Original Article

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Investigating the effect of enzymatic elimination of endocannabinoids inhibitors on tonic- colonic seizure provoked by PTZ

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ABSTRACT

BACKGROUND AND OBJECTIVE: In recent years, numerous attempts were done to find new treatment methods for patients with drug-resistant epilepsy. Anandamide (Anandamides; AEA) and di-Arachidonoylglycerol (2-Arachidonoylglycerol; 2-AG) are two major ligands of endocannabinoid system can be produced or deleted by a certain enzymatic pathway. Given the frequency and significance of these endocannabinoid ligands, it seems that endocannabinoid system in the brain can be changed with pharmacologically manipulating in the pathway of these two main ligands. Therefore, the aim of this study was to evaluate the effect of enzymatic elimination of endocannabinoids inhibitors on tonic-clonic seizure caused by PTZ.

METHODS: In this exprimental study 35 Adult male wistar rats were used in 4 groups. Tonic-colonic seizure was induced through single intra-peritoneal injection of PTZ (80 mg/Kg). Latency and duration of each five behavioral seizure stages were monitored for 30 minutes. To inhibit Anandamides elimination, URB and LY (URB: 1 mg/kg, LY: 2.5 mg/kg, i.p), to inhibit 2-2-Arachidonoylglycerol degradation WWL and JJKK (JJKK: 1 mg/kg, WWL: 5 mg/kg, i.p), were used, all dissolved in DMSO and injected 15 minutes before PTZ injection. In sham group, PTZ was injected after DMSO.Time and duration of all five behavioral stages of seizure were recorded for 30 minutes.

FINDINGS: Delay to stages 4 and 5 in DMSO+PTZ group were 206+39 and 209+39, respectivly. While in JJKK+WWL+DMSO+PTZ group delay to stages 4 and 5 were 630+159 and 726+360, respectivly, which reveald significant increase ($p<0.05$). In addition, percentage of stage 5 incidence and mortality rate were 91% in DMSO+PTZ group, while both indexes were decreased to 50% in (JJKK+WWL+DMSO) group.

CONCLUSION: Contemporary using both WWL and JJKK as inhibitors of 2-Arachidonoylglycerol elimination effectivly reduced tonic- clonic seizure.

KEY WORDS: *2-Arachidonoylglycerol, Anandamide, Dimethylsulfoxide, Epilepsy.*

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Introduction

Epilepsy is a neurological syndrome with a prevalence of about 1% in humans (1, 2). People with epilepsy experience seizures that are epileptic seizures could be iteratively develop (1, 3). One of the most common animal models of acute seizure created by injection of PTZ (PTZ) which is an antagonist of GABAA receptors. According to reports, about 30% of patients do not respond to existing drugs and have drug-resistant epilepsy (4,5). In recent years, numerous attempts were carried out to find new treatment methods as well as the treatment of patients with drug-resistant epilepsy.

Anandamides and 2-Arachidonoylglycerol are two major ligands of endocannabinoid system, which are produced and removed through certain enzyme pathways (6-8). The presence of these compounds in the environment can cause neuronal and synaptic connections and can lead to modulatory effects on existing practices (8,9).

Much of anandamides is produced by the enzyme NAPE-PLD (N-acyl-phosphatidyl ethanolamide-phospholipase D) within the cell and then transported to the extracellular space. The enzyme FAAH (fatty-acid amide hydrolase) is responsible for the removal of anandamides (8,10). Di-arachidonoylglycerol is mainly produced by the enzyme DAGL (Diacylglycerol Lipase) within the cells and then transported outside the cell.

Enzymes MAGL (Monoacylglycerol Lipase) and ABHD (Alpha Beta Hydrolase Domain) are responsible for the AG-2 breakdown (11,12). The AEA and 2-AG also picked up from the synaptic surroundings into the cell by a cannabinoid carrier (6). Given the prevalence and importance of these two major ligands of endocannabinoid system and main mentioned routes on their synthesis and removal, it seems that the level of these two main ligands of endocannabinoid system in the brain can be changed and manipulated pharmacologically. In a study using MAGL enzyme inhibitor which plays a role in the decomposition of 2-AG increased seizures indicators (13), while in another study using ABHD enzyme inhibitor which is also involved in the decomposition of 2-AG improved seizure indicators (14). In this study the effect of selective inhibitors of both enzymes involved in the removal of 2-AG is studied together. In addition, the effect of enzymatic elimination of endocannabinoids inhibitors have been investigated in PTZ induced seizure model. The aim of this study was to evaluate the effect of enzymatic elimination of endocannabinoids inhibitors on the tonic-clonic seizure caused by PTZ.

