Original Article

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Zoledronic Acid-Induced Insulitis in Rats

Heresh Rezaei[1,2];PhD$_{Candidate}$, Akram Jamshidzadeh[1,2];PhD, Abdollah Arjmand[3];PhD$_{Candidate}$, Narges Abdoli[4];PhD, Asma Najibi[1,2];PhD, Forouzan Khodaei[1];PhD, Sepideh Alidaee[1];PharmD, Negar Azarpira[5];PhD, Mohammad Mehdi Ommati[1,6,7*];PhD, Reza Heidari[1*];PhD

[1]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[2]Department of Toxicology and Pharmacology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
[3]Department of Toxicology and Pharmacology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[4]Food and Drug Administration, Iran Ministry of Health and Medical Education, Tehran, Iran.
[5]Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[6]Henan Key Laboratory of Environmental and Animal Product Safety, College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471000, Henan, China.
[7]College of Life Sciences, Shanxi Agricultural Sciences, Taigu, Shanxi, China.

Abstract

Insulitis is the inflammation of beta cells of the Langerhans islets. It is well-known that insulitis is a prevalent complication of diabetes. A series of xenobiotics, including drugs, could also induce insulitis. The current study evaluated the effect of zoledronic acid (ZLD) on the pancreas in an animal model. Actually, in an attempt to evaluate the adverse effects of ZLD on the kidney, we noticed severe morphological alterations in the pancreas. Therefore, the effects of ZLD on the pancreas tissue were further investigated. Rats received ZLD (10 and 15 mg/kg, single dose, i.p) and pancreas weight index, serum biomarkers of pancreas injury, the level of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), oxidative stress biomarkers in this organ, and pancreas histopathological alterations were assessed. A significant increase in pancreas weight index was detected in ZLD-treated animals. ZLD also significantly increased serum amylase and lipase levels. No significant changes in serum glucose were detected in this study. A significant increase in reactive oxygen species, lipid peroxidation, decreased glutathione levels, and antioxidant capacity was also evident in the pancreas of ZLD-treated rats. Histopathological findings indicate the insulitis lesions of the islets of Langerhans at both doses of 10 and 15 mg/kg of ZLD. The data obtained from this study revealed insulitis as a serious adverse effect associated with high doses of ZLD. Clearly, further studies are warranted to evaluate the effects of other doses and/or patterns of administration of ZLD on pancreas tissue and, finally, the clinical significance of these data.

Keywords: Diabetes, Glucose metabolism, Inflammation, Insulitic lesion, Oxidative stress

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1. Introduction

Insulitis is the inflammation of the Langerhans islet (LI) cells (1-3). Various inflammatory cells (e.g., T and B lymphocytes) infiltrate the LI during insulitis (1-4). The infiltration of inflammatory cells to LI is associated with increased levels of cytokines and chemokines in these cells (5). It is believed that these mediators could induce cell death in LI (5, 6). The inflammation of LI could significantly damage these cells, interrupt insulin

Corresponding Author: Reza Heidari & Mohammad Mehdi Ommati, Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
Email: rheidari@sums.ac.ir & mehdi_ommati@sxau.edu.cn

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secretion, and impair glucose metabolism (7, 8). On the other hand, it has also been found that insulitis is a prevalent complication in human type 1 (≈up to 31%) and type 2 (≈up to 28%) diabetes (1, 2). Various pathologies could induce insulitis (1, 9-11), and consequently, diabetes might either develop or progress. Based on these data, insulitis could be the cause or consequence of diabetes.

As mentioned, insulitis is the hallmark of diabetes (12-14). However, several xenobiotics (e.g., drugs) are also identified to induce insulitis (15, 16). Xenobiotics-induced insulitis could develop into impaired glucose metabolism or even diabetes. Therefore, identifying insulitis as an adverse drug effect and investigating the mechanisms involved in its pathogenesis are critical steps that could help developing more safe pharmaceuticals and expanding preventive/therapeutic options against this complication.

Zoledronic acid (ZLD) is a bisphosphonate drug used in various pathological conditions, including hypercalcemia of malignancy, bone metastases from solid tumors, osteoporosis, and multiple myeloma (17, 18). Several adverse effects have been reported to be associated with ZLD administration (19, 20). Renal toxicity, gastrointestinal (GI) toxicity, and osteonecrosis of the jaw (ONJ) are the most common adverse effects related to ZLD (21-23).

