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Levetiracetam Protects Against Glutamate-Induced Excitotoxicity in SH-SY5Y Cell Line

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Abstract

The latest research has shown that the new generation of antiepileptic drugs has neuroprotective on nervous system. On the other hand, the effect of levetiracetam, a new generation antiepileptic drug, on GIC in SH-SY5Y cells remains uncertain. This research aims to investigate the effect of levetiracetam on GIC and oxidant and antioxidant levels in SH-SY5Y cells. It is utilized SH-SY5Y cell line at this research. Four groups were formed to assess the impact of levetiracetam on SH-SY5Y cell death following GIC. While no treatment was administered to the control group, 10 mM glutamate was administered to the glutamate group for 24 hours (10, 25, 50 and 100 µg/ml). LEV at different concentrations was given to the levetiracetam for 24 hours. The levetiracetam + glutamate was pretreated with levetiracetam at several concentrations for 1 hour (10, 25, 50, and 100 µg/ml), which was followed by a 24-hour exposure to 10 mM glutamate. TAS and TOS levels in cells and cell viability were examined. Following the GIC, a 25 µg/ml-Levetiracetam improved cell viability in neuroblastoma cells dramatically ($p < 0.05$). LEV (25 µg/ml) + glutamate while enhanced TAS levels in neuroblastoma cells in comparison to the glutamate ($p < 0.05$), significantly reduced TOS levels ($p < 0.05$). Levetiracetam improves cell survival by reducing cell death following GIC in neuroblastoma cells. In the acute process, levetiracetam exerts a protective effect.

Key words: Levetiracetam; Glutamate; Toxicity; SH-SY5Y; Oxidative stress

1. Introduction

Neuroblastoma is a malignancy caused by neuroblasts during embryonic development and is one of the most common cancers in children aged 1-4 years (Suebsoonthron et al., 2017). This disease is usually difficult to treat and is resistant. Although there are various treatments such as surgery, chemotherapy and radiation, development of an alternative effective treatment and new drugs is an urgent need due to the resistance and recurrence of neuroblastoma (Filiz et al., 2021). SH-SY5Y and IMR-32 cells as Neuroblastoma cell lines normally contain a population of side cells that exhibit stem cell properties, and these cells may explain the treatment and resistance of neuroblastoma (Xing et al., 2015). The bone marrow biopsy of a 4-year-old girl with the disease was used to obtain Neuroblastoma cells, known as SH-SY5Y cells. Neuroblastoma cells are commonly used in in vitro in conditions such as neuronal function and differentiation. (Biedler et al., 1973).

Excitotoxicity is a type of neurotoxicity mediated by glutamate (Filiz and Öztürk, 2021). Glutamate, the most important stimulator of the central nervous system, is a neurotransmitter that has a key role in the execution of several cognitive functions such as synaptic plasticity, learning and memory (Ergül and Taşkıran, 2021). Glutamate is an amino acid that is found in excess in the brain (Zhou and Niels, 2014). Glutamate is involved in the pathogenesis of some diseases such as Alzheimer's disease, ischemic or hemorrhagic strokes, autism, and amyotrophic lateral sclerosis (Lau and Tymianski, 2010). It has two groups of receptors: ionotropic and metabotropic. The ionotropic receptor family consists of three groups: N-methyl-D-aspartate (NMDA) receptors, alpha amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) receptors, and kainate receptors (Mayer, 2005). During synaptic activity, there is an increase in the concentration of glutamate in the synaptic cavity, but the concentration of extracellular glutamate is removed by glutamate carriers, preventing excessive activation of glutamate receptors (Zhou and Niels, 2014; Ergül and Taşkıran, 2021). Excessive calcium ion entry into the cell as a result of excessive glutamate release leads to excitotoxicity, which leads to mitochondrial dysfunction and an rise in reactive oxygen species (ROS), which triggers neuronal cell death (Lewerenz and Pamela, 2015; Weita et al., 2014).

