RNA from the excised kidney tissues were isolated using the TRIzol extraction reagent (Invitrogen, 15596026), according to the manufacturer's recommendations. The integrity of mRNA was confirmed by electrophoresis in a denaturing 1% agarose gel. First strand cDNA was synthesized from total RNA with random hexamer primers using the RevertAid H Minus cDNA synthesis kit (Biofact, W2569-100). Quantitative Real time-PCR of GAPDH (reference gene) and CHOP were carried out using specific primers are listed in Table 1. The reaction mixture of 20 µl was consisted of 2× ABI SYBR Green PCR Master Mix, 2 µl cDNA and 0.2 µL of each primer. Amplifications were performed on the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with 40 cycles of denaturation; 95°C for 30 s, annealing and extension at 60°C for 30 s and data collection at 80°C for 20 s. The mRNA levels are reported as relative fold changes which were normalized to those of the GAPDH product. The comparison of relative gene expression between different groups was evaluated through the CT method ($2^{-\Delta\Delta CT}$)[18].

Table1. Specific primers for real time PCR

Genes	Forward primer (5' → 3')	Reverse primer (5'→3')
CHOP	acggaaacagagtggtcagt	agacagacaggaggtgatgc
GAPDH	gagacagccgcatcttcttg	tgactgtgccgttgaacttg

Preparation of histopathological slides and evaluation of renal tissue fibrosis

The prepared slides are stained with Hematoxylin and Eosin (H and E); and Masson´s Trichrome; which are the specific staining method for tissue fibrosis detection. In Masson´s Trichrome staining, the fibrotic tissue areas are blue and normal tissue areas are red. From each microscopic slide, 5 of them were randomly selected and the ratio of blue to red tissue areas was calculated by Image J software, and the average of these 5 was considered as fibrosis severity. They were divided into 4 degrees according to the severity of the fibrosis: grade 0 was interpreted if fibrosis was not observed and grades 1, 2, 3 were interpreted according to the severity of the fibrosis.

Statistical Analysis

Data are expressed as means ± SD. Using SPSS version 16 software; the data were statistically computed by one-way ANOVA procedure and subsequently Duncan's test. A P value < 0.05 was considered statistically significant.

Results

Blood and urinary biochemical variables

According to data in Table 2 (page 12), there was no statistically significant difference in serum and urine urea, creatinine and total protein concentration in groups 2-5 in comparison with normal control group. Fig. 1 (page 13) indicates that NaCl in groups 2-4 had no statistically significant effect in urinary KIM-1 concentration compared to normal control group. It should be added that, urinary KIM-1 concentration increased significantly in group 5 compared to normal control group (p-value < 0.05).

Relative gene expression of CHOP in renal tissue

It can be inferred from Fig. 2 (page 14); NaCl in group 2 had no statistically significant effect in relative gene expression of CHOP in kidney tissue compared to normal control group. It should be noted that, the relative gene expression of CHOP in Kidney tissue in groups 3-5 increased significantly in comparison with normal control group (p-value < 0.05)

Findings from H&E staining of kidney tissue

According to Fig.3 (page 15) in animals of group 1, glomeruli and renal tubules showed normal tissue structures. In animals of group 2, hydropic degeneration, cell swelling, and bleeding were prominent pathological features. In only one case was a limited number of fibrotic foci observed in the cortical region of the kidney as well as mild non-purulent perivasculitis. In animals of group 3, vascular congestion, vascular degeneration, and cell swelling were observed in most cases. In only one case were foci of infiltration of inflammatory cells (multinucleated cells and mononuclear cells), focal coagulation

necrosis, and mild focal fibrosis. In animals of group 4, hyperemia, hemorrhagic foci, and degenerative changes were observed in the tubules. In some cases; evidence of Bowman's dilatation, fibrosis, and mild inflammatory cell accumulation was also observed. In animals of group 5, evidence of epithelial cell membrane destruction, hydropic degeneration, glomerular fibrosis, accumulation of inflammatory cells, decreased glomerular space, coagulation necrosis, fibrosis foci, degenerative changes in some glomeruli, hyperemia and mild to moderate bleeding were observed. Outstanding features of histopathology in most cases belonged to this group.As shown in Fig.4 and Fig.5 (page 17-18); the fibrotic score of kidney tissue in group 5 had a significant increase compared to normal control group (p-value < 0. 05).

It is noteworthy that the degree of fibrosis is mild to moderate, which is expected to have no significant effect on kidney function.

