The study of diazepam, pregabalin and glucose effect on glutamate toxicity: In vitro study

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Abstract
In this study we used pregabalin, diazepam and glucose for evaluating which one are more effective agents against glutamate induced neurotoxicity. After cortex culture preparation glutamate toxicity induced by adding 10-5 mM glutamate to each wells except negative control group. After 10 min different dose of diazepam, pregabalin and glucose were added for 24 h and then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Total Antioxidant Status (TAC) and Total oxidant status (TOS) assays were done. Diazepam, dose dependently shows neuroprotective effects and low dose of diazepam did not increase cell viability higher than 80 %. Combination groups only in high dose increased neurons viability. Total Antioxidant capacity of Pregabalin also show correlation with diazepam groups and highest dose of both show nearest value to negative control group. In addition, lowest and highest oxidant level were gained by the negative and positive control groups respectively. Except high dose of combination group, all groups show statically difference in compare with the negative control group P<0,05. According to our data combination of diazepam, pregabalin and glucose are more effective than pure drug to reduce glutamate toxicity in cortex neuron culture.

Keyword: Diazepam, pregabalin, glucose, cortex neuron, total antioxidant capacity and total oxidant status

Introduction
Glutamate is the main excitatory neurotransmitter in the mammalian brains. In addition, it has important role like memory and learning. The excitatory action of glutamate in mammalian has been known since the 1950s [1]. Glutamate acts mainly on post-synaptic neuron via ionotropic (ligand-gated ion channels) and metabotropic (G protein) receptors [2]. The all possess in ionotropic receptors were done by ion channels that are permeable to cations, although the relative permeability to Na⁺ and Ca²⁺ varies according to the family and the subunit composition of the receptor [3]. NMDA is the mammalian biggest body receptor and have permeability to Ca²⁺ ions also have role in long term memory. AMPA receptor is Na⁺ ion channels and have important role in short term memory [4-6].

Glutamate is a potent neurotoxin, and have a pivotal role in the pathogenesis of many devastating human neurological diseases such as depression, stroke, amyotrophic lateral sclerosis and enough long to cations cross the channel and induced toxicity and apoptosis to neurons [8,9].

Revers to glutamate GABA inhibits neurons function by opening Cl⁻ channels. Negative ions take neuron action potential voltage from -90 to -110 -120 mv the excitation of neuron have been difficult and need to strong stimulation (5,10,11). Pregabalin and diazepam are GABA agonist drugs and widely are using for epilepsy, sizer control, sedation and pain killer to patients. Pregabalin is anti-epileptic drug and diazepam is benzodiazepine type (12).

Diazepam widely are using for treatment of anxiety and central nervous system disorders. In present study we used Pregabalin and diazepam for evaluating which one are effective drug against glutamate toxicity (13-16). In addition, we added the glucose to well plate for evaluating is glucose can tolerate glutamate toxicity by regulation energy pathways or not. Tyler Barnes and colleagues show glutamate increased glucose uptake by AMPK mechanism. In brain hypoxia and physiological condition glutamate have been secrete to protect neurons (17). In brain hypoxia and physiological condition glutamate have been secrete to protect neurons (18,19).

In current study we hypothesized, increase in glucose level and then regulation of energy consumption can increase neuroprotection status against induced glutamate toxicity or not. In addition, we investigate are diazepam and pregabalin able to protect neuron against glutamate by activating GABA mechanism. For this aim, pure drugs (diazepam, pregabalin and glucose) and combination of those drugs in different dose were added to culture for 24 hrs and
then MTT, TAC and TOS assays were done for evaluation of cell viability, Total antioxidant capacity and total oxidant status.

**Materials and Methods**

**Chemicals and reagents**
Pregabalin was purchased from Roche (Basel, Switzerland) and Diazepam was purchased from Pfizer (New York, USA). Glucose, Dulbecco modified eagle’s medium (DMEM), Fetal calf serum (FCS), Neurobasal medium (NBM), MTT, phosphate buffer solution (PBS), antibiotic antimitotic solution (100×), L glutamine, B27 and trypsin–EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). TAC and TOS obtained from Rel assay diagnostics (Turkey).

**In vitro studies**

**Cell cultures**
Cortex cell cultures were obtained from department of medical pharmacology of Ataturk University (Erzurum, Turkey). Briefly, the cells after centrifuged in 1200 rpm for 5 min were seed in 24 well plate (Corning, USA) by fresh medium (Neurobasal medium, FBS %10, B27 %2 and antibiotic %0,01 and store at incubator (5% CO2; 37°C)(20). Figure 1.

