Zoledronic acid-induced mitochondrial impairment, inflammation, and oxidative stress in the rat kidney

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Abstract

Zoledronic acid (ZLD) is a bisphosphonate drug widely administered against pathological conditions such as hypercalcemia of malignancy, osteoporosis, bone metastases from solid tumors, and multiple myeloma. Unfortunately, renal injury is a serious and dose-limiting adverse effect of ZLD. There is no specific mechanism for ZLD-induced renal damage. The current study aimed to assess the effects of ZLD (10 and 15 mg/kg, i.p., single dose) on the rat kidney. In this regard, several parameters, including oxidative stress biomarkers, serum level of BUN and creatinine, inflammatory cytokines, kidney histopathology, and indices of mitochondrial function were assessed. A significant increase in serum Cr and BUN revealed renal injury. Moreover, kidney histopathological changes, including interstitial inflammation, tissue necrosis, and tubular atrophy, were detected in ZLD-treated rats. Biomarkers of oxidative stress, including a significant increase in reactive oxygen species (ROS), depletion of kidney glutathione (GSH) stores, increased lipid peroxidation, and suppression of the total antioxidant capacity, were detected in ZLD-treated animals. ZLD also significantly increased renal levels of TNF-α, IL-6, and IL-1β. ZLD exposure was also associated with significantly decreased mitochondrial dehydrogenases activity, mitochondrial depolarization, mitochondrial permeabilization, and ATP depletion. These data highlight mitochondrial dysfunction, inflammatory response, and oxidative stress as potential mechanisms in ZLD-induced kidney injury.

Keywords: Bisphosphonates; Drug safety; Mitochondrial dysfunction; Kidney Injury; Renal Failure; Pharmacotherapy

1. Introduction

Zoledronic acid (ZLD) is a bisphosphonate drug widely administered against various pathologies, including hypercalcemia of malignancy, bone metastases from solid tumors, osteoporosis, and multiple myeloma (1). However, some serious adverse effects, such as renal injury, are associated with ZLD administration (2-5). ZLD-induced kidney injury could lead to acute renal failure (3, 6). Unfortunately, adverse effects such as renal injury could limit the effectiveness of ZLD or its use in clinical settings. Hence, finding potential mechanisms of organ injury induced by this drug could pave the way for preventing/managing this profound adverse effect.
Although there is no well-known mechanism(s) for ZLD-induced kidney injury, some studies mentioned that oxidative stress and its associated complications could be involved in this adverse drug reaction (7, 8). It should be noted that all findings on the effects of oxidative stress in renal cells are obtained from in vitro studies (HEK-293 and HTK-2 cell lines) (7, 8). In this regard, we decided to evaluate the effects of ZLD on several biomarkers of oxidative stress in vivo. These data could help identify the mechanism of kidney injury induced by this drug and develop therapeutic strategies against this complication.

Mitochondria play a pivotal role in renal tissue (9, 10). Actually, these organelles provide a high level of ATP, which is critical for processes such as the reabsorption of several compounds from the renal tubule (11-13). Hence, several diseases or various xenobiotics could affect mitochondrial function in the kidney tissue (9). Therefore, the mechanism of renal damage induced by xenobiotics (e.g., drugs) could rely on mitochondrial injury and mitochondria-related cell death (10, 14). Moreover, it should be mentioned that mitochondrial damage and oxidative stress are interactive events (9, 14-16). In the current study, the effect of ZLD on renal mitochondria has been investigated to shed light on the mechanism of adverse reactions induced by this drug.

As mentioned, there is no specific mechanism for ZLD-induced renal damage in the in vivo experimental models or human cases. Hence, in the current study, we evaluated the effects of oxidative stress, inflammatory response, and mitochondrial impairment in the pathophysiology of ZLD-induced renal damage. These data could help progress preventive/therapeutic options against ZLD-induced kidney injury.

2. Materials and methods
2.1. Reagents
3-(N-morpholino) propane sulfonic acid, dithiothreitol, bovine serum albumin, malondialdehyde, 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), reduced glutathione, methanol HPLC grade, 4,2 Hydroxyethyl,1-piperazineethanesulfonic acid (HEPES), iodoacetic acid, potassium hydroxide, dimethyl sulfoxide, 2’,7’-Dichlorofluorescein diacetate, sodium chloride, sucrose, ethylene glycol-bis (2-aminoethyl ether)-N, N, N’, N’-tetraacetic acid, and Rhodamine 123, were obtained from Sigma Chemical Co. Hydroxymethyl aminomethane hydrochloride, zoledronic acid monohydrate, trichloroacetic acid (TCA), thiobarbituric acid, acetonitrile HPLC grade, and glacial acetic acid were purchased from Merck Co.