Methods

Animals: In this experimental study, male Wistar rats weighing 170 to 200 grams were used. Animals in standard conditions (temperature 22-25°C and 12 hours lighting-12 hours of darkness) were maintained. Food and water except during tests was the free form. Animals were randomly in the experimental groups and sample size was 8-5 mice in each group similar to previous studies (15, 16). The ethics of working with laboratory animals were considered according to the approved protocol based on Regional Ethics Committee at the Arak University of Medical Sciences.

Induction and assessment: For evaluation of the seizure related behaviors, PTZ (Sigma-Aldrich) was injected once at the concentration 80 mg/kg intraperitoneally (17) alone in PTZ group or in drug and solvent groups 15 minutes after drug or solvent. After injection of PTZ, animals were placed in a Plexiglas box (dimensions: 35 x 35 x 35 cm) and animal behaviors were monitored for 30 minutes and the incidence of behavioral seizure stages were recorded. All injection process and record of behavioral observations were done by a trained person.

Division of seizure stages (18,19): stage 1: Contraction of the mouth and face muscles. Stage 2: contraction and movements of the head and neck muscles. Stage 3: contraction of hands. Stage 4: Contraction of hands and stand on two legs. Stage 5: stand on two legs with falling sideways. Time to reach each stage, duration and length of stages 3 to 5, the frequency of occurrence of each stage and the death of the animals was measured and recorded for analysis.

Drugs and experimental groups: Used drugs included enzymatic elimination of anandamide inhibitors and 2-AG. To increase the level of anandamide used inhibitor were: URB597 (Santa Cruz Biotech, US) as an inhibitor of FAAH enzyme that plays a key role in enzymatic degradation of anandamide.

This inhibitor was used as 1 mg/kg (20, 21). In addition, LY2183240 (Tocris, US) was used as anandamide reuptake transporter inhibitor in the amount of 2.5 mg/kg with URB597 (22, 23). Two inhibitors used to increase levels of 2-AG were:

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JJKK048 (Tocris, US) as MAGL enzyme inhibitor in the amount of 1 mg/kg (24). In addition, WWL70 (Tocris, US) was used as an inhibitor of the ABHD enzyme in the amount of 5 mg/kg (25, 26). These enzymes have a vital role in breakdown of 2-AG, especially MAGL. All four inhibitor dissolved in (DMSO) dimethyl solfoxide (99.9% Sigma-Aldrich) as a solvent and were used by intraperitoneal injection 15 minutes before PTZ. Injection volume in all groups for each animal was about cc 0.8. Groups included: The first group (PTZ injection): single dose injection of PTZ 80 mg/kg and immediately evaluation of seizure stages for 30 minutes.

The second group (DMSO+PTZ): an injection of DMSO (concentration 99.9%, 0.8 cc) was done intraperitoneally. PTZ injection was performed after 15 minutes and continued like the first group.

The third group (URB+LY+DMSO+PTZ): an injection of URB (1 mg/kg) and LY (2.5 mg/Kg) dissolved in DMSO was done and then single dose injection of PTZ was done after 15 minutes and continued similar to first group.

The fourth group (JJKKK+WWL+DMSO+PTZ): an injection of JJKKK (1 mg/kg) and WWL (5 mg/kg) dissolved in DMSO was performed and then after 15 minutes a single dose of PTZ was injected and continued similar to the first group.

The number of samples in PTZ group, DMSO+PTZ group and in the experimental groups were 12, 11 and 6, respectively.

Statistical analysis of data: GraphPad Prism statistical software was used for statistical analysis. The parametric data were analyzed and compared by using One-way ANOVA test and Bonferroni test. Nonparametric data were compared and analyzed by Kruskal-wallis test and Dunns test, and $p<0.05$ was considered significant. Data are presented as Mean±SEM.

Results

JJKK+WWL injection before PTZ injection caused a delay in the onset of stage 4 and 5 of seizures, while the URB+LY injection increased the duration of stage 5. The results showed that DMSO is effective in the arrival time to stage 1 and 2 of seizure so that arrival time to first seizure phase (44.0±5.3 seconds) in the group (DMSO+PTZ) significantly increased compared to (PTZ) (11.9±1.2 seconds) ($p<0.001$ for S1L). In addition, arrival time to stage 2 of seizure (98.7±18.7 seconds) in the group (DMSO+PTZ) has increased significantly compared to PTZ group (32.2±4.3 seconds) ($p<0.05$ for S2L) (Fig A, B-1). However DMSO had no significant effect on the arrival time to step 3-5 and duration of this stage.

