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The antioxidant and neuroprotective effects of Zolpidem on acrylamide-induced neurotoxicity using Wistar rat primary neuronal cortical culture

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ABSTRACT

Zolpidem is an introduced medication for the therapy of sleeping disorders. Its pharmacological effects are consequently characterized by a quick onset and a half-life of 2.4 h. Previous studies revealed the antioxidant and neuroprotectant effects of zolpidem. In this research, we wanted to demonstrate the exact sub-cellular/molecular mechanism of this medication using the primary neuronal cortical culture.

For this purpose, firstly, the cortical neurons were isolated from the postnatal Wistar rat pups. Thereafter, different neural toxicity endpoints caused by acrylamide including ROS formation, lipid peroxidation, mitochondrial membrane potential collapse, lysosomal membrane integrity, and apoptosis were determined. All of these parameters are upstream events of cellular apoptosis which justifies neurodegeneration involved in many diseases such as Alzheimer's and Parkinson's.

Our results demonstrated that zolpidem at concentrations of 1 and 2 mM prevented all the acrylamide-induced above referenced neural toxic events leading to neuronal apoptosis.

These results revealed that zolpidem has the antioxidant and neuroprotectant properties that make it a promising prophylactic agent for preventing neurodegenerative complications. Considering the important role of oxidative stress in the development or progression of diseases, if the medication used as a treatment of a disease has antioxidant properties at the same time, it will certainly have much greater healing effects.

1. Introduction

The brain is vulnerable to oxidative stress because of high oxygen consumption and an unsaturated lipid-rich environment. The mitochondria, calcium, glutamate, and neurotransmitter auto-oxidation are also involved [1]. Oxidative stress occurs when the balance between the pro-oxidant and antioxidant in favor of the pro-oxidants. Therefore, the intracellular reactive oxygen species (ROS) elevated. In this unbalanced situation, the overproduction of ROS and/or the deficiency of the antioxidant defence mechanisms lead to many complications including mitochondrial and lysosomal damages, protein oxidation, loss of sulfhydryl groups and alterations of amino acids, which changes the protein functions, causing per-oxidative damages to lipid peroxidation and proteolysis with a consequent cell degeneration. This fact ends up in tissue damages, which are mainly irreversible [2–8]. Many studies demonstrated that oxidative stress has a critical role in the most of the brain and neuronal complications such as Parkinson's disease [9] and Alzheimer's disease [10]. Nunes, et al. demonstrated that there is a direct link between the lowered quality of life in mood disorders and an increased neuro oxidative stress [11]. Many efforts were conducted to find ways for preventing neuronal diseases [12–15]. Many studies suggest that antioxidant therapy could enhance neuroprotection [16–18]. Vitamin E and C, Omega-3 fatty acids, coenzyme Q10, GSH, rutin, melatonin, quercetin, lycopene, and crocin are such antioxidants that used as a neuroprotective agent against neurodegenerative disorders [19–23].

Zolpidem, a non-benzodiazepine hypnotic medication, has an imidazopyridine structure [N, N, 6-trimethyl-2-p-tolyl-imidazo (1, 2-a) pyridine 3-acetamideL-(+)]. Zolpidem is known to act with binding to the omega site of GABA benzodiazepine chloride channel receptor and create a complex with a Kd of 6–8 nM [24,25]. Zolpidem structure is similar to melatonin (amine heterocyclic structure). Therefore, it seems that zolpidem imitates melatonin properties such as its role in jet lag and its antioxidant effect [26–28]. Fig. 1 demonstrated the molecular structure of zolpidem and melatonin. Heterocyclic indole moiety that exists in both zolpidem and melatonin is known as an antioxidant

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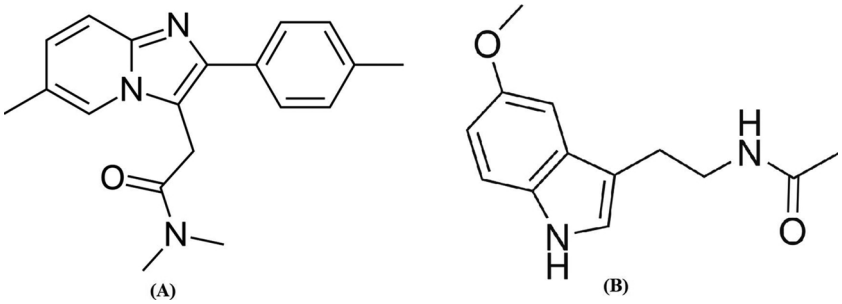


Fig. 1. Molecular structure of zolpidem (A) and melatonin (B).

