



Insight into the Mechanism of Doxorubicin-induced Nephrotoxicity through Gene Expression Analysis of Oxidative Stress, Kidney Injury and Inflammation Markers

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ABSTRACT

Doxorubicin (DOX), a valuable anti-cancer drug can cause multi-organ toxicities. Among several suggested mechanisms, the role of oxidative stress remains obscure, though it is the most probable mechanism for its cardiotoxic effect. The main focus of this study is to analyze the mechanism of renal toxicity caused by this chemotherapeutic drug. As reactive oxygen species are significant regulators of transcription factors and gene expression, this study was aimed at analyzing the expression levels of genes that are induced in response to oxidative stress following DOX administration to rats. DOX was administered to rats intraperitoneally at 3 mg/kg at alternate days for two weeks. Kidney tissues were harvested and the mRNA levels of glutathione peroxidase-1 (Gpx1), NAD(P)H quinone dehydrogenase 1 (Nqo1), 24-dehydrocholesterol reductase (Dhcr24), dual oxidase 2 (Duox2), and isocitrate dehydrogenase-1 (Idh1) were analyzed in the kidney tissues by RT-PCR. Extent of injury was analyzed by the change in expression of kidney injury biomarkers, kidney injury molecule-1 (KIM-1) and osteopontin (OPN), while the effect of DOX was also analyzed in terms of changes in the anti-inflammatory cytokine, interleukin-10 (IL10). Of the oxidative stress induced genes analyzed, the expression of Gpx1 was found to be increased significantly. Significant increase was also observed in case of IL10 gene, while kidney injury biomarker level was not increased in response to DOX. It is concluded from this study that DOX treatment can modulate the expression levels of Gpx1 and IL10, while injury markers may have differential expression depending upon the duration following toxicity.

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Authors' Contribution

UJ conducted the experiments with technical and analytical assistance from NN and IK. UJ wrote the first draft. AS conceived the idea, critically analyzed the results and wrote the manuscript in its final form. RM reviewed the manuscript and gave technical input.

Key words

Chemotherapeutic drug, Cytotoxicity, Interleukin-10, Glutathione peroxidase-1, Kidney injury biomarkers, Osteopontin, Oxidative stress

INTRODUCTION

Doxorubicin (DOX), a quinone-containing compound, is used as a potent anti-cancer drug in many cancer therapies. However, its clinical use results in multi-organ toxicity leading to liver and kidney damage as well as cardiotoxicity (Carvalho *et al.*, 2014). DOX belongs to the anthracyclines class of chemotherapeutic drugs which causes increase in oxidative stress and as a result produce organ toxicity (Kumral *et al.*, 2015). Cardiomyopathy, one of the side effects of DOX, is related to free radical formation (Minotti *et al.*, 2004). One of the mechanisms involved

in DOX-induced myocardial toxicity is oxidative stress caused by an imbalance between reactive oxygen species (ROS) and endogenous antioxidants (Songbo *et al.*, 2019). Further molecular insights can be gained from studies including DOX-induced oxidative stress in mitochondria, disruption of oxidative phosphorylation, and permeability transition, contributing to altered metabolic and redox circuits in cardiomyocytes, with increase in apoptosis (Wallace *et al.*, 2020). DOX affects renal function through several mechanisms including free radical production as well as inflammatory, apoptotic and hyperuricemic effects (Chmielewska *et al.*, 2015; Khames *et al.*, 2017). In a recent report, DOX-induced nephrotoxicity was determined by different histopathology and biochemical analyses. The results showed increased levels of serum urea, creatinine, albumin and total protein along with renal kidney injury

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markers, oxidative stress parameters, mediators of inflammation, and apoptosis markers (Khames *et al.*, 2019).

