



Research Article

# Mitochondrial Transplantation Attenuates Toxicity in Human Lymphocytes Caused by Clozapine and Risperidone

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## Article Info

### Article History:

Received: 18 Feb 2023

Accepted: 3 May 2023

ePublished: 22 Jul 2023

### Keywords:

- Clozapine
- Human Lymphocytes
- Mitotherapy
- Mitochondria
- Risperidone

## Abstract

**Background:** Clozapine (CLZ) and risperidone (RIS) are drugs that have the ability to disrupt mitochondrial function. Also, these drugs increase the level of free radicals. Mitochondrial dysfunction plays a role in the etiology of various diseases. Replacement and treatment of defective mitochondria with healthy mitochondria have been considered. Mitochondrial therapy (mitotherapy) or exogenous mitochondria transplantation is a method that can be used to replace dysfunctional mitochondria with healthy mitochondria. This method can help in the treatment of diseases related to mitochondria.

**Methods:** In this study, we investigated the transplantation effect of isolated lymphocyte mitochondria on the toxicity induced by CLZ and RIS on human blood lymphocytes. Lymphocytes were isolated using the Ficoll standard method. Mitochondria of human lymphocytes were used for mitotherapy. This study was conducted in 6 groups. After treatment, the level of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), reduced glutathione (GSH) content, oxidized glutathione (GSSG) content, and adenosine triphosphate (ATP) content were evaluated.

**Results:** Our data showed that CLZ (70  $\mu$ m) and RIS (24 nM) caused cytotoxicity on human blood lymphocytes which are associated with ROS generation, collapse in MMP, decrease in GSH content, increase in GSSG content and change in ATP content. Mitochondria transplantation results showed that adding mitochondria of lymphocytes could protect the lymphocytes against the toxicity effects caused by CLZ and RIS. Furthermore, the results showed that pre-incubation with cytochalasin D considerably reserved the protective effects of mitotherapy in the human lymphocytes.

**Conclusion:** We proposed that mitochondria transplantation or mitotherapy-affected blood lymphocytes with exogenous mitochondria could be used to treat CLZ and RIS-induced toxicity.

## Introduction

Clozapine (CLZ) and risperidone (RIS) are drugs that are used to treat patients with behavioral disorders.<sup>1,2</sup> Research has shown that these drugs can cause disruption in mitochondrial function.<sup>3,4</sup> CLZ and RIS can increase the level of reactive oxygen species (ROS), cause disruption in the mitochondrial membrane, decrease the level of intracellular antioxidants, and change the energy content. Also, reports show that these compounds cause the beginning of the process of oxidative stress through an increase in the level of lipid peroxidation (LPO) and a decrease in the level of reduced glutathione (GSH),

resulting in oxidative damage in cell.<sup>3-6</sup> Oxidative stress is involved in the pathophysiology of many diseases.<sup>7,8</sup> Mitochondrial dysfunction is involved in the etiology of many diseases, which shows the importance of mitochondria.<sup>9,10</sup> Mitochondria are known as one of the most critical intracellular organelles and are involved in many important intracellular processes. They play a role in ROS production, energy production, growth, and cell death. Defects in mitochondrial function are associated with disturbances in many intracellular physiological processes.<sup>11,12</sup>

Today, mitochondrial therapy (mitotherapy)

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is considered a new strategy for the treatment of mitochondrial diseases.<sup>13,14</sup> The purpose of mitotherapy is to transfer exogenous healthy mitochondria into defective mitochondria in cells to prevent and improve mitochondrial diseases.<sup>12,15</sup> Studies have shown that mitochondria can significantly improve mitochondrial dysfunction caused by various compounds. In *in vivo* and *in vitro* studies, the mitochondrial transfer is done by simple co-incubation in the culture system or by intravenous injection into animals.<sup>12,15</sup> Reports show that 1 hour after simple co-incubation in most cell types, the process of mitochondrial internalization is confirmed, and it is also significantly increased after 4 and 24 hr.<sup>15</sup> Mitotherapy has been associated with a decrease in the level of ROS and oxidative stress, an increase in the level of intracellular GSH, an improvement in the level of energy production and mitochondrial function, and also maintaining the MMP.<sup>16,17</sup> Studies have shown that mitochondria isolated from female animals are more sensitive to stress and give better performance and efficiency in harmful conditions.<sup>12</sup> In this study, in order to select a novel therapy method to improve human lymphocytes toxicity, we evaluated whether co-incubation of functional mitochondria isolated from the human lymphocytes could ameliorate mitochondrial dysfunction-related cytotoxicity in human lymphocytes affected by CLZ and RIS treatment.