2.2. Animals and treatments
Mature male rats (Sprague-Dawley, 200±20 g, n=18) were obtained from Shiraz University of Medicine, Shiraz, Iran. Rats were housed in a standard environment (22-24 °C; 12:12 h, photoschedule; and 44±1% relative humidity). All processes on experimental animals were conducted in agreement with the ethical guidelines approved by the Shiraz University of Medical Sciences ethics committee. The ARRIVE guidelines for the care and use of experimental animals were also followed in the current investigation (17). Animals were allocated to three groups (n = 6 rats/group) and treated as follows: A) Control (vehicle-treated); B) ZLD (10 mg/mL, s.c, single dose); C) ZLD (15 mg/kg, s.c, single dose). Twenty-four hours after ZLD administration, rats were anesthetized (thiopental, 100 mg/kg, i.p), and serum and kidney samples were collected.

2.3. Sample collection
Urinary samples (≈400 µL) were collected when animals were handled. Subsequently, samples were centrifuged (17000 g, 10 min, 4 °C), and the supernatant was used for urinalysis. Animals were anesthetized (100 mg/kg of thiopental, i.p). Blood was collected from the abdominal aorta in VACUSERA® tubes for serum preparation. The kidney tissue was excised and washed in ice-cooled normal saline (4 °C) and used for further measurements. Renal weight index was determined as [wet weight of organ (g) / body weight (g)] × 100.

2.4. Reactive oxygen species (ROS) formation
Renal tissue ROS level was determined using dichlorofluorescein diacetate (DCF) (18). Briefly, 10 µL of DCF (10 µM final concentration) was added to 900 µL of KCl, and the supernatant was used for urinalysis. Animals were anesthetized (100 mg/kg of thiopental, i.p). Blood was collected from the abdominal aorta in VACUSERA® tubes for serum preparation. The kidney tissue was excised and washed in ice-cooled normal saline (4 °C) and used for further measurements. Renal weight index was determined as [wet weight of organ (g) / body weight (g)] × 100.
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2.5. Renal tissue lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) test assessed renal tissue lipid peroxidation in the rat kidney (18). Briefly, 500 µL of the renal tissue homogenate was treated with 1 mL of a reaction mixture composed of thiobarbituric acid (0.4 %, w/v), trichloroacetic acid (50 % w/v), and meta-phosphoric acid (1 % w/v solution); pH=2 (18). The mixture was incubated in a water bath (100 °C) for 40 minutes. Then, 0.5 mL of n-butanol was mixed with samples. Next, samples were centrifuged (10000×g, 15 min), and the absorbance of the n-butanol was assessed at λ=532 nm (EPOCH® multifunctional plate reader, US) (18).

2.6. Renal glutathione levels

A method based on the colorimetric assessment of the reaction of Ellman’s reagent (dithiobis-2-nitrobenzoic acid) with GSH was used to measure renal GSH content (18). Briefly, 500 µL of the renal tissue homogenate was treated with 20 µL of 50 % w/v trichloroacetic acid (4 °C) and incubated on ice (5 min). Then, samples were mixed well and centrifuged (10000 g, 4 °C, 20 min). The supernatant was treated with 200 µL of Tris buffer (40 mM, 4 °C, pH=8.9) and 20 µL DTNB solution (20 mg in 5 mL methanol, protected from light) and mixed well. Finally, the absorbance was measured at λ=412 nm (18).

2.7. Ferric-reducing antioxidant power (FRAP)

Renal tissue's total antioxidant power was assessed using the FRAP test (18). The working FRAP solution was prepared (freshly) by mixing acetate buffer (300 mM; pH=3.6) with TPTZ (10 mM in 6 N HCl) and ferric chloride (20 mM FeCl₂.6H₂O). Then, 100 µL of tissue homogenate was added to 1000 µL of the mentioned FRAP reagent and incubated in a shaker incubator (37 °C, 5 min, protected from light). Finally, sample absorbance was assessed at λ=593 nm (18).

2.8. Renal histopathological alterations

Tissue samples were fixed in a formalin-containing solution (0.4 % w/v NaH₂PO₄, 0.64 % w/v Na₂HPO₄, and 10 % w/v formaldehyde) in distilled water (pH=7.4). Next, paraffin-embedded samples were cut (4 µm) and stained with H&E. Renal histopathological alterations were evaluated using a light microscope (Olympus BX53; Japan) by a pathologist.

