# Risperidone Toxicity on Human Blood Lymphocytes in Nano molar Concentrations

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**ABSTRACT**

Risperidone is an atypical antipsychotic drug used for the pharmacotherapy of psychiatric disorders. Some reports indicate that risperidone is toxic to various systems of the body, including the immune system. This study evaluated the toxicity effect of risperidone on human blood lymphocytes. To achieve this aim, lymphocytes were isolated using Ficoll paque plus. The results showed that risperidone (12, 24 and 48 nM) causes toxicity in human blood lymphocytes by increasing the level of intracellular reactive oxygen species (ROS), damage to lysosomal membrane, the collapse of the mitochondrial membrane potential (MMP), and increased extracellular oxidized glutathione (GSSG). Also, exposure of human blood lymphocytes to risperidone is associated with a decrease in intracellular glutathione (GSH) levels. Finally, it could be concluded that oxidative stress is one of the mechanisms of risperidone-induced toxicity in human blood lymphocytes.

## Introduction

The immune system plays an important role in the protection of the body against harmful microorganisms and toxins [1]. Immunotoxicants are environmental contaminants and drugs that disrupt the immune system's balance and cause damage to living organisms [2]. Common environmental agents that contribute to immune deficiency are stress, some bacterial and fungal toxins, pesticides, alcohol and tobacco abuse, or pharmaceuticals such as antibiotics, chemotherapeutic agents, birth control pills, and neuropsychiatric drugs [3–5].

The atypical antipsychotics risperidone is FDA-approved first-line therapy for the treatment of schizophrenia, bipolar disorder,

and irritability associated with autism [6]. This medication may present a different set of adverse effects, including weight gain, cardiotoxicity, tardive and withdrawal dyskinesia, iron deficiency anemia, pituitary tumors, hyperprolactinemia, diabetes mellitus, and hyperlipidemia [7]. Clinical reports showed that risperidone treated patients are more susceptible to infections. Furthermore, laboratory studies reported immunosuppression, myeloid dysplasia in the bone marrow, and thymus involution by the risperidone, however, the exact molecular mechanisms for risperidone-associated immune dysregulation are unknown [8, 9].

Previous studies have shown that risperidone can cause cytotoxicity. The cytotoxicity induced by risperidone can be caused by an increase in the level of reactive oxygen species (ROS) and also dysfunction of vital organs including mitochondria and lysosomes. In addition, risperidone has the ability to decrease the level of intracellular antioxidant enzymes, including glutathione (GSH), and increase the level of lipid peroxidation (LPO). These events indicate oxidative stress [10–12]. Reports indicate that some antipsychotic drugs, such as risperidone, have the ability to inhibit and impair mitochondrial electron transport chain (mETC) function. Also, risperidone has higher toxicity effects on the mitochondrial than other atypical antipsychotics [11, 13, 14]. Mitochondria are known as one of the essential organelle that play a role in many physiological functions. In addition, its dysfunction is associated with many consequences, including an increase in the level of ROS and the induction of apoptosis [15–17].

In previous research, the cellular and molecular mechanism(s) of risperidone-induced human blood lymphocytes toxicity has not been evaluated. Therefore, the aim of our *in-vitro* study was to evaluate the toxic effect of risperidone on human blood lymphocytes by evaluating cytotoxicity, intracellular ROS generation, collapse of mitochondrial membrane potential (MMP), lysosomal membrane damage, intracellular reduced GSH and extracellular oxidized glutathione (GSSG).

## Materials and Methods

### Chemicals

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2 ′,7 ′-dichlorofuorescin diacetate (DCFH-DA), acridine orange, Rhodamine 123 (Rh 123) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO USA) (Cambridge. UK). Furthermore, all materials used in this study were purchased with the analytical grade.

### Blood samples and ethics statement

The study obtained ethical approval from the Shahid Beheshti University of Medical Science Ethics Committee (ID Number: IR.SBMU.PHARMACY.REC.1397.062). Lymphocytes were obtained from 20 healthy, non-smoking volunteers in the age range of 18 to 30 years old, who showed no signs of infection disease symptoms. After becoming aware of our investigation donors are asked to fill out the approval form.