The current study reported the insulitis induced by ZLD as a novel adverse effect of this drug. Moreover, we tried to evaluate biomarkers of tissue injury as well as histopathological evaluations to clear potential mechanisms of this side effect. These data could help the development of preventive/therapeutic strategies against ZLD-induced pancreas injury as a serious inmportant effect of this drug.

2. Materials and methods

2.1. Reagents and chemicals

2,4,6-tripyridyl-s-triazine, reduced glutathione (GSH), 2′,7′-dichlorofluorescein diacetate, hydrogen peroxide, methanol, zoledronic acid, and ethylenediaminetetraacetic acid (EDTA), trichloroacetic acids, hydroxymethyl aminomethane hydrochloride (Tris-HCl), m-phosphoric acid, potassium chloride, sodium chloride, ferric chloride (Fe2Cl3), and hydrochloric acid were obtained from Merck (Merck KGaA, Darmstadt, Germany). Kits for evaluating serum biochemistry were obtained from ParsAzmoon® (Tehran, Iran). Kits for assessing pro-inflammatory cytokines were purchased from Shanghai Jianglai Biology® (China).

2.2. Animals

Sprague-Dawley rats (n=48, w=200-250 g) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Rats were maintained in a standard environment (12 h photo schedule, ≈43±3% relative humidity, and 23±1 ºC temperature) with free access to tap water and a regular rodent diet (RoyanFeed®, Iran). The institutional ethics committee of Shiraz University of Medical Sciences approved laboratory animal care and use (Approval code: IR.SUMS.REC.1399.1344). The current study also followed the ARRIVE protocol for using and caring laboratory animals.

2.3. Experimental setup

Rats received a single dose of ZLD intraperitionaly (i.p). Then, serum biomarkers of organ injury, pancreas tissue histopathological alterations, pro-inflammatory cytokines, and oxidative stress biomarkers were evaluated 24 hours after drug administration. The experimental groups were as follows (16 rats/group): 1) Control (2.5 ml/kg, normal saline); 2) ZLD (10 mg/kg, i.p., in normal saline); and 3) ZLD (15 mg/kg, i.p., in normal saline).

2.4. Serum biomarkers of pancreas injury

Animals were deeply anesthetized using thiopental (80 mg/kg, i.p). Then, blood samples were collected from the abdominal vena cava and centrifuged (4000 g, 10 min, 4 °C). Serum levels of amylase and lipase as biomarkers of pancreas tissue injury as well as serum glucose, were assessed using commercial kits (Pars-Azmoon, Tehran, Iran) and an autoanalyzer (Mindray® BS-200 autoanalyzer, Guangzhou, China).

2.5. Reactive oxygen species in the pancreas of ZLD-treated rats

The level of reactive oxygen species (ROS) formation in the pancreas tissue was as-

sessed using 2′, 7′ dichlorofluorescein diacetate (DCF-DA) (24). For this purpose, pancreas tissue (≈700 mg) was homogenized in 5 mL of ice-cooled Tris-HCl buffer (40 mM, pH=7.4). Then, 100 µL of the resulting tissue homogenate was added to 1 mL of Tris-HCl buffer (40 mM, pH=7.4) containing 10 µM of DCF-DA and incubated in a 37ºC shaker-incubator (10 min, protected from light). Finally, the fluorescence intensity was assessed (FLUOstar Omega® fluorimeter at λ_{excit}=485 nm and λ_{emiss}=525 nm) (24).

2.6. Pancreas tissue lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) test assessed lipid peroxidation in the pancreas tissue (24). Briefly, 500 µL of the tissue homogenate (homogenized in 40 mM Tris-HCl buffer, pH=7.4) was treated with 1 mL of TBARS assay reagent (a mixture of 1 mL of 0.375 % w: v thiobarbituric acid, 1 mL of 50 % w: v of trichloroacetic acid, pH=2). Samples were vortexed well (1 min) and heated (100 °C water bath, 45 min). Afterward, 0.5 mL of n-butanol was added, and samples were mixed and centrifuged (10000 g, 15 min, 4 °C). Finally, the absorbance of the n-butanol phase was assessed (λ=532 nm, EPOCH® plate reader, USA) (24).

