The Effect of Melatonin on Cisplatin Induced-Kidney Injury in Rats: Possible Role of Autophagy

OLA M. TORK, M.D. and NASHWA ELTABLEWY, M.D.
The Department of Physiology, Faculty of Medicine, Cairo University

Abstract

Background: Acute kidney injury by nephrotoxin; the chemotherapeutic cisplatin is a typical condition of renal stress, leading to cell death, tissue damage, and loss of renal function or renal failure. Melatonin hormone possess free-radical scavenging activity and it has been shown that it protects against cisplatin toxicity. Autophagy which is a cellular process of bulk degradation of damaged macromolecules in the cytoplasm, is thought to be contributing to cell death, or it might act as a cytoprotective mechanism. However, the mechanism of the protective effects of melatonin against cisplatin-induced nephrotoxicity and its relation to autophagy is still essentially unknown.

Aim: The present study was designed to investigate the effects of acute kidney injury induced by cisplatin in adult male rats on autophagy and apoptosis after 19 days of its administration and to clarify the possible protective mechanisms of melatonin against acute kidney injury and its relation to both autophagy and apoptosis.

Material and Methods: The study was conducted on 24 male adult albino rats. The experimental animals were divided into 3 groups, 8 rats each.

Group I: Control treated with vehicle, Group II: Cisplatin treated (7mg/kg b.w., i.p.once), Group III: Melatonin+cisplatin co-treatment, melatonin (4mg/kg b.w., i.p. at 17:00hr. for 3 weeks - 5 days/week) and cisplatin (7mg/kg b.w., i.p. once). Melatonin administration was started two days before the single i.p. injection of cisplatin.

At the end of the experimental period, Blood samples were taken for the determination of serum urea nitrogen and creatinine then all rats were sacrificed and kidneys were excised for the determination of Tumor necrosis factor α (TNFα), Hemeoxygenase-1 (HO-1), LC3-II (an autophagic marker) and Bcl2 (antiapoptotic marker) in the kidney tissues, in addition to examine histological changes in the kidney.

Results: It was observed that melatonin co-treated group showed a significant low extent and severity of the histological and biochemical signs of kidney injury than untreated one. The main significant biochemical changes between melatonin co-treated and cisplatin treated rats were (i) significant decrease in the elevated inflammatory marker (TNFα), (ii) significant decease in HO-1 activity which indicate reduction of the oxidative stress, as well as (iii) significant increase of LC3-II and Bcl2 gene expression in the kidney.

Conclusion: Melatonin may be one of the future therapeutic possibilities to overcome the side effects of anti-cancer drugs induced acute renal injury through various mechanisms including stimulation of autophagy in addition to its anti-inflammatory, antioxidant and antiapoptotic effects.

Key Words: Melatonin – Cisplatin – Nephrotoxicity – Hemeoxygenase-1 (HO-1) – Tumor necrosis factor α (TNFα) – Autophagy – LC3-II.

Introduction

ONE of the major side effects of cisplatin chemotherapy, used for several solid tumors, is nephrotoxicity, or toxic acute kidney injury (AKI). The cellular and molecular mechanisms responsible for drug-induced nephrotoxicity to renal tubular epithelial cells (RTEC) are not completely understood [1].

Exposure of cisplatin to RTEC activates several signal transduction pathways mediated by ROS. DNA damage, cell cycle inhibitors, MAPKs (mitogen-activated protein kinases), and TNFα may contribute to cisplatin-induced RTEC injury [1]. Cisplatin has been shown to induce mitochondrial membrane permeabilization and the release of the mitochondrial inter-membrane proapoptotic molecules including cytochrome c [2], Omi/HtrA3 [3] Smac/Diablo20 and AIF [4].

Autophagy has emerged as another major “programmed” mechanism to control life and death, much like “programmed cell death” is for apoptosis in eukaryotes [8].

Autophagy is known to play a role in both physiological as well as pathophysiological conditions. In normal physiological conditions, basal autophagy maintains cellular energy homeostasis and quality control by the recycling of aged or
damaged macromolecules. Similarly, in stress conditions due to nutrient and growth-factor depletion, autophagy generates both nutrients and energy needs to meet the metabolic substrate demands of the cells for survival [4].