### Isolation of Human Lymphocytes

Human Blood lymphocytes were isolated using the Ficoll standard method with some modification from fasting blood collected from volunteer donors. Five milliliters of the whole blood was diluted 1:1 with PBS and layered on Ficoll paque plus, centrifuged for 20 min at 2500 rpm, and lymphocytes layer were collected, suspended in erythrocyte lysis, and incubated for 5 min at 37°C. The gradient-separated lymphocytes were diluted 1:1 with PBS and centrifuged a second time at 1500 rpm for 10 minutes. The supernatant was eliminated, and the cells were washed twice with RPMI1640 with L-Glutamine and 10 % fetal bovine serum (FBS) at 2000 × g for 7 min. The cells were re-suspended in RPMI1640 medium with L-glutamine and 10 % FBS. Cell viability was determined using the trypan blue dye exclusion method, and only cell suspensions with viabilities of more than 96 % were maintained at 37°C in a humidified atmosphere with 5 % $CO_2$. The lymphocyte density used in the experiments was $10 \times 10^6$ cells/ml [18].

### Cell Viability Assay

The cell viability of isolated human lymphocyte was assessed by MTT staining. Lymphocytes were plated onto 96 well plate ($1 \times 10^4$ cells/ml) and incubated with risperidone at concentrations of 12.5, 25, 50, 100, and 200 nM for 6 hours for assessing probable cytotoxicity. In the following, MTT (20 µl) was added to each well and incubated for an additional 4 hours at 37°C. The purple-blue MTT formazan precipitate was dissolved in DMSO (100 µL) and the absorbance was measured at 570 nm on ELISA reader [19].

### ROS level assay

The intracellular ROS level was measured using DCFH-DA probe. Human blood lymphocytes were treated with risperidone at concentrations of 12, 24 and 48 nM for 2, 4, and 6 hours. Then, the medium was replaced by 10 µmol DCFH-DA containing medium, after 15 min incubation, the medium was removed, then, the cells were rinsed twice with PBS. The fluorescence intensity (DCFH-DA) was measured by fluorescence spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 495 nm and the emission wavelength of 530 nm [20].

### Lysosomal membrane damage assay

Human blood lymphocyte lysosomal membrane integrity was assessed by redistribution of the lipophilic dye acridine orange. Human blood lymphocytes were treated with risperidone (12, 24 and 48 nM) for 2, 4, and 6 hours. Then, the medium was replaced by an acridine orange (5 µM). After 10 min incubation, the fluorescence intensity (acridine orange) was measured by fluorescence spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 470 nm and the emission wavelength of 540 nm [21].

### MMP collapse assay

The alteration in the MMP collapse in human blood lymphocytes was measured by using the cationic fluorescent dye Rh123. Human lymphocytes were treated with risperidone (12, 24 and 48 nM) for 2, 4, and 6 hours. In the following, the medium was replaced by Rh123 (1 µmol), and after 15 min incubation the medium was removedand the cells were rinsed twice with PBS. The fluorescence intensity (Rh 123) was measured by fluorescence spectrophotom-

eter (Shimadzu RF5000U) at the excitation wavelength of 470 nm and the emission wavelength of 540 nm [22, 23].

### GSH and GSSG levels assay

After treatment of human blood lymphocytes with risperidone (12, 24 and 48 nM), cells were lysed with 0.5 ml of TCA 10 % and centrifuged at 11,000 × g for 2 min. For evaluation of GSH, the supernatant was diluted with phosphate-EDTA buffer and incubated with 100 µl of the o-phthalaldehyde (OPT) solution for 15 min at room temperature. For determination of GSSG, the supernatant of the cells was diluted with NaOH 0.1 N solution and before incubation with OPT, 200 µl of Nethylmaleimide [24] solution was incubated with supernatant for 30 min. The fluorescence intensity was measured by UV spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 350 nm and the emission wavelength of 420 nm [25, 26].

