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Neuroprotective Effect of Midazolam Low Dose against Glutamate Toxicity Induced to Olfactory, Cortex and Cerebellum Neurons Culture: *In vitro* Study

Muhammet E. Naldan[1]* and Ali Taghizadehghalehjoughi[2]

[1] *Department of Anesthesia and Reanimation, Turkey Health Sciences University, Erzurum Regional Training and Research Hospital, Erzurum, Turkey*

[2] *Department of Pharmacology and Toxicology, Atatürk University, Erzurum, Turkey*

**Corresponding e-mail: muhammetnaldan@gmail.com*

ABSTRACT

Different neuronal cells are found in olfactory bulb, cortex and cerebellum regions. Olfactory regulates complex behaviors, cerebellum has a great role in body balance and cortex mainly coordinate complex motor and sensory behaviors. The anesthetic is given to all patients (from birth to death) who undergo surgery. Midazolam is GABA agonist and used as an anesthesia. Glutamate an excitatory neurotransmitter can reach toxic levels in traumatic brain injury to cause schizophrenia and behavior disorders. In this study, we obtained 3 different olfactories, cortex and cerebellum cell culture. Glutamate 5-10 mM was added to all culture dishes except the negative control group. Midazolam was added in 4 different doses for 24th and then evaluation was done by MTT, TAC (Total antioxidant capacity), TOS (Total oxidant status) and flow cytometry (Annxin V-apoptosis marker). Our results show that although ketamine is NMDA antagonist there is an increased toxicity level of glutamate and reduce cell viability.

Keywords: Midazolam, Olfactory, Glutamate, Neurotoxicity, Cortex, Cerebellum

INTRODUCTION

Different neuronal cells are found in olfactory bulb, cortex and cerebellum regions. Olfactory regulates complex behaviors, cerebellum has a great role in body balance, and cortex mainly coordinate complex motor and sensory behavior's [1-3]. Those neurons majorly work by glutamate and GABA neurotransmitter [4,5]. Glutamate is main excitatory mediator and GABA in reverse has an inhibitory effect. Extracellular elevated glutamate levels induce neuronal damage [6]. In cerebral hypoxia/anoxia, the glutamate transporter did not work properly and extracellular glutamate level increases and cause irreversible neuronal damage [7]. Also, glutamate by attaching to NMDA, AMPA receptors for a long time causes Ca^{++} and Na^{+} influx. GABA in response by opening CL- channel and influx negative ion into the cytoplasm establishes resting potential [8,9].

Midazolam was discovered in 1955 and was clinically used for first time by Wasser, et al., at 1976 [10]. Midazolam is GABA agonist and the first benzodiazepine that was used as anesthesia [11]. There are many uses of midazolam, including sedation, anesthesia induction, and procedures for diagnosis and treatment in the perioperative period. Midazolam is preferred because of its rapid, painless induction and non-venous irritation [12].

Midazolam has great potential use in patients with neuronal illness background. In the present study, we evaluate midazolam different doses to determine midazolam proper use in glutamate toxicity model. For this, we used MTT, TAC, TOS and apoptosis marker. The evaluation of midazolam at the same time in 3 neuronal base cell line (olfactory, cortex and cerebellum neuron culture) was done for the first time.

Significance Statement

- Olfactory, cortex and cerebellum culture were prepared
- Glutamate 5-10 mM toxicity was induced to culture and then treatment was added in 4 different doses

- MTT, TAC, TOS and apoptosis marker (annexin v-FTCR) test were done to determined cell viability, antioxidant capacity, oxidant level, and apoptosis ratio

PATIENTS AND METHODS

Chemicals and Reagents

Midazolam was purchased from BFARM Company (Berlin, Germany). Dulbecco modified eagle's medium (DMEM), Fetal calf serum (FCS), Neurobasal medium (NBM), MTT, phosphate buffer solution (PBS), antibiotic antimitotic solution (100X), L glutamine and trypsin-EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA).

