



ANTICONVULSANT EFFECT OF RESVERATROL AGAINST PENTYLENETETRAZOLE INDUCED- EPILEPSY IN RATS: ROLE OF NITRIC OXIDE

Amany N. Ibrahim¹MD and Anjuman Gul. Memon²

¹Department of Pharmacology, Faculty of Medicine, Benha University, Egypt.

²Department of Biochemistry, Faculty of Medicine, Qassim University, Saudi Arabia.

ABSTRACT

The present study aims to investigate the involvement of nitric oxide (NO) in the convulsive state induced by pentylenetetrazole (PTZ). Moreover, to evaluate the anticonvulsant effects of resveratrol on nitric oxide alternation in the PTZ induced epilepsy in rats. Adult male albino rats were divided into 8 groups: control group (CN), PTZ induced epileptic non treated rats, epileptic rats treated with L-arginine (NO precursor) 30 min prior to convulsant state with PTZ, epileptic rats treated with N (G) nitro L-arginine (L-NOARG) (NO synthase inhibitor) 30min prior to convulsive state, epileptic rats treated with resveratrol 30 min before induction of experimental epilepsy with PTZ, epileptic rats treated with coadministration of L-arginine and resvestrol 30 min prior to convulsive state induced by PTZ, epileptic rats treated with coadministration of L-NORAG and resveratrol 30 min prior to convulsive state induced by PTZ, and epileptic rats treated with coadministration of L-arginine, L-NORAG and resveratrol 30 min prior to convulsive state. The latency and duration of tonic-clonic seizures were recorded. Present work revealed that convulsive state induced by PTZ resulted in significant elevation ($p \leq 0.05$) of NOx (NO metabolites, NO^{2-} plus NO^{3-} as indices of NO generation), compared with normal rats. L-arginine induced significant elevation ($P \leq 0.05$) in cortical NOx and non-significant increase in the duration of clonic seizures, but both L-NORG and resveratrol significantly reduce ($p \leq 0.05$) both cortical NOx and duration of clonic seizures compared with epileptic treated group. Combined administration of L-arginine and resveratrol resulted in significant increase of both cortical NOx and duration of clonic seizures compared with resveratrol treated epileptic group. Moreover, combined administration of L-NOARG and resveratrol resulted in significant reduction ($P \leq 0.05$) of both cortical NOx and duration of clonic seizures. The last result was reversed by co-administration of L-arginine to L-NOARG and resveratrol which resulted in significant increase of cortical NOx and the duration of clonic seizures. This study reflects that NO has a significant effect on the PTZ- induced seizures. Moreover, the involvement of NO pathway in the mechanism of action of resveratrol seems probable, since the effect of NOSi was reversed by L-arginine. The present data promising anticonvulsant and potent antioxidant effects of resveratrol in reducing nitric oxide and induction of seizures in epileptic animals.

Keywords: epilepsy, resveratrol, Pentylenetetrazole, nitric oxide, nitric oxide synthase inhibitor.

INTRODUCTION

Epilepsy and seizures disorders affect 50 million people around the world and contribute to morbidity and mortality [1]. The use of antiepileptic drugs is limited due to the vast array of adverse effects. Such as cognitive

impairment, effective disorders and recurring seizures [2]. Hence, there is a need for the development of new antiepileptic drugs with fewer adverse effects and high efficacy. Pentylenetetrazole, a prototype chemoconvulsant, is commonly employed for inducing experimental

for the evaluation of antiepileptic agents. A part from blocking the chloride ionopore coupled to GABA receptors, seizures excite toxicity and free radical generation have been implicated in the mechanism of seizures induced by PTZ [3].

