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Clozapine-induced Toxicity via Induction of Oxidative Stress and Mitochondrial Dysfunction in Human Blood Lymphocytes and Protecting role of L-Carnitine

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Abstract:

Clozapine is a useful antipsychotic drug but with serious, life threatening toxicity effects. The aim of this study is to assess the direct cytotoxicity effect of clozapine (CLZ) on human blood lymphocytes and investigate the protective effect of L-carnitine (LC) against clozapine-induced cytotoxicity. Clozapine at 70 µM concentration induced cytotoxicity following 12 h. The Clozapine induced cytotoxicity was associated with intracellular reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP) collapse, lysosomal membrane injury, lipid peroxidation, and depletion of glutathione (GSH) and raising of oxidized glutathione (GSSG). We showed that LC (1 mM) has a beneficial cytoprotective effect against clozapine-induced toxicity. Summery, clozapine causes organelles damages and triggers oxidative stress in lymphocytes. These data suggest that using of L-carnitine could be useful for prevention and treatment of clozapine toxicity. Finally, it could be concluded that LC exerts a beneficial antioxidant and mitochondrial protective effects against oxidative stress induced by CLZ.

Keywords: Clozapine; Cytotoxicity; L-carnitine; Mitochondrial Damages; Oxidative Stress

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1. Introduction

Clozapine as a dibenzodiazepine, is an atypical antipsychotic drug. Also, clozapine is called an antimanic agent and is the most effective agent for affective psychotic mania condition. Clozapine can be used for treatment of refractory schizophrenia. This drug also reduces suicidal behavior in schizophrenia. Because of these beneficial effects of clozapine is used in treatment of schizophrenia, bipolar disorder (BD), acute manic/hypomanic/mixed and depressive episodes [1,2]. Previous studies showed that clozapine has adverse effects on blood and immune system with life-threatening potential, like agranulocytosis and neutropenia [3]. Mild neutropenia occurs more frequently than agranulocytosis. Also, transient neutropenia, occurs frequently in patients. Moreover, clozapine-treated patients may show a daily variation in the number of circulating neutrophils [4,5]. Because of these serious side-effects of clozapine, all patients treated with clozapine should be undergo regular white cell count monitoring. Clozapine is immediately discontinued if a patient develops leucopenia or neutropenia [5]. The incidence of drug-induced agranulocytosis, is estimated to be approximately seven cases per one million people, about 1–2% of patients [6]. Approximately 5 to 10 percent is the mortality rate of drug-induced agranulocytosis which decreases with early

identification and treatment [7]. Clozapine among the antipsychotics, shows the highest tendency to induce agranulocytosis. Sudden late onset of blood dyscrasia has been reported after treatment with clozapine [8]. The exact mechanism of this side effect is not yet known.

L-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid), as an endogenous mitochondrial membrane compound is a small water-soluble organic solute. For the transport of long-chain fatty acids into the mitochondria LC is essential [9]. It has been reported that LC has effective ROS scavenging activity, stopping peroxidation via the chelation of ferrous ions and protective effect on oxidative DNA damage in vitro models [10,11]. Some studies in mammals have reported the antioxidant properties of LC, which effectively prevented the lipid and protein resulting from oxidative stress and damage to DNA [11-13]. LC supplementation increased the levels of glutathione (GSH), enhanced the activities of antioxidant enzymes, and decreased the concentration of malondialdehyde (MDA) under some pathological conditions [11,14].

Fluctuation of the immune system could lead to a variety of adverse effects like enhancement or suppression of the immune response [15]. Suppression of the immune response in turn could lead to decrease in the host resistance to cancerous cells or infectious agents. Evaluation of drug immunotoxicity in humans is complex than in experimental animals and invitro models because of many factors. Moreover, clinical immunotoxicity data cannot be used in the assessment of drug immunotoxicity. Probably, immunotoxicity evaluation in in vitro model would be a good method for assessment of these conditions. The aim of our study is to investigate the toxic effect of clozapine and cytoprotective effect of LC on human lymphocytes by assessing cellular viability, reactive oxygen species (ROS) formation, mitochondrial membrane permeability transition (MMP) collapse, lysosomal membrane damage, intracellular reduced glutathione (GSH) and extracellular oxidized glutathione (GSSG) and lipid peroxidation.