Levetiracetam (LEV), which targets active N-type calcium channels with high-voltage, is an antiepileptic drug that is used commonly. In studies conducted in recent years, LEV has been discovered to also function as a histone deacetylase inhibitor exhibiting anti-inflammatory and antioxidant properties. By binding to synapse vesicle protein 2A (SV2A) and inhibiting N-type Ca²⁺ channels, LEV shows neuroprotective effect by regulating the release of neurotransmitters (Lynch et al., 2004). Ion channel modulation is a practical approach for protecting neurons and glial cells, as is well documented (Vaxman, 2008). According to several in vitro models, blockers of sodium channels, potassium channels, or L-type calcium channels reduce inflammation and stop apoptotic and necrotic cell death (Herzog et al., 2003). The neuroprotective effects of LEV have been reported on Parkinson's disease as well as in vitro damage patterns (Erbaş et al., 2004; Yan et al., 2018). However, its effects on GIC in SH-SY5Y cells and the underlying mechanisms is not certain yet. In this study, SH-SY5Y cell line was used to elucidate the effect of levetiracetam on glutamate excitotoxicity in the cell line and the mechanisms involved in this effect.

2. Material and Methods

2.1. Cell culture

SH-SY5Y (CRL-2266) cell were maintained containing 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine and it was kept in the incubator (atmospheric environment humidified with 37 °C and 5% CO₂) by providing suitable conditions. When the cells reached a density of 80-90%, they were passed. After three passages, the cells were sown at a cell density of 1×10^4 cells in each well with a plate of 96.

2.2. Administration of the drug

Levetiracetam (LEV) and glutamate were dissolved in DMEM and stock solutions were prepared prior to experimentation.

2.3. Glutamate-induced cytotoxicity (GIC)

For the purpose of examining levetiracetam's effects on GIC, four cell groups were created. While the control did not receive any drugs. Glutamate-induced cells were exposed to 10 mM glutamate for 24 hours. LEV at varying doses (10, 25, 50 and 100 µg) was given to cells in the levetiracetam for 24 hours. To the LEV + glutamate group was pretreated with levetiracetam at several concentrations for 1 hour (10, 25, 50, and 100 µg/ml), which was followed by a 24-hour exposure to 10 mM glutamate.

2.4. Assessment of cell viability

XTT test was utilized to determine cell viability. Initially, cells were planted in 96-cavity plaques at a cell density of 1×10^4 in 100 µL of DMEM per cavity and incubated for 24 hours. GIC method was conducted as previously explained. After 24 hours of incubation, DMEM in the plate of 96 was removed, and phosphate-buffered saline (PBS) was used to wash the cavities, which was followed by adding 100 µL DMEM and 50 µL XTT solution free of phenol red to all wells. The plates were then kept at 37 °C for 4 hours. An ELISA microplate reader at 450 nm was used to determine the absorbance values. All the experiments were conducted three times. Besides, cell viability was measured as percentages of viable cells in comparison untreated cells (control group).

2.5. Preparation of cell homogenates

All cells in the study were taken into sterile tubes. After the supernatant was centrifuged at 2000 rpm for about 10 minutes, it was removed. The cell suspension was diluted with PBS (pH: 7.4) to a cell concentration of around 1 million/ml and the cells in the tubes were suspended. Repeated freeze-thaw cycles allowing internal components to come out damaged the cells. The cells were centrifuged at a temperature of 4 °C at 4000 rpm for 10 minutes. Then, for biochemical analysis, the supernatants were collected. The amounts of total protein in the samples were determined using the Bradford protein assay kit Merck Millipore, Darmstadt, Germany).

2.6. Measurement of TAS and TOS activity

The tests were carried out following the manufacturer's instructions provided with Total Antioxidant Status(TAS) Assay Kit (Rel Assay Diagnostics, Turkey) ve Total Oxidant Status(TOS) Assay Kit (Rel Assay Diagnostics, Turkey). Total antioxidant status levels in the supernatants of the cells were assessed using a previously described automated assay method by Erel (Erel, 2004). The technique relies on the detection of colored dianisidil radical's absorbance during free radical reactions that start with the creation of hydroxyl radicals in the Fenton reaction in order to track f free radical's reaction rates. Antioxidants in tissue samples might reduce colour in proportion to their concentration (Erel, 2004). The findings were presented as micromolar Trolox equivalents per milligram of tissue protein (mol Trolox Eq/mg protein). Erel's automated test method (Erel, 2005) was utilized to measure tissue TOS levels in cell supernatants. When sufficient amounts of oxidants are present in the environment, since iron ions are oxidized to iron ions, the method allows TOS levels to be measured by measuring tissue levels of iron ions with the use of xylene orange. Calibration of the test was performed using Hydrogen peroxide (Erel, 2005). The test results were expressed using Micromolar hydrogen peroxide equivalents per milligram of tissue protein (mol H₂O₂ Eq/mg protein).

