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RESEARCH ARTICLE

The effect of Nrf2-Keap1pathway on the oxidative stress and inflammations in acute kidney injury patients

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Abstract

Background: Inflammation and oxidative stress are always considered as key players in the pathophysiology of acute kidney injury (AKI). Nuclear factor erythroid-2-related factor-2 (Nrf2) pathway confers protection against tissue injury by orchestrating antioxidant and detoxification responses to oxidative stress.

Aim: This research aimed to investigate the effect of nuclear factor erythroid-2-related factor-2 - Kelch-like ECH-associated protein 1 (Nrf2-Keap1) pathway on the oxidative stress and inflammations in AKI patients.

Subjects and Methods: This study examined the plasma levels of reduced glutathione (GSH) and nitric oxide (NO) colorimetrically, and high sensitive-C reactive protein (hsCRP) and Kelch-like ECH-associated protein 1 (Keap-1) by ELISA method in 30 AKI patients and 30 matched controls. Plasma heme oxygenase-1 (HO-1) was also assessed by western blotting.

Results: The hsCRP and keap-1 levels were significantly higher ($P < 0.001$), whereas NO and GSH levels were significantly diminished ($P = 0.015$, $P = 0.007$ respectively) in AKI group compared to the control group. The decline in NO and GSH levels were 31.78% and 32.77% respectively, while the rise of Keap-1 was 103.52 % in the AKI group relative to the controls. Western blotting analysis demonstrated overexpression of plasma HO-1 in the AKI patients compared to the controls.

Conclusion: Our study concluded that despite Keap-1 was increased in AKI group, various oxidative stresses that prone to accompany AKI may modify its cysteine residues that could avert proteasomal degradation of Nrf2. Upregulation of Nrf2 target gene products including the antioxidant enzyme HO-1 may be one of the compensatory mechanisms that could ameliorate the effect of oxidative stress in AKI.

Introduction:-

Acute kidney injury (AKI) occurs in up to 7% of hospitalized patients (Lameire et al., 2006). Whereas 25% of patients in the intensive care unit (ICU) develop AKI and 5% of patients in the ICU would need renal replacement therapy (Lameire et al., 2006 and Waikar et al., 2007). Despite the extensive use of both intermittent hemodialysis (IHD) and continuous renal replacement therapy (CRRT), the mortality of AKI is considerably as high as 80% in ICU patients (Liano and Pascual, 1996 and Liano et al., 1998). Recently, Wang et al., (2012) concluded that AKI occurred in over 1 of 5 hospitalizations and was associated with a more than fourfold increased likelihood of death. AKI can result from mechanical trauma, ischemia/reperfusion, sepsis, toxins or nephrotoxic medication (Nath and Norby, 2000 and Pannu and Nadim , 2008). A better understanding of the different mechanisms that underlie AKI, and the biological aspects that determine the balance between renal adaptation and dysfunction, is vital to reduce clinical burden and patient suffering (Shelton et al., 2013).

Kidney is one of the organs that are highly vulnerable to damage caused by reactive oxygen species (ROS), likely due to the abundance of long chain polyunsaturated fatty acids in the composition of renal lipids (Ozbek , 2012). In recent years, oxidative stresses (Oss) have become one of the most popular topics in research of molecular mechanism of renal diseases.

Inflammation is vastly believed to play a major role in the pathophysiology of AKI. Previous reports showed that the initial insult of ischemia, sepsis, and nephrotoxic models of AKI, results in morphological and/or functional changes in vascular endothelial cells and/or in tubular epithelium (Edelstein and Schrier, 2003, 2007). Leukocytes including neutrophils, macrophages, natural killer cells, and lymphocytes would infiltrate later into the injured kidneys. Injury of the kidney induces the generation of inflammatory mediators like cytokines and chemokines by tubular and endothelial cells which contribute to recruiting of leukocytes into the kidneys. Therefore, inflammation is considered to play an important role in the initiation and extension phases of AKI (Akcay et al., 2009).

A likely consequence of the dysregulated inflammatory response in patients with AKI is an increase in oxidative stress. In inflammatory disorders, stimulated phagocytic cells, in addition to producing excess cytokines, are major producers of ROS. Renal failure itself is now recognized as an additional stimulus for increased oxidative stress (Himmelfarb et al., 2002, Descamps-Latscha et al., 2001).