2. Materials *&* Methods

2.1. Chemicals

RPMI1640 and FBS (Fetal Bovine serum) were taken from GIBCO (USA). Ficoll-paque PLUS was obtained from Ge Healthcare Bio-Science Company. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Trypan blue, 2′,7′-dichlorofuorescin diacetate (DCFH-DA), Rhodamine123, bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), acridine orange, L-carnitine and clozapine were obtained from the Sigma Chemical Co.

2.2. Sampling and Ethic statement

This research was done in Shahid Beheshti University of Medical Sciences at faculty of pharmacy and given ethical approval by research ethic committee of Shahid Beheshti University of Medical Sciences. Lymphocytes were obtained from 20 healthy donors in the age range of 18 to 30 years old, which did not exhibit any disease at the time of blood samples collection. After became aware of our investigation donors are asked to fill out the approval form.

2.3. Isolation of Human Lymphocytes

Lymphocytes were isolated using Ficoll standard method with some modification from blood collected. Diluted blood was layered on 3ml ficoll paque, centrifuged for 20min at 2500rpm and lymphocytes layer were collected, suspended in erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, 183 pH 7.4), and incubated for 5 min at 37 °C. Then, PBS was added immediately, and the cells were centrifuged at 1500*g* for 10 min at 20 °C. 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 resuspended in RPMI1640 medium with L-glutamine and 10 % FBS and counted using trypan blue exclusion dye. The viability of the cells was over 95% and cells were maintained at 37° C in a humidified atmosphere with 5% CO_2. The lymphocyte density used in the experiments was 10×10^6 cells/ml [16].

2.4. Cell Viability Assay

The cell viability of isolated human lymphocyte was assessed from the intactness of the plasma membrane as determined by the MTT assay. Lymphocytes were plated onto 96 well plate (1×10^4 cells/ml) and incubated with different concentration of CLZ (0, 50, 100 and 150 µM), for 12 h or IC50 12h CLZ +1 mM LC. After the incubation time, the cell viability was measured with MTT assay [16].

2.5. Measurement of ROS

The rate of ROS generation in human lymphocytes were evaluated by using the probe 2′, 7′- dichlorodihydro-fluorescein diacetate (DCFH-DA). In the presence of ROS, DCFH is oxidized to highly fluorescent

dichlorofluorescein (DCF). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells. Human lymphocytes were treated for 2, 4 and 6 h with different concentrations (35, 70 and 140 µM) of CLZ and IC50 12h CLZ +1 mM LC. After the incubation time, 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 Ca^{2+}-free PBS. The fluorescence intensity was measured by fluorescence spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 495 nm and the emission wavelength of 530 nm [17].

2.6. Measurement of MMP collapse

The alteration in the MMP collapse in human lymphocytes were measured by using the cationic fluorescent dyerhodamine-123. Rho-123 is a highly specific fluorescent dye for mitochondria in living cells. Human lymphocytes were exposed for 2, 4 and 6 h with different concentrations (35, 70 and 140 µM) of CLZ and IC50 12h CLZ +1 mM LC. After the incubation time, medium was replaced by 1 µmol rhodamine-123 containing medium, after 15 min incubation, the medium was removed, and then, the cells were rinsed twice with Ca^{2+}-free PBS. The fluorescence intensity was measured by fluorescence spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 470 nm and the emission wavelength of 540 nm [18].

2.7. Measurement of lysosomal membrane destabilization

Human lymphocyte lysosomal membrane integrity was assessed from the redistribution of the lipophilic dye acridine orange. Human lymphocytes were exposed for 2, 4 and 6 h with different concentrations (35, 70 and 140 µM) of CLZ and IC50 12h CLZ +1 mM LC. After the incubation time, medium was replaced by 5µM acridine orange containing medium. After 10 min incubation, the fluorescence intensity was measured by fluorescence spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 470 nm and the emission wavelength of 540 nm [19].