## Methods

### Blood samples

To conduct the study, blood was collected from 15 healthy volunteers (Female). In order to collect blood, volunteers must have criteria such as age between 18 and 22, not smoking, and having no signs of infectious diseases. The present study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences as ID IR.SBMU.PHARMACY.REC.1397.062. After becoming aware of our study donors are asked to fill out the approval form.

### Isolation of human lymphocytes

Human blood lymphocytes were isolated according to our previous report. Briefly, the Ficoll standard method has been used to isolate human lymphocytes from the blood of healthy volunteers. First, collected blood (5 ml) was diluted with PBS and layered on Ficoll paque plus. Then, the centrifugation (2500 rpm/20 min) was carried out, and the lymphocytes layer was collected and then suspended at the erythrocyte lysis buffer. Finally, were incubated at 37°C for 5 min. After dilution with PBS, a centrifuge was performed at 1500 rpm for 10 min. Subsequently, the supernatant was removed and the human lymphocytes were washed with the RPMI1640 (twice) and were centrifuged at 2000 × g (7 min). Finally, the human lymphocytes were re-suspended in RPMI1640 medium with L-glutamine. In the tests, the density of lymphocytes was  $10 \times 10^4$  cells/ml. The concentrations used by CLZ and RIS in this study are based on our previous reports.<sup>6,18</sup>

### Mitochondrial isolation

Lymphocyte mitochondria of human blood were isolated according to our previous study. Briefly, mitochondria were isolated from lymphocytes using mechanical lysis and centrifugation (Hettich, Universal 320R, Germany). To perform this isolation, PBS was used for washing. After washing, centrifugation ( $450 \times g$  for 20 min) was performed. Next, the obtained pellet was re-suspended in the isolation buffer. Homogenization was used for the cell disruption and centrifugation ( $750 \times g$  for 20 min) was performed again. Next, the supernatant was centrifuged twice ( $10000 \times g$  for 10 min) and the pellet was suspended in the assay buffer.<sup>19</sup> In this study, the dose of isolated mitochondria (mitochondrial protein concentration) was done based on the Bradford test.<sup>20</sup> Mitochondria isolated from lymphocytes were used for mitotherapy. Isolation of mitochondria from human lymphocytes was done simultaneously with treatment of lymphocytes to CLZ and RIS for 2 hr. After 2 hr, human lymphocytes in different groups were immediately treated with freshly isolated mitochondria for 4 hr.

### Experimental scheme

The investigational groups were classified into six different groups. Group 1: Control group lymphocytes were treated with a vehicle for 2 hr. Group 2: lymphocytes were treated with CLZ (70  $\mu$ M) for 2 hr. Group 3: lymphocytes were treated with RIS (24 nM) for 2 hr. Group 4: Group 1 (2hr) + fresh exogenous mitochondria for 4 hr. Group 5: Group 2 (2hr) + fresh exogenous mitochondria for 4 hr. Group 6: Group 3 (2 hr) + exogenous mitochondria for 4 hr.

### Mitochondrial transformation into cells

The human lymphocytes were seeded in a 24-well plate at a density of  $5 \times 10^4$  cells/well at 37 °C. Then, CLZ (70  $\mu$ M) and RIS (24 nM) were added into cell media for 2 hr incubation. After the media were replaced by fresh RPMI 1640 (the washing process has been done 3 times with fresh RPMI 1640), exogenous mitochondria (160  $\mu$ g/ml) were added into the media for 4 hr incubation. Then, the desired tests were performed.<sup>21</sup> The concentrations of CLZ and RIS were determined based on our previous studies.<sup>5,6</sup> In order to perform the transplantation mitochondria were isolated from healthy human lymphocytes.