In vitro Studies

Cell cultures: Olfactory, cortex and cerebellum cell cultures were obtained from Department of Medical Pharmacology at Ataturk University (Erzurum, Turkey). Briefly, the cells were centrifuged in 1200 rpm for 5 min and were then seeded in 24 well plates (Corning, USA) by fresh medium (Neurobasal medium, FBS 10%, B27 2%, and antibiotic 0.01%) and then stored in incubator (5% CO_2; 37°C) (Nuaire, USA) [13] (Figure 1).

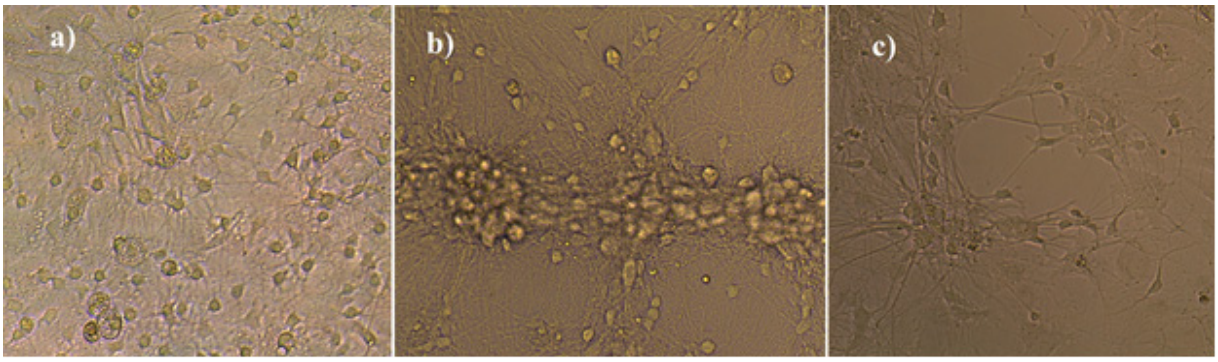

Figure 1 Harvested cell line 10X: a) cerebellum neuron cells; b) cortex neuron cells; c) olfactory neuron cells

Glutamate toxicity: By 10th day the cells have adequate branches. All medium and glutamate 5-10 mM for inducing toxicity were added to each well except negative controls. After 10 min midazolam (50 µM, 100 µM, 200 µM and 400 µM) final concentration was added to each well except negative control (NC) group and was incubated for 24 hours (5% CO_2; 37°C). As a negative control, 150 µL of NBM was only added to each well and positive control contained only 5-10 mM glutamate for 24 hours.

MTT Assay

Then, MTT assay was carried out by a commercially available kit (Sigma alderich, USA). Briefly, MTT reagent (10 µL) was added to the well and the plate was incubated (5% CO_2; 37°C) for 4 hours. Then, the medium was discarded and 100 µL of dimethylsulfoxide (Sigma, USA) was added to each well. The optical density was determined at 570 nm using Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA) and the cell viability (%) was calculated [14].

$$\text{Viability \% Ratio} = \frac{\text{Sample absorbance value}}{\text{control group absorbance value}} \times 100$$

Total Oxidant Status (TOS)

In total oxidant status (TOS) assay, the assessment is done by measuring spectrophotometrically the density of the color related to the number of oxidants in the sample. In the present study, TOS (Total Oxidant Status) kits manufactured by Rel Assay Diagnostics® Company (Turkey) were used.

The components in the kit were reactive 1 solution, reactive 2 solution, standard 1 solution, and standard 2 solution. In order to determine the TOS level; 500 µl reactive 1 solution was added to the wells in which 75 µl plasma sample was present and after reading the initial absorbance value at 530 nm, 25 µl reactive 2 solution was added in the same well and second absorbance was read at 530 nm at the end of the waiting period of 10 minutes at room temperature.

Standard 2 solution in the kit was used for standard 2. By using the absorbance values obtained and the following formula, TOS levels were determined in mmol Trolox Equiv./L [15].

$$TOS = \Delta sample / \Delta ST2 \times 20$$

ΔST2 (Δstandard 2=ST2 second reading-ST2 first reading),

ΔSample (ΔSample=sample second reading-sample first reading)

Total Antioxidant Status (TAS)

In TAS assay, antioxidant capacity was determined by inhibiting the formation of the 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate=ABTS+) radical cation. In the assay process, Rel Assay Diagnostics® Company (Turkey) commercial kit was used.