2.7. The total antioxidant capacity of the pancreas tissue

The pancreatic tissue's ferric-reducing antioxidant power (FRAP) was assessed as an index of the total antioxidant capacity (24). Briefly,100 µL of the tissue homogenate was added to 1 mL of a freshly-prepared working FRAP mixture (ten parts of 300 mM acetate buffer, one part of 10 mM 2, 4, 6-tripyridyl-s-triazine, and one part of 20 mM ferric chloride, pH=3.6). Samples were incubated in a shaker incubator (37 ºC) for five minutes (protected from light). Finally, the absorbance was assessed at λ=593 nm (EPOCH® plate reader, USA) (24).

2.8. Pancreas glutathione (GSH) levels

Pancreatic tissue GSH content was assessed using Ellman's reagent (dithiobis-2-nitrobenzoic acid, DTNB) (24). Briefly, 500 µL of the prepared tissue homogenate was treated with 100 µL of trichloroacetic acid (50 % w: v; 4 ºC). Samples were mixed well and centrifuged (16000 g, 4 °C, 15 min). Afterward, the supernatant was mixed with 1 mL of Tris-HCl buffer (pH = 8.9; 4 °C) and 100 µL of DTNB solution (20 mg in 5 mL methanol, protected from light). Finally, the optical density was measured at λ = 412 nm using an EPOCH® plate reader (24).

2.9. Bradford's method of protein measurement

To standardize the obtained data, the protein content of each sample was assessed based on the Bradford method, as previously reported (25).

2.10. Histopathological assessments

Tissue samples were collected for histopathological evaluations and fixed in a buffered formalin solution (0.4% w: v of NaH2PO4, 0.64% w: v of Na_2HPO_4, and 10% v: v formaldehyde in distilled water; pH=7.4). Afterward, paraffin-embedded samples were prepared (5-µm sections) and stained with hematoxylin-eosin (H&E). The insulitis index was used as a quantitative parameter for each group, as previously reported, based on the formula: insulitis index=((0×n0)+(1×n1)+(2×n2)+(3×n3)+(4×n4))/(4×(n0+n1+n2+n3+n4)) , where n0, n1, n2, n3, and n4 are the number of islets scored in grades 0 (no inflammatory cell infiltration), 1 (pre-insulitis), 2 (up to 25% inflammatory cells infiltrated), 3 (>25% and up to 75% inflammatory cells infiltrated), and 4 (>75% inflammatory cells infiltrated), respectively (26).

2.11. Statistics

Data are given as mean ± SD. Data comparison was carried out by the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test as the post hoc. Values of $P<0.05$ were considered statistically significant.

3. Results

Compared to the control group, the pancreas weight index was significantly increased in ZLD-treated rats (10 and 15 mg/kg) (Figure 1). Changes in pancreas weight index were more prominent at the higher dose of ZLD (15 mg/kg) (Figure 1).

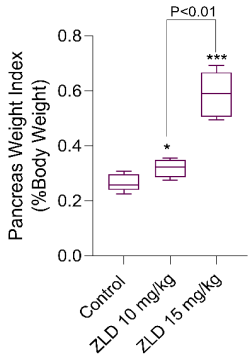

Figure 1. Pancreas weight index in zoledronic acid (ZLD)-treated animals. Data are represented as box and whisker plots. Asterisks indicate significantly different from the control group (*P<00.05 and ***P < 0.01). ZLD caused a significant increase in the pancreatic weight index at both doses administered in the current study. The higher dose of ZLD had a more significant effect on the rat pancreatic index.

Serum levels of amylase and lipase as biomarkers of pancreatic damage were compared with the control animals. The lower dose of ZLD caused no significant changes in serum lipase and amylase levels in the current model (Figure 2). On the other hand, these enzymes were significantly higher in ZLD-treated animals when the drug was administered at 15 mg/kg (Figure 2). Evaluation of serum glucose levels revealed no significant changes in this parameter in control and ZLD-treated (10 and 15 mg/kg) rats (Figure 2).

The level of pro-inflammatory cytokines (TNF-α, IL-6, and IL-β) was also elevated in the pancreas tissue of ZLD-treated animals compared to the control group (Figure 3). It was found that both doses of ZLD caused a significant elevation in the pancreas levels of pro-inflammatory cytokines (Figure 3). The higher dose of ZLD (15 mg/kg) caused a more significant increase in the pro-inflammatory cytokines in the pancreas tissue (Figure 3).