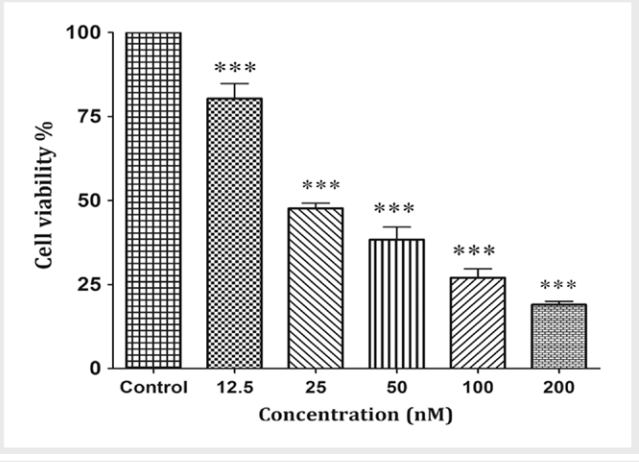
### Statistical analysis

Results are presented as mean ± SD (n = 3). Statistical significance was determined using the one-way and two-way ANOVA tests, followed by the post hoc Tukey and Bonferroni tests, respectively. Statistical significance was set at $P < 0.05$. One-way ANOVA test was used for the assessment of cell viability. Furthermore, two-way ANOVA test was used for the assessment of ROS generation, MMP collapse, lysosomal membrane integrity, and GSH and GSSG content.

## Results

### Risperidone decreased cell viability

After 6 hours of exposure, risperidone at concentrations of 12.5, 25, 50, 100 and 200 nM was able to reduce cell viability in human blood lymphocytes. The reduction in cell viability occurred in a concentration-dependent pattern (▶ **Fig. 1**). According to the MTT test, the $IC_{50}$ concentration was 48 nM. For subsequent experiments, $IC_{50}$ concentration (48 nM) and lower concentrations (12 and 24 nM) of it were used.




▶ **Fig. 1** Risperidone and of human blood lymphocytes viability. Cell viability determined by MTT assay after exposure of human blood lymphocytes with risperidone (12.5, 25, 50, 100 and 200 nM) for 6 hour ( $^{***}p < 0.001$ vs control group).

### Risperidone increased ROS level

Initially, ROS levels were assessed in human blood lymphocytes at 2, 4, and 6 hours after risperidone exposure (12, 24 and 48 nM). As shown in ▶ **Fig. 2**, risperidone was able to increase the level of ROS in human lymphocytes at all concentrations (12, 24 and 48 nM) and exposure times (2, 4 and 6 hours). The results showed a positive correlation between exposure of human lymphocytes to risperidone and an increase in the level of ROS (▶ **Fig. 2**).

### Risperidone induced lysosomal membrane damage

In ▶ **Fig. 3**, the results showed that risperidone in a concentration (12, 24 and 48 nM) and time (2, 4 and 6 hours) dependent pattern caused damage to the lysosomal membrane in human blood lymphocytes. An increase in fluorescence intensity indicates damage to the lysosome membrane (▶ **Fig. 3**).

### Risperidone induced MMP collapse

In the following, MMP collapse were assessed in human blood lymphocytes at 2, 4, and 6 hours after risperidone exposure (12, 24 and 48 nM). As shown in ▶ **Fig. 4**, risperidone was able to increase the MMP collapse in human lymphocytes at all concentrations (12, 24 and 48 nM) and exposure times (2, 4 and 6 hours) (▶ **Fig. 4**).

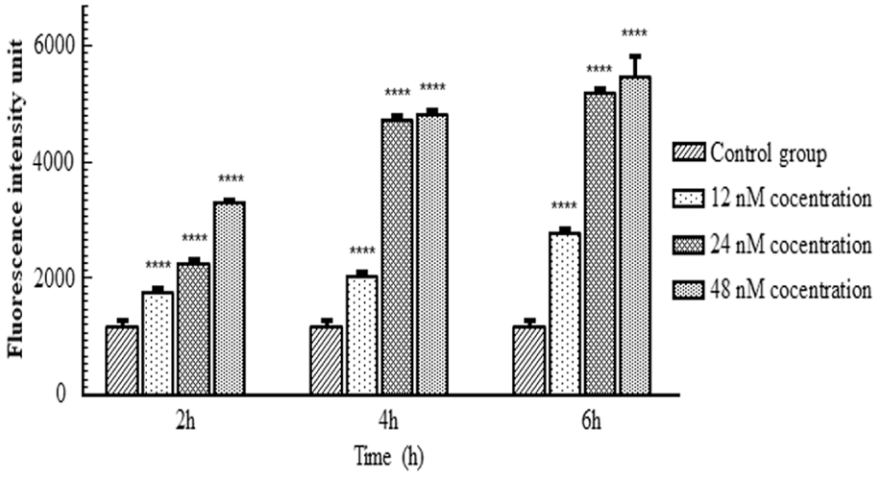
### Risperidone increased GSH level and decreased GSSG level