Table 2. The effect of high salt diet on concentration of urea, creatinine and total protein in blood and urine

	Control (Group 1)	NaCl 0.5% (Group 2)	NaCl 1% (Group 3)	NaCl 1.2% (Group 4)	NaCl 1.5% (Group 5)	p-value
Blood urea concentration	69.3± 6.25	68.35± 5.75	71.78± 6.12	70.53± 5.81	72.48± 6.73	> 0.05
Blood creatinine concentration	0.72± 0.03	0.78± 0.07	0.78± 0.02	0.76± 0.08	0.71± 0.05	> 0.05
Urine urea concentration	123.51± 10.81	125.11± 12.43	127.21± 13.66	125.18± 11.74	125.15± 11.21	> 0.05
Urine creatinine concentration	33.81± 3.82	34.71± 4.11	34.71± 2.93	35.55± 4.38	36.56± 4.61	> 0.05
Urine total protein concentration	27.11± 2.55	29.22± 3.04	28.24± 2.83	29.95± 2.71	27.11± 3.10	> 0.05

NaCl was dissolved in distilled water. Data are Mean ±SD concentration of urea, creatinine and total protein in blood and urine.

Discussion

The results of the present study indicated that consumption of NaCl in group 5 caused a statistically significant increase in the expression of the CHOP gene as well as mild fibrosis in kidney tissue. Biochemical indicators of renal function such as urea, creatinine in blood and urine, and total protein in urine were not significantly different in comparison with normal control group; however, the concentration of KIM-1 increased significantly in the urine.

Results of present study indicated that osmotic stress due to prolonged excessive salt consumption increased expression of apoptotic CHOP gene in kidney tissue. In 1998, a study was designed on the mIMCD cell line. The cells were cultured in two isosmotic media (300 mosmol / kg) and hyperosmotic media (300 mosmol / kg + 150 mM NaCl) for 48 hours. Consistent with results of our study; in cells exposed to hyperosmotic media, the expression of GADD45 and GADD153 (CHOP) genes increased significantly [21].

Findings of present research demonstrated that urea, creatinine in blood and urine, and total protein in urine of rats received different concentrations of NaCl were not significantly different in comparison with normal control group. In the study carried out in 2007, spontaneously hypertensive rats (SHR), were divided into four groups with 0.6% (normal control), 4%, 6%, and 8% NaCl in the diet for 8 weeks. In contrast to results of our research; in animal groups received 6% and 8% NaCl in the diet; serum creatinine concentration, proteinuria and albuminuria increased significantly compared to the normal control group. Additionally; statistically significant decreased GFR was observed due to mild renal tubular degeneration in the same groups[22].

Based on results of present study, mild renal fibrosis was observed in rats treated with 1.5% NaCl compared to the control group. According to the study designed in 2011 on SHR rats; in animal groups that received 8% NaCl in the diet for 4 weeks, consistent with results of our study, significant glomerular damage and interstitial fibrosis were observed in comparison with group that received 8% NaCl in the diet with Losartan. It means that modulating osmotic stress with Losartan; could prevent from developing renal tissue fibrosis[23].

Results of present study demonstrated that, 1.5% NaCl in drinking water for 8 weeks in male Wistar rats, increased the expression of CHOP gene in kidney tissue. In 2015, researchers carried out a study on Sprague Dawley rats; weighing 250 to 300 g. To induce osmotic stress; the animals were deprived of drinking water for 3 days. In the next step; For 7 days, the animals consumed drinking water containing 2% NaCl. In both conditions of water depriving and consumption of water containing 2% NaCl, consistent with findings of our research, expression of CHOP gene in hypothalamus tissue was significantly increased in comparison with the normal control group [10].

In present research we observed mild renal fibrosis in rats that received 1.5% NaCl in drinking water for 8 weeks. In 2015, the study conducted on mice that had 5/6 kidneys removed. In this study, the animals were divided into three groups and exposed to high-salt (4% NaCl) with Hydralazine, low-salt (0.02% NaCl), and normal (0.4%Nacl) diet for two weeks. At the end of the study, consistent with results of our research, it was found that salt induces stable renal fibrosis while blood pressure is normalized with