Statistical analysis showed that the group receiving JJKK+WWL (JJKK: 1 mg/kg, WWL: 5 mg/kg) dissolved in DMSO for about 15 minutes before the injection of PTZ arrived significantly later to stage 4 and 5 of tonic-clonic seizure compared with the group received only DMSO before PTZ ($p<0.05$) (Fig D, E-1). However, a recipient group (URB: 1 mg/kg, LY: 2.5 mg/kg) URB+LY dissolved in DMSO prior to injection of PTZ had significantly a longer tonic - clonic seizure compared with the group received only DMSO before PTZ (Fig H-1).

JJKKK+WWL injection prior to PTZ decreased the incidence of stage 5 of seizure. The percent of stage 3 in four groups (PTZ), (DMSO+PTZ), (URB+LY+ DMSO+PTZ) and (JJKK+WWL+DMSO+PTZ) was 91%, 100%, 83% and 100%, respectively (Fig a-2) that there was not a significant difference between these groups. The percent of stage 4 in the above mentioned groups was 83%, 91%, 83% and 100%, respectively (Fig b-2) that there was also not a significant difference between these groups. The percent of stage 5 in the above mentioned groups was 41%, 90%, 66% and 50%, respectively.

Reduction of 40% stage 5 in the (JJKK+WWL+ DMSO+PTZ) group compared to (DMSO+PTZ) group was significant (Fig C-2). As the presented data indicates, apparently injection of JJKK + WWL before the PTZ is effective on the occurrence of tonic-clonic seizure stage. JJKKK+WWL and URB+LY intraperitoneal injection before PTZ had no effect on the incidence of seizure. Average stage of the incident seizure in four groups (PTZ), (DMSO+PTZ), (URB + LY+DMSO+PTZ) and (JJKK+WWL+DMSO+PTZ) was (4.1±0.3), (4.8±0.2), (4.3±0.5) and (4.5±0.2), respectively.

There was no significant difference between groups (Fig 3). Incidence of death in four groups (PTZ), (DMSO+PTZ), (URB+LY+DMSO+PTZ) and (JJKK+WWL+DMSO+PTZ) was (33%), (90%), (50%) and (50%), respectively. As numerical values show, the mortality rate in (URB+LY+DMSO+PTZ) and (JJKK+WWL+DMSO+PTZ) groups compared to (DMSO+PTZ) significantly decreased 40% (Fig 4).

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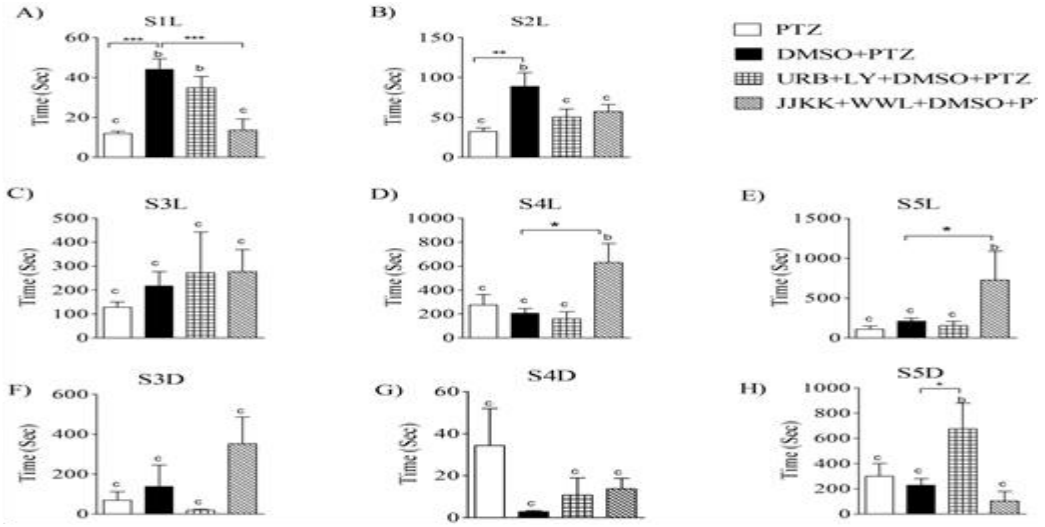

Figure 1. JJKK+WWL injection before PTZ injection caused a significant increase in the delay of stage 4 and 5 of seizure. Dissimilar letters on the columns show significant values and similar letters show insignificancy between groups. p<0.05*, p<0.01 ** and p<0.001 *. N in the PTZ group is 12 in the DMSO + PTZ group is 11 and in experimental groups is 6.**