The components of the kit were reactive 1 solution, reactive 2 solution, standard 1 solution, and standard 2 solution. In order to determine the TAS level; 500 µl reactive 1 solution was added in the wells containing 30 µl sample and the first absorbance was read at 660 nm. Then, 75 µl reactive 2 was added to the same wells and allowed to wait at room temperature for 10 minutes. At the end of the waiting period, the second absorbance value was read at 660 nm. While distilled water was used for standard 1, and standard 2 solution in the kit was used for standard 2. The absorbance values obtained were placed according to the following formula and TAS levels were determined in mmol Trolox Equiv/L [16].

TAS=(ΔST1-ΔSample)/(ΔST1-ΔST2)

ΔST1 (Δstandard 1=ST1 second reading-ST1 first reading), ΔST2 (Δstandard 2=ST2 second reading-ST2 first reading), ΔSample (ΔSample=sample second reading-sample first reading)

Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) staining assay

The cells (105) were collected, washed and stained after treatment with midazolam at final concentrations of 50 mM, 100 mM, 200 mM, and 400 mM after 24 hours, according to the manufacturer's protocol (Biovision, USA). Briefly, cells were washed with PBS and after adding 500 µL binding buffer, annexin v-FITC and PI were added in the dark for 10 min at room temperature. The stained samples were then analyzed on a CytoFLEX flow cytometer as instructed by the manufacturer (Beckman Coulter, USA) [17].

Statistically Analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's HSD using the SPSS version 20.0 software. The $p<0.05$ was considered as statistically significant for all tests.

RESULTS

MTT Assay

Olfactory, cortex and cerebellum culture were prepared. After 24 hours midazolam (50 µM, 100 µM, 200 µM and 400 µM) exposing time, the experiment was finished by adding MTT solution. The data were analyzed and showed in Figure 2.

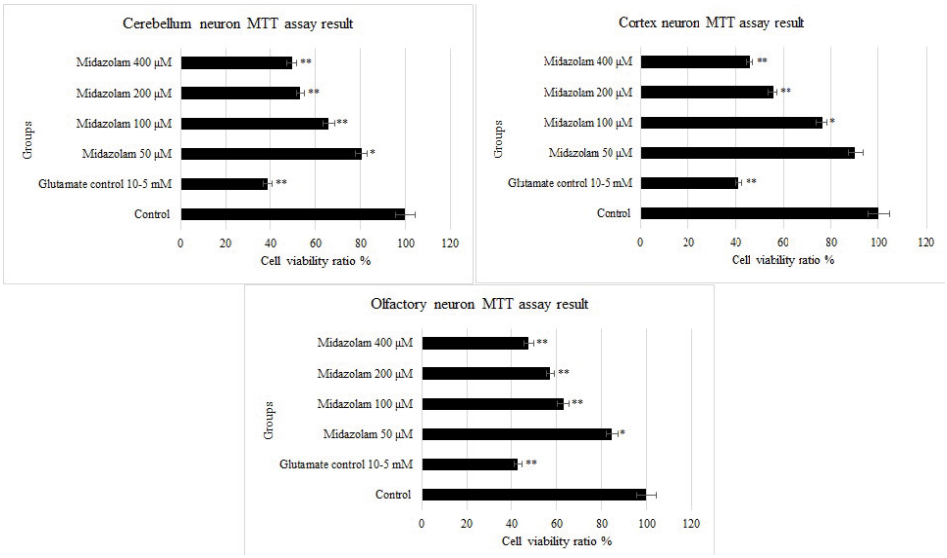

Figure 2 MTT assay result of cerebellum, cortex and olfactory cell line after 24 hours treatment by midazolam; *(p<0.05), **(p<0.001)

According to our result cerebellum neuron show highest and cortex neurons show the lowest viability ratio. Also, positive control group (only have 10-5 mM glutamate) have viability ratio near 40%. In all treatment and all cell type data is significantly different in comparison with the control group. Midazolam in 400 mM shows lowest viability ratio in all neuron cultures. According to our data midazolam low dose effectively reduce glutamate toxicity effect in cortex culture but cerebellum and olfactory, 50 mM midazolam did not increase viability higher than 85%. It means neuron cortex can tolerate toxicity by midazolam till 50 mM but olfactory bulb and cerebellum neurons viability is highly reduced (Figure 2).