### ROS level assay

A fluorescent dichlorofluorescein diacetate (DCFH-DA) dye was used to assay changes in ROS levels in the human lymphocytes. Initially, human lymphocytes ( $10 \times 10^4$  cells/ml) were treated with CLZ (70  $\mu$ M) and RIS (24 nM) for 2 hours. For mitotherapy, exogenous mitochondria isolated from lymphocytes were added to treated lymphocytes and incubated for 4 hours. Then, all groups were incubated with the DCFH-DA probe at a concentration of 1  $\mu$ M for 20 min. The ROS level (fluorescent intensity) was evaluated through a fluorescence spectrofluorometer (Shimadzu RF5000U, Japan) at  $\lambda_{ex}=485$  nm and  $\lambda_{em}=535$  nm.<sup>22</sup>

### Mitochondrial membrane potential (MMP) assay

A fluorescent Rh 123 dye was used to assay changes of MMP in the human lymphocytes. Initially, human lymphocytes ( $10 \times 10^4$  cells/ml) were treated with CLZ (70  $\mu$ M) and RIS (24 nM) for 2 hours. For mitotherapy, exogenous mitochondria isolated from lymphocytes were added to treated lymphocytes and incubated for 4 hours. Then, all groups were incubated with the rhodamine 123 (Rh123) probe at a concentration of 1  $\mu$ M for 20 min. The MMP (fluorescent intensity) was evaluated using a fluorescence spectrofluorometer (Shimadzu RF5000U, Japan) at  $\lambda_{ex}$ =470 nm and  $\lambda_{em}$ =540 nm.<sup>6,23</sup>

### Reduced glutathione (GSH) and oxidized glutathione (GSSG) content assay

5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) reagent based on the Hissin and Hilf method has been used to evaluate GSH and GSSG content in human lymphocytes. Briefly, human lymphocytes ( $10 \times 10^4$  cells/ml) were treated to CLZ and RIS at concentrations of 70  $\mu$ M and 24 nM, respectively. 2 hr after treatment, the lymphocytes were incubated with exogenous mitochondria for 4 hr. Then, all groups were washed with PBS (twice). After centrifugation, the supernatant part was collected, and then the supernatant of the previous step and DTNB (10 mM) were used to assay GSH and GSSG content. In the following, the optical density was assayed at 412 nm (Beckman DU-7 spectrophotometer).<sup>24</sup>

### Adenosine triphosphate (ATP) content assay

The ATP content was evaluated by the luciferase enzyme. Lymphocytes ( $10 \times 10^4$  cells/ml) were treated to CLZ (70  $\mu$ M) and RIS (24 nM) for 2 h. For mitotherapy, exogenous mitochondria isolated from lymphocytes were added to treated lymphocytes and incubated for 4 hours. Finally, bioluminescence intensity was assayed using a Sirius tube luminometer (Berthold Detection System, Germany).<sup>25</sup>

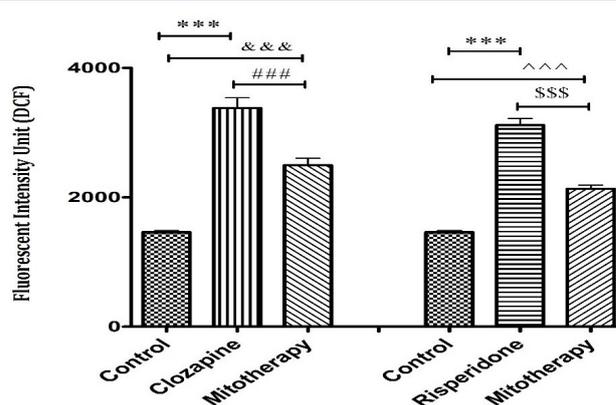
### Statistical analysis

The process of data expression was carried out as mean  $\pm$  SD. The findings were analyzed through the analysis of variance (ANOVA) and appropriate post-tests. Also,  $p < 0.05$  was considered as a significant level. We conducted the statistical analysis and graphics creation through GraphPad Prism 6 (GraphPad, La Jolla, CA, USA).

## Results

### Mitotherapy decreased ROS production induced by CLZ/RIS

The results indicated that ROS generation of CLZ and RIS-treated cells increased ( $p < 0.001$ ) (Figure 1). Furthermore, after the CLZ and RIS-treated human lymphocytes were incubated with exogenous mitochondria for 4 hr, intracellular ROS production significantly decreased ( $p < 0.001$ ). These results indicate the role of mitotherapy in the reduction of ROS production and subsequent oxidative stress caused by these drugs in human lymphocytes.