Biomarkers of oxidative stress were significantly changed in ZLD-treated rats (Figure 4). It was found that ZLD caused significant ROS formation and lipid peroxidation in the pancreas tissue (Figure 4). Moreover, pancreas GSH content and antioxidant capacity were significantly decreased in drug-treated rats (Figure 4). The higher dose of ZLD caused a more significant increase in oxidative stress biomarkers in the pancreas tissue (Figure 4).

Insulitis (the infiltration of inflammatory

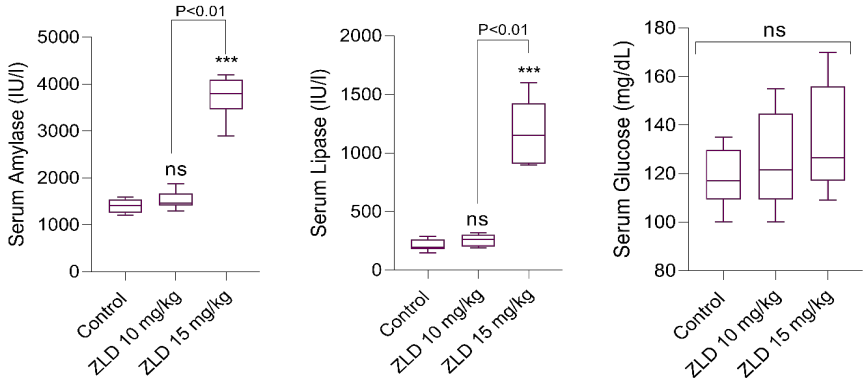

Figure 2. Serum amylase and lipase levels in zoledronic acid (ZLD)-treated rats. Data are given as box and whisker plots. ***Indicates significantly different compared to the control group (P < 0.01). ns: not significant. No significant changes in the serum amylase, lipase, and glucose were detected in the 10 mg/kg dose of ZLD compared to the control animals. However, the higher dose of this drug (15 mg/kg) significantly increased serum amylase and lipase.

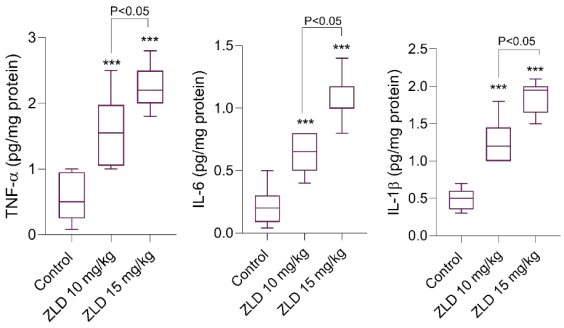

Figure 3. The level of pro-inflammatory cytokines in the pancreas tissue of rats exposed to zoledronic acid (ZLD). Data are represented as box and whisker plots. ***Indicates significantly different as compared with the control group ($P < 0.01$). ZLD (10 and 15 mg/kg) caused a significant increase in the pancreatic level of pro-inflammatory cytokines. On the other hand, it was found that these cytokines' levels were significantly higher when animals received a higher dose of ZLD (15 mg/kg).

cells to the islets of Langerhans) was a prominent histopathological alteration of the pancreas of ZLD-treated animals at both doses of 10 and 15 mg/kg of this drug (Figure 5). It seems that ZLD-induced inflammatory cell infiltration of Langerhans islet was more prominent at the higher dose of this drug (15 mg/kg) (Figure 5).