The formation of the phagophore and the autophagosomes requires 18 distinct Autophagy related (Atg) proteins initially identified in yeast [6]. The process of autophagosome formation involves initiation, nucleation and elongation/enclosure [7]. The initiation step is controlled by the ULK1-Atg13-FIP200 complex and the nucleation step requires the Beclin-1-class III phosphatidyl inositol 3-kinase (PI3K) complex. The two conjugation systems are divided in the elongation/enclosure step. The first is the conjugation of Atg12-Atg5 mediated by two ligases Atg7 and Atg10 and the second involves cleavage of microtubule-associated protein 1 light chain 3 (LC3 or Atg8) by Atg4 releasing the soluble form LC3-I, which is then conjugated to phosphatidyl ethanolamine (PE) via participation of Atg7 and Atg3. This lipid conjugation forms the autophagic double membrane-associated LC3-II protein allowing the closure of autophagic vacuole [7]. LC3-II is used as a marker of autophagosomes [8]. The main inhibitor of autophagy is the serine/threonine kinase mammalian target of rapamycin (mTOR), a component of the mTORC 1 complex [7]. It is to be noted that the term autophagy encompasses several processes including not only survival or death mechanisms, but also pexophagy, mitophagy, ER-phagy or ribophagy, depending on which organelles are targeted for specific autophagic degradation [9].

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized and released into the circulation and especially into cerebrospinal fluid by the pineal gland in a circadian rhythm [10] and is also produced by immune system cells, brain, airway epithelium, bone marrow, gut, ovary, testes, skin and likely other tissues [11].

Melatonin and its metabolites possess free-radical scavenging activity [12]. Melatonin has both receptor-mediated and receptor independent actions and is believed to affect all cells [13].

Is autophagy induced during acute kidney injury? Does autophagy contribute to renal cell injury and death, or survival under this pathological condition? Is melatonin has an effect on autophagy?.

In this study, we investigated: (I) the effects of acute kidney injury induced by cisplatin on autophagy and apoptosis after 19 days of its administration and (II) the possible protective mechanisms of melatonin against acute kidney and its relation to autophagy and apoptosis.

**Material and Methods**

**Experimental animals:**
24 adult male albino rats with initial body weight range between 150-200g were used in this study. They were kept in the animal house of Physiology Department in Kasr Al-Aini, Faculty of Medicine, Cairo University. They were kept at 22±1°C temperature at normal dark-light cycles. They had free access to laboratory rat chow and top water throughout the study for 3 weeks. The experiments were carried out during October 2013.

**Animal groups:**
- **Group I:** Control treated with vehicle,
- **Group II:** Cisplatin (from Sigma) treated (7mg/kg b.w., i.p. once) and Group III: Melatonin+cisplatin co-treated; melatonin (from Sigma), (4 mg/kg b.w., i.p. daily at 17:00hr. It was given for 3 weeks-5 days-week). And cisplatin (7mg/kg b.w., i.p. once).

Melatonin administration was started two days before the single i.p. injection of cisplatin.

Melatonin was dissolved in ethanol and diluted in saline. Final ethanol concentration was 1% [14].

At the end of experimental period, blood samples were taken for the determination of serum urea nitrogen and creatinine then all rats were sacrificed and kidneys were excised for the determination of tumor necrosis factor α (TNF-α), Hemeoxygenase-1 (HO-1), LC3-II (autophagic marker) and Bcl2 (antiapoptotic cytokine) in addition to examine histological changes in the kidney.

**Biochemical measurements:**

**Measurement of urea and creatinine:**
Serum urea and creatinine levels were measured by conventional colorimetric method using QuantiChrom TM assay kits according to manufacturer’s instruction.

**Measurement of TNF-α:**
TNF-α was measured by using ELISA (quantikine R&D system USA) according to the manufacturer’s instructions [15].

**HO enzyme activity assay:**
Kidney tissues were homogenized with 2.5 volume Tris-HCl buffer (10mmol/L, pH 7.6) containing 250mmol/L sucrose and 0.4mmol/L phenylmethysulfonyl fluoride. The homogenate was centrifuged at 800g for 10 minutes and then at 13 500g for 20 minutes to produce the mitochondrial
pellet. The supernatant was withdrawn. The protein content was determined by the method of Lowry et al., [16]. The activity of HO in the supernatant was determined as previously described [17,18]. Briefly, the supernatants were incubated at 37 °C for 1 hour with heme (50mmol/L), rat liver cytosol (5mg/mL), MgCl2 (2mmol/L), glucose-6-phosphate dehydrogenase (1 unit), glucose-6-phosphate (2mmol/L), and NADPH (0.8mmol/L) in 0.5mL of 0.1mol/L phosphate buffer saline (pH 7.4). Reaction was stopped by putting the tubes on crushed ice, then the bilirubin generated was extracted by chloroform and its amount was determined with a scanning spectrophotometer and was defined as the difference between the absorbance at 463 and 520nm, while using a standard bilirubin curve.

