Molecular and Immunohistochemical Evidences for Cardiovascular Damage in Addition to Renal Toxicity of Cisplatin in Rats

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Abstract: Renal dysfunction is a well-known dose-limiting toxicity of the chemotherapeutic drug Cisplatin (CP). CP was also reported to trigger a degenerative process of endothelium that contributes to vascular abnormalities. We aimed to investigate the molecular and biochemical mechanisms contributing to cisplatin-induced vascular damage by evaluating mRNA expression of genes related to leukocytes adhesion (ICAM-1), inflammation (TNF-α and TNFR-1), oxidative stress (HO-1), and basement membrane molecules (desmin, and nephrin). Endothelial damage was evaluated in aorta sections by immunohistochemical staining with von Willebrand factor (vWF). Rats injected with CP (4 mg/kg) i.p. per week for 4 weeks showed significant deterioration in biomarkers of kidney and heart functions, increased oxidative stress (NO and MDA), and decreased antioxidant enzymes of kidney and heart tissue homogenates. CP-injected rats showed also a prominent increase in mRNA of the studied genes compared with control rats. CP-group also showed a significant decrease in vWf expression in aorta sections in comparison to aorta from healthy rats. In conclusion, our data indicated that cisplatin induces cardiovascular changes in addition to renal dysfunction, and that increased leukocytes adhesion, oxidative stress and inflammation may account for these abnormalities.

[Faheim E. Wehaish, Yousef Y. Elseady, Rasha M. Saleh and Mohamed F. Elshal. **Molecular and Immunohistochemical Evidences for Cardiovascular Damage in Addition to Renal Toxicity of Cisplatin in Rats.** *Life Sci J* 2014;11(6):1-8]. (ISSN:1097-8135). http://www.lifesciencesite.com. 1

Keywords: cisplatin, kidney, heart, aorta, vascular damage, gene expression, immunohistochemistry, oxidative stress, inflammation.

1. Introduction

Cisplatin (CP) as a potential drug for solid tumors produces nephrotoxicity and disturbs endothelial function. CP induced nephrotoxicity is a multifactorial process as it activates several signaling process pathways that lead to tubular toxicity, inflammation, oxidative stress, and change in the renal circulation (Demkow and Stelmaszczyk-Emmel, 2013; dos Santos et al., 2012). In addition, histopathological examination showed that CP injury can cause endothelial cell dysfunction and leukocytes infiltration (Yu et al., 2008). Furthermore, animal experiments and clinical reports have also demonstrated that the vascular effects of CP could contribute to renal dysfunction (Daher and Yeh, 2008). The mechanisms of CP-induced vascular toxicity might involve oxidative stress, leukocytes infiltration and proinflammatory state (El-Naga, 2014). Clinical studies also demonstrated that CP triggers a degenerative process of medium-thickness vessel walls, thus causing occlusive vascular disease in the long term and development of hypertension (Morlese et al., 2007).

The present work was aimed to investigate the molecular mechanisms associated with the deleterious effect of cisplatin on vasculature in rats by evaluating some biochemical parameters in the serum, and tissue homogenate, and mRNA expression of genes related to inflammation (tumor necrosis factor-alpha and its receptor-1 (TNF-α, TNFR-1)), oxidative stress (heme oxygenase-1 (HO-1)), leukocytes adhesion (intracellular adhesion molecule (ICAM-1)), and basement membrane molecules (desmin, and nephrin). Endothelial damage was evaluated in aorta sections by immunohistochemical staining with von Willebrand factor (vWF).

2. Materials and methods

2. 1. Animals

Thirty six healthy male rats (initially weighing between 200 and 220 g body weight) were used in this study. The animals were purchased from animal house in Helwan, and housed in Department of Physiology, Faculty of Veterinary medicine, Mansoura University Animals were left for one week to acclimatize the place. Rats were kept in cages in a rate of six rats per cage in a controlled environment, maintained under a 12 hours light:dark cycle, 24^{o}C (± 3^{o}C) and 50-70% humidity. Rats were provided with standard diet and water ad-libitum. This study was approved by the Animal Care Committee of Faculty of Veterinary medicine, Mansoura University, Egypt.

2.2. Experimental procedure

Rats were randomized into six groups (each n=6). Animals of control group received a single dose of 0.9% saline i.p. and three times of distilled water by

oral gavage; animals in the CP-group were received a single dose of CP (4 mg/kg) i.p. per week for 4 weeks the duration of the experiment besides three times of distilled water by oral gavage.