An increasing body of evidence indicates that Nrf2 (nuclear factor erythroid 2–related factor 2) and Keap1 (Kelch-like ECH-associated protein 1; also referred to as inhibitor of Nrf2 (iNrf2)) regulate critical cytoprotective processes in mammals (Copple, 2012). Encoded by the NFE2L2 gene, Nrf2, a cap ‘n’ collar basic leucine zipper (bZip) transcription factor, is sequestered in the cytoplasm by its physical interaction with the zinc-finger protein Keap1. Nrf2 comprises six functional Neh domains; the Neh2 domain contains critical ‘DLG’ and ‘ETGE’ amino acid motifs that permit binding of the transcription factor to the C-terminal kelch domain of Keap1 (Tong et al., 2006). Nrf2 has a relatively short half-life of 10–30 min in the absence of stress (Itoh et al., 2003). The structural integrity of Keap1 is critical for the efficient regulation of Nrf2 activity, and the modification of these and other cysteines in Keap1 likely underlies the induction of Nrf2 under conditions of chemical or oxidative stress (Sekhar et al., 2010).

Nrf2 is shown to play a critical part in basal activity and coordinated induction of genes encoding numerous antioxidant and phase II detoxifying enzymes and related proteins such as catalase, superoxide dismutase (SOD), UDP-glucuronosyltransferase, NAD (P) H:quinoneoxidoreductase- 1, heme oxygenase-1 (HO-1), glutamate cysteine ligase, glutathione S-transferase, glutathione peroxidase, and thioredoxin, among others (Li et al., 2008).

Numerous animal- and cell-based studies have demonstrated a critical role for Nrf2 in regulating susceptibility to a wide variety of chemical and environmental insults affecting multiple organs and cell types, including the kidney (Copple et al., 2008).

This research aims to investigate the effect of Nrf2-Keap1 pathway on the oxidative stress and inflammations and its possible role in protection against AKI.

Subjects and methods:-

Subjects:-

The study was conducted on 60 subjects; 30 patients having acute kidney injury (sepsis-induced) admitted to Alexandria Main university hospital and 30 healthy subjects as controls. Patients were 9 males and 21 females, their age ranged between 34 years and 65 years with mean of 49.15 ± 15.64 years. Healthy subjects were 10 males and 20 female, their age ranged between 33 and 67 years with a mean of 50.85 ± 17.76 years. Both groups were comparable in age and sex. Smokers, patients with established diagnosis of CKD and subjects receiving anti-inflammatory or antioxidant therapy were excluded from the study. An Informed consent was contracted from all participants in the study. This study was conducted with approval of the Institutional Review Board of the Ethics Committee at the Faculty of Medicine, Alexandria University.

Ten ml blood samples were collected on EDTA- coated plastic tubes and centrifuged at 3000 rpm for 10 minutes at 4 °C to separate plasma. Samples were stored at – 80°C until analysis of creatinine, high sensitive C-reactive protein (hsCRP), reduced glutathione (GSH), nitrite and Keap-1, whereas fresh plasma samples were used for western blotting of HO-1.

Methods:-

Measurement of creatinine (Zilva et al., 1987 and Weber and van Zanten, 1991).

Creatinine concentrations were estimated by colorimetric determination method using kit supplied by (BioVision's Creatinine Assay Kit)

As creatine is enzymatically converted to sarcosine, it specifically oxidized to generate a product that converts a colorless probe to an intensely red color (OD 570 nm) product that was measured by spectrophotometer.

Measurement of high sensitive C- reactive protein (hsCRP) by ELISA (van Weemen and Schuurs, 1974).

hsCRP ELISA assay kit purchased from Calbiotech, life science company was used to measure C-reactive protein.

The CRP ELISA kit is a solid phase direct sandwich method. 10 µL of standard and sample and 100 µl anti-CRP-HRP conjugate were added to the wells coated with monoclonal antibody (MAb) to CRP (this coating was done by manufacturer) and mixed well. The plate was incubated for 60 minutes at room temperature. CRP in the patient's plasma bound to anti-CRP MAb on the well and the anti-CRP second antibody then bound to CRP. Unbound protein and HRP conjugate were washed off three times by 300 µl of 1X wash buffer. 100 µl of TMB substrate was added to all wells and incubated for 15 minutes at room temperature. 50 µl of stop solution was added to all wells and plate was shaked gently to mix the solution, the intensity of color was proportional to the concentration of CRP in the samples. The absorbance was read on ELISA reader at 450 nm within 15 minutes after adding the stopping solution. A standard curve was prepared relating color intensity to the concentration of the CRP.