Hydralazine. As a result, a prolonged high salt diet causes renal tissue fibrosis and chronic progressive renal disease independent of blood pressure[24]. According to the study carried out in 2017, Dahl salt-sensitive (DSS) rats were divided into two groups with 2% NaCl and 8% NaCl in the diet for 5 weeks. In contrast to results of our research, a statistically considerable kidney tissue fibrosis was not seen and there was no statistically significant difference in serum creatinine and urea levels between the two animal groups. After 15 weeks statistically significant difference in serum creatinine and urea levels and notable kidney tissue fibrosis was observed between two animal groups. Also, at the end of the 15^{th} week, consistent with results of our study, the expression of the KIM-1 gene in kinney tissue of group received 8% NaCl was significantly higher than the group received 2% NaCl[25]. In studies conducted in 2017 and 2018 on male Wistar albino rats; consistent with results of our research, significant tubular degeneration and renal tissue fibrosis were observed in the group received 8% NaCl diet for 8 weeks; compared to the normal control group [26, 27].

Conclusion

Osmotic stress due to prolonged excessive salt consumption can result in fibrosis through increased expression of apoptotic CHOP gene in kidney tissue. At the onset of apoptosis; KIM-1 protein appears in urine. In other words; KIM-1 can be detectable in urine before fibrosis of significant part of kidney tissue and impaired renal function.

Abbreviations

CKD: Chronic kidney disease

RF: Renal failure

GFR: Glomerular filtration rate

ER: Endoplasmic reticulum

CHOP: C/EBP Homologous protein

KIM-1: Kidney injury molecule 1

H and E : Hematoxylin and Eosin

SHR: Spontaneously hypertensive rats

DSS : Dahl salt-sensitive

Declarations

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prior publication: Neither this manuscript nor one with substantially similar content under our authorship has been published or is being considered for publication elsewhere. W certify that all the data collected during the study is presented in this manuscript and no data from the study has been or will be published separately.

Conflicting Interest: All contributing authors declare no conflicts of interest.

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Figures

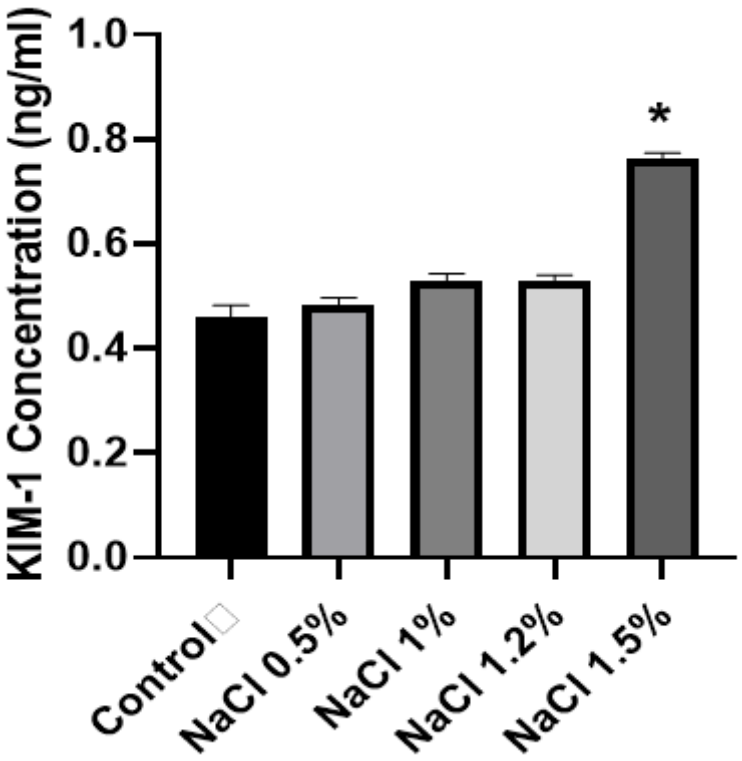

Figure 1

The effect of high salt diet on concentration of urinary KIM-1 protein. KIM-1 concentration in urine was measured by ELIZA method. NaCl was dissolved in distilled water. Data are Mean ±SD concentration of urinary KIM-1 in ng/ml. * : p-value < 0.05 when compared to normal control group.