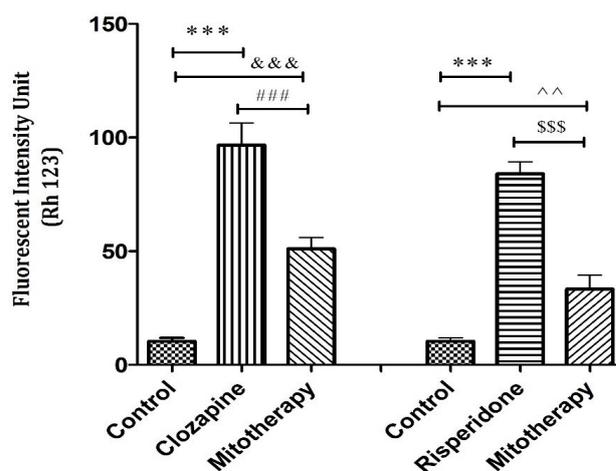
**Figure 1.** ROS assay. Effects of clozapine (70  $\mu$ M) and risperidone (24 nM) treatment and mitotherapy on ROS generation. \*\*\* $p < 0.001$  control vs clozapine/risperidone. \*\*\* $p < 0.001$  control vs mitotherapy. ###  $p < 0.001$  clozapine vs mitotherapy. \*\*\*  $p < 0.001$  control vs mitotherapy. \$\$\$  $p < 0.001$  risperidone vs mitotherapy.

### Mitotherapy improved MMP collapse induced by CLZ/RIS

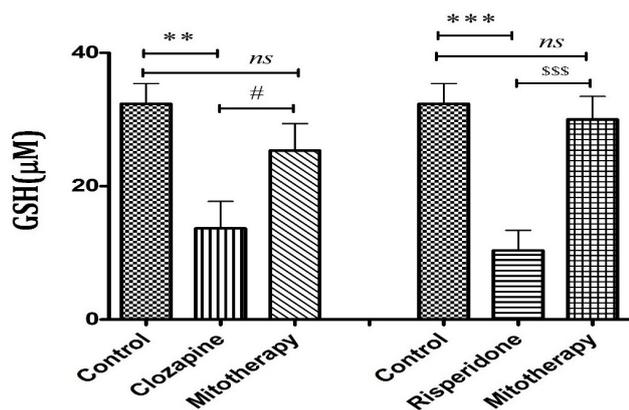
MMP assessment test revealed that CLZ (70  $\mu$ M) and RIS (24 nM) treatment resulted in MMP collapse in human lymphocytes ( $p < 0.001$ ) (Figure 2). The results showed that exogenous mitochondria, given at 2 hr after CLZ (70  $\mu$ M) and RIS (24 nM) treatment, could prevent CLZ and RIS-induced MMP collapse in human lymphocytes ( $p < 0.001$ ).

### Mitotherapy improved GSH content decreased by CLZ/RIS

The results indicated that the GSH content of CLZ ( $p < 0.01$ ) and RIS ( $p < 0.001$ ) treated cells decreased (Figure 3). Furthermore, after the CLZ ( $p < 0.05$ ) and RIS ( $p < 0.001$ ) treated human lymphocytes were incubated with exogenous mitochondria for 4 hr, GSH content significantly increased. These results indicate the role of mitotherapy in maintaining GSH content as one of the most important intracellular antioxidants.



**Figure 2.** MMP assay. Effects of clozapine (70  $\mu$ M) and risperidone (24 nM) treatment and mitotherapy on MMP collapse. \*\*\* $p < 0.001$  control vs clozapine/risperidone. \*\*\* $p < 0.001$  control vs mitotherapy. ###  $p < 0.001$  clozapine vs mitotherapy. \*\*  $p < 0.01$  control vs mitotherapy. \$\$\$  $p < 0.001$  risperidone vs mitotherapy.



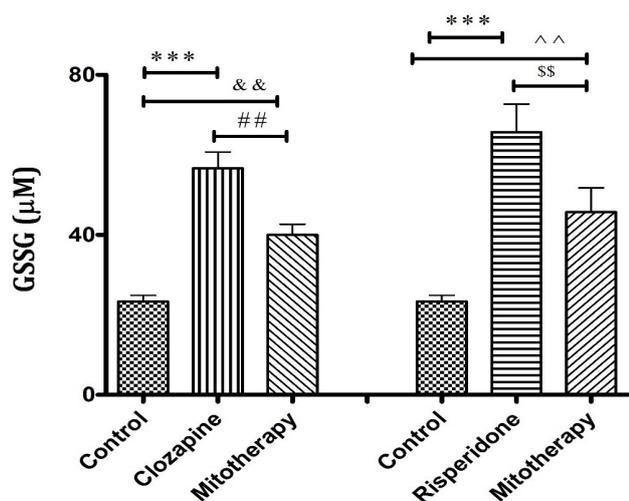
**Figure 3.** GSH assay. Effects of clozapine (70 µM) and risperidone (24 nM) treatment and mitotherapy on GSH content. \*\* $p < 0.01$  control vs clozapine. \*\*\* $p < 0.001$  control vs risperidone. #  $p < 0.05$  clozapine vs mitotherapy. \$\$\$  $p < 0.001$  risperidone vs mitotherapy.