Measurement of reduced glutathione (GSH) (Moron et al., 1979).

GSH (the most important nonprotein sulphydryl antioxidant in the cell) was estimated using Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid; DTNB) according to the method described by Moron et al., (1979) with some modification. Briefly, plasma samples were deproteinized by adding equal volume of 25% TCA and then centrifuged at 4°C at 3000 rpm for 10 minutes, 0.5 mL of supernatant was then added to 4.5 mL of Ellman's reagent. The produced yellow color was then measured using spectrophotometer at 412 nm against reagent blank.

Measurement of Nitrite (An Index of Nitric Oxide, NO) (Kleinbongar et al., 2002).

Biologically produced NO is rapidly oxidized to nitrite and nitrate, thus, nitrite concentrations can reflect NO production, and may be measured colorimetrically using Griess reagent. 100 µL of plasma samples were added to 100 µL Griess reagent (1:1 mixture of 1% sulfanilamide in 2.5% orthophosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine in distilled water). After 10 minutes of color development at room temperature, absorbance was measured by spectrophotometer at 540 nm.

Estimation of Kelch-like ECH-associated protein 1 (Keap-1) by ELISA (van Weemen and Schuurs, 1974).

KEAP-1 ELISA assay kit purchased from Cusabio Biotech was used to estimate Keap-1.

Antibody specific for Keap-1 had been pre-coated onto a 96 well plate (this was done by manufacturer). 100 µl of standard and sample were added per well and incubated for 2 hours at 37°C, any Keap-1 present was bound by the immobilized antibody. After removing any unbound substances (the liquid of each well was removed, but not washed). 100 µl of a biotin-conjugated antibody specific for Keap-1 (1x) was added to the wells and incubated for 1 hour at 37°C After washing three washes, any remaining wash buffer was removed by aspiration. The plate was inverted and blotted against clean paper towels. 100 µl of avidin conjugated Horseradish Peroxidase (HRP) (1x) was added to each well and incubated for 1 hour at 37°C. Following five times wash to remove any unbound avidin-enzyme reagent, 90µl of TMB substrate solution was added to the wells and incubated for 15-30 minutes at 37°C, and color then develops in proportion to the amount of Keap-1 bound in the initial step. Color development was stopped by adding 50µl of stop solution and the intensity of the color was measured at 450 nm. The concentrations of the samples were read from the standard curve.

Western blotting of plasma heme oxygenase-1 (HO-1) (Burnette, 1981).

Samples preparation:-

Blood samples from patients and controls were collected in EDTA-coated plastic tubes. The plasma was immediately separated by centrifugation at 3000 rpm for 15 minutes at 4 °C. The total protein content was then quantified by the Bradford method according to the manufacturer's instructions (Bio-Rad).

Western blotting of HO- 1 in plasma

Four samples from AKI group and four samples from the control group were randomly chosen. Eight lanes were loaded with the samples and the controls. The HO-1 intensity of each lane from each sample was then analyzed by Western blot.

As for the Western blot procedure, plasma samples were diluted with 0.1 M phosphate-buffered solution (PBS) and 5x Laemmli buffer (10% [w/v] sodium dodecyl sulfate [SDS], 50% [v/v] glycerol, 50 mM dithiotreitol [DTT], 500 mM Tris–HCl [pH 6.8], and 0.5% [w/v] traces of bromophenol blue) to a protein concentration of 5 µg/µl and then heated at 99 °C for 5 minutes. Samples (5 µg/ lane) were run on a 10% SDS–PAGE (polyacrylamide gel electrophoresis) gel, and then the protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Then, the membranes were blocked in a 5% (w/v) skimmed milk solution for 1 hour at room temperature and incubated overnight at 4 °C with primary rabbit polyclonal anti-HO-1 (1:3000, Anti-Heme Oxygenase 1 antibody ab13243, purchased from abcam). After three 30 minutes washings in 100 mM Tris–HCl buffer (pH 7.5) with 150 mM NaCl and 0.05% Tween 20 (TTBS buffer), the membranes were incubated at 37 °C for 60 minutes with HRP-Goat conjugated anti-rabbit immunoglobulin G (IgG) , ab6721 purchased from abcam ,and washed three times for 30 minutes with TTBS buffer. Then, the membranes were developed with enhanced chemiluminescence (ECL) reagents and the chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad). The immunoblots were quantified using Quantity One software (Bio-Rad).