TAC Assay

The total antioxidant capacity of neurons examined the data is shown in Figure 3. NC showed highest antioxidant capacity compare to treatment. TAC status in cortex and olfactory in 50 mM are near to NC group but in the cerebellum, there is a statistical difference ($p<0.05$). High dose of midazolam did not increase antioxidant capacity higher than 3.8 trolox equiv/mmol^{-1}. In glutamate control group antioxidant capacity is lowest in an amount in compararison to treatments (Figure 3).

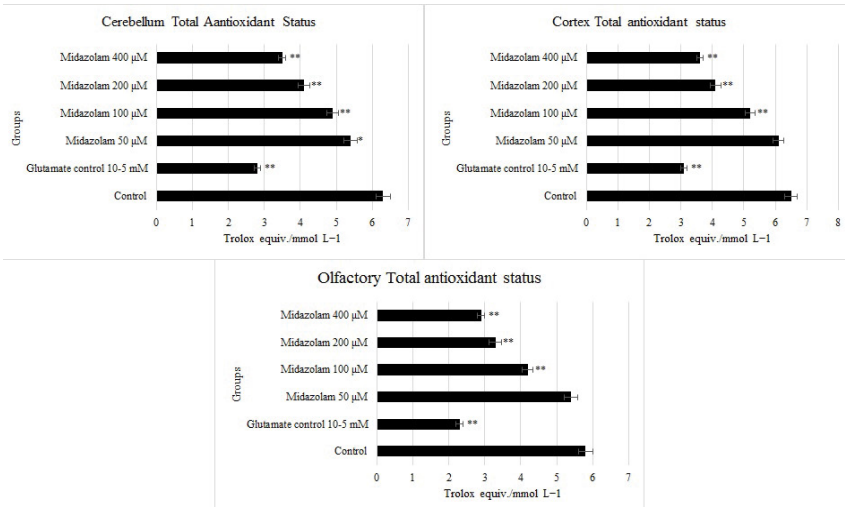

Figure 3 Total antioxidant capacity assay result of cerebellum, cortex and olfactory cell line after 24 h treatment by midazolam; *(p<0.05), **(p<0.001)

TOS Assay

Total oxidant level of cells is shown in Figure 4. Our data shows the lowest oxidant level gained by the negative control group, also the highest level of oxidant was obtained by glutamate control groups. According to our data midazolam, 50 mM in all culture did not show any significant statistical difference in comparison to the control group. But treatment in all culture was higher than 50 mM treatments which show statistical difference ($p<0.001$) in comparison to negative control group (Figure 4).

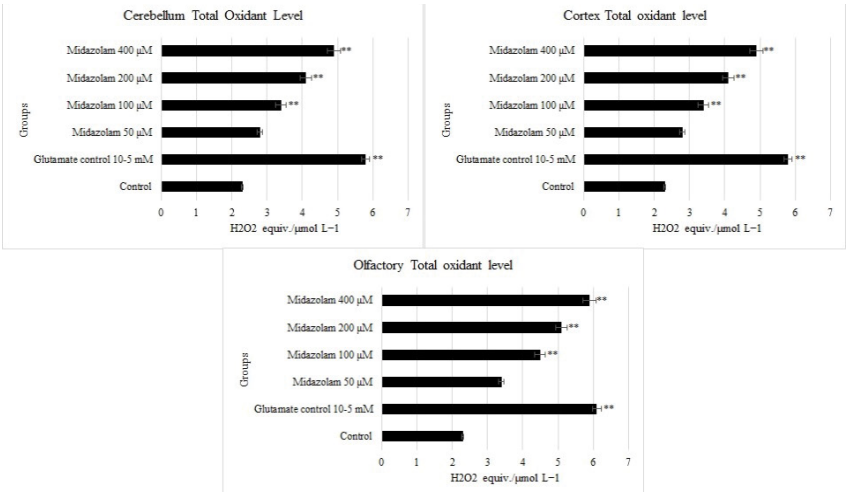

Figure 4 Total oxidant status assay result of cerebellum, cortex and olfactory cell line after 24 hours treatment by midazolam; *($p<0.05$), **($p<0.001$)