2.9. Kidney mitochondria isolation

Rat renal mitochondria were isolated using a differential centrifugation procedure (18). Briefly, kidneys were washed with 4 °C saline (NaCl 0.9% w/v) and minced in 4 °C isolation buffer (75 mM mannitol, 0.5 mM EGTA, 220 mM sucrose, 2 mM HEPES, and 0.1 % w/v bovine serum albumin) (pH=7.2). Subsequently, minced tissue was homogenized in fresh isolation buffer (1 g tissue: 10 mL buffer) (18). In the first round of the centrifugation (10000 g, 4 °C, 20 min). Subsequently, the supernatant was centrifuged at 10000 g (20 min at 4 °C) to pellet the mitochondria fraction. The second centrifugation phase was repeated thrice using a fresh buffer medium. Finally, isolated mitochondria were suspended in a buffer (1 g tissue: 5 mL buffer) containing 225 mM sucrose, 75 mM mannitol, and 2 mM HEPES (pH=7.4). The isolated kidney mitochondria were used for further analysis.

2.10. Mitochondrial dehydrogenases activity

A colorimetric technique using MTT was applied to estimate mitochondrial dehydrogenase activity (18). Briefly, a 0.5 mL of isolated mitochondria preparation was incubated with 40 µL of a 0.4 % w/v MTT solution and incubated at 37 °C for 30 minutes (protected from light) (18). Then, samples were centrifuged (17000 g, 10 min), and the pellet was dissolved in 1 mL of dimethyl sulfoxide. Finally, the absorbance at λ=570 nm was measured (18).

2.11. Mitochondrial ATP levels

An HPLC protocol was used to assess mitochondrial ATP levels (19). Briefly, 1000 µL of the isolated renal mitochondria were treated with 100 µL of 4 °C perchloric acid, incubated on ice (10 min), and centrifuged (20 min, 17000 g, 4 °C). Subsequently, the supernatant was treated with 100 µL of 4 °C KOH (1 M) and filtered (19). Finally, 25 µL of the prepared sample was filtered (0.45 µm filters) and injected into an HPLC system (µ-Bondapak C-18 column, 25 cm as the stationary
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phase and a mixture of 100 mM potassium hydrogen phosphate mono-basic, 2.5% v/v acetonitrile, and 1 mM tetrabutylammonium hydroxide, pH=7 as the mobile phase; flow rate was 1 mL/min). The UV detector was set at λ=254 nm (18, 19).

2.12. Mitochondrial swelling

Alterations in samples’ absorbance at λ=540 nm is a technique to evaluate isolated mitochondria permeabilization and swelling (18). Briefly, fractions of isolated kidney mitochondria were suspended in the pre-warmed (37 °C) mitochondria permeabilization buffer (10 mM HEPES, 70 mM KCl, 125 mM Sucrose, pH=7.2). The absorbance was recorded at a constant temperature of 30 °C during 30 minutes of incubation (EP-OCH-BioTek® multifunctional plate reader with orbital shaking) (18). Ca²⁺ (100 µM) was used as an inducer of mitochondrial permeability. Finally, the maximal mitochondrial swelling amplitude was calculated as maximal swelling amplitude (ΔOD)= (OD1 at λ=540 nm) – (OD2 at λ=540 nm) (18).

2.13. Mitochondrial depolarization

The cationic dye rhodamine 123 (Rhd 123) uptake by mitochondria was applied to assess mitochondrial depolarization (18). Briefly, 0.5 mL of mitochondrial samples were incubated with 20 µL of Rhd 123 (10 µM concentration) and incubated in the dark (30 min, 37 °C) (18). Subsequently, samples were centrifuged (10000 g, 10 min, 4 °C), and the fluorescence intensity of the supernatant was measured (λexcitation=485 nm and λemission=525 nm) (18).

2.14. Statistics

Data are represented as mean±SD. Data analysis was accomplished by the one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test as the post hoc. Renal tissue scores of histopathological alterations are represented as median and quartiles. The Kruskal-Wallis test was used for the analysis of renal histopathological alterations. A P<0.05 was considered a statistically significant difference.

3. Results

The current study investigated the nephrotoxic profile of the ZLD as a severe adverse effect of this drug. As shown in Figure 1, ZLD (10 and 15 mg/kg) caused no significant changes in the kidney weight index compared with the control rats (Figure 1). On the other hand, serum BUN was significantly higher in rats that received 15 mg/kg of ZLD (Figure 1). Both doses of ZLD used in the current study caused a substantial increase in serum creatinine levels compared to the control animals (Figure 1). The analysis of the serum of ZLD-exposed animals also revealed significant hypophosphatemia, hypokalemia, and decreased serum Na⁺ at 15 mg/kg of ZLD (Table 1). The lower dose of ZLD (10 mg/kg) caused hypokalemia (Table 1). Urinalysis in ZLD-treated rats revealed a considerable increase in urine glucose, ALP, protein, and γ-GT (Table 1). It was found that the lower dose of ZLD (10 mg/kg) caused a surge in urine levels of γ-GT and ALP (Table 1).