Seizures discharge are initiated in the parietal area, then generalized through the hippocampus. It is well established that alternation in excitatory and inhibitory aminoacids play a crucial role in the initiation, spread and termination of epileptic activity [4]. Oxidative stress and free radicals production are of the most important mechanisms by which neurological disorders such as epileptic seizure occur [5]. NO is known as a neurotransmitter in the brain that has shown paradoxical role in seizure modulation, as an inhibitor [6,7] and promoter [8] in different cases. Nitric oxide (NO) has recently been shown to be a novel class of neurotransmitter and cell to cell signaling agent [9]. It is produced in neurons and glia by the enzyme nitric oxide synthetase (NOS) and induces the formation of guanylate cyclase and it has been shown to contribute to glutamate excitotoxicity. Its role in epileptic activity is unclear. NO is formed from L-arginine by the enzyme NO synthase (NOS) [10] and the involvement of NO in epileptic disorders has been shown in experiments with systemic injection of NOS inhibitors [11,12]. However, NOS inhibitor treatment has been reported to augment [13] or to inhibit experimentally induced seizures.

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenolic phytoalexin that occurs naturally in many plant parts and products, such as grapes, berries, red wine, and peanut skins [14] and has numerous beneficial health effects. Evidence indicates that resveratrol exerts neuroprotective effects against diabetes-induced oxidative damage [15,16]. Resveratrol is also cardioprotective [17] anti-inflammatory [18] prevents certain cancers [19].

The present study aims to investigate the involvement of NO in the convulsive state induced by pentylenetetrazole. Moreover, to evaluate the possible anti-epileptogenic effects of resveratrol on nitric oxide alternation in the PTZ treated rats.

MATERIAL AND METHODS

Animals used

Adult male albino rats, weighing 140±20 gm were used for the experiment brought from (Experimental Animal Breeding Farm, Helwan - Cairo). All animals were housed in controlled laboratory condition at 20 -25C in a 12h light/dark cycle and had free access to standard laboratory chow (El-Nasr Company, Abou-Zaabal, Cairo, Egypt) and water. They have acclimatized for one week and were caged (6/cage) in fully ventilated room (at room temperature) in pharmacology department, Benha Faculty of Medicine. All experimental protocols were approved by the committee of Benha University.

Drugs:

- Resveratrol (Sigma-Aldrich, MO, USA).
- L-arginine (Winlab, Leicestershire U.K)
- Pentylenetetrazole (Leptazole) El-Nile Pharmacochem. Co.
- N(G) Nitro-L-arginine (Sigma Chemical Co. St. Louis, Mo, USA)

Design study

To investigate the role of NO in epileptogenesis of rats and its involvement of the antiepileptic effects of resveratrol, an experimental model of epilepsy was created by injection of PTZ in a dose 60 mg/kg I.P [20] in rats. Pentylenetetrazole (PTZ) was prepared daily by dissolving in normal saline. Test materials were administered in a volume not exceeding 10mg/kg, 30 min. before the seizure induction by PTZ. Animals were observed for myoclonic jerk latency and the occurrence of generalized tonic seizures up to 30 min. after PTZ injection [21]. The ability of a drug to prevent the seizures or delay/ prolong the latency or onset of the tonic hind-limb extensions was considered as an indication of anticonvulsant activity. Epilepsy was induced in 8 groups of rats (n= 12 in each group):

Group (1): Control group, this group did not receive any drug they were given equivalent amount of drug vehicle I.P.

Group (2): Epileptic non treated rats.

Group (3): Epileptic rats were treated with L-arginine (150mg/kg I.P) 30 min prior to convulsive challenge with PTZ (60mg/kg I.P).

Group (4): Epileptic rats were treated with N (G) nitro L-arginine (120 mg/kg I.P) 30 min prior to convulsive state induced with PTZ (60 mg/kg I.P).

Group (5): Epileptic rats were treated with resveratrol (80 mg/kg I.P) 30 min before induction of experimental epilepsy with PTZ (60mg/kg I.P).

Group (6): Epileptic rats treated with coadministration of L-arginine (150mg/kg I.P) and resveratrol (80mg/kg I.P) 30 min prior to convulsive state induced by PTZ (60mg/kg I.P).

Group (7): Epileptic rats treated with coadministration of L-NORAG (120mg/kg I.P) and resveratrol (80 mg/kg I.P).