Flowcytometry

MTT test shows the viability ratio but we need to determine the apoptosis level in early apoptosis or late apoptosis stage. For evaluation apoptosis, we use annexin v-FITC and PI. Early apoptosis stage can be reversible and cells can come back to normal but in late apoptosis, cells go under the irreversible stage and some literature changes the name of late apoptosis to early necrosis stage. Our result is shown in Figures 5-7.

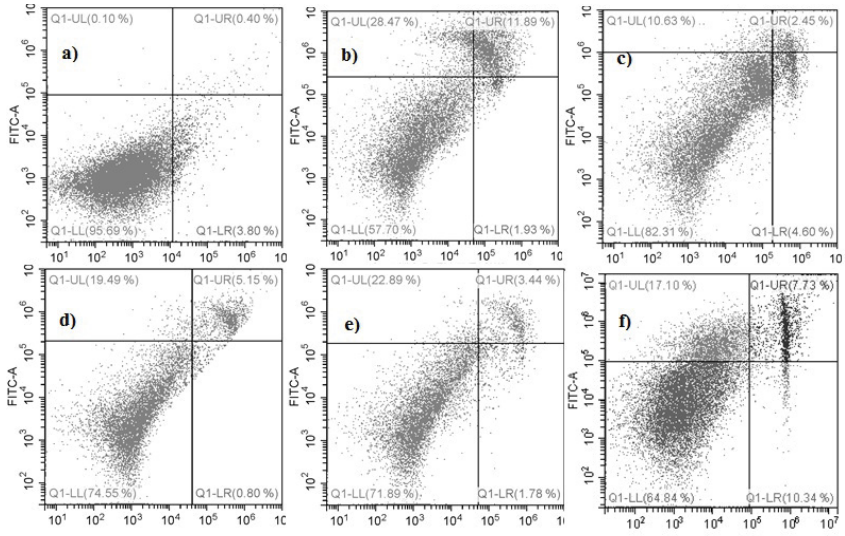

Figure 5 Flowcytometry result of cerebellum neurons stained by annexin-V and PI after 24 h treatment by midazolam. a) NC group; b) glutamate control 10-5 mm; c) midazolam 50 µm; d) midazolam 100 µm; e) midazolam 200 µm and f) midazolam 400 µM

Cerebellum neuron culture after 24 hours were stained (Figure 5). According to our result NC group show cell viability in 95.69% with early and late apoptosis 0.1% and 0.4%, respectively. Glutamate control shows 57.%7 viability with 28.47% and 11.89% early and late apoptosis ratio. Also, our data shows a correlation with MTT result. According to

our data, early apoptosis level are higher than late apoptosis in all treatments. Among groups, 200 mM have higher early apoptosis level and 50 mM have lower late apoptosis level.

Neuron flowcytometry data was shown in Figure 6. According to cortex neuron result cell viability was 89.11% with early and late apoptosis 6.72% and 2.66% respectively. Glutamate 5-10 mM cell viability in 52.56% with late apoptosis rate in 24.91% and necrosis 5.30%. The highest necrosis and early apoptosis ratio were seen in the midazolam 400 mM group among treatments. Also, highest late apoptosis ratio was seen in 100 mM group.