Toxicity by reactive oxygen species (ROS) may in turn induces alterations at the genome level affecting cellular homeostasis (Noreen *et al.*, 2018). We recently reported change in the expression levels of certain oxidative stress induced genes in liver and heart tissues in DOX treated rats (Jabeen *et al.*, 2019). In this study, expression of oxidative stress genes, kidney biomarkers, and anti-inflammatory gene was analyzed in kidney tissues following DOX injection. The genes analyzed in this study include kidney injury molecule-1 (KIM-1), osteopontin, glutathione peroxidase-1 (Gpx1), NAD(P)H quinone dehydrogenase-1 (Nqo-1), 24-dehydrocholesterol reductase (Dhcr24), dual oxidase 2 (Duox2), isocitrate dehydrogenase-1 (Idh-1), and interleukin 10 (IL10). To the best of our knowledge, this is the first report regarding the molecular analysis of DOX toxicity in terms of expression of certain oxidative stress induced genes. Targeting specific genes modulated by DOX would be helpful in understanding the mechanism of renal toxicity and to design a management strategy for this chemotherapeutic drug.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (SD rats) weighing 180-200g were purchased from Aga Khan University, Karachi and housed in the Animal House of Federal Urdu University of Arts, Science and Technology with 12-h light: 12-h dark cycle. All animal procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals. Animals were allowed to acclimatize for a period of 3-4 days prior to the start of the experiment. The animals were provided with sterile water and food.

Experimental groups

The animals were divided into two groups: (1) Untreated control: Rats received normal saline (i. p.); (2) Test: Rats were injected DOX (i. p). DOX was dissolved in saline and injected as 3 mg/kg every alternate day for two weeks. Rats were sacrificed and kidney tissues were dissected and stored at -80°C for gene expression analysis.

Gene expression analysis

Analysis of *KIM-1*, Osteopontin (*OPN*), *Gpx1*, *Nqo1*, *Dhcr24*, *Duox2*, *Idh1*, and *IL10* genes was performed in the harvested kidney tissues in both control and treated groups. Total RNA isolation was done with 30 mg of

kidney tissues by SV total RNA isolation kit (Promega, USA) according to the manufacturer's instructions. The concentration and purity of total RNA samples were determined by measuring the absorbance at 260 and 280 nm using UV-VIS spectrophotometer. Absorbance ratio (260:280) of ~ 2.0 is considered as pure RNA. Total RNA (2 µg) was reverse-transcribed using reverse transcription kit (Promega, USA) according to the manufacturer's instructions. PCR amplification was performed using GoTaq PCR kit (Promega, USA) with specific primers of *KIM-1*, *OPN*, *Gpx1*, *Nqo1*, *Dhcr24*, *Duox2*, *Idh1*, and *IL10* genes (Table I). cDNA was denatured for 1 min at 94°C, followed by 30 cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 59 – 63°C, and 1 min extension at 72°C) and final extension of 10 min at 72°C. The primer sequences and their expected product sizes and calculated annealing temperatures are listed in Table I. Rat *GAPDH* was used as an internal standard. Finally, the PCR products were detected on 1% agarose gel electrophoresis and visualized through ultraviolet gel documentation imaging system. Comparison of the gene expression was done by normalizing the gene bands with that of *GAPDH*.

Table I. Forward and reverse primer sequences are shown with the expected product sizes and annealing temperatures used during RT-PCR.

Gene	Primer sequence (5'→3')	Annealing temperature (°C)	Product size
<i>GAPDH</i>	(F) GGAAAGCTGTGGCGTGATGG	60	414
	(R) GTAGGCCATGAGGTCCACCA		
<i>Gpx1</i>	(F) ATAGAAGCCCTGCTGTCCAA	56	216
	(R) GAAACCGCCTTTCTTTAGGC		
<i>Dhcr24</i>	(F) AAGGGTTGGAGTTCGTCT	53	243
	(R) TGAGACAGTGAGCCATCCAG		
<i>Idh1</i>	(F) GCTTCATCTGGGCCTGTAAG	58	246
	(R) GCTTTGCTCTGTGGGCTAAC		
<i>Nqo1</i>	(F) GCCCGATATTGTAGCTGAA	56	202
	(R) GTGGTGATGGAAAGCAAGGT		
<i>KIM-1</i>	(F) AGAGAGAGCAGGACACAGGCTT	57	225
	(R) ACCCGTGGTAGTCCCAACA		
<i>OPN</i>	(F) AAGGCGCATTACAGCAAACTCA	55	243
	(R) CTCATCGGACTCCTGGCTCTTCAT		
<i>IL10</i>	(F) CACTGCTATGCTGCCTGCTC	56	181
	(R) TGGCCTTGTAGACACCTTGG		

Statistical analysis

All data are expressed as the means ± standard error of the mean. Data were subjected to *t*-test to determine significant differences in gene expression levels; values

corresponding to $P < 0.05$ is considered statistically significant.