4. Discussion

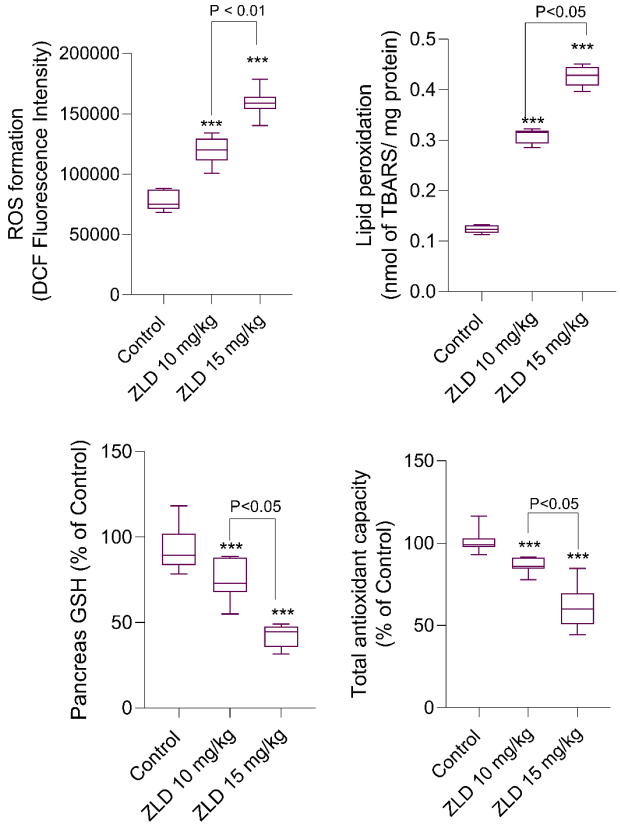

Figure 4. Pancreas tissue oxidative stress biomarkers in the zoledronic acid (ZLD)-treated animals. Data are represented as box and whisker plots. ***Indicates significantly different as compared with the control group ($P < 0.01$). Both doses of ZLD administered in this study significantly increased biomarkers of oxidative stress. It was found that the higher dose of ZLD (15 mg/kg) could cause more significant oxidative stress in the pancreas of rats.

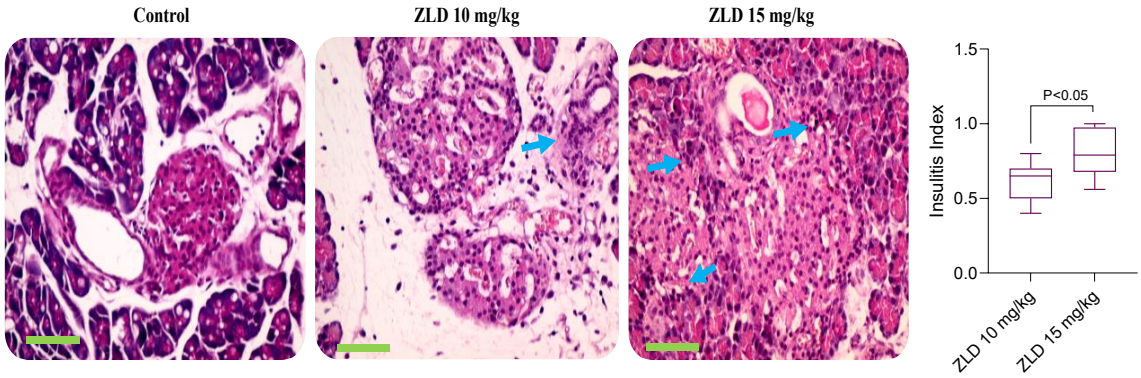

Figure 5. Insulitis (blue arrow) induced by zoledronic acid (ZLD) in rat pancreas. ZLD caused significant insulitis in rats, especially at the higher dose of this drug (15 mg/kg, i.p, single dose) 24 hours after drug administration. Scale bar =100 µm. The insulitis index was calculated as described in the materials and methods section and represented as a box and whisker plot. The insulitis index in the control group is zero. The higher dose of ZLD (15 mg/kg) leads to a higher insulitis index.

ZLD is a bisphosphonate drug clinically used to manage several complications from hypercalcemia of malignancy, bone metastases from solid tumors, osteoporosis, and multiple myeloma (17, 18, 27, 28). However, several adverse effects, such as kidney injury, GI toxicity, hypersensitivity, and ONJ, are reported in association with ZLD administration (19-23). The current study found that ZLD could induce insulitis shortly (24 hours) after drug administration. This is a novel adverse effect of this drug which could significantly influence its applications in clinical settings.

In a recent study by Cheung et al., the authors found that ZLD did not affect insulin resistance in men receiving this drug (29). In this study, men (n = 39) received a single dose (5 mg) of ZLD, and the glucose tolerance test was assessed at different time intervals (0, 3, 12, and 24 months) (29). The authors found that ZLD did not influence these patients' glucose metabolism and insulin resistance. In the current study, we found that ZLD (10 and 15 mg/kg) significantly altered pancreas histopathology (Figure 5) and increased serum amylase and lipase levels (Figure 2). Although serum glucose tended to increase at higher doses of ZLD, this drug did not significantly affect glucose levels 24 hours after administration (Figure 2). Comparing these data reveals the importance of drug doses in developing adverse effects such as insulitis in ZLD therapy.