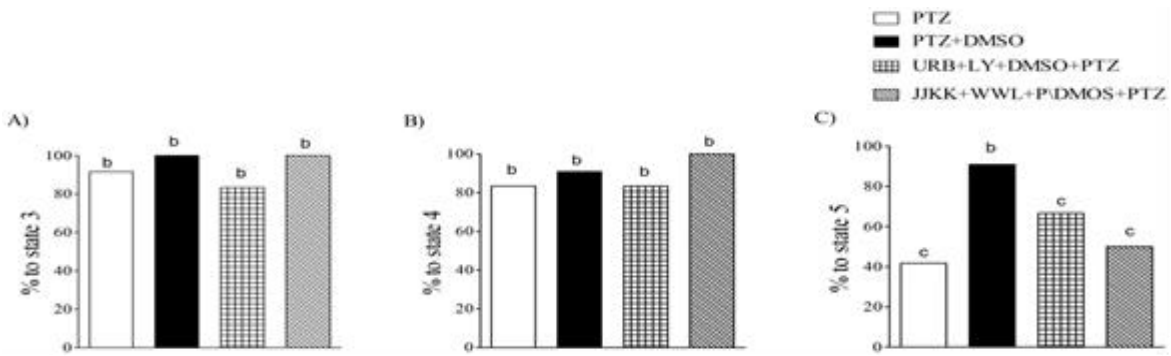

Figure 2. JJKK+WWL injection before PTZ injection reduced the percentage of tonic-clonic attacks. In the section C, the percentage of stage 5 of seizure dramatically decreased in (JJKK + WWL + DMSO + PTZ: 50%) and (URB+LY+DMSO+ PTZ: 66%) groups compared to (DMSO+PTZ: 91%). Dissimilar letters on the columns show significant values and similar letters show insignificant values between groups. N in the PTZ group is 12 in the DMSO+PTZ group is 11 and in experimental groups is 6.

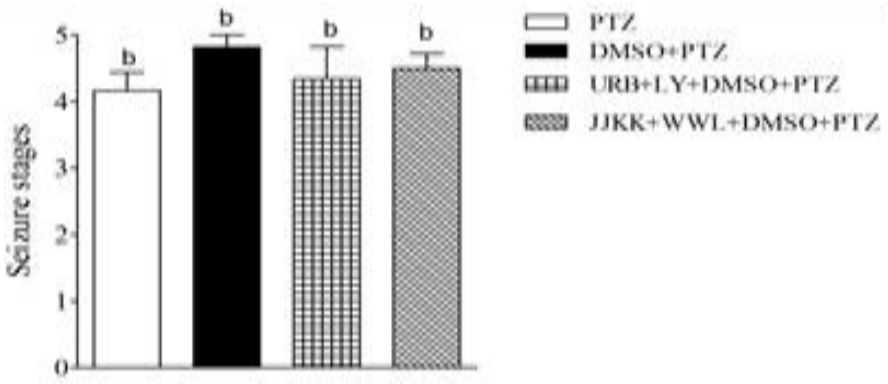

Figure 3. JJKK+WWL and URB+LY intraperitoneal injection 15 minutes before PTZ injection had no significant effect on the occurrence of seizures. Comparing the mean jerky stage in three groups together through Kruskal-Wallis and Dunn test is indicated. Data are presented as Mean ± SEM. Dissimilar letters on the columns show significant values and similar letters show insignificant values between groups. N in the PTZ group is 12 in the DMSO + PTZ group is 11 and in experimental groups is 6.

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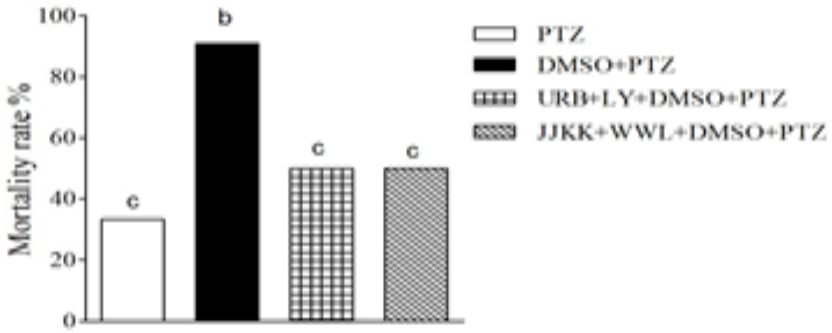

Figure 4. JJKK+WWL and URB + LY injection before PTZ injection reduced the amount of death after the tonic - clonic attack. Percentage of death in four groups is shown. Percent of death decreased from 91% in the (DMSO + PTZ) group to 50% in the group receiving JJKK + WWL and URB+LY. The data is expressed as a percentage. Dissimilar letters on the columns show significant values and similar letters show insignificant values between groups. N in the PTZ group is 12 in the DMSO + PTZ group is 11 and in experimental groups is 6.