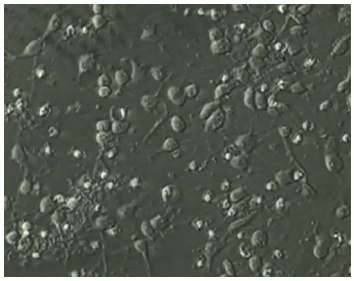


Fig. 2. Morphological feature of the isolated neurons after 24 h of incubation in 37 °C.

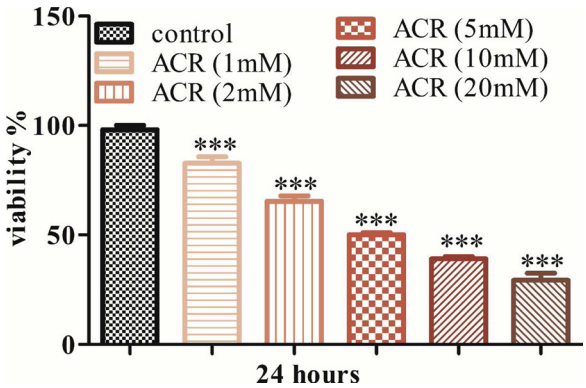


Fig. 3. Calculation of IC50 for acrylamide against isolated cortical neurons. Determination of cytotoxicity was done as the percentage of cells that absorb trypan blue. Values are shown as mean ± SD of three separate experiments (n = 3). *** $p < 0.001$ significant difference in comparison with non-treated neurons (control).

pharmacophore and trials in search of new antioxidants carrying this group were published [29–31]. The neuroprotective and antioxidant effects of zolpidem have recently been studied. Zolpidem illustrated a neuroprotective effect against neuronal death induced by glutamate in hippocampal cells. Its neuroprotective activities may be also attributed to the GABA-mimetic action [28]. There is one case report that zolpidem was useful in the patients with antipsychotic-induced refractory Parkinsonism symptoms [32]. Hasanvand, et al. evaluated the efficacy of zolpidem on the rat model of cisplatin-induced nephrotoxicity. Results showed that zolpidem exhibits its nephroprotective effect by decreasing oxidative stress and increasing the activity of the endogenous antioxidant system. So, it could prevent apoptosis in renal cells [33]. Bortolia, et al. inferred that zolpidem with its antioxidant capacity could prevent oxidative stress-related psychiatric disorders [34].

In this research, we used primary neuronal cortical culture for investigating zolpidem neuroprotective effects. The primary neuronal culture has many benefits. The greatest rapture of primary nerve cell culture is it makes living neurons immediately accessible to observation and manipulation. We used Kaech and Banker protocol for preparing the primary neuronal culture [35] and then we used them for toxicological evaluations.

As mentioned above, some researchers investigated the antioxidant efficacy of zolpidem, but the exact sub-cellular and molecular mechanism associated with that medication does not evaluated yet.

In this study, we wanted to know whether zolpidem, as an antioxidant and neuroprotective agent, could protect neurons against acrylamide-induced neurotoxicity in the primary neuronal culture model. Besides, we also wanted to find out the exact mechanisms of the antioxidative effect of zolpidem.

2. Materials and methods

2.1. Chemicals

Zolpidem tartrate BP was purchased from Ipca Co. 10 × Hank's balanced salt solution (HBSS), 1 M HEPES buffer, minimal essential medium (MEM) with Earle's salts and L-glutamine, penicillin-streptomycin, fetal bovine serum (FBS), neuro basal medium, glutaMAX-I supplement, B27 serum-free supplement, trypsin, cell-freezing medium from was purchased from Invitrogen. Acrylamide, D-glucose, sodium pyruvate, putrescine, progesterone, selenium dioxide, bovine transferrin, insulin from Sigma. Crocin was quantified through the authentic method in an aqueous saffron extract [36]. Crocin was 95% pure.

2.2. Animals

All experiments and procedures in this study were performed in full compliance with the NIH Guide for the Care and Use of Laboratory Animals guidelines for animal research and Shahid Beheshti University of Medical Sciences Animal Ethics Committee. Experiments were held on young (1-day-old pups) male Wistar rats.