2.8. Measurement of Lipid Peroxidation

Lipid peroxidation was measured by using the thiobarbituric acid assay. This assay based on reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA). Human lymphocytes were exposed for 2, 4 and 6 h with different concentrations (35, 70 and 140 µM) of CLZ and IC50 12h CLZ +1 mM LC. After the incubation, lymphocytes were washed with PBS, and then lysed with PBS contain 2% triton. 100 µl of cell lysate was mixed with 200 µl of TBA reagent (containing 3.75% TCA and 0.0925% TBA) and the mixture was incubated at 90 °C for 60 min. After cooling, the mixture was centrifuged at 1000 × g for 10 min. Calorimetric absorption was measured at 530 nm [20]. Malondialdehyde standard curve was created using the stable MDA precursor, MDA bis dimethyl acetal. The amount of protein was evaluated by Bradford method [21].

2.9. Measurement of GSH and GSSG

GSH and GSSG levels in clozapine-treated human lymphocytes were measured by Hissin and Hilf method [22]. After treatment of human lymphocytes with clozapine, cells were lysed with 0.5 ml of TCA 10% and centrifuged at 11,000 × g for 2min. For assessment of reduced glutathione (GSH), 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 oxidized glutathione (GSSG), cells supernatant was diluted with NaOH 0.1N solution and before incubation with OPT, 200µl of N-ethylmaleimide (NEM) solution was incubated with supernatant for 30min. The fluorescence intensity was measured by UV spectrophotometer (Shimadzu RF5000U) at the excitation wavelength of 350 nm and the emission wavelength of 420 nm [22].

2.10. Statistical analysis

Each data presented as the Mean ± SEM of three separate experiments. Data were analyzed by GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using one and two-way analysis of variance followed by post hoc Tukey and Bonferroni test. P value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Effect of Clozapine on Cell Viability

Cytotoxic effect of clozapine on human lymphocytes shown in the Figure 1. Clozapine caused dose dependent cytotoxicity on human lymphocytes and significantly ($P < 0.05$) reduced cell viability in all used concentrations (0, 50, 100 and 150 µM). The IC50 observed was 70 µM in the RPMI1640 culture medium used in this study. The presented data demonstrated LC, as an antioxidant and mitoprotective agents prevent of cytotoxicity induced by CLZ.

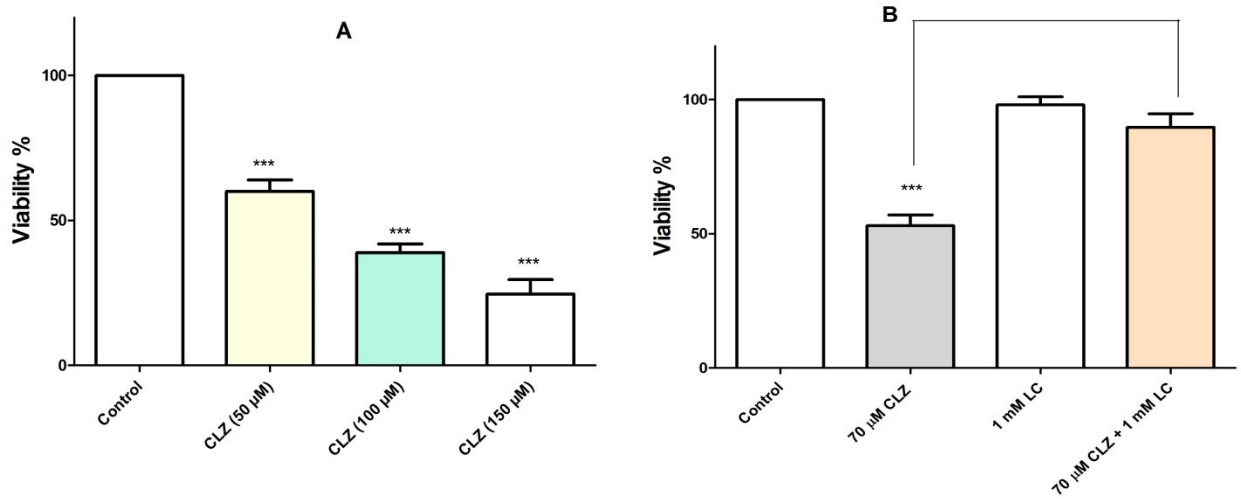

Figure. 1. Viability of human lymphocytes following treatment with CLZ for 12h (A) and cytoprotective effect of LC (B). Cell viability determined by MTT assay after incubation of lymphocytes with different concentration of CLZ (0, 50, 100 and 150 µM) for 12h. CLZ decrease lymphocyte viability in a dose-dependent manner and this decrease is significant at concentration higher than 50 µM. Presented data showed LC significantly decreased cell death compared to treated group with CLZ alone. (***p<0.001 vs. control, ###p<0.001 vs. treated groups with CLZ).