Beta cells have low protection against toxic insults such as oxidative/nitrosative stress or elevated levels of inflammatory cytokines (30-32). Hence, many pathogenic factors could quickly induce β cell injury and death. In the current study, we found that ZLD caused a significant increase in biomarkers of oxidative stress in the rat pancreas (Figure 4). These findings indicate that oxidative stress could result from ZLD-induced insulitis, which finally induces beta cell death. Notably, in the current study, we assessed the reduced glutathione level (GSH; Figure 4). However, the reduced/oxidized glutathione (GSH/GSSG) could give a better insight into the occurrence of oxidative stress in tissues. This point could be kept in mind for further research in this field. Based on these data, the administration of robust and safe antioxidant agents might blunt xenobiotics (e.g., ZLD) or diseases (diabetes)-induced insulitis (33, 34). These points are worth further studies in various experimental models.

Insulitis also has been reported as an adverse effect of other drugs in experimental models or humans. Streptozocin is an excellent example of drug-induced insulitis in animal models (15, 35). Interestingly, several cases of ZLD-induced increase in serum amylase, as well as disturbances in serum glucose, have been reported in patients receiving this drug (36, 37). However, there is no precise mechanism for these adverse effects so far. In an attempt to evaluate the mechanisms of ZLD-induced renal injury, we found significant changes in animals' pancreatic tissue. Hence, the pancreas was excised and weighed. Moreover, specimens

were excised and maintained in formalin solution (10% v: v) for histopathological evaluations. Biomarkers of oxidative stress were also assessed in the rats' pancreas.

Although ZLD is usually used at low doses and in a short-term manner, some diseases (e.g., metastatic prostate cancer) need repeating and high doses of ZLD acid (e.g., 4 mg/4 weeks) (38). Therefore, ZLD-induced insulitis and maybe diabetes could be a severe adverse effect of this drug that needs urgent attention. It should be mentioned that this study was conducted on a high dose of ZLD in a short time frame (24 hours after drug administration). It is well-known that the types of immune cells that infiltrate the Langerhans islet and the histopathological features of insulitis lesions are different in human cases compared to animal models. Therefore, more studies are needed to extrapolate the data from this *in vivo* study to human cases.

Further studies on this drug's lower doses and chronic administration patterns might produce different results. However, the data obtained from this study highlight insulitis as a severe adverse effect of ZLD (e.g., in case of a drug overdose), which needs monitoring and clinical intervention. The potential molecular mechanisms of zoledronic acid-induced insulitis also warranted further investigations to be precisely cleared. Moreover, using other precision technologies (e.g., immunohistochemical analysis) to evaluate the type of immune cells infiltrated in LI in ZLD-treated animals could help identify factors involved in the pathogenesis of drug-induced insulitis and the development of preventive/therapeutic strategies against this complication.

Several reports of the adverse effects of ZLD on various components of mitochondria and mitochondria-mediated cell death exist (39-42). On the other hand, it has also been found that mitochondrial impairment is also associated with insulitis as an adverse effect of xenobiotics (e.g., drugs) (43, 44). Although not investigated in the current study, it is suggested to evaluate the role of mitochondrial impairment as a potential mechanism of ZLD-induced insulitis. Moreover, safe and clinically-applicable mitochondrial protecting agents might mitigate ZLD-induced insulitis (45-48).

Unfortunately, insulitis induced by drugs or various pathologies has no specific treatment protocol. As mentioned in previous parts, mechanisms such as severe inflammatory response and oxidative stress could play a central role in the pathogenesis of insulties. Therefore, anti-inflammatory and immune suppressant drugs, mitochondrial protecting agents, as well as antioxidant molecules could be potential agents for the treatment of insulitis with various etiologies.

5. Conclusion

ZLD is a widely administered drug against a variety of human pathologies. Therefore, it is essential to investigate its adverse effects and their mechanisms of action. Collectively, the data obtained from this study revealed that insulitis is a severe complication of ZLD in rats. These data could be used to develop different drug treatment regimens, dose adjustment, and monitoring of patients for the adverse effects of ZLD in clinical settings.

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Conflict of Interest

None declared.

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