2.3. Isolating the cortical neurons

Postnatal rat pups were killed after anesthetization. Under a laminar flow hood, the brains were removed and placed in a dish containing CMF-HBSS (Calcium-, magnesium-, and bicarbonate-free Hank's balanced salt solution). Under a microscope, the cerebral hemispheres and hippocampus were removed carefully and transferred to a drop of CMF-HBSS and chopped the tissues as finely as possible. The tissue pieces were transferred to a 50 ml conical centrifuge tube in a final volume of 12 ml CMF-HBSS containing 1.5 ml each of 2.5% trypsin and 1% (wt/vol) DNase and incubated for 5 min at 37°C. After triturating the suspension, the cell suspension passed through a cell strainer to remove chunks of undissociated tissue. For removing enzymes and lysed cells centrifugation was done (10 min at 120 g) and the supernatant was discarded. For determining the cell density a hemocytometer was used (the yield should be approximately 10^7cells per brain). Cells were suspended at a density of 10^6 cells/mL in the flasks in the Glial Medium. Each flask contained 10 ml of isolated neurons suspension. After one day of incubation, the neurons was used for toxicological tests [37].

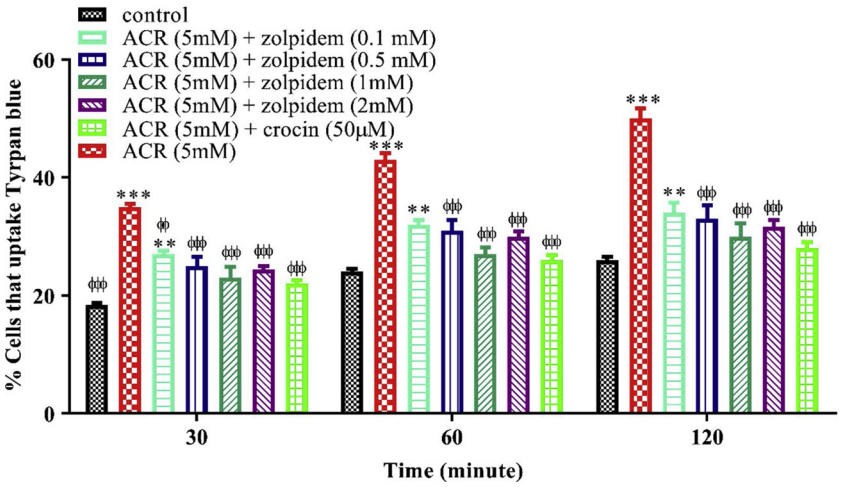


Fig. 4. Effect of different concentrations of zolpidem on acrylamide-induced neuronal death using primary neuronal cortical culture. Determination of cytotoxicity was done as the percentage of cells that absorb trypan blue. Values are shown as mean ± SD of three separate experiments (n = 3). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). ϕϕ $p < 0.01$, ϕϕϕ $p < 0.001$, significant difference in comparison with neurons that just received acrylamide (ACR 5 mM).

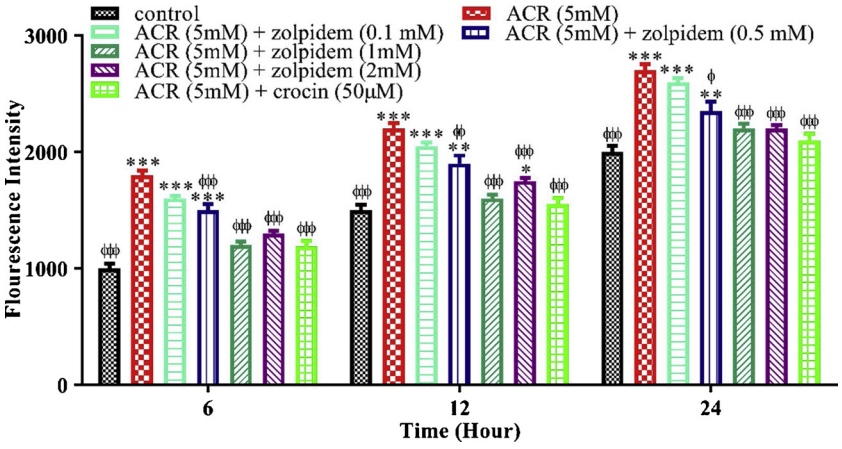


Fig. 5. Effect of different concentrations of zolpidem on acrylamide-induced ROS formation using primary neuronal cortical culture. Reactive oxygen specious were determined spectrofluorometrically by the measurement of highly florescent DCF. Values are shown as mean ± SD of three separate experiments (n = 3).* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). ϕ $p < 0.05$, ϕϕ $p < 0.01$, ϕϕϕ $p < 0.001$, significant difference in comparison with neurons that just received acrylamide (ACR 5 mM).