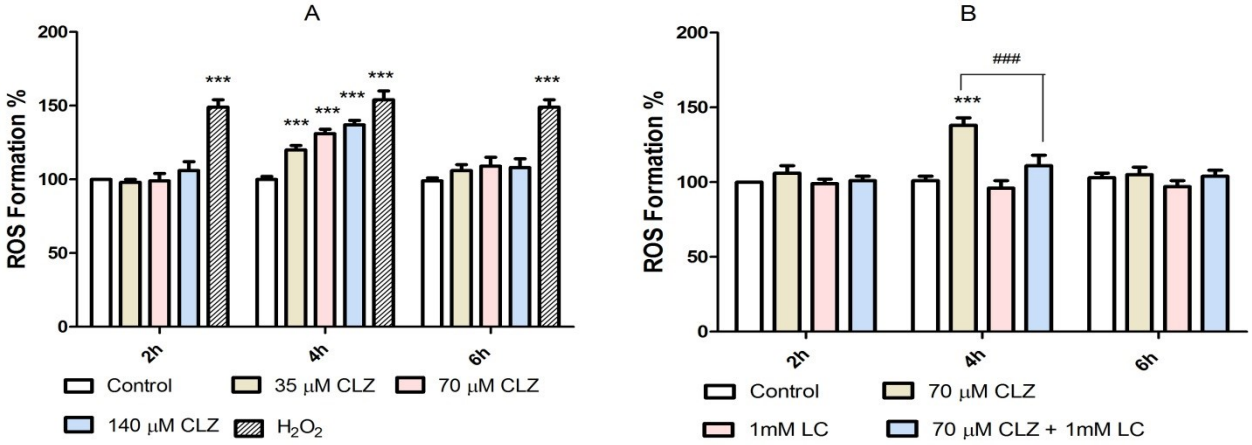

Figure. 2. ROS Generation in human lymphocyte after incubation with CLZ for different concentration (35, 70 and 140 µM) and time (2, 4 and 6 h) intervals (A) and protective effect of LC (B). CLZ has induced ROS production only in 4 h in all used concentrations. LC, inhibited CLZ-induced generation of ROS in isolated lymphocytes. (***p<0.001 vs. control, ###p<0.001 vs. treated groups with CLZ). DCF: fluorescent dichlorofluorescein.

3.2. Effect of Clozapine on ROS Productio

The effects of clozapine on the formation of ROS in human lymphocytes are shown in Figure 2. Clozapine has induced ROS production only in 4h. When the isolated lymphocytes were treated with CLZ + LC, the mean fluorescence intensities were significantly decreased compared to treated groups with CLZ.

3.3. Effect of Clozapine on MMP

The effects of clozapine on the MMP collapse of lymphocytes were investigated. The fluorescent dye rhodamine-123 was used to measure changes in MMP in humane lymphocytes. As shown in Figure3. Clozapine induced statistically MMP collapse at concentrations of 70 and 140 µM at 4 and 6h. As shown in the Figure 3, collapse of mitochondrial membrane potential was inhibited after treatment of human lymphocytes with CLZ by LC at toxic doses.

3.4. Effect of Clozapine on Lysosomal Membrane Destabilization

Figure 4 shows clozapine caused lysosomal membrane damage in human lymphocytes following 2, 4 and 6 h of exposure. All used concentrations of clozapine (35, 70 and 140 µM) caused significant (P <0.001) lysosomal membrane leakage in dose and time dependent manner. Clozapine-induced leakage of lysosomal membrane, inhibited by pretreatment of lymphocytes with LC (1 mM).