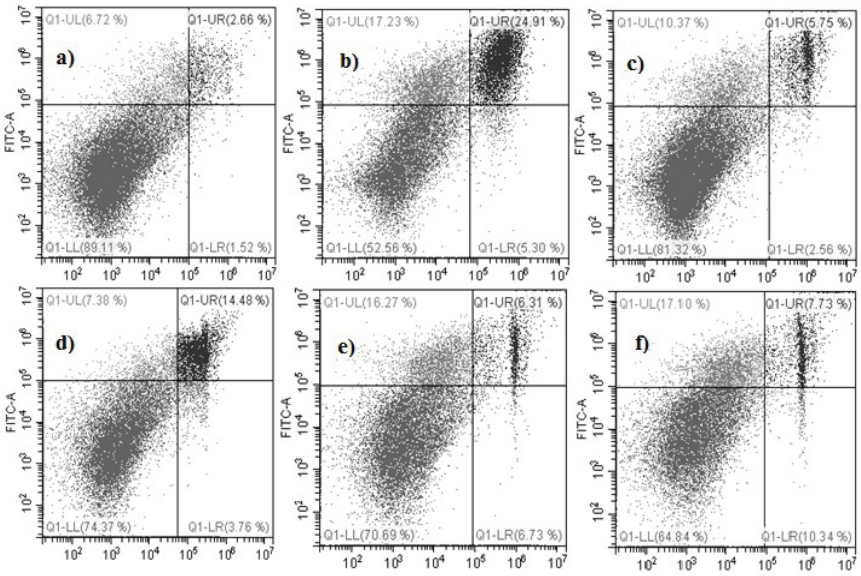

Figure 6 Flowcytometry result of cortex neurons stained by annexin-V and PI after 24 h treatment by midazolam. a) neg control; b) glutamate control 10-5 mm; c) midazolam 50 µm; d) midazolam 100 µm; e) midazolam 200 µm and f) midazolam 400 µM

Our olfactory annexin-V/Pİ result is shown in Figure 7. NC and glutamate control viability ratio was 96.43% and 50.49% respectively. Our data shows olfactory neuron exposure to midazolam which shows lowest early apoptosis except for 400 mM group. The late apoptosis level also is lowest among all cell type. The highest necrosis level was seen in 200 mM in comparison to other groups (Figure 7).

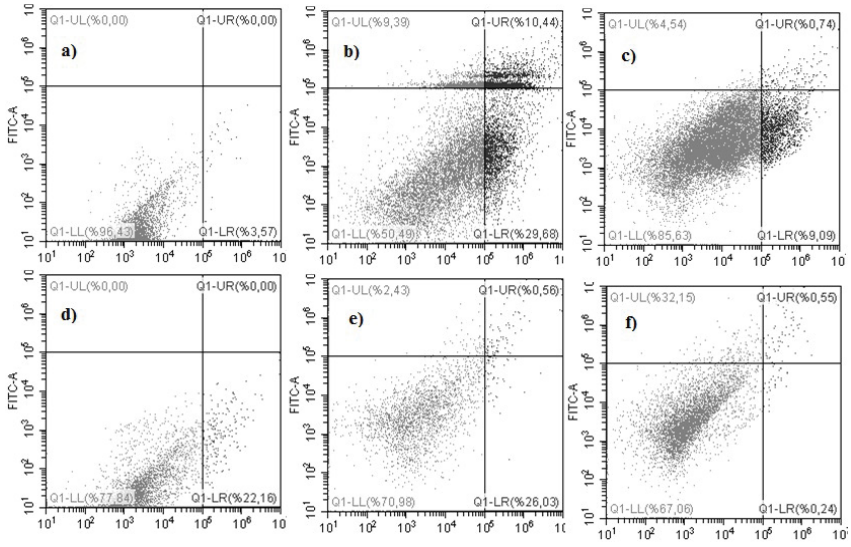

Figure 7 Flowcytometry result of olfactory neurons stained by annexin-V and PI after 24 h treatment by midazolam. a) neg control; b) glutamate control 10-5 mm; c) midazolam 50 µm; d) midazolam 100 µm; e) midazolam 200 µm and f) midazolam 400 µM

DISCUSSION

Neurons have different type and function in olfactory bulb, cortex and cerebellum regions. Olfactory neurons regulate complex behaviors, cerebellum neurons have a great role in body balance and cortex neurons mainly coordinate complex motor and sensory behavior's. Those neurons mainly work by glutamate and GABA neurotransmitters. Benzodiazepine specific receptors are associated with cortex neuron that encodes pain related information. Benzodiazepine receptor agonists by increasing GABA efficacy conduct the chloride into cells. Midazolam, a benzodiazepine derivative, depresses nociceptive neurotransmission.