RESULTS AND DISCUSSION

In this study, we analyzed the toxic effect of DOX on rat kidney tissue following intraperitoneal injections of the drug. The effect of the genes in rat kidney tissues was analyzed in order to evaluate the effect of this chemotherapeutic drug on the modulation of certain genes that are expected to cause renal toxicity. We analyzed whether the toxic effect of DOX on kidneys is due to the oxidative stress caused by the drug and what is its effect on oxidative induced genes. We selected *Gpx1*, *Nqo1*, *Dhcr24*, *Duox2* and *Idh1* genes for this purpose. Additionally, we checked what is the extent of injury caused to by the drug to the kidneys by analyzing kidney injury biomarkers, *KIM-1* and *OPN*, and finally we analyzed an anti-inflammatory cytokine, *IL10* to check whether the drug also causes inflammation.

Oxidative stress induced genes

The renal toxicity caused by DOX was evaluated in terms of changes in the expression of oxidative stress induced genes, *Gpx1*, *Nqo1*, *Dhcr24*, *Duox2* and *Idh1*. The results in the form of bar diagram and corresponding gel bands are shown in Figure 1.

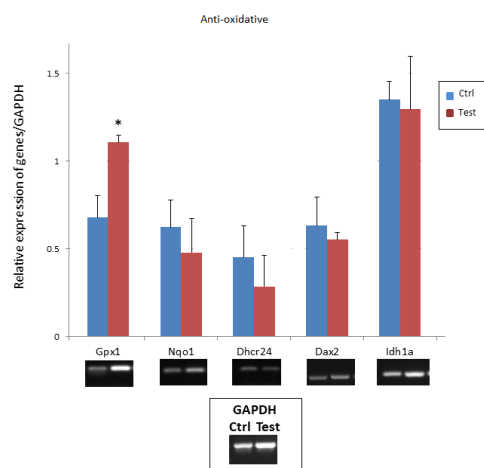


Fig. 1. Agrose gels showing RT-PCR expression analysis of the oxidative stress induced genes, glutathione peroxidase-1 (*Gpx1*), NAD(P)H quinone dehydrogenase 1 (*Nqo1*), 24-dehydrocholesterol reductase (*Dhcr24*), dual oxidase 2 (*Duox2*), and isocitrate dehydrogenase-1 (*Idh1*) in the kidney tissue following DOX injection (3 mg / kg; i.p.) in SD rats. All data are expressed as the means \pm standard error of the mean. Data were subjected to Student's *t*-test and values corresponding to $P < 0.05$ is considered statistically significant.

Glutathione peroxidase-1 (*Gpx1*)

Gpx1 gene expression in the kidney tissues was increased significantly in the DOX injected rats as compared to the control. Glutathione acts as a reducing agent in this class of antioxidant enzymes. This enzyme is expressed in all kidney cells (Muse *et al.*, 1994). It prevents cells from oxidative damage by catalyzing the reduction of hydrogen peroxide to water and removes peroxides and peroxynitrite that can cause renal damage. The main target of *Gpx* activity in the kidney plasma cells are proximal tubular cells (Mohammedi *et al.*, 2016). Oxidative stress has been demonstrated to play an important role in the development of renal diseases (de Haan *et al.*, 2005). The increase in expression of *Gpx1* gene can be considered a part of the homeostatic mechanism to deal with the oxidative stress caused by DOX.

24-dehydrocholesterol reductase (*Dhcr24*)

In our study, there was no significant change in *Dhcr24* expression in the kidney tissue of DOX injected and control rats. We analyzed *Dhcr24* gene expression as its upregulation in response to H_2O_2 has been reported in several studies (Battista *et al.*, 2009; Lu *et al.*, 2012). *Dhcr24* is involved in the formation of cholesterol from desmosterol and belongs to a family of FAD-dependent oxidoreductases (Waterham *et al.*, 2001). It has been demonstrated that the FAD-binding domain is crucial for the ROS scavenging role of *Dhcr24*. In our study, however, we did not find any change in the expression of *Dhcr 24*, therefore, it may be possible that DOX does not work via increasing its level in renal cells under oxidative stress.