Detection of LC3-II & Bcl2 gene expression using real time PCR (RT-PCR):

RNA extraction:

Total RNA was isolated from kidney tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturer’s instruction. RNA was quantified spectrophotometrically and the integrity of the RNA was studied by gel electrophoresis.

cDNA synthesis:

First-strand cDNA was synthesized from 4 µg of total RNA using an Oligo(dT) 12-18 primer and SuperscriptTM II RNase Reverse Transcriptase, This mixture was incubated at 42 °C for 1 h, the kit was supplied by Super Script Choice System (Life Technologies, Breda, the Netherlands).

Real-time quantitative polymerase chain reaction (PCR):

Real-time PCR (RT-PCR) amplification was carried out using 10 µmol/L amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8ng of reverse-transcribed RNA and 300nM primers, the sequences of PCR primer pairs used for each gene are shown in Table (1). Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) PCR reactions consisting of 95 °C for 10min (1 cycle), 94 °C for 15s, and 60 °C for 1min (40 cycles), Data were analyzed with the ABI Prism sequence detection system software and quantified using the v 1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to house keeping gene beta actin [19].

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC3-II Forward 5'-ACGGTGAGTCTAATGAC-3'</td>
<td>Reverse 5'GAAGAATGTGTGGCTGGT-3'</td>
</tr>
<tr>
<td>Bcl2 Forward 5' CAGGAGAACAGGGTATGA 3'</td>
<td>Reverse 5' CAGGGTGGAGAAGAT 3'</td>
</tr>
<tr>
<td>Beta actin Forward 5' TCT GGC ACC ACA CCT TCT ACA ATG 3'</td>
<td>Reverse 5' AGC ACA GCC TGG ATA GCA ACG 3'</td>
</tr>
</tbody>
</table>

Histological changes in the kidney:

Immediately following sacrification, sections of the kidney were fixed in 10% formalin, embedded in paraffin wax and cut at 5 micrometer thickness. Kidney sections were then processed and stained with hematoxylin and eosin dye (H&E) [20]. The sections were then examined under light microscope for histopathological changes at 200x magnification.

Data analysis and statistics:

The data was coded and entered using the statistical package SPSS version 15. The data was summarized using descriptive statistics: Mean ± Standard deviation for: Serum urea and creatinine and kidney tumor necrosis factor α and Hemeoxigenase-1, while the median, minimal and maximum values for the kidney LC3II, and Bcl2 variables. Statistical differences between groups were tested using ANOVA (analysis of variance). When a significant F was obtained, subsequent multiple comparisons between the different groups were analyzed by post hoc test: Bonferroni in normally distributed quantitative variables. Correlations were done to test for linear relations between variables. Statistical differences between groups were tested using Nonparametric Mann Whitney test for quantitative variables, which aren't normally distributed. Correlations were done to test for linear relations between variables. p-values less than or equal to 0.05 were considered statistically significant.

Results

The level of serum urea (mg/dl) and creatinine (mg/dl) were summarized in Table (2):

The serum urea in cisplatin treated rats (Group II) was significantly higher than that of the control (p<0.001). Although it was significantly lowered in the melatonin and cisplatin co-treated rats (Group III) (p<0.001), but it was still significantly higher than the control animals (p<0.001). The serum creatinine mg/dl was significantly higher in group
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II rats compared to the control \((p<0.001)\). While it was significantly lowered in group III rats when compared to the control group. But there was insignificant difference in between group III & control as regard serum creatinine level. These observations confirm the ability of melatonin to improve kidney function parameters.

Table (2): Level of serum urea (mg/dl) and creatinine (mg/dl) in the studied groups in Mean±SD \((n=8\) in each group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea</th>
<th>Creatinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I)</td>
<td>41.91±10.69</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>Group (II)</td>
<td>92.81±11.05</td>
<td>1.19±0.34</td>
</tr>
<tr>
<td>Group (III)</td>
<td>61.36±3.87</td>
<td>0.34±0.11</td>
</tr>
</tbody>
</table>

\@ = Statistically significant as compared to control (Group I).
\# = Statistically significant as compared to cisplatin treated (Group II).

Table (3): Kidney tumor necrosis factor \(\alpha (TNF\alpha)\) pg/ml and Hemeoxygenase-1 (HO-1) in pmol/bilirubin/mg. ptn./min in the studied groups in Mean±SD \((n=8\) in each group).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TNF(\alpha)</th>
<th>HO-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I)</td>
<td>30.66±2.83</td>
<td>11.63±1.99</td>
</tr>
<tr>
<td>Group (II)</td>
<td>112.76±13.38@</td>
<td>43.89±9.38@</td>
</tr>
<tr>
<td>Group (III)</td>
<td>67.90±9.30@#</td>
<td>22.93±9.10@#</td>
</tr>
</tbody>
</table>

\@ = Statistically significant as compared to control (Group I).
\# = Statistically significant as compared to cisplatin treated (Group II).