Statistical analysis of the data (Kotz et al., 2006).

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (Kirkpatrick and Feeney, 2013). Quantitative data were described using mean and standard deviation (SD). Comparison between control and AKI groups was done using independent student (t-test). Significance of the obtained results was judged at the 5% level.

Results:-

Plasma creatinine showed a percentage increase of 233.3 % from the base line, while the mean urine output was about 300 cc /day in the AKI group. Whereas the concentration of hsCRP, in AKI group ranged between 11.82 and 54.35 mg/L with a mean ± SD of 32.08± 9.76 mg/L, it was significantly higher than the control group (<0.001), which CRP concentration ranged between 2.30 and 5.21 mg/L with a mean ± SD of 2.95± 0.68 mg/L as shown in tab. 1.

A significant decrease of NO and GSH levels in the AKI group (mean; 30.63± 2.68 µmol/L, 23.65± 2.81 µg/ml respectively) was detected compared to the control group (mean± SD; 44.94± 3.96 µmol/L, 35.18 ± 4.15 µg/ml respectively) (P= 0.015, P= 0.007 respectively) as shown in tab. 2, fig.1. There were percentage decrease of 31.78% for NO and 32.77% for GSH in the AKI group relative to the control group (tab. 2, fig. 2). On the other hand there was significant increase in the keap-1 levels in the AKI group (mean ± SD; 98.73 ± 5.88 ng/ml) as compared to the control group (mean ± SD; 48.51 ± 4.05 ng/ml) (P <0.001) with percentage increase of 103.52 % in the AKI group relative to the control group (tab. 2, fig. 1 and 2).

Western blotting analysis has shown that HO-1 was highly expressed in the plasma of AKI patients as compared to the control samples (fig. 3).

Discussion:-

Oxidative stress and inflammation are constant features associated with advanced renal disease and play a major role in progressive deterioration of renal function and structure. hsCRP was used in this study as a marker of inflammation. Our study demonstrated a highly significant increase of the hsCRP in the AKI group as compared to the control group, indicating acute stressful condition, as its level rise dramatically during inflammatory processes. Pegues, et al., (2013) reported that CRP might play an active role in AKI, with CRP worsening the damage caused by renal ischemia-reperfusion injury (IRI) (Pegues et al., 2013).

Our study showed a significant increase in the keap-1 levels in plasma of the AKI group as compared to the control group with percentage increase of 103.52% in the AKI group relative to the control group. Previous study of Kim and Vasari (2010) reported a significant elevation of Keap1 in the remnant kidneys of the chronic renal failure (CRF) animals. However, to our knowledge, this is the first study that indicates significant elevation of Keap-1 in sepsis-induced -AKI.

On the basis of the previous studies by Sekhar et al., (2010) and Surh et al., (2008) septic acute kidney injury (S-AKI) animals prove to have greater inflammatory and oxidative stress, we can speculate that oxidative or covalent modification of thiols in some of the Keap 1 cysteine residues could results in dissociation of Nrf2 from Keap1 and its translocation to the nucleus. In the nucleus, Nrf2 binds to the regulatory sequences, termed antioxidant response elements or electrophile response 2 elements, located in the promoter region of genes encoding the antioxidant and phase 2 detoxifying enzymes, up-regulates the expression of Nrf2 target genes. These genes include heme oxygenase 1 (HO-1), NQO1, and catalase.

Experimental studies in kidney cells and animal models, as well as limited data in humans, have implicated an increase in Nrf2 as beneficial in a range of kidney diseases, including acute kidney injury, chronic kidney disease, diabetic nephropathy and hypertension (Liu et al., 2009 and Pedruzzi et al., 2015).

Given the critical role of HO-1 in Nrf2 pathway, we tested the plasma HO-1 levels using western blotting which reflects the elevation of HO-1 expression in the AKI as compared to the control. Our findings were in agreement with other investigators, Zager et al., (2012) who noted that plasma and urinary HO-1 were increased as early as 4 hours after I/R- or glycerol-induced injury, indicate that extracellular HO-1 release is an early AKI event, and one with potential AKI biomarker utility.