Dual oxidase 2 (*Duox2*)

In our study, there was no significant change in *Duox2* gene expression in the kidney tissue of DOX injected and control rats. Dual oxidases are part of NADPH oxidase (Nox) protein family. There are seven isoforms that generate ROS in biological systems (Ueyama *et al.*, 2015) but complex formation between the *Duox1* A-loop and *DuoxA1* results in tighter control of H_2O_2 released by the enzyme complex. Involvement of NADPH oxidase was observed in renal pathology. The precise function of NADPH oxidase-derived ROS and specific isoforms in the kidney is poorly understood. As we found no change in the expression of *Duox2* gene, therefore, it may be possible that DOX does not work via increasing its level in renal cells under oxidative stress.

NAD(P)H quinone dehydrogenase 1 (*Nqo1*)

We observed no significant change in the *Nqo1* gene expression level in the kidney tissue following DOX injection. *Nqo1* gene is an endogenous antioxidant enzyme.

It catalyzes reactions having cyto-protective effect against redox cycling and oxidative stress (Sharma *et al.*, 2016). It was revealed that after free radical burst, Nqo1 causes synthesis of antioxidants, e.g. vitamin E and ubiquinone. Nqo1 is a chemo-protective enzyme and has potential to defend cells from oxidative challenge and the capability to decrease quinones by using two electron mechanism, which prevents production of reactive oxygen radical (Ross and Saigel, 2004; Wu *et al.*, 2016). In our study, however, we did not observe any significant change in its expression. It may be possible that DOX did not work via upregulating this gene.

Isocitrate dehydrogenase 1 (Idh1)

No change in the *Idh1* gene expression level was observed in the kidney tissue of DOX injected rats. Isocitrate dehydrogenases (ICDHs) are key metabolic enzymes that catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate. In the tricarboxylic acid cycle, *Idh1* catalyzes the reversible conversion of isocitrate to alpha-ketoglutarate and CO₂ in a two-step reaction (Stoddard *et al.*, 1993). ICDHs belong to two distinct subclasses, one of which utilizes NAD(+) as the electron acceptor and the other NADP(+). Five ICDHs have been reported: three NAD(+)-dependent ICDHs, which localize to the mitochondrial matrix, and two NADP(+)-dependent ICDHs, one of which is mitochondrial and the other predominantly cytosolic (Cairns and Mak, 2013). NADP⁺-dependent oxidative decarboxylation as their major function in nonmalignant cells, contributes substantially to the control of mitochondrial redox balance and the prevention of oxidative damage (Jo *et al.*, 2001). *Idh1* gene expression has a physiological role in protecting cells from oxidative stress by regulating the intracellular NADP(+)/NADPH ratio. Protection of the kidney injury is associated with an upregulation of *Idh1* gene expression which increases NADP(+)/NADPH and GSH/total glutathione ratios, as a result of which oxidative stress is reduced (Kim *et al.*, 2011). In our study, however, we did not observe any significant change in its expression. It may be possible that DOX did not work via upregulating this gene.

Kidney injury biomarkers

The extent of kidney injury caused by DOX was evaluated in terms of changes in the expression of kidney injury biomarkers, KIM-1 and osteopontin. The results in the form of bar diagram and corresponding gel bands are shown in Figure 2.

Kidney injury molecule-1 (KIM-1)

Gene expression of *KIM-1* was found to have no

change in the kidney tissue of DOX injected rats and that of the control. KIM-1 is a type 1 membrane glycoprotein that is not expressed in normal kidney but is noticeably upregulated in the injured proximal tubular epithelial cells of the human and rodent kidney in ischemic and toxic conditions (Ichimura *et al.*, 1998, 2004). It was reported that after injury, kidney injury molecule-1 gene functions in the development of renal graft fibrosis (Nogare *et al.*, 2015). In our study, as we found no change in the expression of *KIM-1* gene, it may be possible that DOX either cause toxicity without causing change in the structural integrity of the kidneys, or the change in KIM-1 may be time dependent. Thus, a temporal gene expression analysis is required to be performed to know the changes in gene expression of this important indicator of kidney injury.