2.3. Sample collection

Blood was drawn after 12-h fasting at one, two, and four weeks post CP injection; blood samples were collected via retro-orbital bleeding for serum separation. Blood samples were left in plain test tube at room temperature for 1 hour and then centrifuged for 10 min at 3000 rpm to obtain the serum. Serum samples were stored at −80°C for further analysis. Then rats were sacrificed by decapitation. Right and left kidneys, aorta and heart were rapidly excised from all rats. Part from the kidneys, aorta and heart slices were fixed in 10% neutral buffered formalin. Fixed specimens were processed for routine histopathological examination and immunohistochemical staining.

2.4. Biochemical blood analyses:

Serum creatinine, urea, cardiac troponin-I (cTn-I), and lactate dehydrogenase (LDH) concentrations were measured by a colorimetric method (Tietz et al., 1990) using commercial kits (Biodiagnostic, Cairo, Egypt). All measurements were performed according to the manufacturer's instructions.

2.5. Oxidant/Antioxidant Status Analyses

For estimation of the different oxidative stress parameters as well as the antioxidants, a part of kidney (0.25 g) was ice cooled, homogenized in 2.5 ml phosphate buffer (pH 7.4), and then centrifuged at 3000xg for 15 min at 48°C. The supernatant was collected for the following biochemical analysis. Lipid peroxidation was determined in homogenate by estimating level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA), according to the method of Mihara and Uchiyama (1987), using commercial kits (Biodiagnostic, Cairo, Egypt). Antioxidant markers glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase were measured in heart and kidney samples by a colorimetric method using commercial kits (Biodiagnostic, Cairo, Egypt) according to the manufacturer procedures. Total nitric oxide (NO) content was estimated in homogenate spectrophotometrically by colorimetric method (Miranda et al, 2001) using commercial kits (Biodiagnostic, Cairo, Egypt).

2.5. Expression of mRNAs of genes in the renal cortex

RNA was extracted from the renal cortex after the kidney was separated into two parts along the sagittal plane. The cortex was separated along the medulla and stored directly at −70°C before real time PCR. Real-time quantitative fluorescence PCR with SYBR Green was used to measure expression of TNF-α, TNFR-1, HO-1, ICAM-1, desmin, and nephrin mRNAs in the renal cortex, with β-actin as an internal reference. The gene sequences were identified in GeneBank for the design of specific primers (Table 1). Total RNA of the renal cortex was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions. Then, 4 μl of total RNA were subjected to reverse transcription with random primers, and M-MuLV reverse transcriptase (Fermentas, #EP0451, European Union) in 20 μl of reaction mixture at 37 ºC for 1 h and then at 95 ºC for 3 min using a PCR instrument, to convert RNA into complementary DNA (cDNA) (El-Magd et al., 2013). Then, 5 μl of cDNA were added to the 50 μl reaction mixture, followed by amplification in an automatic quantitative fluorescent PCR instrument (Model 7500, Applied Biosystems, USA). The PCR conditions were: pre-denaturation at 93 °C for 3 min, 40 cycles of denaturation at 93 °C for 30 sec, annealing at 55 ºC for 45 sec, and extension at 72 ºC for 45 sec. Expression of each target gene was normalized to that of β-actin.

2.6. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the general liner model procedure of SAS (SAS Institute, 2004). F-test (Fisher least significant difference test) was used to compare between parameters at 1, 2 weeks and 4 weeks of treatments. T-test (student T-test) was used to compare between groups. The mean values were significant at $P<0.05$.

Table (1): Primer sequences used for real-time quantitative polymerase chain reaction:

Gene	Forward (5----3)	Reverse (5----3)	Accession number
Nephrin	CGGAGAAGACTGAGGCGCCTT	TCACACCAGATGTCCCCTCAG	AB513652
Desmin	TCAAGGGCACCAACGACT	GGTCTGGATCGGAAGGTTGAT	BC031760
TNF-α	GCATGATCCGCGACGTGGAA	AGATCCATGCCGTTGGCCAG	NM_013693
TNFR1	CCGGGCCACCTGGTCCG	CAAGTAGGTTCCTTTGTG	NM_011609
ICAM-1	AGATCACATTCACGGTGCTG	CTTCAGAGGCAGGAAACAGG	NM_010493
HO-1	GGAAAGCAGTCATGGTCAGTCA	CCCTTCCTGTGTCTTCCTTTGT	NM_010442
VEGF	GATCATGCGGATCAAACCTCACC	CCTCCGGACCCAAAGTGCTC	NM_001287058
β-actin	CATGGATGACGATATCGCT	CATGAGGTAGTCTGTCAGGT	NM_007393

TNF-α, tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor 1; ICAM-1, intracellular adhesion molecule-1, HO-1, hemeoxygenase 1; VEGF, vascular endothelial growth factor receptor.

