DPPD ameliorates renal fibrosis induced by HgCl$_2$ in rats

Mohamed M. Elshemy 1,*, Ahmed E-S AbdEl-mejied 1, Faten Zahran 2, Mohamed M. Omran 3 and Ahmed Nabil 4

1Faculty of Science, Menoufia University, Menoufia, Egypt
2Faculty of Science, Zagazig University, Zagazig, Egypt
3Faculty of Science, Helwan University, Cairo, Egypt
4Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Beni-Suef, Egypt

*Correspondence: mohamed.alshimy@yahoo.com  Accepted: 19 Aug.2018 Published online: 30 Sep. 2018

Mercury (Hg), considered one of the most toxic metals, accumulating mainly in the kidney cause glomerulonephritis, tubulointerstitial injury, fibrosis, fibroblast activation and collagen deposition. So, the present study concerned with N N'-diphenyl-1, 4-phenylenediamine (DPPD) antioxidant activity against mercuric chloride (HgCl$_2$) induced renal fibrosis in rats. This study, carried on 81 female Sprague–Dawley rats divided into three groups (control, HgCl$_2$ and HgCl$_2$+DPPD). In HgCl$_2$ group results showed a significant increase in urea, creatinine, nitric oxide (NO), alpha smooth muscle actin (α-SMA), collagen deposition, histopathological changes and significant reduction in superoxide dismutase (SOD), catalase (CAT) activity. Administration of DPPD significant attenuated hydroxyproline and collagen formation in rats treated with HgCl$_2$+ DPPD (p ≤ 0.001), attenuated the inhibitory effect of HgCl$_2$ on SOD and CAT activity, showed significant decrease in renal fibrosis percent area and α-SMA-positive cells, reduced nephrotoxicity, improved histopathological and biochemical examinations.

Keywords: Antioxidants; Mercury; Kidney; Rat; DPPD; Fibrosis.

INTRODUCTION

Mercury is one of the most toxic non-essential trace metals in the environment, with a high level of persistent and ability to bio accumulates, so mercury negatively affects our health and environmental quality (Buch et al., 2017).

Exposed to mercury in any of its forms by different ways like water, air, soil, and food pose serious threats to our health and the environment, mercury toxicity commonly affecting the skin, circulatory, respiratory, renal, digestive, and nervous systems (Kim et al., 2016). Mercury considered the most common toxic metal cause nephrotoxic effects (Bridges and Zalups, 2017), which mainly accumulated in the renal proximal tubular cells (Bridges and Zalups, 2010). Consequently, cause tubular necrosis, tubulointerstitial nephritis and glomerulonephritis (Miller et al., 2013).

N N'-diphenyl-1, 4-phenylenediamine, a gray or dark gray powder, used as a polymerization inhibitor and antioxidant. DPPD widely used in rubber, oils, and foodstuffs, especially for tires in the rubber industry due to its color and stability (Matsumoto et al., 2013). Mercury chloride-induced renal fibrosis was prevented by an antioxidant DPPD (Ahn et al., 2002). DPPD inhibit interstitial fibrosis by scavenge lipid and peroxylradicals; prevent cellular lipid peroxidation (Colles and Chisolm, 2000), DPPD scavenged free radicals by giving electron(s) to them, protecting cells from oxidative stress (Satoh and Izumi, 2007), DPPD significantly inhibited α-SMA over expression, collagen formation, improved the
histological pictures and reduced the number of apoptotic cell (Kawai et al., 2009; Ahmed et al., 2011).

The present study aims to test DPPD effect on the progression of renal fibrosis induced by HgCl₂ in rats.

MATERIALS AND METHODS

Experimental animals

The study carried on 81 female Sprague–Dawley rats (body weight 170 – 220 gm). Rats bred and housed in temperature-controlled conditions (22 ± 2°C) with 12:12 light: dark cycle, specific pathogen free animal house. They were provided with free access to rat chow and tap water. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) followed. Ethical protocols for the care and use of laboratory animals were approved and followed under supervision of the animal facilities, Medical Experimental Research Centre, Faculty of Medicine, Mansoura University. Rats were randomly divided into three groups as follows:

(1) Control group, where 20 Rats received saline and served as the control.
(2) Mercuric chloride group, where 20 Rats received mercuric chloride (4 mg/kg, i.p.) at the start of the experiments.
(3) Mercuric chloride + DPPD group, where 20 Rats received mercuric chloride (4 mg/kg, i.p.) at the start of the experiments and three days after mercuric chloride administration, rats received DPPD (0.5 g / kg, i.p.) every two days.