#### Mitotherapy decreased GSSG content induced by CLZ/RIS

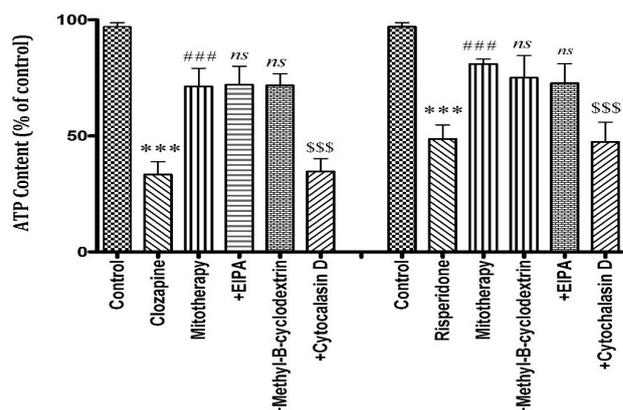
GSSG content assessment test revealed that CLZ (70 µM) and RIS (24 nM) treatment resulted in an increase of GSSG content in human lymphocytes ( $p < 0.001$ ) (Figure 4). The results showed that exogenous mitochondria, given at 2 hr after CLZ (70 µM) and RIS (24 nM) treatment, could prevent CLZ and RIS-induced GSSG content in human lymphocytes ( $p < 0.01$ ).

#### Mitotherapy improved ATP content decreased by CLZ/RIS

ATP content test revealed that CLZ (70 µM) and RIS (24 nM) treatment resulted in decreased ATP content in human lymphocytes ( $p < 0.001$ ) (Figure 5). The results showed that exogenous mitochondria, given at 2 hr after CLZ (70 µM) and RIS (24 nM) treatment, could prevent CLZ and RIS-induced decrease in ATP content in human



**Figure 4.** GSSG assay. Effects of clozapine (70 µM) and risperidone (24 nM) treatment and mitotherapy on GSSG content. \*\*\* $p < 0.001$  control vs clozapine/risperidone. &&  $p < 0.01$  control vs mitotherapy. ##  $p < 0.01$  clozapine vs mitotherapy. ^^  $p < 0.01$  control vs mitotherapy. \$\$  $p < 0.01$  risperidone vs mitotherapy.



**Figure 5.** ATP assay. Effects of clozapine (70 µM) and risperidone (24 nM) treatment and mitotherapy on ATP content. \*\*\* $p < 0.001$  control vs clozapine/risperidone. ###  $p < 0.001$  clozapine/risperidone vs mitotherapy. \$\$\$  $p < 0.001$  mitotherapy vs cytochalasin D (10 µM).

lymphocytes ( $p < 0.001$ ). For the mechanistic study, pre-incubation with cytochalasin D (10 µM) which is a well-known inhibitor of actin-dependent endocytosis, considerably reserved the protective effects of mitotherapy in the human lymphocytes, while the other reputed cellular internalization pathways inhibitors such as methyl-β-Cyclodextrin (caveola/clathrin dependent endocytosis inhibitor) and EIPA (macropinocytosis inhibitor) didn't show any significant effect on the protective effects of mitotherapy in the same lymphocytes (Figure 5), This finding shows that actin-dependent endocytosis plays a role in mitochondrial internalization.