3. Results

3.1. Renal and cardiac function tests

Serum creatinine levels were significantly increased in CP-group when compared with the control group. Serum urea concentrations were significantly increased in CP-group compared to control group. Serum LDH levels significantly (P<0.0001) increased in CP-group when compared with the control group. Tp-I were significantly (P<0.0002) increased in CP-group compared to normal group (Table 2).

3.2. Lipid peroxidation and NO levels

MDA levels in kidney and heart tissue were significantly (P<0.0001, 0.0013) increased in CP-group compared to control group**.** Kidney and heart tissue homogenate NO levels significantly (P<0.0001, 0.0001 respectively) decreased in CP-group compared to control group (Table 3).

3.1.3. Antioxidants markers

CP-group showed significant lower levels of antioxidant enzymes in kidney and heart tissue compared to control group (Table 4).

3.2. Gene expression analyses

Fold change in genes expression in kidney samples were significantly increased in CP-group compared to normal group (Table 5).

3.3. Microscopical examination of vWf

In CP-group after the first week, the microscopical examination of the aorta showed moderate increase in the thickness of tunica media layers compared to the control group (Figures 1A&B). The increase in thickness of intimal layer became also obvious. However, the thickness of tunica media was found to be more prominent than the thickness of tunica intima.

Immunostaining of aorta sections with vWF revealed strong positive staining throughout the wall of the aorta of control group, whereas CP-group showed weak vWF staining throughout the wall of the aorta (Figures 1C&D).

Table (2): Kidney and heart function biomarkers in serum samples:

Group	1st Week	2nd Week	4th Week
Creatinine (mg/dl)			
Control	0.670 ± 0.37^{A}	0.62 ± 0.04^{A}	0.70 ± 0.01^{A}
Cisplatin	2.91 ± 0.12^{Ba}	3.78 ± 0.09^{Bb}	5.09 ± 0.08^{Bc}
Urea (mg/dl)			
Control	32.33 ± 2.33^{A}	27.66 ± 2.02^{A}	24.33 ± 3.84^{A}
Cisplatin	155.66 ± 3.17^{B}	169.33 ± 6.64^{B}	219.66 ± 10.6^{B}
LDH (U/L)			
Control	62.33 ± 6.74^{Aa}	82.66 ± 3.93^{Ab}	70.66 ± 4.26^{Aab}
Cisplatin	396.33 ± 3.7^{Ba}	438.66 ± 11.2^{Bb}	470.66 ± 4.26^{Bc}
cTn-I (ng/ml)			
Control	0.057 ± 0.003^{A}	0.047 ± 0.003^{A}	0.053 ± 0.003^{A}
Cisplatin	0.210 ± 0.006^{Ba}	0.233 ± 0.009^{Ba}	0.317 ± 0.009^{Bb}

- Values are mean ±S.E.
- Values with the different superscript letters in the same column are significantly different at (P< 0.05).

Table (3): Lipid peroxidation and NO in kidney and heart homogenates:

Group	Kidney			Heart		
	1st Week	2nd Week	4th Week	1st Week	2nd Week	4th Week
MDA (nmol/ml)						
Control	18.30 ± 0.36^{Fa}	19.26 ± 0.43^{Fa}	19.266 ± 0.24^{Da}	29.73 ± 0.34^{Ea}	30.53 ± 0.52^{Ea}	29.333 ± 0.33^{DEa}
Cisplatin	40.03 ± 0.20^{Ac}	44.10 ± 0.20^{Ab}	51.100 ± 1.21^{Aa}	47.76 ± 0.17^{Ac}	58.50 ± 0.36^{Ab}	65.10 ± 0.63^{Aa}
NO (µmol/L)						
Control	43.86 ± 0.14^{Aa}	43.46 ± 0.31^{Aa}	44.03 ± 0.14^{Aa}	44.6 ± 0.24^{Aa}	45.0 ± 0.51^{Aa}	45.8 ± 0.11^{Aa}
Cisplatin	20.6 ± 0.34^{Fa}	17.20 ± 0.23^{Fb}	13.70 ± 0.26^{Cc}	31.63 ± 0.34^{Ea}	27.23 ± 1.46^{Eb}	22.46 ± 1.28^{Cc}

- Values are mean ±S.E.
- Values with the different superscript letters in the same column are significantly different at (P< 0.05).