Previous studies on post ischemic mouse kidney revealed that Nrf2 is the master regulator of acute kidney injury stress responses (Leonard et al., 2006). It is assumed that in the presence of reactive oxygen species and other potentially harmful stimuli, Keap1 dissociates from Nrf2, as verified in S-AKI, allowing it to translocate into the nucleus, bind to antioxidant response elements along with other binding partners, and actively transcribe antioxidant and anti-inflammatory genes (Liu et al., 2009). which may explain the HO-1 overexpression in the AKI group in our study.

This elevated level of HO-1 may contribute to the Nrf2 protective effects handled through control over hundreds of antioxidant and xenobiotic genes that are actively transcribed following an insult, including HO-1. This finding is in convention with Noel et al., (2015) who reported that strategies to 'Keap' Nrf2 activated could prove beneficial for the kidney disease patients.

ROS elicit a compensatory response aimed at mitigating the impact of oxidative stress and its constant companion inflammation, via activation of Nrf2 and consequent expression of the antioxidant and detoxifying enzymes (Bea et al., 2003; Chen et al., 2006 and Harvey et al., 2009).

Likewise, experimental models targeting Nrf2 as a therapeutic strategy to amend oxidative stress has shown potential for future studies concerning management of kidney disease in humans (.Ruiz et al., 2013).

Our study demonstrated a significant decrease of NO in AKI group as compared to the control group with a percentage decrease of 31.78% which could be explained by the previous observations of Brodsky et al., (2002) who reported that early endothelial dysfunction might contribute to the pathophysiology of acute renal failure. Likewise, several studies indicate that nitric oxide is fundamentally involved in the regulation of renal hemodynamics and homeostasis and that NO pathways are disturbed in AKI. Thus, decreased NO activity may be casually linked to AKI (Schneider et al., 2003).

Vascular endothelium undergoes structural and functional changes in early ischemic renal failure. Altered NO production and /or decreased bioavailability of NO comprise the endothelial dysfunction in acute renal failure (Conger et al., 1995) . Nitric oxide deficiencies may result from decreased formation or increased degradation, evidence for both mechanisms are provided by Schneider et al., (2003) study.

It has been suggested that the balance between expression and activity of the inducible and constitutive isoforms of NO synthase (NOS) is disturbed in ARF, and that these changes contribute to ARF pathophysiology (Goligorsky and Noiri, 1999). Several studies have indicated imbalance of NOS activity with enhanced expression and activity of inducible NOS (iNOS) and decreased endothelial NOS (eNOS) in ischemic kidneys (Conger et al., 1995). Employing antisense-oligodexynucleotides to iNOS, Noiri et al., (1996) demonstrated that high output NO production by iNOS might suppress the activity of eNOS.

Our results demonstrated that GSH was significantly reduced in the AkI group as compared to the control group which could be attributed to its utilization by glutathione dependent antioxidant process through generation of ROS that is usually accompanying S-AKI. Additionally, selective inhibition of the enzymes of the glutathione redox cycle enhances the susceptibility to ROS-mediated cell injury (Harlan et al., 1984). Likewise, many pathological conditions including, atherosclerosis, diabetes, liver disease, myocardial infarction and stroke are associated with elevated GSSG (oxidized glutathione) and diminished GSH (reduced glutathione) levels (Cho et al., 1999; Morrison et al., 1999; Rosenblat et al., 2002and Chen et al., 2006).

Fig.1.Plasma levels of nitric oxide, reduced glutathione (GSH) and Keap1 in the control and AKI groups.
NO and GSH levels were significantly decreased, while the keap-1 levels was significantly increased in the AKI group as compared to the control group.