The kidney tumor necrosis factor \(\alpha (TNF\alpha)\) pg/ml and Hemeoxygenase-1 (HO-1) in pmol. bilirubin/mg. ptn./min were summarized in Table (3) and Figs. (1,2):

Melatonin co-treated rats showed lower levels of TNF\(\alpha\) than non treated group \((p<0.001)\). This indicated that melatonin can reduce the inflammatory marker (TNF\(\alpha\)) level but not to the control level as there was significant difference between them and the control \((p<0.001)\). Also the indicator of oxidative stress: Hemeoxygenase-1 was significantly reduced in group III as compared to group II \((p<0.001)\), but still significantly higher than that of the control rats \((p<0.001)\).

Table (4): Median and (range) levels of the kidney LC3II, and Bcl2 in the studied groups \((n=8\) in each group).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LC3 II</th>
<th>Bcl 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I)</td>
<td>10.80 (8.40-12.50)</td>
<td>1.92 (1.20-2.40)</td>
</tr>
<tr>
<td>Group (II)</td>
<td>2.70@ (1.90-6.10)</td>
<td>0.16@ (0.08-0.28)</td>
</tr>
<tr>
<td>Group (III)</td>
<td>8.25@# (6.90-9.20)</td>
<td>0.61@# (0.48-0.81)</td>
</tr>
</tbody>
</table>

\@ = Statistically significant as compared to control (Group I).
\# = Statistically significant as compared to cisplatin treated (Group II).

Analysis of the results of autophagic and anti-apoptotic factors (LC3II and Bcl2 respectively) were shown in Table (4) and Figs. (3,4):

Table (3) shows significantly lower level in LC3II expression in cisplatin treated group in the control \((p<0.001)\). LC3II expression was higher in group III (melatonin and cisplatin co-treated rats) as compared to group (II) \((p<0.001)\) But it was significantly lower than that in the control group \((p<0.01)\).

The anti-apoptotic gene expression of Bcl2 decreased significantly in cisplatin treated rats as compared to the control \((p<0.001)\). However, melatonin treatment to these animals caused significant increase in Bcl2 as compared to group II.

Also, there was significant inverse correlation between LC3II gene expression and tumor necrosis factor \(\alpha\) level (in pg/ml) \((r=–0.928 & –0.857, p=0.001 & 0.007)\) in groups II and III respectively.

The anti-apoptotic gene expression of Bcl2 decreased significantly in cisplatin treated rats as compared to the control \((p<0.001)\). However, melatonin treatment to these animals caused significant increase in Bcl2 as compared to group II.

Fig. (1): The kidney tumor necrosis factor \(\alpha (TNF\alpha)\) level in the studied groups \((n=8\) in each group).

Fig. (2): The kidney Hemeoxygenase-1 (HO-1) level in the studied groups \((n=8\) in each group).
Histopathological examination of kidney tissues were shown in Fig. (5):

Renal sections of control rat in the cortex and outer medulla, showed normal parenchyma. Proximal and distal convoluted tubules were also showed no abnormality (Plate A). Histological examination of cisplatin treated kidney revealed destructive changes in kidney tissue: Such as vacuolation, interstitial edema, severe tubular necrosis, and interstitial inflammation in comparison with control (Plate B). However, rats received melatonin treatment revealed some improvement in renal tissue compared with cisplatin treated rats (Plate C).
Discussion

The nephrotoxicity of cisplatin is well documented and considered as the most important dose-limiting factor in cancer chemotherapy [14]. Cisplatin accumulates in the renal tubular cells approximately five times its extracellular concentration leading to cell injury and necrosis [21].

The current histopathological examination of kidneys revealed that there was a significant tubular damage indicated by tubular cell necrosis and interstitial inflammation. These results confirmed that kidney is very sensitive to cisplatin toxicity [22]. These histopathological changes were reduced significantly under melatonin therapy in group III. Our findings were supported by the results of Kilic et al., [14].

In the current work, administration of cisplatin produced a significant increase in levels of serum urea and creatinine than control one, which indicating marked renal dysfunction, however these changes were corrected partially with melatonin therapy. These observations were in agreement with the study of Kilic et al., [14].

Moreover, we observed that hemeoxygenase-1 (HO-1) activity was significantly higher in cisplatin-injured kidneys compared to control one, and these results were in agreement with that of the work of Chirino et al., [23].