Table (4): Antioxidant enzymes in kidney and heart homogenates:

Group	Kidney			Heart		
	1st Week	2nd Week	4th Week	1st Week	2nd Week	4th Week
SOD (U/g)						
Control	22.26±0.27^{Aa}	21.70±0.15^{Aa}	21.46±0.33^{Aa}	3.01±0.04^{Aa}	2.98±0.05^{Aa}	2.83±0.06^{Aa}
Cisplatin	12.73±0.44^{Ea}	12.06±0.16^{Fa}	11.70±0.20^{Da}	1.73±0.03^{Ea}	1.60±0.01^{Eb}	1.44±0.03^{Ec}
Catalase (U/g)						
Control	50.43± 1.2^{Aa}	48.03±1.24^{Aab}	45.70±0.47^{Ab}	18.66±0.87^{Aa}	18.50±0.35^{Aa}	17.93±0.08^{Aa}
Cisplatin	33.66±0.67^{Ea}	30.43±0.59^{Eb}	24.90±0.79^{Ec}	14.30±0.46^{Ea}	12.30±0.25^{Eb}	9.56±0.23^{Dc}
GR (mmol/l)						
Control	2.033±0.03^{Aa}	2.02±0.06^{Aa}	1.97±0.05^{Aa}	3.53±0.03^{Aa}	3.48±0.04^{Aa}	3.59±0.03^{Aa}
Cisplatin	0.90±0.03^{Ea}	0.85±0.03^{Fa}	0.72±0.01^{Fb}	1.25±0.03^{Fa}	1.106±0.06^{Fa}	0.85±0.02^{Fb}
GPx (U/g)						
Control	30.00±0.05^{Aa}	29.46±0.29^{Aab}	28.93±0.12^{Ab}	25.00±0.11^{Aa}	24.23±0.17^{Ab}	23.90±0.11^{Ab}
Cisplatin	19.03±0.14^{Fa}	17.73±0.17^{Eb}	16.83±0.23^{Ec}	10.50±0.20^{Fa}	9.66±0.39^{Eab}	8.56±0.31^{Db}

- Values are mean ±S.E.
- Values with the different superscript letters in the same column are significantly different at (P< 0.05).

Table (5): Fold change of gene expression in CP-group:

Parameter	1st Week	2nd Week	4th Week	F-test	
				F-value	P-value
HO-1	12.33±1.35	13.20±1.27	14.700±0.63	1.11	0.3875
VEGF	10.800±1.15	10.96±0.66	12.63±2.68	0.34	0.7225
ICAM-1	121.03±25.13	228.200±26.08	253.66±26.53	7.38	0.0242
TNF-α	83.26±14.33	710.70±110.97	2616.0±240.78	74.04	0.0001
TNFR-1	300.3±20.4	1418.2±113.4	5312.7±317.3	182.26	0.0001
Nephrin	39.40±3.29	76.96±12.45	84.066±7.11	7.98	0.0204
Desmin	32.56±4.69	59.93±2.52	65.100±13.25	4.51	0.0638

- Values are mean ±S.E.

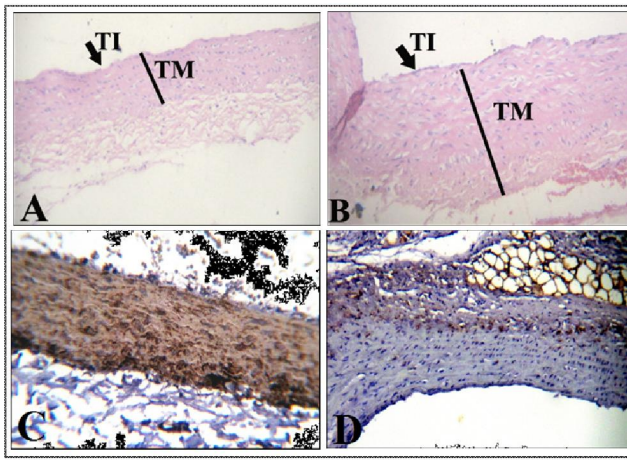

Figure 1: A. Contol group, aorta at first week, shows normal histological picture consisting of tunica intima (TI), tunica media (TM), and tunica adventitia**. B.** CP-group, aorta at first week, shows an increase in the thickness TM layers compared to the control group due to the proliferation of smooth muscle cells in the aortic wall (H&E, x 100). **C.** Control group, shows strong positive vWF staining throughout the wall of the aorta. **D.** CP-group, shows weak positive vWF staining throughout the wall of the aorta (IHC counterstained with Mayer´s hematoxylin, x 100).