2.7. Statistical analysis

Mean \pm SEM was used for the presentation of the data obtained. Biochemical data were statistically analyzed. The groups were evaluated with one-way ANOVA and Tukey HSD (post-hoc test). $p < 0.05$ values defined.

3. Results and Discussion

XTT cell viability test was utilized to analyze effect of levetiracetam on in neuroblastoma cells. This study investigated the effects of increased levetiracetam concentrations (10–100 $\mu\text{g/ml}$) on cell viability in both control and glutamate-induced in neuroblastoma cells. The cells were initially treated with levetiracetam at increasing concentrations (10, 25, 50, and 100 $\mu\text{g/ml}$) for 1 hour and then incubated with and without 10 mM glutamate for the next 24 hours. Figure 1 shows that the treatment concentration of 10 mM glutamate reduced cell viability significantly in comparison to the control ($p < 0.05$). Besides, when LEV+glutamate was co-administered, LEV doses of 10–100 $\mu\text{g/ml}$ increased cell survival in neuroblastoma cells compared with the glutamate group ($p < 0.05$).

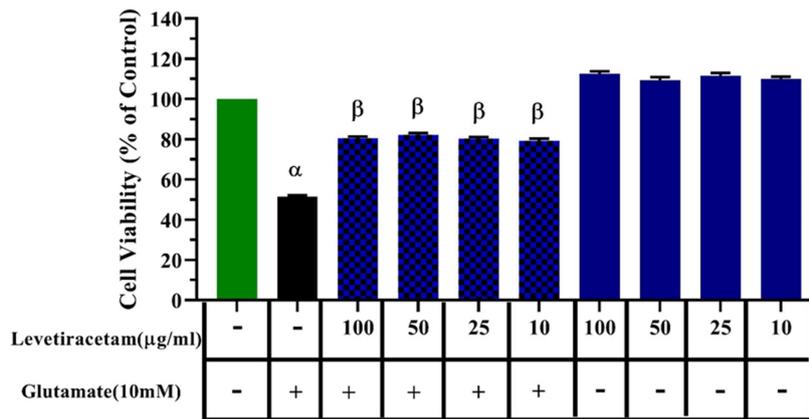


Figure 1. Effect of levetiracetam on cell viability in SH-SY5Y cells following GIC.

TAS levels in neuroblastoma cells were found to reduce in the glutamate and LEV+ glutamate in comparison to the control ($p < 0.05$; Figure 2A). Besides, levetiracetam treatment of 25 µg/ml improved TAS levels in neuroblastoma cells significantly following GIC ($p < 0.05$; Figure 2A). Additionally, levetiracetam treatment of 25 µg/ml alone did not alter TAS levels in comparison to the control and glutamate ($p < 0.05$; Figure 2A). When compared to the glutamate, however, LEV+ glutamate reduced TOS levels significantly ($P < 0.05$) (Figure 2A). Furthermore, 25 µg/ml of levetiracetam alone had no effect on TOS levels in neuroblastoma cells ($P > 0.05$; Figure 2B).

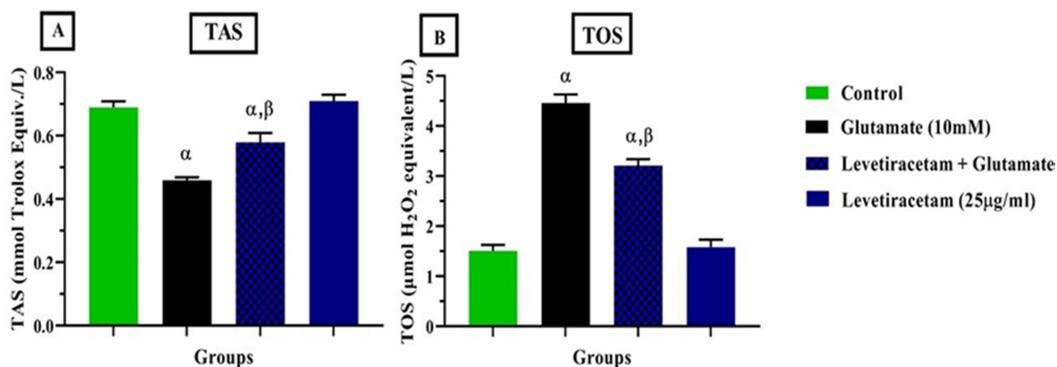


Figure 2. Effect of levetiracetam on TAS and TOS levels in SHSY-5Y cells after GIC.