Mortality:

In this study 21 female Sprague–Dawley rats died after HgCl₂ injection. Samples of kidneys, urine and blood collected from each rat after 8 days of HgCl₂ (or saline) treatment.

Biochemical determination

Serum creatinine, urea, creatinine clearance, total protein content, N-acetyl-β-D-glucosaminidase (NAG), serum magnesium, SOD, CAT, NO and hydroxyproline content in kidney tissues were measured in all rats involved in this study using standard laboratory methods.

Histological evolutions

Histopathology examinations performed on animals from each experimental group, at day 8 rats in all groups sacrificed and both kidneys were quickly removed and fixed at 10% neutral formalin. These samples embedded in paraffin wax and cut into 4 μm section thickness. Slides stained with hematoxylin and eosin (H&E), Masson trichrome and α-SMA immune stain. Then the slides photographed using Olympus® digital camera installed on the Olympus® microscope with 1/2 X photo adaptor, using a 40X objective. The resulting images analyzed on Intel® Core i5® based computer using Image J software with a specific built-in routine for stain quantification and automated area measurement. Five slides prepared from each group, 5 random fields from each slide analyzed. Three analysis protocols performed which summarized as follows:

Protocol-I:
Quantification of fibrosis in slides stained with Masson trichrome.

Protocol-II:
Quantification of α-SMA.

Protocol-III:
Determination percentage of normal renal tubules in the group received HgCl₂ only and the group received HgCl₂ + DPPD stained with (H&E).

Statistical analysis

Data tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 17. Descriptive statistics calculated in the form of Mean ± Standard deviation (SD).

In the statistical comparison between the different groups, significance in difference tested using one of the following tests:-

Student's t-test (Paired):- Used to compare between mean of two related groups of numerical (parametric) data.
ANOVA (analysis of variance):- Used to compare between more than two groups of numerical (parametric) data followed by post hoc Tukey test.
A P value <0.05 was considered statistically significant (S).

RESULTS

Biochemical results

Urinary excretion of NAG

Urinary NGA significant increase in (HgCl₂ and HgCl₂+ DPPD groups) compared to control group with; p < 0.001 (Table 1).
Table (1): Results of kidney injury markers. Data are expressed as mean±SD, n = 20 animals in each group.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control Group</th>
<th>HgCl₂ Group</th>
<th>HgCl₂ + DPPD Group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAAG (μmol/mg creatinine)</td>
<td>10.32±0.39</td>
<td>25.79±8.20</td>
<td>21.57±8.19</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Total protein (mg/dl)</td>
<td>0.50±0.03</td>
<td>2.16±0.81</td>
<td>1.11±0.70</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.45±0.03</td>
<td>1.21±0.42</td>
<td>0.77±0.34</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>23.68±0.73</td>
<td>40.33±10.46</td>
<td>31.34±6.14</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min/100 gm body weight)</td>
<td>0.53±0.04</td>
<td>0.23±0.08</td>
<td>0.42±0.11</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Magnesium (mg/dl)</td>
<td>1.81±0.06</td>
<td>3.40±0.24</td>
<td>2.85±0.18</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Hydroxyproline (μg/mg tissue)</td>
<td>23.10±1.86</td>
<td>73.97±8.24</td>
<td>37.12±7.48</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Nitric oxide (μmol/g tissue)</td>
<td>108.88±7.02</td>
<td>185.08±20.77</td>
<td>116.65±10.44</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>12.46±0.70</td>
<td>6.49±1.13</td>
<td>11.13±1.17</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>CAT (mol/min/gm)</td>
<td>0.65±0.05</td>
<td>0.26±0.05</td>
<td>0.54±0.11</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

SD: standard deviation  P: Probability
*: significance <0.05  **: High significance
Test used: One way ANOVA followed by post-hoc tukey.
a Significance relative to control group (with HgCl₂ and HgCl₂ + DPPD groups).
b Significance between HgCl₂ group & HgCl₂ + DPPD group.

Urinary total protein
Total protein significant increase in HgCl₂ group compared to (control and HgCl₂ + DPPD groups) with; p < 0.001 (Table 1).