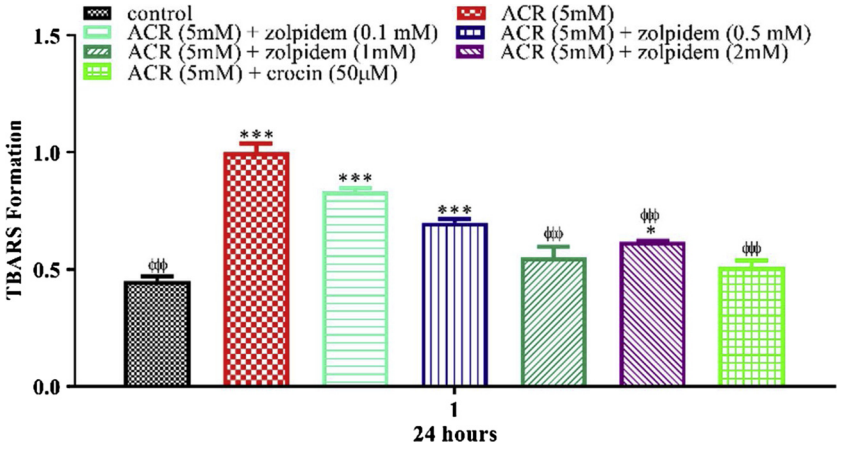


Fig. 6. Effect of different concentrations of zolpidem on acrylamide-induced lipid peroxidation using primary neuronal cortical culture. TBARS formation was measured spectrophotometrically and expressed as μM concentrations. Values are shown as mean ± SD of three separate experiments (n = 3). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). ϕϕ $p < 0.01$, ϕϕϕ $p < 0.001$, significant difference in comparison with neurons that just received acrylamide (ACR 5 mM).

2.4. Drug administration

The isolated neurons were exposed different concentrations of acrylamide (1, 2, 5, 10, 15, 20 mM) for evaluating the IC50 in 24 h (50% inhibitory concentration) by the trypan blue (0.2% w/v) exclusion test. The IC50 was determined by analyzing dose-dependent inhibition using the GraphPad Prism 6 statistical software (data provided in Fig. 2) [17].

After that, in order to assess the effect of zolpidem on acrylamide-induced neurotoxicity, the isolated neurons were cultured in 96-well microplates and incubated with different concentrations of zolpidem (0.1, 0.5, 1, 2, 3, 4, and 5 mM) for 24 h, the isolated neurons exposed to acrylamide 5 mM (final concentration which calculated based on IC50 concentration assay). After 24 h, the trypan blue (0.2% w/v) exclusion test was used for evaluating the cytotoxicity [38].

2.5. Cell viability

The survival of isolated neurons was performed with the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion test. Taking the samples of the incubated neurons were at different time points during 24 h [39].

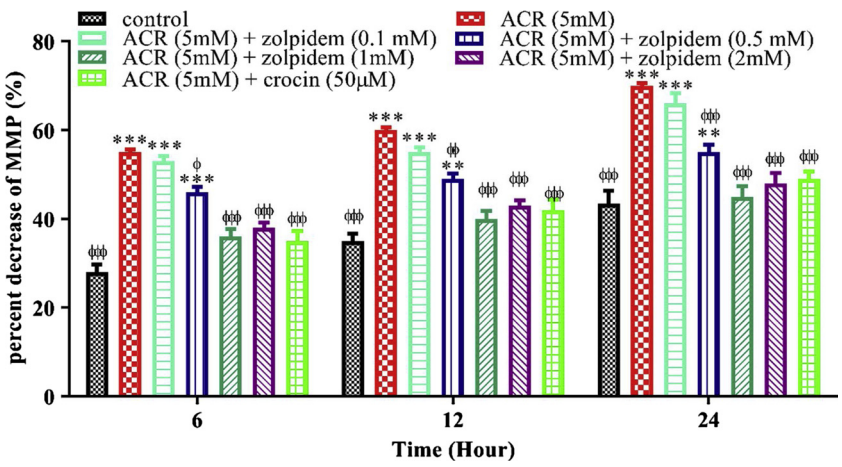


Fig. 7. Effect of different concentrations of zolpidem on acrylamide-induced mitochondrial membrane potential collapse using primary neuronal cortical culture. The difference in mitochondrial uptake of the rhodamine 123 between the untreated control and acrylamide treated cells is the biochemical basis for the measurement of the percentage of mitochondrial membrane potential decline. Values are shown as mean ± SD of three separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). ϕ $p < 0.05$, ϕϕ $p < 0.01$, ϕϕϕ $p < 0.001$, significant difference in comparison with neurons that just received acrylamide (ACR 5 mM).