In ▶ **Fig. 5**, the results showed that risperidone in a concentration (12, 24 and 48 nM) and time (2, 4 and 6 hours) dependent pattern decreased the GSH level in human blood lymphocytes. On the other hand, risperidone in all concentrations and times used has been able to increase the level of extracellular GSSG (▶ **Fig. 6**).

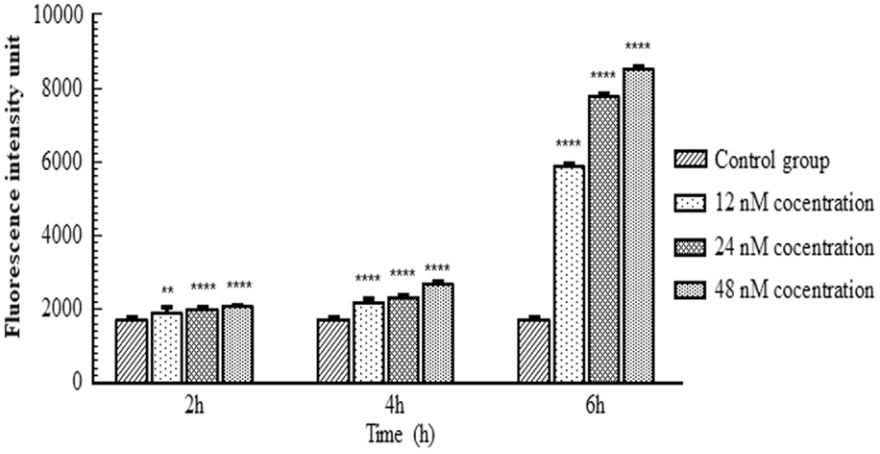
## Discussion

Atypical antipsychotic medications such as risperidone are widely prescribed for diverse psychiatric indications including schizophrenia, bipolar disorder, and depression. These medications have complex pharmacology and are associated with significant side effects [27]. The adverse effects, involving immune alterations, caused by risperidone have been widely studied. Clinical reports indicate that patients treated with the risperidone are more susceptible to infections. It seems the bone marrow compartment may be a key target of risperidone and its active metabolite, paliperidone, resulting in immunosuppression, myeloid dysplasia, leukopenia, neutropenia, lymphopenia, and thrombocytopenia.

Since the aim of this in vitro study was to investigate the effects of risperidone-induced toxicity on human blood lymphocytes, parameters such as cytotoxicity, level of ROS, collapse in the MMP, damage to lysosomal membrane, GSH and GSSG level were evaluated. Evidence showing the relationship between risperidone and decrease in platelet-associated antibodies titers, inhibition of phagocytosis and development of acute eosinophilic pneumonia. Furthermore, immune function pathways related to T cell maturation/differentiation were among the most heavily impacted by risperidone treatment, strongly suggesting that reduced plasma con-




▶ **Fig. 2** Risperidone and human blood lymphocytes ROS level. ROS level determined by DCFH-DA probe after exposure of human blood lymphocytes with risperidone (12, 24 and 48 nM) for 2, 4 and 6 hours ( $^{****}p<0.0001$ vs control group).