The assessment of the renal tissue biomarkers of oxidative stress revealed significant ROS formation and lipid peroxidation, decreased kidney GSH reservoirs, and depleted tissue anti-

Figure 1. Serum biochemical analysis and kidney weight index in ZLD-treated rats. Data sets are represented as mean±SD (n=6). Data sets with alphabetical superscripts differ significantly (P<0.05). ns: not significant.
oxidant capacity in ZLD-treated animals (Figure 2). The greater dose of ZLD (15 mg/kg) caused a more severe change in biomarkers of oxidative stress in the current study (Figure 2).

The current study also found that ZLD (10 and 15 mg/kg) could significantly impair mitochondrial function (Figure 3). In this regard, significant decreases in mitochondrial dehydrogenase activity, mitochondrial membrane potential, depletion of mitochondrial ATP reservoirs, and mitochondrial permeabilization were detected in the kidneys of ZLD-treated rats (Figure 3). The effect of the higher dose of ZLD (15 mg/kg) in impairing mitochondrial indices was more significant, except for mitochondrial swelling (Figure 3).

ZLD caused a significant increase in pro-inflammatory levels of cytokines (TNF-α, IL-6, and IL-1β) in the rats’ kidneys (Figure 4). The effect of 15 mg/kg of ZLD in increasing pro-inflammatory cytokines was more significant in the current study (Figure 4).

Renal histopathological evaluations in ZLD-treated rats revealed significant tubular atrophy, tissue necrosis, and infiltration of inflammatory cells at both doses of this drug evaluated in the current study (Figure 5).

4. Discussion

Zoledronic acid (ZLD) is clinically administered in various pathological conditions. However, nephrotoxicity is a significant adverse effect coupled with this drug (2-5). Unfortunately, the precise mechanism(s) of kidney injury induced by ZLD is unknown. Moreover, there is no therapeutic agent available to blunt this adverse effect. In the current study, we found that ZLD caused significant oxidative stress in the rat kidney, induced mitochondrial impairment, increased

Table 1. Test Urinalysis and some serum biochemical parameters in animals treated with zoledronic acid (ZLD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ZLD 10 mg/kg</th>
<th>ZLD 15 mg/kg</th>
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</thead>
<tbody>
<tr>
<td>Urinalysis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
<td>0.4±0.1</td>
<td>0.58±0.16</td>
<td>1.6±0.3 #</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>1900±344</td>
<td>3455±420 #</td>
<td>3800±456 #</td>
</tr>
<tr>
<td>γ-GT (U/l)</td>
<td>2100±231</td>
<td>3879±230 #</td>
<td>4688±465 #</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>72.0±11</td>
<td>85.0±13</td>
<td>124.0±15 #</td>
</tr>
<tr>
<td>Serum parameters</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Na⁺ (mmol/l)</td>
<td>62.4±5.6</td>
<td>69±6</td>
<td>78±4 #</td>
</tr>
<tr>
<td>Phosphate (mg/dl)</td>
<td>2.7±0.15</td>
<td>2.02±0.13</td>
<td>1.62±0.18 #</td>
</tr>
<tr>
<td>K⁺ (mmol/l)</td>
<td>5.7±1.3</td>
<td>3.24±1 #</td>
<td>3.07±0.49 #</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD (n=6).
ALP: alkaline phosphatase; γ-GT: γ-glutamyl transferase.
# Indicates significantly different from the control group (P<0.01).

Figure 2. Oxidative stress biomarkers in the renal tissue of ZLD-exposed rats. Data are given as mean±SD (n=6). Asterisks differ significantly from the control group (P<0.001).
pro-inflammatory cytokines levels, and caused substantial histopathological alterations in the rat kidney. These findings indicate ZLD as a nephrotoxic agent (e.g., at high dose or short interval use). Therefore, this serious adverse effect should be considered in clinical settings. On the other hand, based on the mentioned mechanisms, therapeutic agents (e.g., antioxidants and mitochondria-protecting agents) could be considered for managing ZLD-induced renal injury.