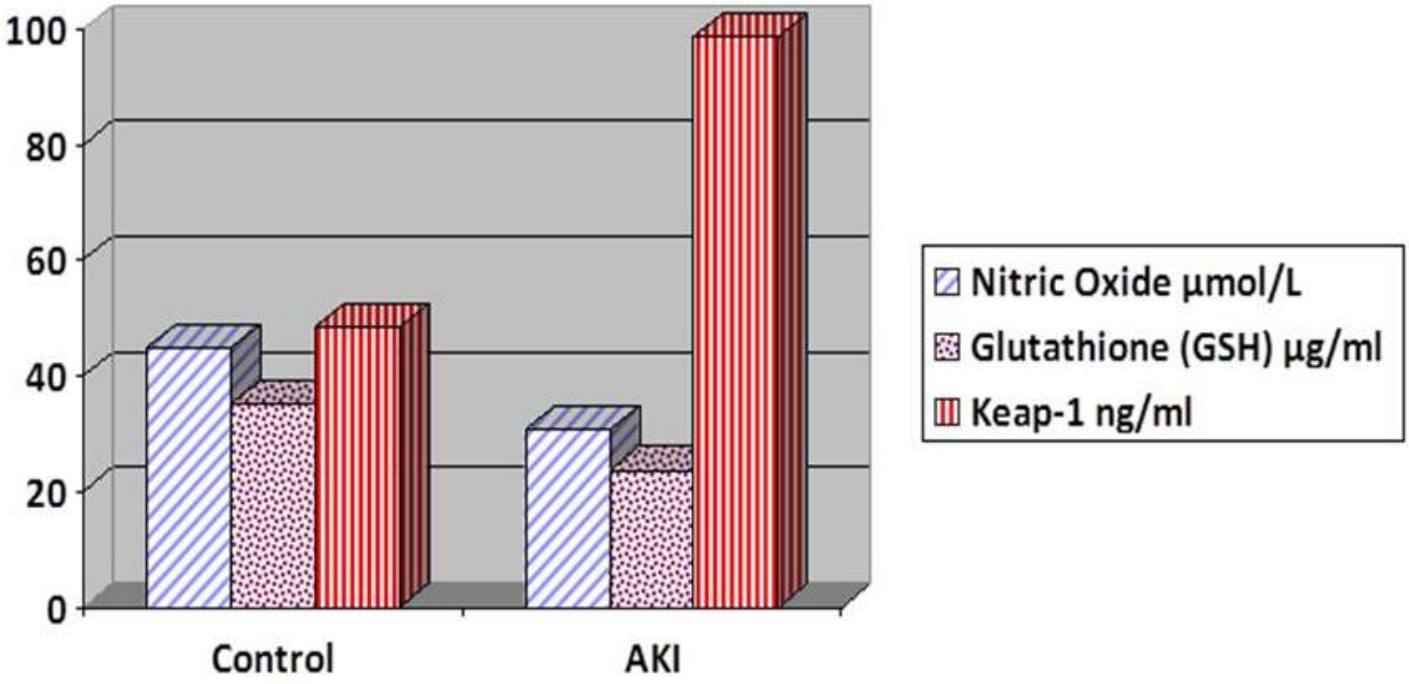

Fig.2.Percentage changes of nitric oxide, GSH and Keap-1 in the control and AKI groups.
The % decrease in NO and GSH levels were 31.78% and 32.77% respectively in the AKI, while the % increase in the Keap-1 level was 103.52 % relative to the control group.

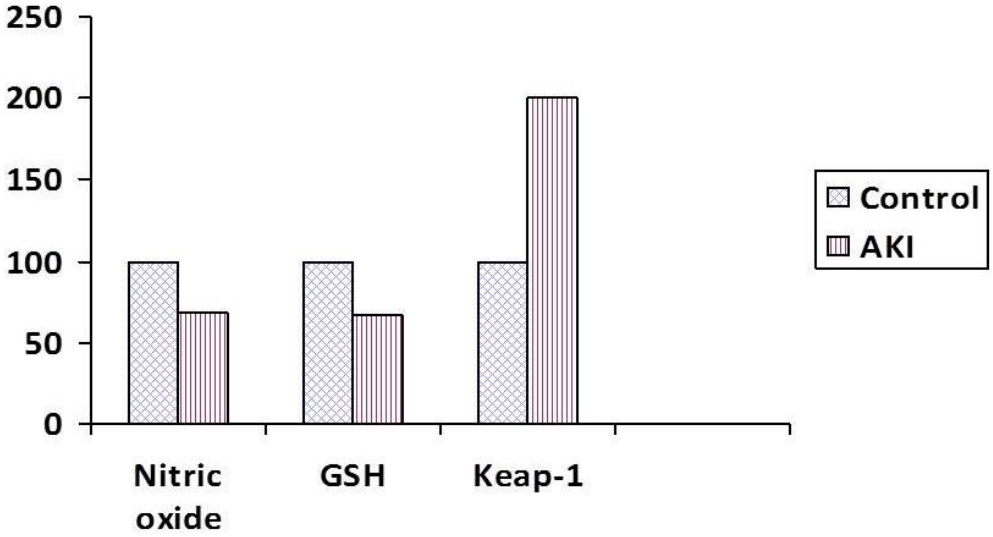

Fig.3.Western blotting analysis of HO-1 in the control and AKI groups.
HO-1 was highly expressed in AKI plasma samples (5, 6, 7, and 8) as compared to control (1, 2, 3, and 4).