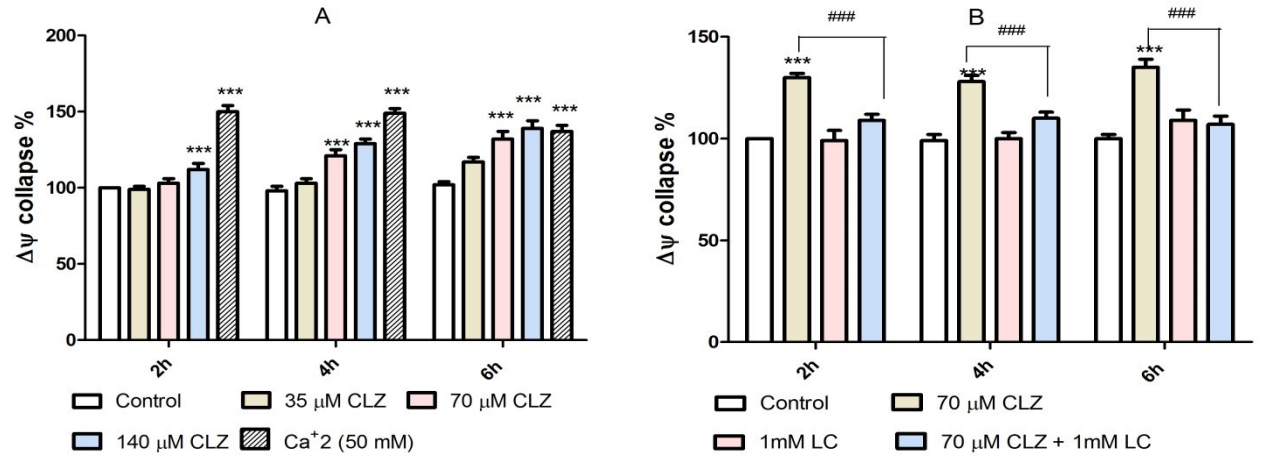

Figure. 3. Collapse of mitochondrial membrane potential (MMP) in human lymphocytes following incubation with CLZ for 2, 4 and 6h (A) and protective effect of LC (B). Collapse in mitochondrial membrane potential started 2 h after treatment of human lymphocytes with CLZ at concentration of 70 μM. CLZ-induced mitochondrial membrane potential collapse was time and concentration dependent. LC prevented CLZ - induced collapse in isolated lymphocytes (B). (***p<0.001 vs. control, ###p<0.001 vs. treated groups with CLZ).

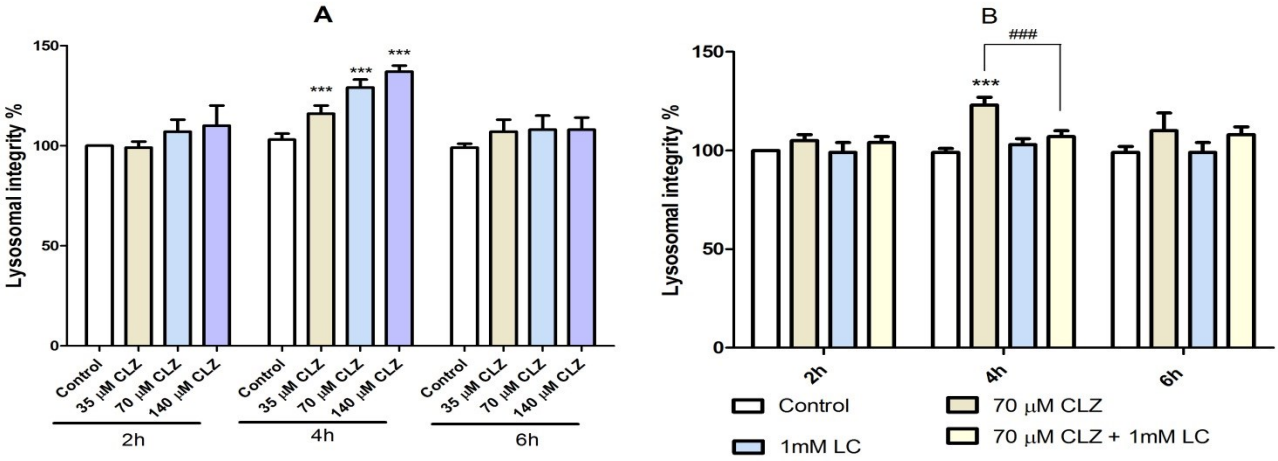

Figure. 4. Lysosomal membrane destabilization in human lymphocyte after incubation with CLZ (A) and protective effect of LC (B). Only after 4 h of treatment, all concentration of clozapine caused significant (P <0.001) lysosomal membrane leakage (***p<0.001 vs. control). LC prevented CLZ - induced lysosomal membrane leakage in human lymphocytes. (***p<0.001 vs. control, ###p<0.001 vs. treated groups with CLZ).