Hemeoxygenase is a microsomal enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism [24]. The HO-1 gene expression is extremely sensitive to up-regulation by oxidative stress in a variety of mammalian tissues. However, the mechanisms behind HO-1-mediated cytoprotection are not fully understood but one of the possible mechanisms is related to the end-products of heme degradation, including biliverdin, bilirubin and CO, which showed an important physiological roles. Both biliverdin and bilirubin are efficient peroxyl radical scavengers that possess potent antioxidant properties [25].

This work showed that the activities of pro-inflammatory marker TNFα, decreased significantly in melatonin co-treated rats compared to the cisplatin treated rats. The changes of inflammatory activity as a result of melatonin co-treatment was supported by the work of Kilic and his colleagues; who found that NF-xB was decreased by melatonin therapy in their study [14]. Thus our results imply that cisplatin generates kidney dysfunction associated with oxidative stress inducing antioxidant enzyme activities and also inflammation biomarker TNFα [26]. This could explain the improved response with melatonin therapy in group III which showed marked decrease in oxidative stress as well as reduction of inflammation biomarker TNFα. This indicates that melatonin has antioxidant and anti-inflammatory activities as supported by several studies [14,27].

This study represented an evidence for the antioxidant properties of melatonin through its significant down regulation of HO-1 expression. These inhibitory effects on oxidative stress were associated with reductions of the kidney injury following cisplatin administration.

Autophagy has been intensely studied during the past few years [28]. Several previous studies were demonstrated increased autophagy in vivo after 24&72h [29] and autophagy and apoptosis in vitro during the first hours after and cisplatin administration [1,30,31]. Induction of autophagy during the initial period of cisplatin insult may efficiently eliminate damaged proteins, organelles, and other macromolecules to establish cellular homeostasis before reaching the threshold for cisplatin-induced apoptosis [32]. It was also reported that caspase activation and apoptosis does not occur until 8-12hr of cisplatin exposure to RTEC suggesting that apoptosis is not an immediate response and a pre-apoptotic lag phase occurs on exposure to cisplatin [23].

Our study examined autophagy and apoptosis in vivo after 19 days of cisplatin administration. We observed that, there was marked decline in kidney’s autophagic marker; LC3II mRNA and antiapoptotic Bcl2 mRNA levels in cisplatin treated rats when compared with the control group. This signifying the marked tendency towards apoptosis at this period. In agreement with our findings is the work of Xu and his colleagues who also observed that Bcl-2 & autophagic vacuoles were decreased in proximal tubular cells at day 14 after kidney injury by unilateral ureteral obstruction (UUO). In addition they showed that H2O2 induced mitochondrial injury leading to interference in Bcl-2 expression in vitro, so, they concluded that oxidative stress leading to mitochondrial damage and induced apoptosis causing tubular epithelial loss and tubular decomposition in obstructive nephropathy [33].

We also observed that anti-apoptotic Bcl-2 significantly elevated under the effect of melatonin therapy as compared to cisplatin treated rats [34].
In this view, we can suggest that impaired autophagic responses and reduced anti-apoptosis in cisplatin induced-acute kidney injury, and their improvement in melatonin co-treated animals further validating that melatonin protection depends on activation of autophagy and inhibition of apoptosis.

As regard to melatonin enhanced effect of autophagy, it was reported in the studies of Jeong, et al., [35] and Guo et al., [36] in other models of cell injury. Jeong, et al., observed in their study that the protective effect of melatonin against mitochondrial dysfunction was related with autophagy activation and Melatonin-treated cells were dose-dependently increased in LC3-II expression [38]. Also Guo et al., observed that the protein levels of LC3III and Beclin1 were remarkably increased in ischemia/reperfusion-injured neuronal cells N2a in the presence of melatonin, suggesting that autophagy is possibly one of the mechanisms underlying neuroprotection of melatonin [36].

The presence of inverse correlation between pro-inflammatory cytokine TNFα and LC3II; in our study, indicates that, the induction autophagy may be involved in regulation of inflammatory response or the reverse. Actually, these results were supported by the work of Fujishima et al., who studied the role of autophagy in intestinal epithelium of mice with a conditional deletion of Atg7 (an autophagy related gene). They showed that the TNFα as well as IL-1β mRNA and NF-kB were markedly enhanced on exposure to LPS compared to control. They concluded that autophagy can attenuate endotoxin-induced inflammatory responses in intestinal epithelium resulting in the maintenance of intestinal homeostasis [37].

Conclusion:

Melatonin may be one of the future therapeutic possibilities to overcome the side effects of anticancer drugs induced acute renal injury through various mechanisms including stimulation of autophagy in addition to its anti-inflammatory, antioxidant and antiapoptotic effects.

References