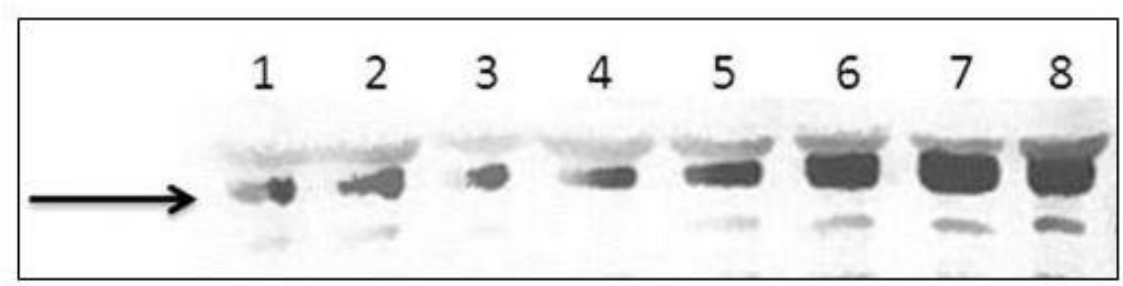

Table (1): Comparison between the two studied groups according to hsCRP levels in plasma.

Parameter	**Control (N= 30)**	**AKI (N= 30)**	***P value***
hsCRP (mg/L) **Mean±SD** **Min** **Max**	2.95± 0.68 2.30 5.21	32.08± 9.76 11.82 54.35	<0.001*

Table 1; hsCRP; high sensitive C- reactive protein was expressed as (Min. – Max., mean ± SD). It describes the independent t-test between the control and acute kidney injury (AKI) groups in hsCRP.

N: number of subjects

*: Statistically significant at $p \leq 0.05$

Table (2): Comparison between the two studied groups according to nitric oxide, glutathione and Keap1 levels in plasma

Parameters	Control (N= 30)	AKI (N= 30)	*P* value	% Change
NO (µmol/L) **Mean± SD** **Min** **Max**	44.94± 3.96 38.23 51.48	30.63 ± 2.68 26.32 38.24	0.015*	- 31.78
GSH (µg/ml) **Mean± SD** **Min** **Max**	35.18± 4.15 27.55 41.36	23.65 ± 2.81 19.75 31.50	0.007*	- 32.77
Keap-1 (ng/ml) **Mean±SD** **Min** **Max**	48.51 ± 4.05 37.47 55.31	98.73 ± 5.88 80.25 110.63	<0.001*	+ 103.52

Table 2; NO; Nitric oxide, GSH; reduced glutathione and Keap-1; Kelch-like ECH-associated protein 1 data were expressed as ((Min. – Max., mean ± SD). It describes the independent t-test between the control and acute kidney injury (AKI) groups in NO, GSH and Keap-1.

N: number of subjects
*: Statistically significant at $p \leq 0.05$

Conclusion:-

Our study concluded that despite the increased Keap 1 in AkI group, various oxidative or electrophilic cellular stresses that prone to accompany S-AKI may modify Keap 1 cysteine residues, resulting in a conformational change of Keap 1. These modifications may avert proteasomal degradation of Nrf2, causing significant upregulation of its target gene products including the antioxidant enzyme HO-1 as shown in our study. Overexpression of HO-1 is one of the compensatory mechanisms that could ameliorate the effect of oxidative stress in AKI.

Even though Nrf 2 is known to induce the expression of the key enzymes in glutathione synthesis, the primary cellular scavenger of electrophiles, reduced glutathione level is shown to be reduced in our study, which may reflect the fact that AKI is associated with severe oxidative stress/lipid peroxidation.

Nrf2 can also reduce RNS and ROS levels by directly controlling the enzymatic formation of such molecules as NO by suppressing the expression of inducible nitric oxide synthase (iNOS; also known as NOS2) which could explain the reduction of NO levels in the AKI group in this study.

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