Serum creatinine and urea levels
Creatinine and urea significant elevate in HgCl₂ group compared with control group (p < 0.001). DPPD significant reduce serum creatinine and urea levels (p < 0.001) (Table 1).

Creatinine clearance
Extremely highly significant decrease found in serum creatinine clearance level in HgCl₂ group compared to (control and HgCl₂ + DPPD groups) with; p < 0.001 (Table 1).

Serum Magnesium level
The level of serum Magnesium significant increase in HgCl₂ group compared to (control and HgCl₂ + DPPD groups) with; p < 0.001 (Table 1).

Kidney tissues Hydroxyproline, Collagen and fibrosis level
Collagen forming and fibrosis estimated by hydroxyproline content examination in the kidney tissues. At day 8, hydroxyproline significant increase in HgCl₂ group compared to control group. DPPD significant attenuated hydroxyproline formation in rats treated with HgCl₂+ DPPD (p ≤ 0.001) (Table 1).

Nitric oxide level and antioxidant enzyme activity in renal tissues
High significant elevation (p < 0.001) of NO level observed in HgCl₂ group compared to (control and HgCl₂ + DPPD groups) (Table 1).

In HgCl₂ injected rats, SOD and CAT activity significant reduced compared to control group with; p < 0.001. Treatment with DPPD significant attenuated the inhibitory effect of HgCl₂ on SOD and CAT activity with; p < 0.001 (Table 1).

3Pathological evaluation for different groups:

Hematoxylin and eosin stain.
Figure 1: show histopathological findings from H&E stained kidney sections. In the control group rats, no glomerular or pathological abnormalities found (A). However, HgCl₂ group rats showed tubular dilatation with degenerative changes, glomerular constriction and fibroblast increase (B). DPPD treatment significant reduced the pathological abnormalities in rats injected with HgCl₂ as evidence for a decreased intensity of tubular cell necrosis, and dilatation (C). Normal tubules percent comparison between rats treated with HgCl₂ only and rats co-treated with HgCl₂ and DPPD (D), rats treated with DPPD showed significant increase in normal tubules percentage compared to rats treated with HgCl₂ only (p <0.001) (Table 2).
Figure 1: Kidney tissue sections of rats from different groups stained with hematoxylin and eosin (400X). (A) A control kidney with normal architecture. (B) Section of rats treated with HgCl₂ only showing tubular dilatation with degenerative changes. (C) Kidney section of rats co-treated with HgCl₂ and DPPD showing attenuated histopathological changes. (D) Comparison among rats treated with HgCl₂ only and rats co-treated with HgCl₂ and DPPD in the Percent of normal tubules.

Table (2): Comparison among different groups in masson trichrome (Percent area), ASMA (Percent area) and comparison between HgCl₂ group & HgCl₂ + DPPD group in the percent of normal renal tubules stained with H/E (Percent area) after 8 days. Data are expressed as mean±SD, n = 20 animals in each group.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group</th>
<th>HgCl₂ group</th>
<th>HgCl₂ + DPPD group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of normal tubules</td>
<td></td>
<td>16.46±4.4</td>
<td>28.67±3.4</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Masson(Percent area)</td>
<td>13.33±4.60</td>
<td>34.71±6.20ₐ</td>
<td>21.43±5.30ₐ,ᵇ</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>ASMA (Percent area)</td>
<td>1.21±0.27</td>
<td>3.99±0.74ₐ</td>
<td>1.64±0.33ₖ</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

SD: standard deviation    P: Probability
*: significance <0.05    **: High significance
Test used: Student’s t-test and one way ANOVA followed by post-hoc tukey.
a Significance relative to control group (with HgCl₂ and HgCl₂ + DPPD groups).
b Significance between HgCl₂ group & HgCl₂ + DPPD group.
Masson’s trichrome stain.

Figure 2: shows Masson’s trichrome stain results. In control group rats, no abnormal collagen deposits observed (A). While marked amounts of collagen deposition (blue) around renal corpuscle and tubules observed in HgCl₂ group rats (B). However, rats treated with HgCl₂+ DPPD showed significant decrease in collagen deposits and renal fibrosis compared to rats treated with HgCl₂ only (C). Comparison among different groups in Masson (Percent area) (D), renal fibrosis percent area significant increase in (HgCl₂ and HgCl₂ + DPPD groups) compared to control group. While rates co-treated with HgCl₂ and DPPD were significant lower in renal fibrosis percent area compared to rats treated with HgCl₂ only (p <0.001) (Table 2).