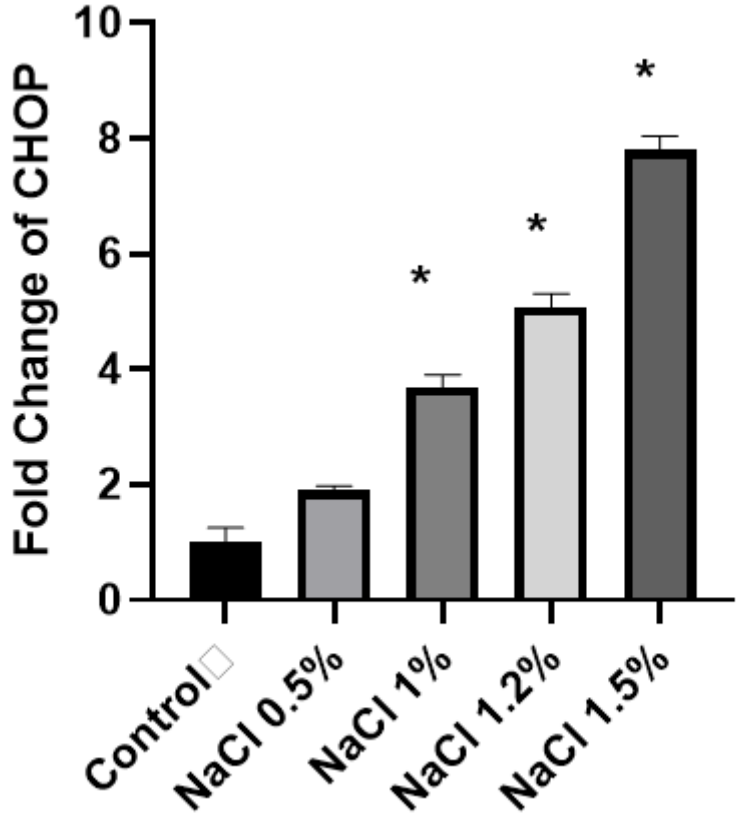

Figure 2

Relative gene expression of CHOP in kidney tissue by qRT-PCR. NaCl was dissolved in distilled water. Data are Mean ±SD of fold change of CHOP gene through Ct and 2 ^ - delta delta Ct method in kidney tissue. * : p-value < 0.05 when compared to normal control group.