4. Discussion

We and other research groups have demonstrated that cisplatin-based chemotherapy induces renal abnormalities and signs of cardiovascular damage (Saleh et al., 2014; Daher and Yeh, 2008; Demkow and Stelmaszczyk-Emmel, 2013). Multiple cellular mechanisms have been suggested underlie these abnormalities. These include oxidative stress-induced tubular epithelial cell toxicity, reduced NO-induced vasoconstriction in the renal microvasculature, and proinflammatory effects (Yu et al., 2008).

In the present study, nephrotoxicity was manifested in the CP-group by significant increase in serum creatinine and urea along the 4 weeks following cisplatin administration. This increased urea, and creatinine during cisplatin toxicity was suggested to be a result of the renal tubular damage (Sinha et al., 2013). Whereas vascular damages as manifested by the loss of vWF in aorta sections.

To further study CP-induced vascular and renal alterations at the molecular level, we assessed the mRNA levels of two renal-related genes, namely nephrin and desmin. Nephrin is a homologous molecule expressed in the podocyte slit diaphragms that are essential for normal glomerular ultrafiltration. It was demonstrated that an increased mRNA levels nephrin is associated with the initial stages of the loss of the permeability barrier in nephropathy (Aaltonen et al., 2001). Importantly, cytokine-induced upregulation of nephrin expression was also confirmed in primary human podocytes (Huwiler et al., 2003). In agreement with these observations, our study showed an increased expression of nephrin mRNA in kidney's tissue homogenates of the cisplatin group.

Cisplatin has also been reported to induce an increase in glomerular desmin expression, which has been reported previously in models of renal injury (Nagase et al., 2006). Desmin is cytoskeletal proteins distribution in normal and diseased glomeruli; and the increase of desmin in glomerular epithelial cells was found associated with glomerular epithelial damage (Zou et al., 2006). In the CP-group, we found an increase in the mRNA of desmin in the kidney homogenate. These results confirm that cisplatin responsible for the development of renal injury in this model.

At the molecular levels, we found that the expression of the oxidative stress related gene hemeoxygenase-1 (HO-1) was increased in samples from CP-group. HO-1 is a microsomal enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism to biliverdin, bilirubin and CO, which are efficient peroxyl radical scavengers that possess potent antioxidant properties (Abraham et al., 2007). HO-1 has been found to exert an important physiological role in mediating cytoprotection related to the end-products of heme degradation (Colin-Gonzalez et al., 2013). Therefore it is considered that HO-1 gene expression is extremely sensitive to up-regulation by oxidative stress in a variety of mammalian tissues (Elmarakby et al., 2012). In the present work, we found the expression of HO-1 gene is significantly higher in cisplatin-group than in healthy control-group. HO-1 gene expressions were significantly higher in CP-induced nephritic rats than in healthy control one.

In addition, several studies demonstrated that TNF-α is implicated in the pathogenesis of CP-induced renal cell injury (Ramesh and Reeves, 2006). Moreover, TNF-α was found to stimulate the inflammatory response in vivo leading to exacerbating cisplatin nephrotoxicity (Uehara et al., 2011). In the other side, inhibition of TNF-α activity and deficiency of TNF-α has demonstrated protection from cisplatin toxicity (Benedetti et al., 2013). In agreement with these data, we found that the expression of TNF-α is significantly increased in the kidney tissues of rats injected with cisplatin. The physiological role of TNF-α is based on its direct engaging with TNF-receptors especially TNFR-1 on renal epithelial cells (Yu et al., 2011). White et al. (2012) also confirmed that TNFR1 pro-apoptotic signaling induces NF-kB activation. In addition, Geering et al. (2011) demonstrated that pro-apoptotic pathways after TNFR1 stimulation are initiated by p38 and PI3K, but not by caspase-8. Furthermore, it was found that loss of TNFR-1 could be a mechanism to limit inflammation in response to apoptotic cell death (Madge et al., 1999). In the present work, TNFR-1 gene expression in kidney tissue was significantly increased in CIS-group compared to normal group.