The impact of levetiracetam on glutamate-induced cytotoxicity in SHSY-5Y cells is being examined for the first time in this investigation. Levetiracetam pre-treatment increased SH-SY5Y cell survival and decreased cell death following GIC. On the other hand, SH-SY5Y lowered TOS levels in cells and increased TAS levels following GIC. Numerous neurotoxic cellular processes, including the production of free oxygen radicals and the release of calcium, are induced by glutamate. Increased glutamate production represents a pathological mechanism that leads to neuron death (Doble, 1999). In conclusion, the main therapeutic strategies include the use of substances that can impede glutamate pathology (McCulloch, 1992). The in vitro model toxicity concentration of glutamate, which has been shown to trigger excitotoxicity in SHSY-5Y cells depending on the

dose and time, varies between 8 and 80 mM according to the experimental culture conditions. (Kritis et al., 2015) 10 mM glutamate was used in our study. Preliminary studies were conducted to determine the optimal glutamate concentration before cells were treated to determine the concentration. In this context, after the glutamate concentrations in the range determined in accordance with the literature were applied to the cells, the cytotoxic concentration was determined in half of the cells.

LEV can block the flow of Ca^{2+} to neuronal cells (Lukyanetz et al., 2002). Yoshii et al. investigated the neuroprotective effects of levetiracetam on spinal motor neurons in glutamate-treated neonatal rat culture. Their research has shown that levetiracetam prolongs the lifespan and function of spinal motor neurons against glutamate-induced neurotoxicity in culture (Yasuhiro et al., 2012). Another study on purified Schwann cells from dorsal root ganglia prepared from ex vivo newborn P3 mice and E15 mouse embryos found to reduce levels of proinflammatory tumor necrosis factor alpha, matrix metalloproteinase 9 (MMP-9) and caspase 6, as well as LPS and glutamate-induced oxidative stress model has been shown to protect cells against oxidative stress (Stettner et al., 2011). LEV, one of the next generation antiepileptic drugs, has been shown to be able to prevent hypoxia-induced neuronal damage in cultured hippocampal neurons (Sendrowski et al., 2011). In a study conducted by Miyazaki et al. in hemi-parkinsonian mice, treatment with LEV was shown to significantly increase cystine/glutamate exchange transporter (xCT) expression in striatal astrocytes. According to the results of this study, LEV showed neuroprotective effects by preventing neurodegeneration in astrocytes through up-regulation of xCT and GSH (Miyazaki et al., 2015).

Oxidative stress is the reaction that creates the possibility of organic damage due to an imbalance of oxidant and antioxidant defenses. According to certain research, oxidative stress actively contributes to the development of neurodegenerative disorders. (Chang and Yu, 2010). Genetic searches and various studies on animal models have shown an increase in cell damage after both mitochondrial dysfunction and recurrent seizures (Li-Ping and Patel, 2006; Gluck et al., 2000; Filiz and Öztürk, 2021). Numerous illnesses or exposure to specific substances can cause oxidative stress. According to some theories, seizures may occur more frequently due to the increase in the quantity of active oxygen metabolites or a decrease in the function of the body's antioxidative defense mechanisms (Jesberger and Richardson, 1991). Antioxidant systems are in charge of regulating this oxidative stress. This mechanism guards against the damaging effects of chemicals on the organism (MaCord, 2000; Tutanç et al., 2015). The function of the antioxidant effect in the treatment of anti-epileptic drugs is controversial. The experimental study conducted by Ekici et al. reported the effect of valproic acid pre-treatment, an anti-epileptic drug, on increasing DNA damage before epileptic seizures (Ekici and Taşkıran, 2020). In their study, Filiz and Öztürk showed that pre-treatment with carbamazepine enhanced TOS and MDA levels in glutamate-derived cells (Filiz and Öztürk, 2021).

In this study, TAS levels reduced and TOS levels enhanced after GIC in SH-SY5Y cells. Besides, LEV lowered TOS levels and elevated TAS levels following GIC in SH-SY5Y cells. Previous research (Marini et al., 2004; Oliveira et al., 2016) has demonstrated that antiepileptic medications have antioxidant effects in several tissues and also lower oxidative stress. These research back up what we do.