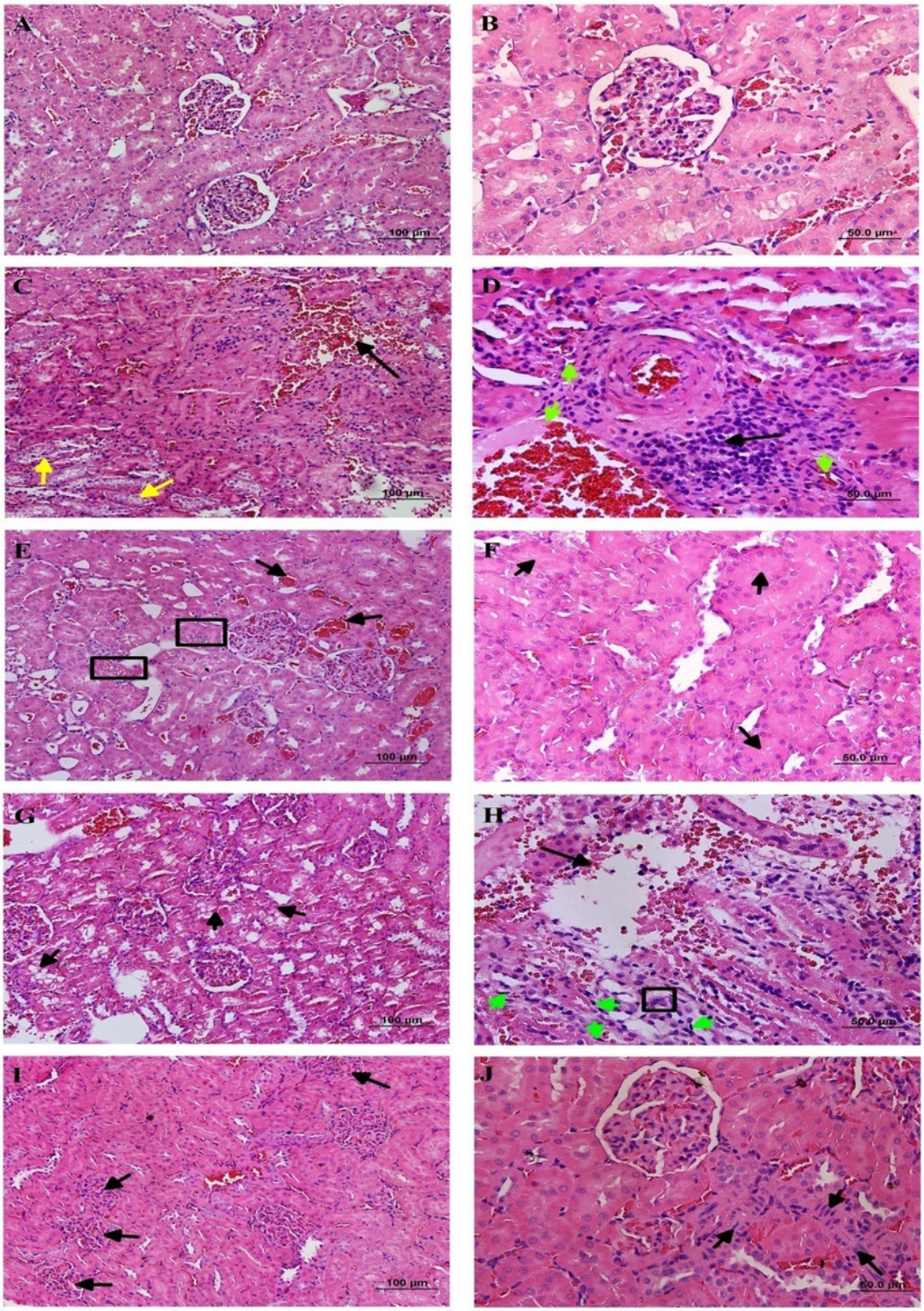

Figure 3

H&E staining of kidney tissue (H&E, scale bar: A, C, E, G, I: 100 µm, B, D, F, H, J: 50 µm) Guide to Fig.3: A and B) control group (Group 1) C and D) 0.5% NaCl is dissolved in distilled water (Group 2) E and F) 1% NaCl is dissolved in distilled water (Group 3) G and H) 1.2% NaCl is dissolved in distilled water (Group 4) I and J) 1.5%NaCl is dissolved in distilled water (Group 5). A and B): Normal renal tissue structure is observed. C): Degenerative changes (yellow arrow) and bleeding foci (black arrow) are observed in the

parenchyma of kidney tissue. D): hyperemia (green arrow) and perivasculitis (black arrow) are observed. E): Hydropic degeneration (rectangular) and vascular congestion (black arrow) are observed. F): Swelling of progressive tubular cells (black arrow) is observed. G): Degenerative changes (black arrow) are visible. H): The empty space indicates necrosis in the tubules and extracellular red blood cells (black arrow) are observed. Accumulation of inflammatory cells (rectangular) and fibroblasts (green arrow) can also be seen. I): decrease Bowman spaces (black arrow) is visible. J): Accumulation of fibroblasts is observed in the interstitial space (black arrow).