In cerebral hypoxia/anoxia, the glutamate transporter did not work properly and extracellular glutamate level increases and cause irreversible neuronal damage. Also glutamate by attaching to NMDA, AMPA receptor for a long time than physiological level, cause Ca^{++} and Na^{+} influx. GABA in response by opening Cl^{-} channel and influx negative ion into the cytoplasm established resting potential.

Sakai, et al., showed midazolam by activating hGlt1 which decreases glutamate toxicity at 3 μM dose in cell culture. This data is very important because midazolam not only by increasing reuptake rate but also by increasing GABA activity can tolerate glutamate toxicity [18]. In this relation, co culture may be more effective for such study but our culture is pure neuron culture and we cannot evaluate astrocyte glutamate transporter performance (re-uptake mainly was done by astrocyte approximately up to 90%). Also, midazolam effects are dose dependent and high dose did not increase cell viability.

Felix, et al., investigate propofol, ketamine and midazolam neuroprotective effect after ischemic brain injuries [19]. According to this study, midazolam does not have neuroprotective effect at 1 ng, 5 ng, 10 ng. This data has some difference with our work, the dose of midazolam is too low and also we found the neuroprotective effect in 50 μM dose. In ischemic brain injury, glutamate release increased to a toxic level and sedation mediate with GABA Cl^{-} channel has a neuroprotective effect [20].

Sinner, et al., studies show midazolam have dose dependent neuroprotective effect, also have a high dose of midazolam induced apoptosis to cells. This data have a correlation with our study, olfactory, cortex, and cerebellum by increasing midazolam dose increase apoptosis ratio [21]. In addition, our study was evaluating midazolam effect on neuronal culture with glutamate toxicity background. In this relation glutamate by opening Na^{+} and Ca^{++} channel cause positive ion influx.

Cheung So, et al., proofed midazolam in micro molar (30 μM) level inhibit K^{+}, $Na^{+,}$ and Ca^{++} dependent k^{+} channels. That means midazolam by inhibition of positive ion channel mainly in glutamate toxicity protects neurons, but in high dose and longtime induce apoptosis to neuron cells [22].

CONCLUSION

Midazolam dependent on dose has a neuroprotective effect. It is very important because the patients have a different complication with glutamate background and when we use drugs specially benzodiazepine, it may involuntarily induce irreversible neuronal degradation by midazolam high dose.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

[1] Cho, Hyung-Ju, et al. "Newly developed method for mouse olfactory behavior tests using an automatic video tracking system." *Auris Nasus Larynx,* Vol. 45, No. 1, 2018, pp. 103-10.

[2] Lawrenson, Charlotte, et al. "The mystery of the cerebellum: clues from experimental and clinical observations." *Cerebellum and Ataxias,* Vol. 5, No. 1, 2018, p. 8.

[3] Arce-McShane, Fritzie, et al. "Primary sensorimotor cortex exhibits complex dependencies of spike-field coherence on neuronal firing rates, field power, and behavior." *Journal of Neurophysiology*, 2018.

[4] Meyerhoff, Dieter J., et al. "Brain GABA and glutamate concentrations following chronic gabapentin administration: a convenience sample studied during early abstinence From alcohol." *Frontiers in Psychiatry* 9, 2018, p. 78.

[5] Carlson, Verginia C. Cuzon. "GABA and glutamate synaptic coadaptations to chronic ethanol in the striatum." 2018, pp. 1-34.

[6] Heimfarth, Luana, et al. "Astrocyte-neuron interaction in diphenyl ditelluride toxicity directed to the cytoskeleton." *Toxicology,* Vol. 379, 2017, pp. 1-11.