Discussion

Results of the study showed that systemic injections of JJKK+WWL can substantially decrease the tonic-clonic seizures indicators. Delay in arrival time to stages 4 and 5 of seizures had significant increase. In addition, the percentage of stage 5 of tonic-clonic seizure and the percentage of deaths caused by seizure by injection of JJKK+WWL was significantly reduced. In this study, WWL70 was used as a powerful inhibitor of the ABHD enzyme and JJKK048 as strong -specific inhibitor of MAGL. ABHD and MAGL enzymes are two key components in the removal of 2-AG.

Therefore, using these inhibitors together can increase the levels of 2-AG. The results of this study showed that intraperitoneal injection of JJKK+WWL before injection of PTZ have a significant influence on delayed onset of stages 4 and 5 of tonic-clonic seizures, however, had no effect on the duration of these stages.

Increased latency in stages of seizures, especially in the tonic-clonic attacks meaning greater efforts inhibitory systems in the brain to prevent the spread of seizures in the brain, in other words seizures will be later generalized and have less stability (27). According to inhibition of key enzymes pathways involved in the removal of 2-AG by two inhibitor (JJKK + WWL), it is possible the increased time delay in reaching to tonic-clonic attacks as the result of an increase in 2-AG in the brain. Recent investigations have shown the effectiveness of herbal cannabinoids in inhibition of epileptic seizures occurrence in laboratory models (28-30). In a study, systemic use of a cannabinoid plant called Cannabidiol significantly decreased the stages 4 and 5 of tonic-clonic seizure in penicillin-induced seizures and pilocarpine-induced seizure (31).

Despite differences in methods of seizure induction in the mentioned studies and our study, reduction of tonic-clonic attacks in two studies are similar. In another study on the PTZ induced seizure, phytocannabinoid compounds produced in the laboratory, could be able significantly to reduce the severity of the seizure and death rates after seizures (32). Our results also indicated reducing the death after seizure following administration of JJKK+WWL that confirms the ability of brain endocannabinoid system to control seizures attacks. In a recent study, seizure was induced using PTZ in mice genetically prone to seizure and pharmacological inhibition of ABHD6 enzyme, one of the enzymes involved in the removal of 2-AG, the severity of seizure attacks was improved (14). This report, despite using genetically predisposed mice to seizures, is consistent with our study findings. It is necessary to note that ABHD6 undertakes only a small part of the removal of 2-AG's, while MAGL is responsible for major removal of 2-AG (8).

In our study, ABHD enzymes family and MAGL enzyme were inhibited. In this study also aimed to increase anandamide levels and URB597 was used as a strong inhibitor of the FAAH enzyme which is the key enzyme for the removal of anandamide. In addition, LY2183240 was used as re-uptake carrier inhibitor of anandamide. The simultaneous use of two inhibitors resulted in a significant increase in stage 5 of tonic-clonic attack. However, the deaths caused by seizures decreased. Increased levels of anandamide in the brain

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seems to be different from 2-AG functions that are not necessarily in line with the 2-AG and can have different consequences. In a study it was shown that mice with the FAAH enzyme defect and therefore increase level of anandamide in them are more prone to seizures and epilepsy (33).

Another finding in this study was the effect of DMSO solvent on seizure indices. Some recent reports have shown that DMSO can change electrophysiological parameters in animal models of epilepsy as dose-dependent. This report shows a decrease of electrical irritability and registered index and therefore its antiepileptic effects is suggested (34, 35). Based on the present results, DMSO as solvent of organic compounds can be effective in occurrence of PTZ-induced seizure. However this solution increased delay in the phase 1 and 2, but the percentage of stage 5 of seizure and the percentage of deaths due to tonic-clonic attacks increased. The reason for this phenomenon can be nervous mechanisms involved in different stages of seizures. Therefore, more investigations are needed to clarify the mechanisms of DMSO on neurons and neural circuits in future. Overall, this study is in line with previous studies (6,32,36,37) and confirms the ability of 2-AG as a component of endocannabinoid system to control tonic-clonic seizures induced by PTZ and it seems that increased levels of anandamide has a different effect from increased levels of 2-AG on indices of seizure induced by PTZ.

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