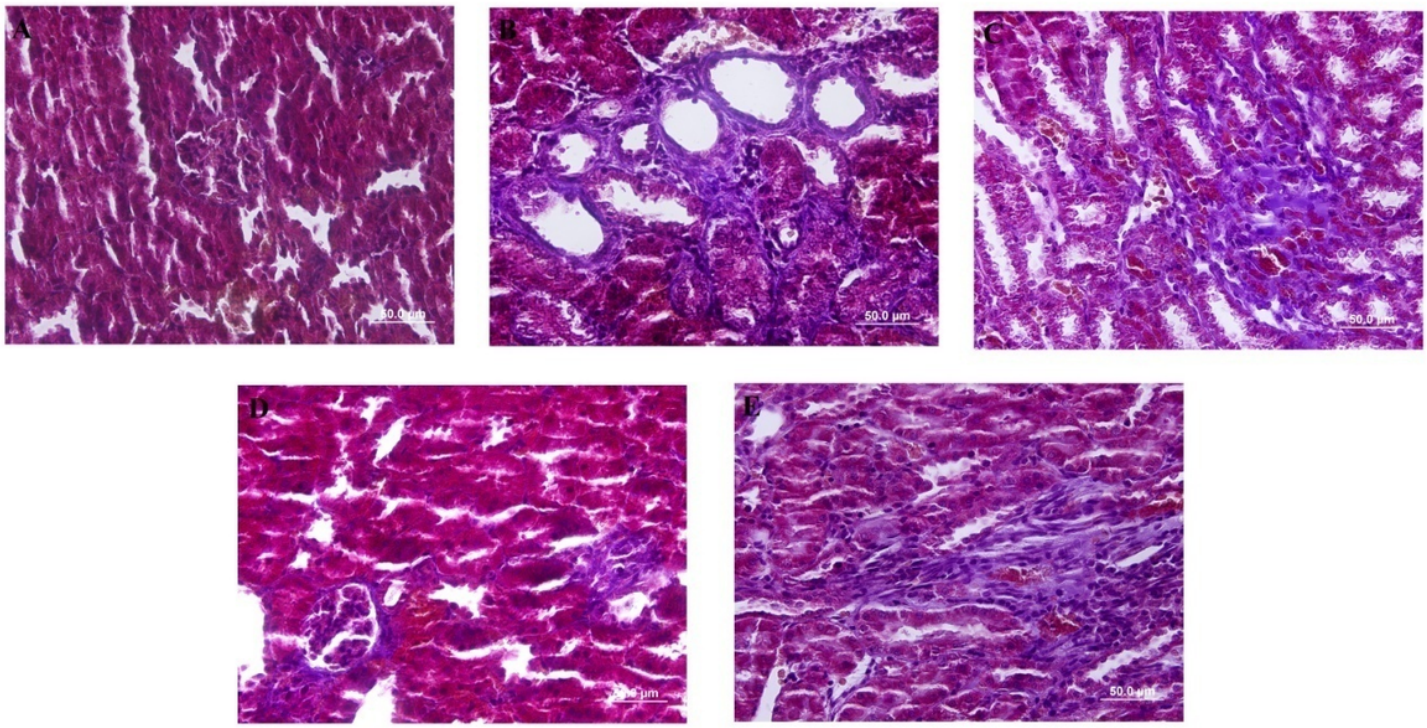

Figure 4

Masson's Trichrome staining of kidney tissue. A) group 1, B) group 2, C) group 3, D) group 4, E) group 5. Collagen fibers in fibrtic tissue are blue.

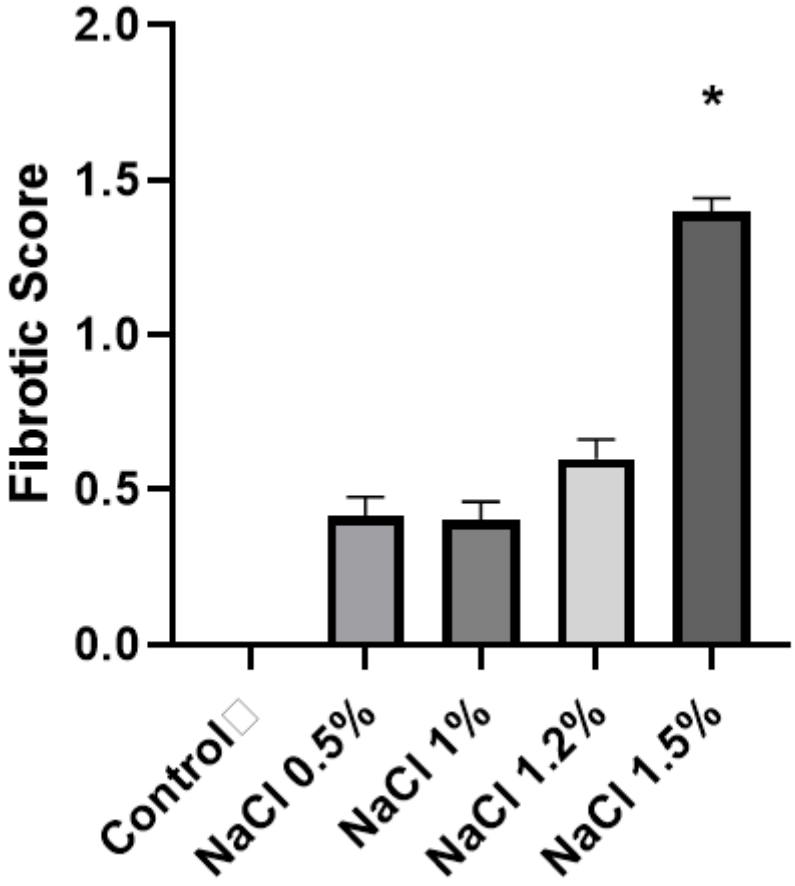

Figure 5

Quantitative analysis of fibrotic score in kidney tissue by Masson's Trichrome staining. NaCl was dissolved in distilled water. Data are Mean ±SD of fibrotic score in kidney tissue. * : p-value < 0.05 when compared to normal control group.