[7] Lu, Fuxin, et al. "Hypoxia-ischemia modifies postsynaptic GluN2B-containing NMDA receptor complexes in the neonatal mouse brain." *Experimental Neurology,* Vol. 299, 2018, pp. 65-74.

[8] Simões, Ana P., et al. "Glutamate-induced and NMDA receptor-mediated neurodegeneration entails P2Y1 receptor activation." *Cell Death and Disease,* Vol. 9, No. 3, 2018, p. 297.

[9] Zhao, Lei, et al. "NSAIDs modulate GABA-activated currents via Ca^{2+}-activated Cl-channels in rat dorsal root ganglion neurons." *Experimental and Therapeutic Medicine,* Vol. 11, No. 5, 2016, pp. 1755-61.

[10] Walser, Armin, Gladys Zenchoff, and R. Ian Fryer. "Quinazolines and 1, 4-benzodiazepines. 75. 7-Hydroxyaminobenzodiazepines and derivatives." *Journal of Medicinal Chemistry,* Vol. 19, No. 12, 1976, pp. 1378-81.

[11] Reves, J. dG, et al. "Midazolam: pharmacology and uses." *Anesthesiology,* Vol. 62, No. 3, 1985, pp. 310-24.

[12] Shader, R. I., and D. J. Greenblatt. "The use of benzodiazepines in clinical practice." *British Journal of Clinical Pharmacology,* Vol. 11, 1981, pp. 5-9.

[13] Minovi, Amir, et al. "Isolation, culture optimization and functional characterization of stem cell neurospheres from mouse neonatal olfactory bulb and epithelium." *European Archives of Oto-Rhino-Laryngology,* Vol. 274, No. 8, 2017, pp. 3071-85.

[14] Abel, Sean DA, and Sarah K. Baird. "Honey is cytotoxic towards prostate cancer cells but interacts with the MTT reagent: considerations for the choice of cell viability assay." *Food Chemistry,* Vol. 241, 2018, pp. 70-78.

[15] Rowicka, Grażyna, et al. "Total oxidant and antioxidant status in prepubertal children with obesity." *Oxidative Medicine and Cellular Longevity,* 2017.

[16] Makedou, Kali G., et al. "Total antioxidant capacity, haematological and coagulation parameters after orthodox christian fast." *Open Access Macedonian Journal of Medical Sciences,* Vol. 6, No. 2, 2018, p. 284.

[17] Eray, Mine, et al. "Flow cytometric analysis of apoptotic subpopulations with a combination of Annexin V□ FITC, propidium iodide, and SYTO 17." *Cytometry,* Vol. 43, No. 2, 2001, pp. 134-42.

[18] Sakai, Fumio, and Keisuke Amaha. "Midazolam and ketamine inhibit glutamate release via a cloned human brain glutamate transporter." *Canadian Journal of Anesthesia,* Vol. 47, No. 8, 2000, pp. 800-06.

[19] Ulbrich, Felix, et al. "Propofol, but not ketamine or midazolam, exerts neuroprotection after ischaemic injury by inhibition of Toll-like receptor 4 and nuclear factor kappa-light-chain-enhancer of activated B-cell signalling: A combined in vitro and animal study." *European Journal of Anaesthesiology,* Vol. 33, No. 9, 2016, pp. 670-80.

[20] Lima, Andréa C. Paula, et al. "Neuroprotection against Aβ and glutamate toxicity by melatonin: Are GABA receptors involved?." *Neurotoxicity Research,* Vol. 5, No. 5, 2003, pp. 323-27.

[21] Sinner, Barbara, et al. "Toxic effects of midazolam on differentiating neurons in vitro as a consequence of suppressed neuronal Ca^{2+}-oscillations." *Toxicology,* Vol. 290, No. 1, 2011, pp. 96-101.

[22] So, Edmund Cheung, et al. "Effects of midazolam on ion currents and membrane potential in differentiated motor neuron-like NSC-34 and NG108-15 cells." *European Journal of Pharmacology,* Vol. 724, 2014, pp. 152-60.