In the current study, we found that oxidative stress and its associated events could play a pivotal role in ZLD-induced renal injury (Figure 2). The effect of ZLD on oxidative stress markers in the in vitro experiments (e.g., HTK-2 and HEK-293) is also reported (7, 8). Our data align with these studies, indicating the role of oxidative stress in the cytotoxicity of ZLD in vitro. However, the current study might be one of the few investigations that propose the occurrence of oxidative stress induced by ZLD as a critical mechanism of toxicity caused by this drug in vivo. As oxidative stress plays a fundamental role in the pathogenesis of ZLD-induced renal injury, administering safe and clinically applicable agents (e.g., N-acetylcysteine) could find therapeutic significance in this complication (19-23). It should be mentioned here that oxidative stress is a mechanism related to other events, such as mitochondrial impairment and inflammatory response. The role of mitochondrial dysfunction and renal inflammation in the mechanism of ZLD nephrotoxicity are discussed in the following sections.

Mitochondria are crucial intracellular targets for xenobiotics-induced organ injury (23-26). Renal mitochondria seem to be critical targets for ZLD-induced organ injury. In the current study, we found that renal mitochondrial indices, including ATP levels, permeability, membrane potential, and dehydrogenase activity, were significantly different from the control group (P<0.001).

**Figure 3.** Mitochondrial indices in the kidney of zoledronic acid-treated animals. Data are given as mean±SD (n=6). Asterisks differ significantly from the control group (P<0.001).

**Figure 4.** Kidney level of pro-inflammatory cytokines in the kidney of zoledronic acid (ZLD)-treated rats. Data are given as mean±SD (n=6). Data sets with different superscripts are significantly different (P<0.05).
impaired by ZLD (Figure 3). The kidney contains numerous mitochondria that are critically involved in providing sufficient ATP, which is critically needed for vital processes such as chemical reabsorption from renal tubules (13, 19). Therefore, impairing renal mitochondrial function with any etiology (e.g., induced by drugs or diseases) could cause the waste of chemicals such as electrolytes, proteins, glucose, and amino acids in urine (4, 13, 19, 27). This situation is usually known as Fanconi syndrome (13, 19). Several drugs, such as valproic acid, lithium, and ifosfamide, could induce Fanconi syndrome in experimental and clinical settings (13, 19). In the current study, we found that some markers, such as glucose and proteins, and enzymes, such as ALP and γ-GT, were significantly higher in the urine of ZLD-treated rats (Table 1). Moreover, ZLD-treated rats showed hypokalemia and hypophosphatemia (Table 1). These events could be associated with impaired renal mitochondrial function and lack of energy (ATP) for the reabsorption of these chemicals from the renal tubules to the circulation. Interestingly, abnormal mitochondrial shapes were detected in the TEM evaluation of renal biopsies taken from ZLD-treated animals (28). Based on these data, ZLD-induced mitochondrial impairment could play a critical role in its mechanism of nephrotoxicity. In this regard, using mitochondria-protecting agents in ZLD-treated patients with renal injury could serve as a preventive/therapeutic strategy (29, 30). It should also be mentioned that mitochondria are principal sources of intracellular ROS (31). Therefore, ZLD-induced mitochondrial dysfunction in the kidney could hasten ROS formation and oxidative stress in this organ.

Severe histopathological alterations also have been detected in other experimental models investigating ZLD nephrotoxicity (28). In the current study, we also found that ZLD caused significant tubular atrophy, necrosis, and penetration of inflammatory cells into the renal tissue (Figure 5). These events could be associated with severe oxidative stress induced by this drug. On the other hand, we found that the level of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) was significantly increased. These data could suggest using anti-inflammatory agents against ZLD-associated renal injury.

5. Conclusion

Collectively, the data presented in the current study revealed nephrotoxicity as a serious adverse effect associated with ZLD. It should be
mentioned that although ZLD was used in short-term and high doses in the current investigation, in many clinical cases, this drug is used chronically (e.g., for years) (4, 32). Moreover, many other factors, such as background renal diseases as well as patients’ age (e.g., >60 yo), are risk factors involved in the development of ZLD-induced renal injury (3, 32). Interestingly, case reports indicate significant renal damage and serum electrolyte disturbances induced by long-term use of ZLD (4, 32). These findings mention that investigating the mechanisms of renal dysfunction caused by this drug is noteworthy and might pave the way for developing preventive/therapeutic strategies in future studies in clinical settings.

Acknowledgments
The authors thank the Pharmaceutical Sciences Research Center of Shiraz University of Medical Sciences for the technical support of this study. This study was financially supported by the Vice Chancellor of Research Affairs, Shiraz University of Medical Sciences, Shiraz, Iran (Grant: 23195/17231/16457/17231).

Conflict of Interest
The authors declare no conflict of interest.

References


2018 Nov 2. PMID: 30396066.