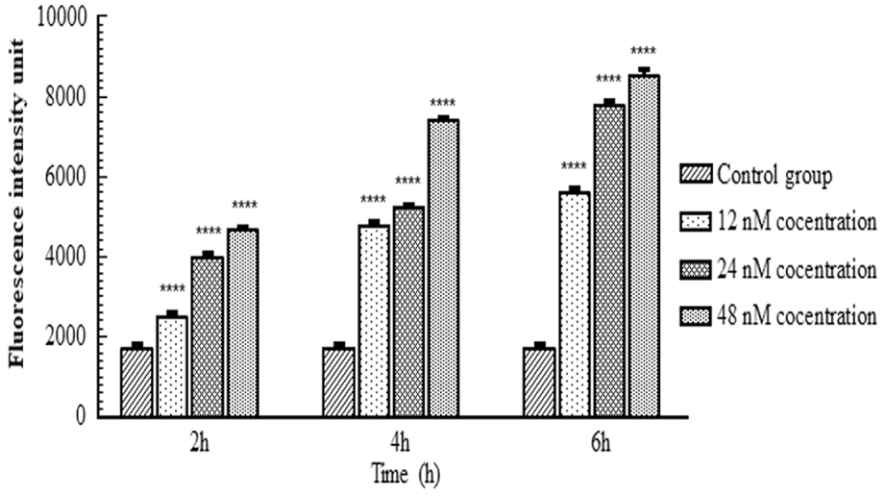



▶ **Fig. 3** Risperidone and lysosomal damage in human blood lymphocytes. Lysosomal damage determined by acridine orange probe after exposure of human blood lymphocytes with risperidone (12, 24 and 48 nM) for 2, 4 and 6 hours ( $^{**}p<0.01$ and $^{****}p<0.0001$ vs control group).

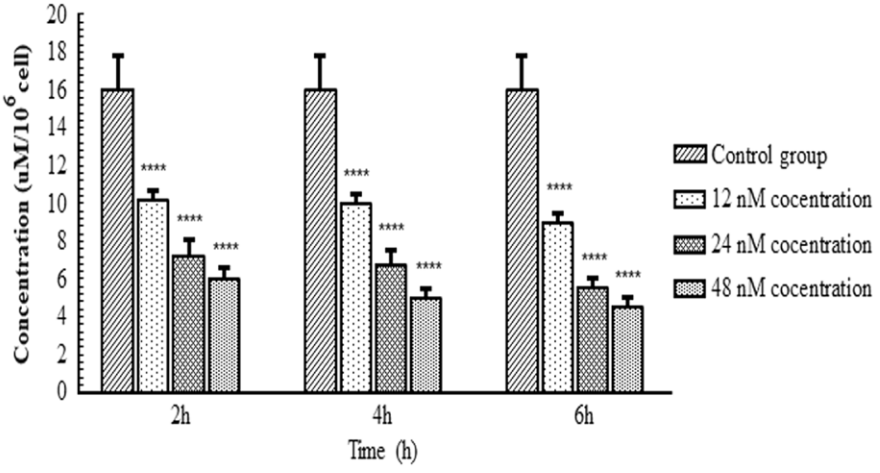
centrations of multiple cytokines and immune modulators is a mechanism for the risperidone induced immunosuppression [28, 29]. Previous experimental evidences have established risperidone can damage to the different cells by inducing oxidative stress [10, 11, 24].

Lymphocytes are the important cells of the immune system as they recognize the foreign antigens and mount an immune response; an increase or decrease in the number of these cells can lead to impaired immune system homeostasis [30]. At first and according to the MTT test results, risperidone (25, 50, 100, and 200 nM) leads to a decrease in human blood lymphocytes viability. The $IC_{50}$ of risperidone was 48 nM. A decrease in the lymphocytes viability might be associated with the direct toxicity of risperidone. These results are consistent with previous studies that have shown that risperidone has cytotoxicity on isolated hepatocytes [11]. Previous studies have shown that exposure of isolated hepatocytes to risperidone is associated with an increase in ROS generation, and this drug has been suggested as a ROS generator [11]. The results showed that risperidone increased the level of ROS in mitochondria at all concentrations and times used.