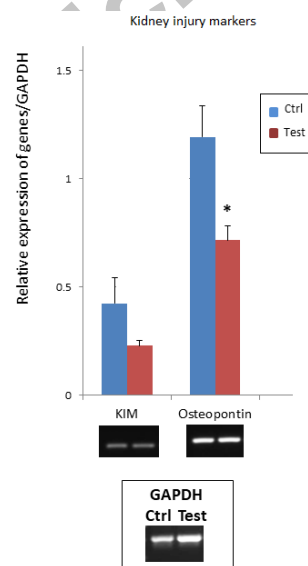


Fig. 2. Agrose gels showing RT-PCR gene expression analysis of the kidney injury biomarkers, kidney injury molecule-1 (KIM-1) and osteopontin (OPN) in the kidney tissue following DOX injection (3 mg / kg; i.p.) in SD rats. All data are expressed as the means \pm standard error of the mean. Data were subjected to Student's *t*-test and values corresponding to $P < 0.05$ is considered statistically significant.

Osteopontin (OPN)

OPN gene expression level was significantly reduced in the kidney tissue of DOX injected rats as compared to the control. *OPN* is involved in different biological functions including cell death inhibition and plays a significant role in the defense mechanism of the kidney. It has been shown that *OPN* not only takes part in the normal kidney function, but it is also involved in the protection of the kidney from

acute cisplatin toxicity in rats (Schmitt and Rubel, 2013). Phosphoglycoprotein secreted by OPN is involved in tissue homeostasis, lesion healing, immune regulation, and stress responses (Rabenstein *et al.*, 2015). In our study, as we found reduced expression of OPN. It is most likely that DOX may have caused increase in the level of OPN very early in the course of injury, while the level gradually decreases as the time to the injury advances. A temporal change would likely give a better picture in this regard.

Anti-inflammatory gene

We finally analyzed whether the effect of DOX-induced toxicity also involves inflammation. For this purpose, we selected IL10 gene and analyzed its expression in kidney tissue following DOX injection. The results in the form of bar diagram and corresponding gel bands are shown in Figure 3.

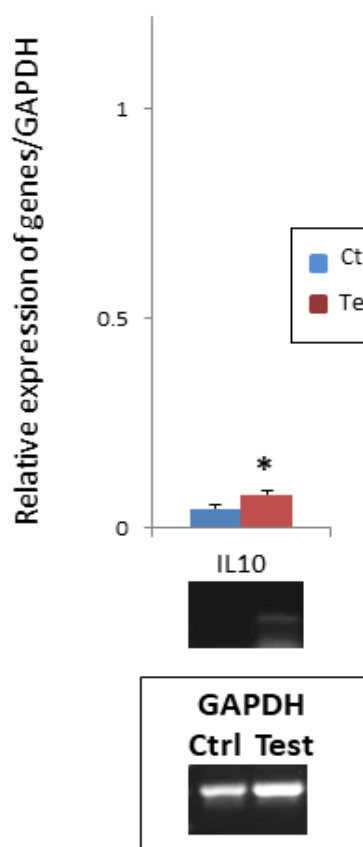


Fig. 3. Agrose gels showing RT-PCR gene expression analysis of the anti-inflammatory cytokine, interleukin-10 (IL10) in the kidney tissue following DOX injection (3 mg / kg; i.p.) in SD rats. All data are expressed as the means \pm standard error of the mean. Data were subjected to Student's *t*-test and values corresponding to $P < 0.05$ is considered statistically significant.

Interleukin (IL10)

IL10 gene expression was significantly increased in the kidney tissue of DOX injected rats as compared to the control; however, both groups have shown an overall low level of expression. IL10 is a small protein of 18 kDa. It is a cytokine having anti-inflammatory immune-regulating property and is expressed in immune active cells, mainly monocytes and lymphocytes. It has been reported that oxidative stress and inflammation share common and overlapping signaling pathways. By damaging macromolecules, ROS can initiate inflammation (Ungvari *et al.*, 2010; Sallam and Laher, 2016). IL10 plays a critical role in suppression of the inflammatory response (Stenvinkel *et al.*, 2005).

CONCLUSIONS

We can conclude from this study that toxicity in kidney tissues due to DOX is variable in terms of expression of certain oxidative stress induced genes and more likely involves glutathione peroxidase-1. The toxicity also involves inflammation as it induces IL10, an anti-inflammatory cytokine. The extent of the injury could not be ascertained and further analysis with temporal gene expression should be performed. Genes upregulated by DOX as a homeostasis mechanism can be used to overcome toxicity and stress caused by this chemotherapeutic agent, so that targeted therapy can be achieved.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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