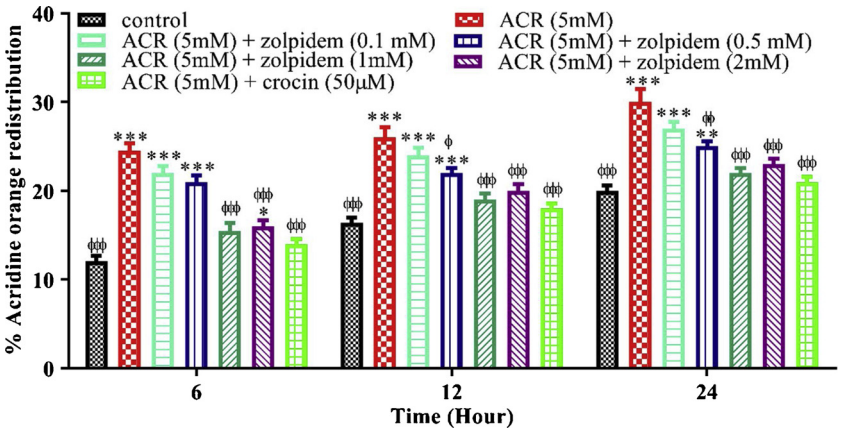


Fig. 8. Effect of different concentrations of zolpidem on acrylamide-induced lysosomal membrane degradation using primary neuronal cortical culture. The redistribution of acridine orange from lysosomes into cytosol in acridine orange loaded neurons was assigned as a biochemical basis for the measurement of lysosomal membrane injury. Highly florescent acridine orange redistribution was determined spectrofluorometrically in treated neurons and shown as the percentage of neurons lysosomal membrane leakage in all groups in three different time intervals. Values are shown as mean ± SD of three separate experiments (n = 3). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). ϕ $p < 0.05$, ϕϕ $p < 0.01$, ϕϕϕ $p < 0.001$, significant difference in comparison with neurons that just received acrylamide (ACR 5 mM).

2.6. Determination of reactive oxygen species

To determine the rates of ROS formation induced by acrylamide, dichlorofluorescin diacetate (1.6 μM) was added to the isolated neurons containing flasks. ROS were determined spectrofluorometrically by the measurement of highly florescent DCFH. The results were expressed as fluorescent intensity per 10^6 cells [40].

2.7. Lipid peroxidation assay

Isolated neuron lipid peroxidation was investigatedby measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides. The absorbance measured spectrophotometrically [41].

2.8. Intracellular GSH and extracellular GSSG assessment

GSH and GSSG were evaluated according to the spectrofluorometric method [42]. This method is based on a reaction between orthophthaldehyde and GSH (in pH 8) and GSSG (in pH 12) and formed a fluorescent substance.

2.9. Mitochondrial membrane potential (MMP) assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine123, has been used for the estimation of mitochondrial membrane potential [43]. Our data were shown as the percentage of MMP collapse (%ΔΨm) in all groups.

2.10. Lysosomal membrane integrity assay

Isolated neuron lysosomal membrane stability was evaluated by redistribution of the fluorescent dye, acridine orange. Our data were shown as the percentage of lysosomal membrane leakiness in all groups [44].

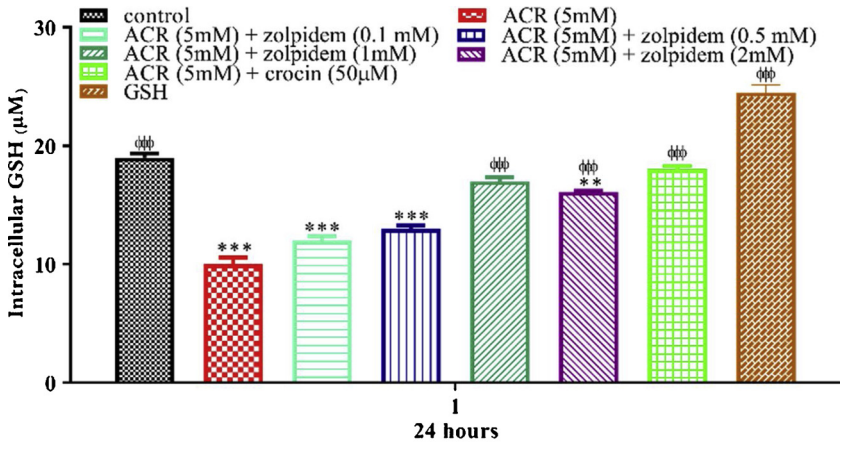
In all tests, crocin was used as a control antioxidant at the concentration of 50 μM based on previous researches [45].