#### Discussion

To the best of our knowledge, there is no report on the effect of exogenous healthy mitochondria co-incubation on human lymphocyte toxicity induced by CLZ and RIS. CLZ and RIS are drugs that cause damage to mitochondria. They can also increase the level of free radicals and reduce the level of intracellular antioxidant enzymes.<sup>3,4,26</sup> CLZ has the ability to oxidize proteins involved in energy metabolism in schizophrenic patients. In an animal study, it has been shown that CLZ has the ability to change mitochondrial function, energy metabolism in the brain of mice and rats. Also, this drug can change the expression of electron transport chain proteins.<sup>3</sup> In another research, it has been shown that CLZ has the ability to inhibit succinate dehydrogenase (SDH) enzyme in the brain. Also, this drug can disrupt the energy metabolism in the brain.<sup>27</sup> It has been reported that RIS caused a decrease in the activity of complex I in the mitochondrial respiratory chain. Also, it has been able to significantly inhibit the activity of complexes II and III.<sup>28</sup> In addition, RIS caused a decrease in the level of MMP, and cytochrome c in brain areas.<sup>29</sup>

Our study tested the effects of the co-incubation of exogenous healthy mitochondria on mitochondrial function, ROS production, MMP changes, GSH and GSSG content and, ATP content in human blood lymphocytes treated with CLZ and RIS. Mitochondria is an intracellular organelle with multi-functions.<sup>30</sup> This

organelle is an essential factor in regulating inflammatory and immune responses.<sup>17</sup> Mitochondrial dysfunction is involved in the development of more than 100 human diseases.<sup>30</sup> Mitochondrial transplantation or mitotherapy is a procedure in which mitochondria are transferred into cells that have defective mitochondria. Its purpose is to restore cell viability and ultimately prevent the progression of the disease.<sup>30,31</sup> In most studies, mitotherapy has shown positive results in various diseases and conditions, which can be promising from a therapeutic point of vision.<sup>13,16,31,32</sup>

In the testing of chemical substances, human lymphocytes are considered one of the important systems. As primary cells, they play a role in the initiation and progression of hematological related malignancies.<sup>33</sup> Free radicals and the resulting oxidative stress play a role in the development of toxicity in many organs. During the process of oxidative stress, the balance between ROS and intracellular antioxidants (such as GSH) is disrupted. Also, this process plays a role in the pathophysiology of many diseases.<sup>7,8,34</sup> Our results displayed that CLZ and RIS are able to rise ROS levels in the human lymphocytes following exposure. While, mitotherapy was able to reduce the ROS generated by CLZ and RIS, which indicates the effects of mitotherapy in the prevention of oxidative stress. MMP is known as one of the mitochondrial markers that are involved in the regulation of energy (ATP) production, oxidative phosphorylation, and ROS production.<sup>16</sup> Our results showed that the lymphocyte MMP level significantly declined in the groups treated with CLZ and RIS compared to the control group. However, an improved MMP level has been observed in CLZ and RIS-treated lymphocytes incubated with exogenous healthy mitochondria.

Research has shown that mitochondrial administration has been associated with an increase in GSH content and a decrease in the ROS level, which indicates the importance of using exogenous mitochondria in improving cell viability and function.<sup>17</sup> Furthermore, an improved GSH level and decreased GSSG level have been observed in human lymphocytes after incubation with exogenous healthy mitochondria. Glutathione as one of the most important intracellular antioxidants exists in two forms of GSH and GSSG, and its GSH form is more dominant. GSH plays an important role in regulating the oxidative stress process, and a decrease in its level increases the risk of cellular oxidative damage.<sup>35,36</sup> The results revealed that mitotherapy was able to increase the ATP content decreased by CLZ and RIS in human lymphocytes, which indicates the effects of mitotherapy in the prevention of ATP depletion.

### Conclusion

Our results showed that exogenous mitochondria transplantation improved mitochondrial dysfunction induced by CLZ and RIS in human lymphocytes by improving mitochondrial membrane changes, mitochondrial ROS level, GSH, and GSSG content, and also ATP production.

### Ethical Issues

The present study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences with the approval ID; IR.SBMU.PHARMACY.REC.1397.062.

### Acknowledgements

All the experiments were carried out in Department of Toxicology and Pharmacology at School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

### Author Contributions

Abdollah Arjmand: Investigation, Methodology, Formal Analysis. Elaheh Azizi Javan: Investigation, Formal Analysis. Jafar Shahraki: Conceptualization, Supervision. Rozhin Shaboustani: Investigation, Formal analysis. Enayatollah Seydi: Methodology, Formal Analysis, Writing – Review & Editing. Jalal Pourahmad: Conceptualization, Methodology, Formal Analysis, Supervision, Writing – Review & Editing.

### Conflict of Interest

The authors report no conflicts of interest.

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