Recent researches suggested that CP-induced inflammation and oxidative stress may also affects cardiovascular system (Demkow and Stelmaszczyk-Emmel, 2013; Sekijima et al., 2011). Furthermore, previous reports indicated that CP-induced vascular dysfunction has been attributed to a reduction in nitric oxide (NO) bioactivity and an increase in reactive oxygen species (ROS) formation (de Haro Miralles et al., 2009). As ROS are known to quench NO with formation of peroxynitrite leading to vascular dysfunction (Freed and Gutterman, 2013). In agreement with these data we found that the levels of NO in kidney and heart tissue homogenates reduced significantly in the cisplatin-group compared to the normal-group. Meanwhile, an elevation in the concentrations of serum MDA and reduction of serum GR levels were recorded. Higher MDA and lower GR in the serum indicated the elevated lipid peroxidation and exhausted antioxidant activities in cisplatin treated rats. These results confirm previous study by Chirino and Pedraza-Chaverri (2009) who suggested that endothelial dysfunction is related to oxidative stress. To further confirm these data, we examined sections from aorta samples from CP-group. The analysis of aorta sections showed that CP injection increases the thickness tunica media layers compared to the control group, suggesting an increase in the proliferation of smooth muscle cells in the aortic wall. The increase in thickness of intimal layer was also observed. However, the thickness of tunica media was found to be more prominent than the thickness of tunica intima. After the second week post cisplatin injection, marked increase in the thickening of the tunica media and narrowing in the aortal lumen were seen that became visible after the fourth week. These observations confirm earlier studies documenting the impact of cisplatin on proliferation of endothelial cells in vitro and in vivo (Morlese et al., 2007). In addition, Daher and Yeh (2008) suggested that chemotherapy-induced

vascular toxicity occurred due to cumulative effects on endothelium leading to endothelial dysfunction.

vWf is a known marker for the assessment of vascular endothelial cells. vWF is a glycoprotein required for normal homeostasis that is produced by endothelial cells and megakaryocytes throughout the body (Sadler, 2013). Cellular vWf is stored in rod shaped cellular structures known as Weibel-Palade (WP) bodies and is distributed within the sub endothelial matrix (Knittel et al., 1995). Levels of circulating vWf are increased following endothelial cell damage leaving endothelial cells empty from vWf (Laursen et al., 2013). In agreement with these data we found that the kidney, large arteries, medium and small sized arterioles were strongly positive and the fenestrated endothelium of the glomeruli was only focally positive or completely negative for vWF. In the heart, all the endothelial cells lining the endocardium and coronary arteries in the heart and aorta were strongly positive. Meanwhile, weak to negative staining for vWF was recorded in the kidneys, heart and aorta from the cisplatin group. These observations also confirmed previous study reported that vWf is released from endothelial cells upon damage induced by cisplatin (Dieckmann et al., 2011).

Vascular endothelial growth factor (VEGF) is another major cytokine protein involved in the development of vascular diseases and is also a potent mediator of endothelial cell proliferation and vascular permeability (Jin et al., 2013). The relation between oxidative stress and VEGF levels was previously reported (Pawlak et al., 2008). The present work demonstrated an increased expression of VEGF gene in heart samples, which was found associated with increased HO-1 expression and MDA level, reflecting the positive association of VEGF with oxidative stress.

Cisplatin may also induce vascular damage through induction of endothelial cells adhesion molecules and endothelial growth factors (Yu et al., 2008). Moreover, Endemann and Schiffrin (2004) reported that ROS raised adhesion molecules including ICAM-1. In addition, Yu et al. (2008) reported that cisplatin injection increased the expression level of ICAM-1 through a nuclear factor kappa-B (NF-κβ) dependent pathway, which in turn promotes leukocyte-endothelium interactions leading to endothelial cell apoptosis and thus arterial damage. In agreement with these data, our results showed a significant increase in ICAM-1 after cisplatin treatment.

Conclusions:

From this study it can be concluded that cisplatin induces vascular damages as manifested by the loss of vWF in aorta sections; and by alterations in gene expression of endothelial function-related genes VEGF and ICAM-1. These changes occurred in addition to the renal damage as demonstrated by proteinuria and changes in the expression of genes regulating glomerular basement membrane; nephrin and desmin genes. These events were associated with alterations in TNF-α and TNFR-1 inflammatory genes as well as HO-1 oxidative stress related gene. Finally, we postulate that the molecular and cellular abnormalities in leukocytes adhesion, oxidative stress and inflammation induced by cisplatin not only affected renal physiology, but also cardiovascular system.

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3/19/2014