2.11. Quantification of apoptosis

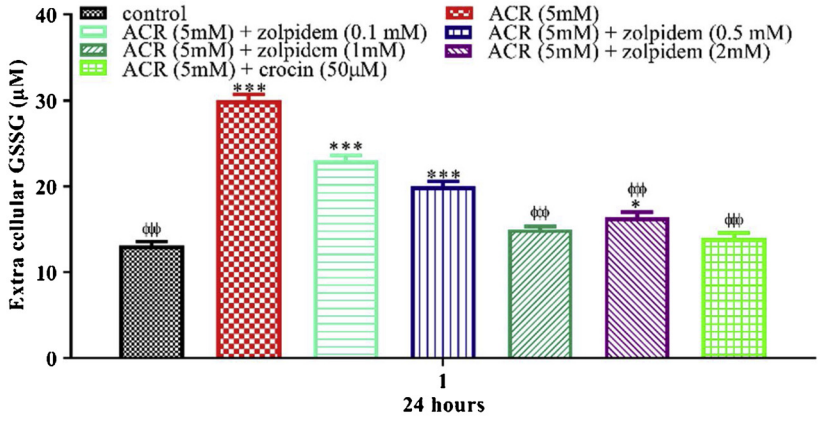
For evaluation the % apoptosis versus necrosis by flowcytometric analysis (Cyflow Space-Partec), Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) stained cells was applied. Annexin V/PI assay was done using a commercial kit (Immunotech; Beckman Coulter, Dubai, United Arab Emirates) according to the manufacturer's instructions [46].

2.12. Statistical analysis

The statistical analysis was done using the Graph Pad Prism software, version 8.0.2 (Graph Pad Software, San Diego, CA, USA). Data are reported as mean ± standard deviation (SD) of three separated tests. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test as the post hoc test. The normality was checked with the Kolmogorov-Smirnov test. This part was missed from the statistical analysis, Methods. The minimal level of significance chosen was $p < 0.05$.



(A)



(B)

Fig. 9. Effect of different concentrations of zolpidem on acrylamide-induced GSH depletion and the net values of GSH (A) and GSSG (B) using primary neuronal cortical culture. Intracellular GSH and extra cellular GSSG were measured spectrofluorometrically. Values are shown as mean ± SD of three separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). ϕϕϕ $p < 0.001$, significant difference in comparison with neurons that just received acrylamide (ACR 5 mM).

3. Result

3.1. Isolated cortical neurons

The morphological feature of the isolated neurons after 24 h of incubation in 37 ˚C has shown in Fig. 2.

3.2. Effect of acrylamide on cell viability in isolated neuron culture

As shown in Fig. 3, the treatment of cells with acrylamide for 24 h significantly reduced cell viability in a dose-dependent manner ($p < 0.001$). IC50 for acrylamide after 24 h exposure was 4.96 ± 0.87 mM.

3.3. Effect of zolpidem on acrylamide-induced cytotoxicity in isolated neuron culture

Fig. 4 revealed that the acrylamide-induced cytotoxicity was significantly ($p < 0.001$) prevented by zolpidem in concentrations of 1 and 2 mM. Zolpidem in concentrations of 0.1 and 0.5 mM had not a significant effect on cytotoxicity induced by acrylamide.

3.4. Effect of zolpidem on acrylamide-induced ROS formation in isolated neuron culture

As shown in Fig. 5, acrylamide produced a marked increase in ROS generation ($P < 0.001$) compare with control. Zolpidem at concentrations of 1 and 2 mM significantly ($p < 0.01$) prevented acrylamide-induced ROS formation.

3.5. Effect of zolpidem on acrylamide-induced lipid peroxidation in isolated neuron culture

As demonstrated in Fig. 6, acrylamide significantly increased the TBARS ($p < 0.001$) compare with control. Zolpidem at concentrations of 1 and 2 mM significantly ($p < 0.01$) prevented acrylamide-induced lipid peroxidation.