According to our results, levetiracetam SH-SY5Y has an important role in cell viability. Our study contains some potential limitations. This method falls short of fully explaining all the mechanisms behind the effects of levetiracetam on SH-SY5Y cells. While it exerts a protective effect in the short term, it can cause cell death by increasing oxidative damage in the long term. Therefore, more investigation is required to determine levetiracetam's effects on SH-SY5Y cells.

4. Conclusion

Levetiracetam was found to lessen GIC death in SH-SY5Y cells, according to the study's findings. Levetiracetam, however, decreased TOS levels while raising TAS levels. This protective effect of levetiracetam has been demonstrated by acute treatment. However, more research is needed to respond to the possible potential mechanisms involved.

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Conflicts of Interests

Authors declare that there is no conflict of interests

Statement contribution of the authors

This study's experimentation, analysis and writing, etc. all steps were made by the authors.

References

- Biedler, J. L., Helson, L., & Spengler, B. A. (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Research*, 33(11), 2643-2652.
- Chang, S. J., & Yu, B. C. (2010). Mitochondrial matters of the brain: mitochondrial dysfunction and oxidative status in epilepsy. *Journal of Bioenergetics & Biomembranes*, 42(6), 457-459.
- Doble, A. (1999). The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacology & Therapeutics*, 81(3), 163-221.
- Ekici, M., & Taşkıran, A. Ş. (2020). The effect of valproic acid on DNA damage and apoptosis after pentylenetetrazole-induced epileptic seizure generated in the hippocampus and cortex in rats. *Journal of the Institute of Science & Technology*, 10(4), 2924-2931.
- Erbaş, O., Yılmaz, M., & Taşkıran, D. (2016). Levetiracetam attenuates rotenone-induced toxicity: A rat model of Parkinson's disease. *Environmental Toxicology & Pharmacology*, 42, 226-230.
- Erel, O. (2004). A novel automated method to measure total antioxidant response against potent free radical reactions. *Clinical Biochemistry*, 37(2), 112-119.

- Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry*, 38(12), 1103-1111.
- Ergül, M., & Taşkıran, A. Ş. (2021). Thiamine protects glioblastoma cells against glutamate toxicity by suppressing oxidative/endoplasmic reticulum stress. *Chemical & Pharmaceutical Bulletin*, 69(9), 832-839.
- Filiz, A. K., & Öztürk, A. (2021). The effect of carbamazepine against glutamate-induced cytotoxicity in the C6 cell line. *International Journal of Scientific & Technological Research*, 7(8), 67-76.
- Filiz, A. K., Joha, Z., & Yulak F. (2021). Mechanism of anti-cancer effect of β -glucan on SH-SY5Y cell line. *Bangladesh Journal of Pharmacology*, 16(4), 122-128.
- Gluck, M. R., Jayatilleke, E., Shaw, S., Rowan, A. J., & Haroutunian, V. (2000). CNS oxidative stress associated with the kainic acid rodent model of experimental epilepsy. *Epilepsy Research*, 39(1), 63-71.
- Herzog, R. I., Liu, C., Waxman, S. G., & Cummins, T. R. (2003). Calmodulin binds to the C terminus of sodium channels Nav1. 4 and Nav1. 6 and differentially modulates their functional properties. *Journal of Neuroscience*, 23(23), 8261-8270.
- Jesberger, J.A., & Richardson, J.S. (1991). Oxygen free radicals and brain dysfunction. *International Journal of Neuroscience*, 57(1-2), 1-17.
- Kritis, A. A., Stamoula, E. G., Paniskaki, K. A., & Vavilis, T. D. (2015). Researching glutamate-induced cytotoxicity in different cell lines: a comparative/collective analysis/study. *Frontiers in Cellular Neuroscience*, 9, 91.
- Lau, A., & Tymianski, M. (2010). Glutamate receptors, neurotoxicity and neurodegeneration. *Pflügers Archiv-European Journal of Physiology*, 460(2), 525-542.
- Lewerenz, J., & Maher, P. (2015). Chronic glutamate toxicity in neurodegenerative diseases—what is the evidence?. *Frontiers in Neuroscience*, 9, 469.
- Liang, L. P., & Patel, M. (2006). Seizure-induced changes in mitochondrial redox status. *Free Radical Biology & Medicine*, 40(2), 316-322.
- Lukyanetz, E. A., Shkryl, V. M. & Kostyuk, P. G. (2002). Selective blockade of N-type calcium channels by levetiracetam. *Epilepsia*, 43(1), 9-18.
- Lynch, B. A., Lambeng, N., Nocka, K., Kensel-Hammes, P., Bajjalieh, S. M., Matagne, A., & Fuks, B. (2004). The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. *Proceedings of the National Academy of Sciences*, 101, 9861-9866.
- MaCord, J. M. (2000). The evolution of free radical and oxidative stress. *American Journal of Medicine*, 108, 652-659.
- Marini, H., Costa, C., Passaniti, M., Esposito, M., Campo, G. M., Ientile, R., ... & Squadrito, F. (2004). Levetiracetam protects against kainic acid-induced toxicity. *Life Sciences*, 74(10), 1253-1264.
- Mayer, M. L. (2005). Glutamate receptor ion channels. *Current Opinion in Neurobiology*, 15(3), 282-288.
- McCulloch, J. (1992). Excitatory amino acid antagonists and their potential for the treatment of ischaemic brain damage in man. *British Journal of Clinical Pharmacology*, 34(2), 106-114.