3.5. Effect of Clozapine on GSH and GSSG Content

As shown in Figure 5 collapse in intracellular GSH and raise in extracellular GSSG levels were measured in human lymphocytes after exposure with clozapine. Intracellular GSH levels significantly (P<0.001) decreased at 2, 4 and 6h following treatment of human lymphocytes with all clozapine concentrations (Figure 5A). Figure 6B shows the effects of clozapine on extracellular GSSG content. These results showed a significant (P <0.001) increase in lymphocytes extracellular GSSG level in concentration-dependent manner. Pretreatment of human lymphocytes with LC inhibited both collapse of intracellular GSH and increase in extracellular GSSG.

3.6. Effect of Clozapine on-Lipid Peroxidation

Lipid peroxidation as an indicator of oxidative damage to the lipids was measured in human lymphocyte using thiobarbituric acid reactive substances (TBARS) assay as a byproduct of lipid peroxidation. The amount of intracellular MDA as the result of lipid peroxidation significantly increased when cells incubated for 4 and 6h (70 and 140 μM) with clozapine (Figure 6). Pretreatment of human lymphocytes with LC decreased the lipid peroxidation level.

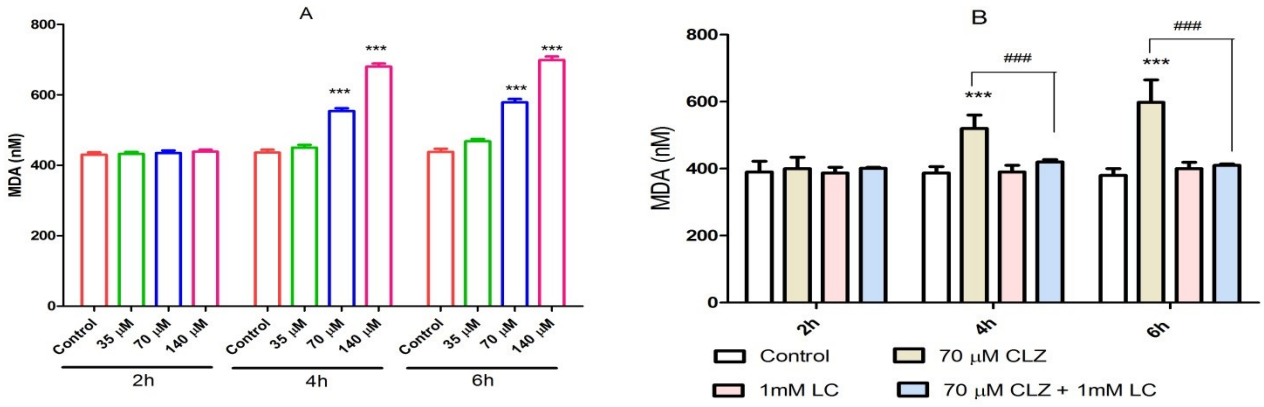

Fig. 5. Induction of lipid peroxidation in human lymphocyte after incubation with CLZ for 2, 4 and 6h (A) and protective effect of LC (B). Lipid peroxidation significantly increased when cells were incubated for 4 and 6 h (70 and 140 µM CLZ) with CLZ. LC prevented CLZ -induced lipid peroxidation in human lymphocytes. (***p<0.001 vs. control, ###p<0.001 vs. treated groups with CLZ).