3.6. Effect of zolpidem on acrylamide-induced mitochondrial membrane potential collapse in isolated neuron culture

As a result of neuronal ROS formation, acrylamide induced a rapid decline of mitochondrial membrane potential, an apparent marker of

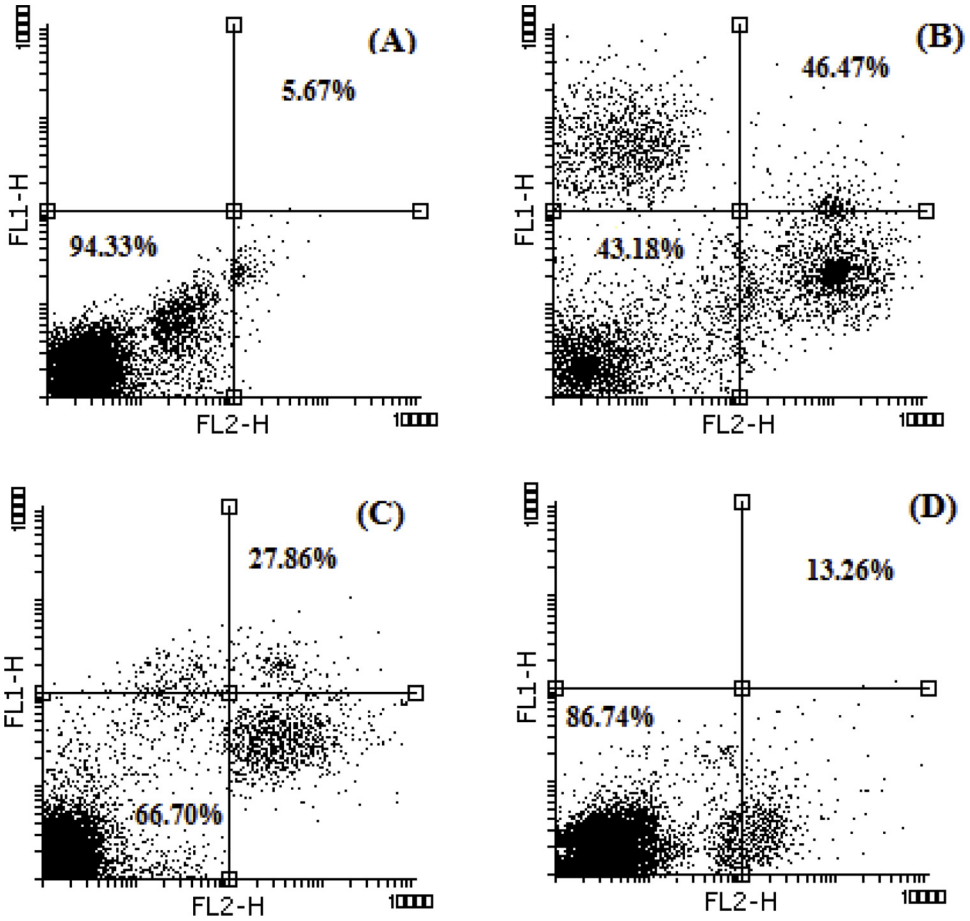


Fig. 10. Evaluation of apoptosis using flow cytometry in primary neuronal cortical culture. Values are expressed as mean ± SD of three separate experiments (n = 3). (A) untreated neurons, (B) neurons that incubated with acrylamide (5 mM), (C) neurons that incubated with acrylamide after incubation with zolpidem 1 mM, (D) neurons that incubated with acrylamide after incubation with zolpidem 2 mM (E).

mitochondrial dysfunction. Acrylamide-induced mitochondrial membrane potential was significantly collapsed when compared with control ($p < 0.001$). Mitochondrial membrane potential decrease was prevented by zolpidem at concentrations of 1 and 2 mM significantly ($p < 0.001$) (Fig. 7).

3.7. Effect of zolpidem on acrylamide-induced lysosomal membrane degradation in isolated neuron culture

When the isolated neurons were loaded with a lysosomotropic agent (acridine orange), severe oxidative damage to lysosomal membrane demonstrated a significant release of acridine orange into the cytosolic fraction ensued within 24 h of incubation with acrylamide (Fig. 8). Acrylamide-induced acridine orange release was significantly prevented by zolpidem at concentrations of 1 and 2 mM ($p < 0.01$).