It is possible that the ROS generated by risperidone could inhibit the function of immune cells as a result of the peroxidation of lipids in membranes, which could then result in decrease of membrane integrity and impairment reactions of lymphocytes. In a most cell types, mitochondrial ROS are generated by the respiratory chain. Also, mitochondria are known as the most important source of ROS generation in cells. The balance of ROS is maintained by cellular processes that are involved in regulating its generation and antioxidant defense. The increase in the level of ROS is associated with the process of oxidative stress. This process is associated with



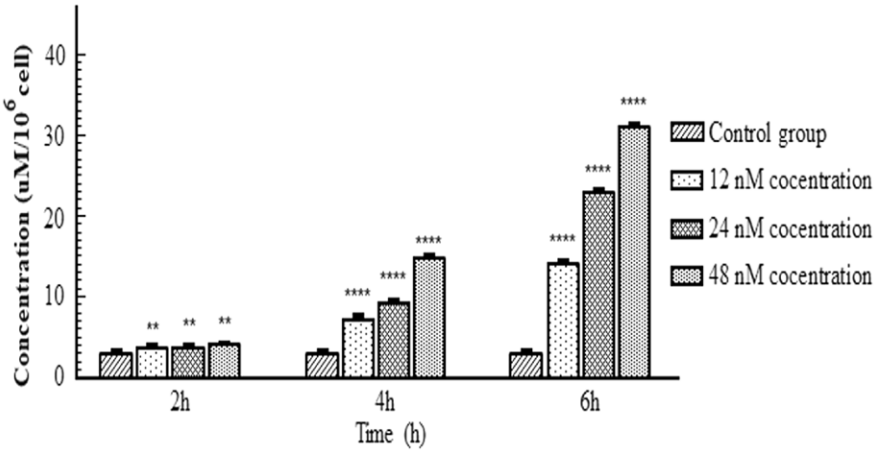

▶ **Fig. 4** Risperidone and MMP collapse in human blood lymphocytes. MMP collapse determined by Rh 123 probe after exposure of human blood lymphocytes with risperidone (12, 24 and 48 nM) for 2, 4 and 6 hours ( $^{****}p<0.0001$ vs control group).




▶ **Fig. 5** Risperidone and GSH level in human blood lymphocytes. GSH level determined after exposure of human blood lymphocytes with risperidone (12, 24 and 48 nM) for 2, 4 and 6 hours ( $^{****}p<0.0001$ vs control group).

irreversible consequences in different cells [31–33].The GSH is a tri-peptide that plays critical roles in important biological functions, including antioxidant defense and regulation of intracellular redox homeostasis. So, cellular decrease of GSH levels and increase of oxidized GSSG were evaluated as the indicator of oxidative stress. Our study showed a decrease in GSH levels and an increase in GSSG levels in risperidone-exposed lymphocytes., which is in line with previous studies [10, 11].

Mitochondrial dysfunction is defined as decreased mitochondrial biogenesis, altered membrane potential, and the fall in mitochondrial number and changed activities of oxidative proteins due to the accumulation of ROS in cells and tissue [34]. This study indicated that treatment of lymphocytes with risperidone lead to MMP collapse. These results are in agreement with previous studies that reported antipsychotic medications such as risperidone are potent mitochondrial toxicants by inhibition mETC function and collapse in mitochondria [35–37]. Furthermore, the results showed that risperidone caused damage to the lysosome membrane in treated lymphocytes. Lysosomal damages are associated with ROS especially superoxide radicals and hydrogen peroxide. Available a high iron (ferruginous) pool in the lysosomes, allows reactions yielding powerful oxidative species such as highly reactive hydroxyl radical's generation via Fenton-type reaction leads to LPO of membrane and lysosomes leakiness with subsequent release of the its digestive proteases. This destructive pathway leads to augmentation of oxidative stress from mitochondria and redox-active iron-rich lysosomes [38].




▶ **Fig. 6** Risperidone and GSSG level in human blood lymphocytes. GSSG level determined after exposure of human blood lymphocytes with risperidone (12, 24 and 48 nM) for 2, 4 and 6 hours ( $^{**}p<0.01$ and $^{****}p<0.0001$ vs control group).

## Conclusion

Finally, it could be concluded that risperidone causes mitochondrial and lysosomal damage and triggers oxidative stress in human blood lymphocytes.

## Acknowledgements

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### Conflict of Interest

The authors declare that there is no conflicts of interest.

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