Immunohistochemistry of α-SMA.

Figure 3: shows α-SMA expression in the kidney caused by HgCl₂ using immunohistochemistry. Expression of α-SMA used as marker for myofibroblast activation in the kidney, which plays a pivotal role in fibrogenesis. Normal control group rats showed normal contents of α-SMA-positive cells (A). However, HgCl₂ group rats showed a significant increase in α-SMA-positive cells (B). Rats treated with HgCl₂ + DPPD showed significant decrease in α-SMA-positive cells compared to the rates received HgCl₂ only (C). Comparison among different groups in ASMA (Percent area) (D) we found a significant increase in α-SMA-positive cells in HgCl₂ group compared to (control and HgCl₂+ DPPD treated groups) with; p < 0.001.
Also, DPPD treated groups showed no significant difference in α-SMA-positive cells percent area compared with control group (Table 2).

DISCUSSION

Mercury ions bind with sulfur, like a thiol group of amino acids, which transfer mercury ion via sodium ion channels to the kidney tubules (Zalups, 2000). So, mercuric ions mainly accumulated in the renal proximal tubular cells (Bridges and Zalups, 2010), increasing free radicals formation (Patel and Rao, 2015). DPPD scavenged free radicals by giving electron(s) to them, protecting cells from oxidative stress (Satoh and Izumi, 2007).

Urinary NAG had a relative higher molecular mass (>130 kD), so it is not filtered by the glomeruli, and only release in urine as a result of kidney tubule damage (Liangos et al., 2007). Thus, urinary NAG may act as an index shows tubular injury in the kidney. In this study, we demonstrated an obvious elevation in urinary NAG in the group injected with HgCl₂ compared with the control group. Also, (Xu et al., 2007) and (Liu et al., 2011) observed an increase of urinary NAG in nephrotoxicity caused by HgCl₂.

Proteinuria (Khazim et al., 2013) suggested that urinary protein level increased due to disorders in glomerular filtration barrier resulted from damage in podocytes, decreased reabsorption of filtered protein. We observed a significant increase in the urinary protein level of HgCl₂ group; also (Boroushaki et al., 2014) reported the same result.

In the present study, elevation of serum creatinine and urea levels in rats treated with HgCl₂ indicates nephrotoxicity. Elevation in serum creatinine and urea resulted from injury in renal tubular cells, which confirmed by obvious alterations in kidney cells of HgCl₂ group compared with control group (Fig. 1B and 1A). (Glaser et al., 2010), (Gado and Aldahmash, 2013), (Boroushaki et al., 2014), (Salman et al., 2016) and (Apaydin et al., 2016) reports suggest
the same histological abnormalities with an obvious increase in serum urea, creatinine and urinary protein levels as a result of HgCl$_2$ toxic effect on kidneys.

The increase in the level of magnesium in plasma associated with a decline in kidney function because urinary excretion is the only magnesium regulating system (Wyskida et al., 2012). Our results suggested a significant increase in the serum magnesium level of mercuric chloride group compared with the control group. Also, (Natochin et al., 1994) reported hypermagnesemia in rats injected by HgCl$_2$.

The creatinine clearance test used to check kidney disease progression which causes eventual reduction in the excretion of creatinine by both the glomeruli and the tubules leads to decrease in creatinine clearance (Gowda et al., 2010). Our study shows significant reduction in creatinine clearance in rats treated with HgCl$_2$ compared to control group. Previous studies by (Zalups, 1995) and (Hazelhoff et al., 2012) documented significant decreases in creatinine clearance in rats after HgCl$_2$ administration.

Hydroxyproline present in the tissue of vertebrate thought to confine exclusively to collagen (Némethy and Scheraga, 1986). Thus, present of hydroxyproline in tissues or serum used as a measure of collagen (Reddy and Enwemeka, 1996). Collagen has about 12.7% hydroxyproline by weight (Kivirikko et al., 1967). Mercuric chloride disturbs collagen metabolism in the body which reflected by altering hydroxyproline fractions (Siddiqi and Alhomida, 2005). Our results were parallel to (Sharma et al., 2017) and (Yuan et al., 2017) who concluded that renal interstitial fibrosis induced by HgCl$_2$, demonstrated by remarkably increased hydroxyproline contents and excessive collagen deposition in kidney tissues.