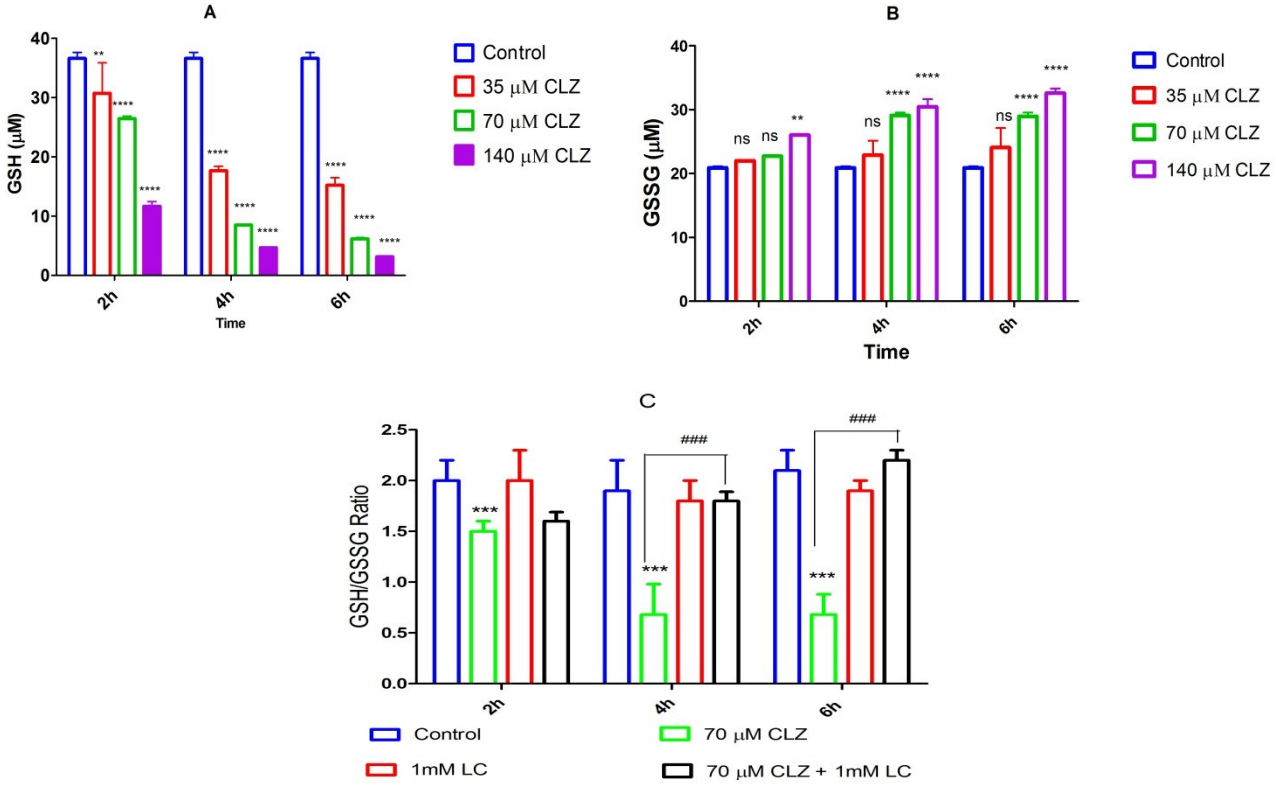

Fig. 6. Effect of CLZ on GSH and GSSG content of human lymphocytes (A and B) and protective effect of LC (C). As demonstrated in part A and B, a significant decrease (P<0.001) in GSH contents and a significant increase (P<0.001) in GSSG contents was observed at 2, 4 and 6h after treatment with CLZ. As demonstrated, significant (P<0.001) GSH/GSSG ratio decrease was found after treatment with CLZ. LC prevented depletion of GSH (***p<0.001 vs. control, ###p<0.001 vs. treated groups with CLZ).

4. Discussion and Conclusion

Clozapine is a second-generation antipsychotic, after introduction to market withdrawn due to fatal events of agranulocytosis and was subsequently reintroduced as a third-line drug for treatment of resistant schizophrenia. Popularity of clozapine has increased over the recent years [23]. The most serious side-effect of clozapine is

agranulocytosis. This condition is defined as a decrease in absolute neutrophil count <500 per cubic millimeter and can be deadly in the presence of an infection [24]. The pathogenesis of clozapine induced agranulocytosis/granulocytopenia (CIAG) has not been fully described [25]. Numerous studies provide an explanation to the pathogenesis of CIAG such as immune-mediated response against haptenized neutrophils [26], direct toxicity against bone marrow stromal cells [7], as well as immaturity of the neutrophil population and involvement of histamine H4 receptors in decline in granulocyte differentiation [27]. The first theory suggests hapten-based process is the background of pathogenesis of blood dyscrasias caused by clozapine. The fact that this condition develops again more rapidly if the patient is exposed to clozapine for the second time supports the theory of an immune mediated mechanism of CIAG [28]. The second described mechanism is the direct toxicity of clozapine against blood and immune cells. Therefore, in here for the first time we analyzed toxicity effect of clozapine on human lymphocytes as important cells in immune system. Obtained results showed that clozapine induces cell toxicity in the lymphocytes up to 70 µM (Figure 1). As expected, the toxicity of clozapine was efficiently prevented by pre-treatment with LC. It has been reported that LC (0.001–1 mM for 12 h) significantly increases the viability of HK-2 cells against 0.6 mM H_2O_2 toxicity [29]. Similarly, LC pre-treatment (1 mM) noticeable increased the cell viability in nickel-treated Neuro-2a cells. These data were consistent with our results.