3.8. Effect of zolpidem on intracellular GSH and extra cellular GSSG

As it is clear, incubation of hepatocytes with acrylamide caused another marker of cellular oxidative stress, rapid hepatocyte glutathione (GSH) depletion, Most of the acrylamide induced GSH depletion could be ascribe to the dismissal of GSSG (Fig. 9). Acrylamide induced GSH discharge was prevented by zolpidem at concentrations of 1 and 2 mM significantly ($p < 0.05$).

3.9. Effect of zolpidem on apoptosis/necrosis

The corresponding dot-plots illustrate apoptosis status, which are displayed in Fig. 10. The percentage of apoptotic cells (both early and late apoptosis) was significantly increased in acrylamide treated group ($p < 0.01$) (fig.10B). On the other hand, zolpidem at concentration of 1 and 2 mM significantly reduced the acrylamide-induced apoptosis ($p < 0.01$) (fig.10C and 10D).

4. Discussion

This research focuses on the antioxidant and neuroprotective effects of the non-benzodiazepine hypnotic zolpidem on isolated primary neurons. To the best of our knowledge, this is one of the first applications of these cells for evaluating the sub-cellular/molecular mechanism of a neuroprotective agent. In this study, acrylamide was used as a neurotoxin at the IC50 (5 mM). The results showed that acrylamide increased the level of intracellular ROS formation and lipid peroxidation. Besides, it decreased the amount of intracellular GSH and elevated the amount of extracellular GSSG. In addition, acrylamide (5 mM) caused a significant ($p < 0.05$) decline of mitochondrial membrane potential and a significant ($p < 0.05$) damage to lysosomal membrane within the 24 h of incubation. These results propose oxidative stress as the main mechanism for acrylamide-induced neurotoxicity at the concentration of 5 mM.

On the other hand, zolpidem with its antioxidative and neuroprotective properties significantly ($p < 0.05$) declined the ROS generation and lipid peroxidation. Zolpidem was as effective as crocin in preventing ROS generation and lipid peroxidation induced by acrylamide. Besides, zolpidem significantly ($p < 0.05$) protected isolated primary neurons against the decline of mitochondrial membrane potential and lysosomal membrane damages induced by acrylamide. The percentage of apoptosis increased following the administration of acrylamide. However, zolpidem pre-treatment prevented the apoptosis.

Previous studies demonstrated that zolpidem, dose-dependently reversed the increase in oxidative damage and the changes in neurotransmitters levels induced by haloperidol and chlorpromazine (only at

higher doses) [47]. Chlorpromazine and haloperidol caused an increment in the synaptic release of glutamate in different brain regions, so results in neuronal degeneration. Furthermore, free radical generated by chronic administration of neuroleptics can inhibit presynaptic glutamate uptake [48]. That leads to inactivating enzymatic defence systems against cellular damages such as the reduction of GSH as the main intracellular antioxidant that protects the cell against intracellular ROS formation and lipid peroxidation [49]. Thereafter, the increase in ROS formation, disrupting mitochondrial electron transport chain, and lysosomal membrane damage leading to cellular death [50]. Zolpidem has a heterocyclic amine structure; it is structurally similar to melatonin, a potent neuroprotective and antioxidant agent. It seems that the heterocyclic amine structure is critical for the antioxidant properties of both melatonin and zolpidem. Therefore, zolpidem and its derivatives have antioxidant, neuroprotective and free radical scavenging activities [51]. Bishnoi, et al. demonstrated that zolpidem has comparable antioxidant activity to melatonin but less effective than trolox and estradiol in antipsychotic-induced orofacial dyskinesia [47]. Although the antioxidants have the ability to reduce oxidative stress markers [52–54], the sole antioxidant treatments have not been able to cure any disease; the sole antioxidant treatments have not been able to cure any disease. On the other hand, a medication that can treat either the primary cause of disease and at the same time reduce the oxidative stress associated with that disease might be more effective than a molecule that treats only the primary cause of disease and zolpidem has these both features [47]. Although in our study there was no significant difference between our positive antioxidant control crocin as and zolpidem, we also contemplate that because of the involvement of oxidative stress and apoptosis in the development or progression of neurodegenerative diseases. This medication can be promising with certainly much greater healing effect.

5. Conclusions

In this study, the antioxidant and neuroprotectant effect of zolpidem was evaluated and these effects were compared with crocin. In this respect, we concluded that examining the exact mechanism of action of antioxidant agents are important for specifying their therapeutic effects. Therefore, the determination of antioxidant capacity for common CNS medications could be very valuable for designing novel medication regimens for treating neurodegenerative diseases

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.01.010.

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