Exposure to mercury increases free radical production and hence oxidative stress (Patel and Rao, 2015). Antioxidant enzymes such as SOD and CAT are essential for the cellular protection against reactive oxygen species (ROS) and other oxidative stress (Morakinyoet al., 2012). From the present work, the activities of antioxidant enzymes; SOD and CAT were significant decreases in the kidney tissues of HgCl$_2$ treated rats in comparison to the control group which indicated that HgCl$_2$ has caused severe oxidative stress. The earlier reports by (Ekor et al., 2010), (Apaydin et al., 2016) and (Salman et al., 2016) documented the same results.

Nitric oxide level often reflects tissue oxidation (Ruan et al., 1997). Our results confirmed the previous results by (Karapetlian et al., 2014) and (Othman et al., 2014) which indicated significant elevation of NO in the kidney tissues of HgCl$_2$ treated rats compared to control.

Our results indicated that DPPD administration after 3 days from single injection of HgCl$_2$ significant reduced the effect of HgCl$_2$ on total urinary protein, serum urea, serum creatinine level, serum magnesium, creatinine clearance, antioxidant enzymes activity such as SOD and CAT in kidney tissues, NO level in kidney tissues, hydroxyproline content and collagen deposition in kidney tissues but DPPD administration had no obvious role on NAG excretion in urine induced by HgCl$_2$.

In agreement with our results, studies by (Kawai et al., 2009) reported that DPPD administration did not influence NAG excretion in urine induced by cisplatin after treatment, but DPPD significant blocked interstitial fibrosis expansion and the increase of type III collagen induced by cisplatin. Also, different studies by (Ahn et al., 2002), (Matsunaga et al., 2005), (Ahmed et al., 2011) and (Zahran et al., 2016) reported the same biochemical evidence which showed that DPPD strongly suppress tubulointerstitial fibrosis.

To further support the biochemical evidence of this study, we used histopathological examination for the kidney tissue sections. Observation of morphological variations using H&E stain as in (Fig. 1). The control rats showed no tubular injury and normal glomerular architecture. Mercuric chloride group revealed an increase in renal tubular injury, accumulation of collagen and loss of normal microscopic architecture. Rats treated with HgCl$_2$+ DPPD a showed significant decrease in tubular injury and collagen accumulation as well as a significant increase in the percent of normal tubules (p <0.001) compared with the group injected with HgCl$_2$ only. Our results agree with earlier studies by (Boroushaki et al., 2014) also (Gado and Aldahmash, 2013) which also histologically observed increased tubular necrosis and atrophy of renal tissue in HgCl$_2$ treated group.

We use Masson’s trichrome stain to investigate renal fibrosis and collagen deposition (blue stained area; Fig. 2). No collagen deposition observed in the normal control rats group. Rats treated with HgCl$_2$ showed a significant increase in collagen deposition and renal fibrosis percent area. Rats treated with HgCl$_2$ + DPPD showed a
significant decrease in collagen deposition and renal fibrosis percent area compared to the group treated with HgCl₂ only (p < 0.001).

Interestingly, our results agree with other studies by (Kawai et al., 2009) and (Zahran et al., 2016) strongly supported that DPPD improved the histological pictures and reduced the number of apoptotic cells.

Next, we examined α-SMA expression in the kidney caused by HgCl₂ using immunohistochemistry (Fig. 3). The α-SMA is a marker of myofibroblast activation in the kidney, which plays a pivotal role in fibrogenesis (Veerasamy et al., 2009). The percent area of α-SMA-positive cells significant increase in the group injected with HgCl₂ only compared with (control and HgCl₂+ DPPD groups) with; p < 0.001. Also, DPPD treated group showed no significant change in α-SMA-positive cells percent area compared to the control group.

These results were consistent with those obtained by (Kawai et al., 2009), (Ahmed et al., 2011), and (Zahran et al., 2016) who reported that the administration of DPPD significantly inhibited α-SMA over expression, collagen formation and reduced the number of apoptotic cell.

CONCLUSION
We concluded that an antioxidant DPPD able to retard the progression of renal interstitial fibrosis and collagen formation induced by HgCl₂. Further studies needed to find the role of ROS in pathogenic and progressive mechanisms leading to the fibrosis induced by HgCl₂ also the antioxidant DPPD anti-fibrotic effect.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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AUTHOR CONTRIBUTIONS
All authors contributed equally in all parts of this study.

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