- Miyazaki, I., Murakami, S., Torigoe, N., Kitamura, Y., & Asanuma, M. (2016). Neuroprotective effects of levetiracetam target xCT in astrocytes in parkinsonian mice. *Journal of Neurochemistry*, 136(1), 194-204.
- de Albuquerque Oliveira, A., Linhares, M. I., Chaves Filho, M., Rios, E. R. V., de Carvalho Lima, C. N., Venancio, E. T., ... & de FranÃ, M. M. (2016). Antioxidant properties of antiepileptic drugs levetiracetam and clonazepam in mice brain after in vitro-induced oxidative stress. *African Journal of Pharmacy & Pharmacology*, 10(14), 278-288.
- Sendrowski, K., Boćkowski, L., Sobaniec, W., Ilendo, E., Jaworowska, B., & Śmigielska-Kuzia, J. (2011). Levetiracetam protects hippocampal neurons in culture against hypoxia-induced injury. *Folia Histochemica et Cytobiologica*, 49(1), 148-152.
- Stettner, M., Dehmel, T., Mausberg, A. K., Köhne, A., Rose, C. R., & Kieseier, B. C. (2011). Levetiracetam exhibits protective properties on rat Schwann cells in vitro. *Journal of the Peripheral Nervous System*, 16(3), 250-260.
- Suebsoonthron, J., Jaroonwichawan, T., Yamabhai, M., & Noisa, P. (2017). Inhibition of WNT signaling reduces differentiation and induces sensitivity to doxorubicin in human malignant neuroblastoma SH-SY5Y cells. *Anticancer Drugs*, 28, 469-479.
- Tutanc, M., Aras, M., Dokuyucu, R., Altas, M., Zeren, C., Arica, V., Ozturk, O. H., Motor, S., & Yilmaz, C. (2015). Oxidative Status in Epileptic Children Using Carbamazepine. *Iranian Journal of Pediatrics*, 25(6), 3885.
- Waxman, S. G. (2008). Mechanisms of disease: sodium channels and neuroprotection in multiple sclerosis-current status. *Nature Clinical Practice Neurology*, 4, 159–169.
- Weita, L. T., Zhang S., & Wang Y. T. (2014). Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Progress in Neurobiology*, 115, 157-188.
- Xing, L. L., Sha, Y. L., Wu, Y. M., Hu, J. M., & Zhang, M. (2015). Preliminary analysis of stem cell-like cells in human neuroblastoma. *World Journal of Pediatrics*, 11, 54–60.
- Yan, B. C., Shen, H., Zhang, Y., Zhu, X., Wang, J., Xu, P., Jiang, D., X. & Yu. X. (2018). The antiepileptic drug levetiracetam promotes neuroblast differentiation and expression of superoxide dismutase in the mouse hippocampal dentate gyrus via PI3K/Akt signalling. *Neuroscience Letters*, 662, 84-90.
- Yasuhiro, Y., Ikeda, K., & Iwasaki, Y. (2012). Levetiracetam Protects Spinal Motor Neurons Against Glutamate-Induced Neurotoxicity in Culture. *Journal of Neurology Research*, 2(2), 39-43.
- Zhou, Y., & Niels, C. D. (2014). Glutamate as a neurotransmitter in the healthy brain. *Journal of Neural Transmission*, 121(8), 799-817.