Regardless toxic or immune backgrounds of hematological adverse effect, one of the best confirmed origins of CIAG is the involvement of metabolites of clozapine [30]. Clozapine has several metabolites such as N desmethylclozapine (NDMC), inactive clozapine N-oxide(CNO), and reactive oxygen species – the nitrenium ion [30]. It is generally accepted that NDMC causes toxicity via direct toxicity, rather than activation of immune system [31]. Today, more evidence confirm that clozapine induced CIAG through reactive metabolite like nitrenium ion [32]. Dehydrogenation of the piperazine-ring in clozapine, NDMC and CNO lead to formation of nitrenium ion [33]. In the liver, clozapine is metabolized by CYP450 isoenzymes such as CYP1A2, CYP3A4, CYP2D6, and CYP2C19. The N-demethylation and N-oxidation reactions of clozapine are mostly catalyzed by CYP1A2 and CYP3A4 [33]. Several CYP450 isoenzymes, like CYP3A4 and CYP2D6 are found in human liver as well as in lymphocytes [30]. Our results showed that clozapine induce ROS formation and lipid peroxidation in exposed lymphocytes. These data confirmed that probably presence of CYP3A4 isoenzyme in the human lymphocytes lead to formation of toxic active metabolites of clozapine (Figure 2, 5 and 6). Our data indicated that LC can effectively counteract ROS in human lymphocytes. Similar results on other cells and animal models have proven that LC exhibits strong antioxidant effects and prevents ROS generation [11,34,35]. Also, after the treatment of LC, the MDA values were decreased in presence of clozapine. The results were in accordance with other studies. This finding indicates that LC can protect cells from oxidative stress under our experimental conditions.

Previous studies demonstrated that clozapine treatment in neuroblastoma cells increase ROS and oxidized mitochondrial proteins important for energy metabolism [36]. In addition to direct protein oxidation and ROS formation, clozapine treatment has been associated with increased production of antioxidant proteins [37]. In here we indicated that treatment with 35, 70 and 140 µM clozapine in human lymphocytes incubated with the mitochondria-selective probe, rhodamine 123 at the highest concentration of clozapine (70 and 140 µM), lead to mitochondrial collapse (Figure 3). As mentioned above clozapine causes ROS formation and oxidation of mitochondrial proteins. The net impact of these events is opening of mitochondrial permeability transition pores (MPTPs), which contributes to swelling and death of lymphocytes [38].

Park discovered that clozapine inhibited autophagosome turnover resulting in a dysfunctional autophagic process, such as impaired lysosomal fusion. Their study showed, these results suggest that clozapine negatively affect neuronal viability, possibly through blocking autophagolysosome formation [39]. In here using acridine orang probe we showed that clozapine induces lysosome damages (Figure 4). Lysosomal damages are associated with reactive oxygen species especial hydrogen peroxide as a mobile active metabolite [40]. Zdolsek and Svensson showed that superoxide radicals and hydrogen peroxide are damaging to lysosomes. Available catalytically active iron in Fe(II) form, in the lysosomes, allows reactions yielding powerful oxidative species like hydroxyl radicals formed via Fenton reactions-to take place inducing peroxidation of the lysosomal membranes resulting in dispersal of the proton-gradient and leakage of their enzyme contents [41].

In summary, our results showed that clozapine induces cell death in human lymphocytes through ROS formation, mitochondrial and lysosomal damages. Also obtained findings indicated that using of LC may be promising agent in prevention and treatment of clozapine toxicity.

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Conflict of interest
None.

Ethics

The approved ethical code is:
IR.JAU.PS. REC.1397.389

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