**Glutamate toxicity**
By day 10th the cells have adequate branches. All medium poured and glutamate 10-5 mM for inducing toxicity were added to each wells except negative controls. After 10 min Pregabalin (5, 10 and 20 µgr) [21], Diazepam (1, 5 and 15 µgr) [22], Glucose (5, 10 and 15 µgr) [23] and combination group (P; Pregabalin, D; Diazepam and G; Glucose) were added to each wells except negative control (NC) groups and incubated for 24 h (5% CO2; 37°C). Negative control, received 150 µL of NBM and positive control contained only 10-5 mM glutamate for 24 h [24].

**MTT assay**
Then, MTT assay was carried out by commercially available kit (Sigma alderich, USA). Briefly, MTT reagent (10 µL) was added to the well and the plate was incubated (5% CO2; 37°C) for 4 h. 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 [20, 25].

**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 amount 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.

\[
\text{TOS} = \frac{\Delta \text{Sample} - \Delta \text{Standard} 1}{\Delta \text{Standard} 2} \times 20
\]

Where \(\Delta \text{Sample} = \text{Sample second reading} - \text{Sample first reading}\), \(\Delta \text{Standard} 1 = \text{First reading of Standard 1} - \text{Initial reading of Standard 1} \), and \(\Delta \text{Standard} 2 = \text{Second reading of Standard 2} - \text{First reading of Standard 2}\).

**Total Antioxidant Capacity (TAC)**
In TAC assay; antioxidant capacity was determined by inhibiting 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 TAC level; 500 µl Reactive 1 solution was added in the wells containing 30 µl sample and 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, second absorbance value was read at 660 nm. While distilled water was used for Standard 1, Standard 2 solution in the kit was used for Standard 2. The absorbance values obtained were placed according to the following formula and TAC levels were determined in mmol Trolox Equiv./L [20, 25].

\[
\text{TAC} = \frac{\Delta \text{Sample} - \Delta \text{Standard} 1 - \Delta \text{Standard} 2}{\Delta \text{Sample} - \Delta \text{Standard} 1 - \Delta \text{Standard} 2} \times 20
\]

Where \(\Delta \text{Sample} = \text{Sample second reading} - \text{Sample first reading}\), \(\Delta \text{Standard} 1 = \text{First reading of Standard 1} - \text{Initial reading of Standard 1} \), and \(\Delta \text{Standard} 2 = \text{Second reading of Standard 2} - \text{First reading of Standard 2}\).

**Statistically analysis**
The statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey’s HSD using the SPSS 20.0 software. P<0.05 was considered as statistically significant difference for all tests.

**Result**

**MTT assay**
Cortex culture was prepared. After 24 h Pregabalin (5, 10 and 20...
µgr), Diazepam (1, 5 and 15 µgr), Glucose (5, 10 and 15 µgr) and combination groups (P; Pregabalin, D; Diazepam and G; Glucose) exposing time, the experiment was finished by adding MTT solution. The data were analyzed and showed in fig 2. According to our result negative control show highest viability ratio and glutamate control shows lowest viability ratio among each groups. Diazepam dose dependently shows neuroprotective effects and low dose of diazepam did not increase cell viability higher than 80%. Pregabalin groups show higher viability in 10 µgr groups but 5 and 15 µgr show difference (P<0.05) in compare to control groups. Glucose groups did not show significant protective effect and only in 5 µgr show highest viability ratio in compare highest dose. Combination groups increased viability only in DPG 10105 (D 10, P 10 and G 5 µgr) dose up to 91%. Figure 2

**TAC assay**

When the total antioxidant capacity of neurons examined the data showed in fig 3. NC showed highest antioxidant capacity compare to treatment. TAC status in glutamate 10-5 mM is lowest among all treatments. Diazepam in 5 and 10 µgr did not show any significance different in compare control group. Pregabalin also show correlation with diazepam groups and highest dose 10 and 15 shows nearest amount to control groups. Pregabalin 5 µgr shows P<0.05 difference and lowest antioxidant capacity among the group. Glucose group only in 5 µgr dose increased antioxidant capacity but high dose decreased antioxidant status in cortex neurons. Combination groups only in DPG 10105 (D 10, P 10 and G 5 µgr) dose increased antioxidant and cell viability. Figure 3

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**Figure 2.** MTT assay result of olfactory cell line after 24 h treatment by remifentanil. * shows (P<0.05), ** shows (P<0.001)

**Figure 3.** Total antioxidant capacity assay result of olfactory cell line after 24 h treatment by remifentanil. * shows (P<0.05), ** shows (P<0.001)
TOS Assay
Total oxidant level of cells was shown in fig 4. Our data shows lowest oxidant level gained by negative control group. Also highest level of oxidant obtained by glutamate control groups. According to our data diazepam revers to TAC data only in 10 µgr dose decreased antioxidant status. Lowest dose of 1 and 5 µgr diazepam shows P<0.05 difference with control groups. Pregabalin group only in 10 µgr dose did not show any difference with control group whereas 10 and 15 µgr of pregabalin shows P<0.05 statically difference. Glucose groups increased oxidant level in all doses. Combination groups only in high dos all drugs did not show difference with control group but other combination groups show difference in compare with control group P<0.05. Figure 4

![Graphs showing TOS Status Assay](image)

**Figure 4.** Total oxidant status assay result of olfactory cell line after 24 h treatment by remifentanil. * shows (P<0.05), ** shows (P<0.01).

Discussion

Neurons have different type and function in brain cortex. Cortex neurons regulate complex behaviors pattern and also have role in motor and sensory behaviors. Those neurons mainly work by glutamate and GABA neurotransmitters. Diazepam and Pregabalin are benzodiazepine and anti-convulsion drugs respectively [26]. Those drugs by opening Cl- channels reduced action potential to -110 mv inhibit neuronal function and protects neurons. Cl- ions by tolerating negative and positive ions in neurons soma may be protect neuron [27,28]. Glutamate by attaching AMPA and NMDA receptors long than philological periods induced toxicity to neurons.

Galeffi F et al shows diazepam promotes ATP recovery in ischemic hippocampal neurons. In this study ability of diazepam to prevent early signals of cell injury (before cell death) after in vitro ischemia were studied. According to author data 2 hours after diazepam administration cytochrome c levels significantly decreased and by recovery of ATP protects neurons. This data show correlation with our study diazepam protect neuron but directly glucose adding to culture did not show any protection. Moreover, diazepam has not effect on glucose but by recovery of ATP regulate energy circulation and protect neurons in ischemic conditions [29].

Cunningham MO et al in their studies evaluate Pregabalin and gabapentin effect on glutamate release in entorhinal cortex synapse (21). In this study showed pregabalin reduced glutamate release at cortical synapses. pregabalin by reducing P/Q-type voltage-gated Ca channels and prevention of Ca influx into the presynaptic terminals protect neurons. The author think combination of pregabalin and gabapentin done this effect. This data is same to our data, we found neuroprotection in pregabalin 10 µgr and combination of diazepam, pregabalin and glucose group.

Brekke E et al in their studies investigate Astrocyte-Neuron Interactions Following Neonatal Hypoxia-Ischemia in rats neonatal. Flowing to this study author declare neonatal brain is vulnerable to oxidative stress, depression of mitochondrial metabolism decreased neurotransmitter release and reuptake [30]. Also Author highlights, flowing to ischemic injury and astrocyte malfunction cause failure to upregulate glutamate uptake in response to the massive glutamate release. This result show similarity to our data. According to our result after slight oxidative stress astrocyte lose their function and flowing to energy metabolism failure glutamate uptake reduced and caused excitotoxicity. In addition, glucose cannot revers neuronal injury alone because Krebs cycle did not work properly flowing to astrocyte malfunction.

Rotter Sopasakis V and colleague show high glucose level preserve glucose uptake. In this study the author declares elevated glucose level decreased glucose uptake by chondrocytes [(31]. In addition, low glutamate secretion was seen in high glucose and Interleukin-1β stimulation to chondrocytes. This data has correlation with our data. In our study high glucose level show lowest neuronal viability. But the combination of drugs high dose shows highest viability. This data maybe has relation with pregabalin and diazepam GABA mechanism. Negative ion influx to neuron increased glucose permeability and help to tolerate glutamate toxicity. This mechanism need to future investigate.

Eintrei C and colleague showed diazepam decreased local cerebral
glucose utilization. According to that study diazepam alone decreased whole body glucose metabolism up to 24% [32]. This significant data declaration negative ion by decreasing energy consumption reduced glucose uptake. In contrast, both pregabalin and diazepam combination by regulating glucose increased cell viability and antioxidant level shows neuroprotective effect in cortex neurons.

Conclusion

Diazepam, pregabalin and glucosealone did not have strong neuroprotective effect but combination of all drugs effectively protect neurons against induced glutamate toxicity. According to our data, patient with glutamate toxicity background usage of diazepam, pregabalin and glucose was recommended. Future study are need to investigate relation between diazepam, pregabalin and glucose neuroprotective effects with glutamate receptor and transporters expression levels.

Competing interests

The authors declare that they have no competing interest

Financial Disclosure

The financial support for this study was provided by the investigators themselves.

Ethical approval

Before the study, permissions were obtained from local ethical committee.

Reference