Group (8): Epileptic rats treated with coadministration of L-arginine (150mg/kg I.P), L-NORAG (120mg/kg I.P) and resveratrol (80 mg/kg I.P) 30 min prior to convulsive challenge with PTZ (60mg/kg I.P).

The doses of the drugs used in the present work were according to previous pilot experiments [22,23]. The duration of clonic seizures were detected in each group and represented as mean ±S.E.

At the end of the experiments, rats were decapitated, the skull was opened, cerebral cortex was dissected and its NO was estimated as nitrite (NO²⁻) and nitrates (NO³⁻). Supernatant NO content was assayed by the Griess method [24]. Since NO is a compound with a short

half-life and is rapidly converted to the stable end products of nitrate (NO₃⁻) and nitrite (NO₂⁻), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and *n*-naphthyl ethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer. NO³⁻ was measured in the same samples according the method described by Hashiguchi et al [25].

Statistical analysis: Results of the present work were presented as mean± S.E. P values were calculated by

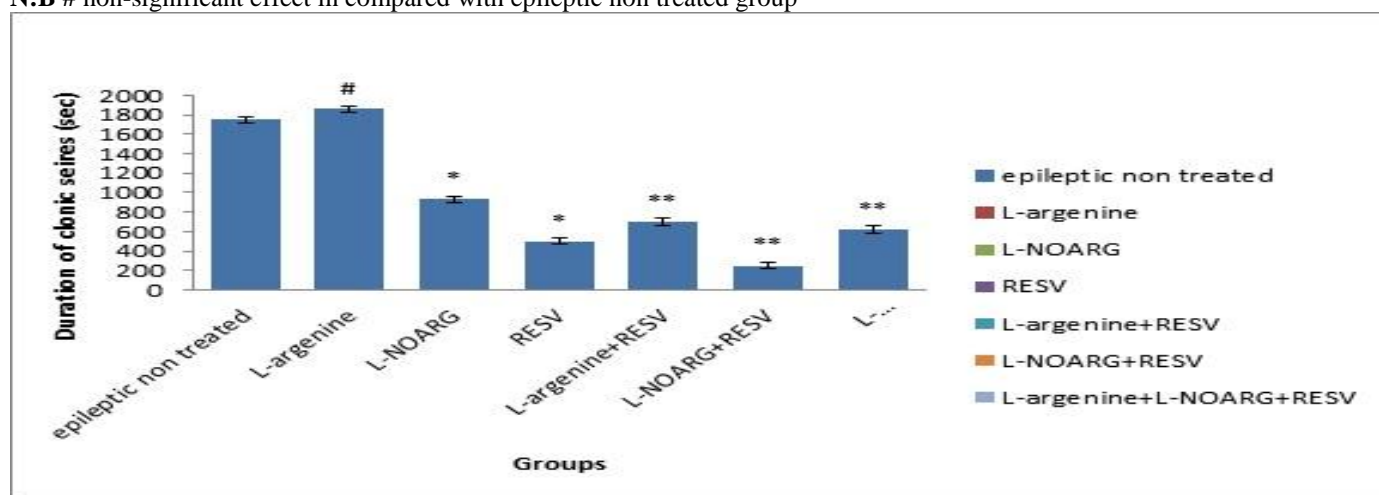
unpaired (t) test, P≤0.05 was taken as the limit of significance.

RESULTS

1- Effect of Resveratrol against PTZ-induced seizures:

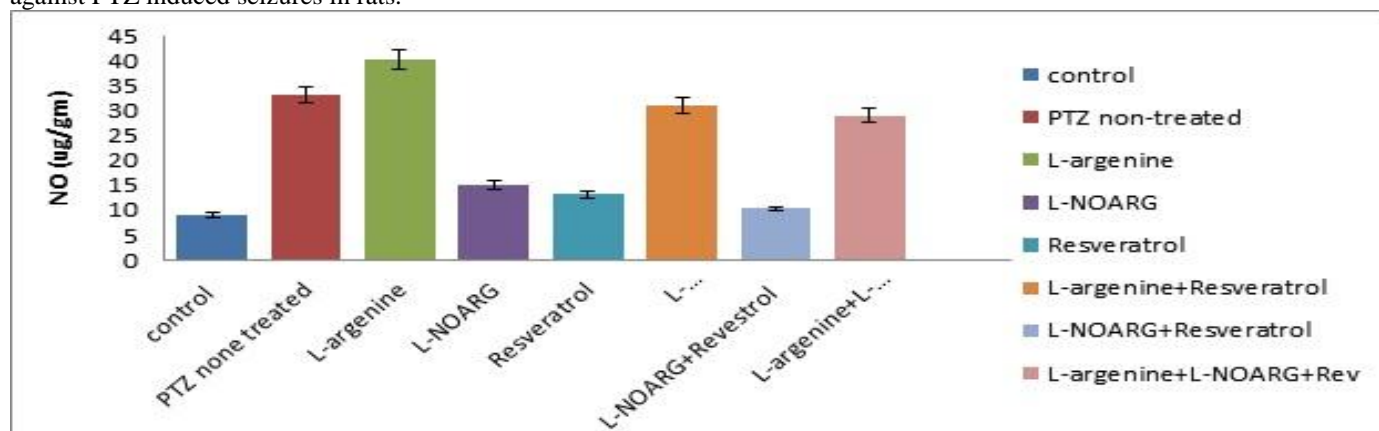
when resveratrol was administered at dose of 80 mg/kg 30 min prior to PTZ challenge, the latency of myoclonic jerks increased significantly (p<0.05) as compared with PTZ. There was also a decrease in the incidences of generalized tonic-clonic convulsions (Figure 1). Moreover, resveratrol decreased cortical level of nitric oxide significantly (p<0.05) as compared with PTZ (Figure2).

Figure 1. Effect of L-arginine (150mg/kg I.P), L-NOARG (120mg/kg I.P), resveratrol (80 mg/kg I.P and co-administration of L-arginine (150mg/kg I.P) + resveratrol (80 mg/kg I.P) and effect of L-NORG (120 mg/kg I.P) + resveratrol (80mg/kg I.P) and combined administration of L-arginine (150mg/kg I.P)+L-NOARG (120 mg/kg I.P) + resveratrol (80 mg/kg I.P) against epilepsy-induced by PTZ (60mg/kg I.P) in male adult rats measured in seconds (means ±S.E)
N:B # non-significant effect in compared with epileptic non treated group



*significant effect in compared with epileptic non treated group. **significant effect compared with resveratrol (RESV) group

Figure 2. shows the concentration of NOx (ug/gm) in cerebral cortex of rat’s brain and the effect of L-arginine (150mg/kg I.P), L-NOARG (120 mg/kg I.P), resveratrol (80 mg/kg I.P), and combined effects of L-NOARG (120 mg/kg I.P.) + Resveratrol (80 mg/kg I.P) and lastly co-administration of L-arginine (150mg/kg I.P) +L-NOARG (120 mg/kg I.P) + Resveratrol (80 mg/kg I.P) against PTZ induced seizures in rats.



DISCUSSION

Epilepsy is a common disorder, affecting roughly 0.5% of the population. The first line antiepileptic drugs (AEDs) such as benzodiazepines and phenytoin used for the treatment of status epilepticus are ineffective in ~40% of patients [26,27]. So, efficient alternative therapies are necessary for preventing status epilepticus-induced mortality and morbidity.

The treatment of epilepsy remain far from adequate primarily due to inadequate understanding of the pathophysiology of seizures whether or not, a disturbance of excitatory or inhibitory transmission is part of the mechanism of epilepsy. Convulsant drugs, such as PTZ are often used. It has been found that activity in inhibiting PTZ induced convulsions is a fairly good index of effectiveness against seizures [28]. NO content in rat's brain cortex was determined by measuring NO metabolites as indices of NO generation.

In the present study L-arginine, substrate for NO generation, significantly increased the duration of clonic seizures. These results were in line with McNamara et al, who reported that L-arginine at different doses (75, 150, and 300 mg/kg) increased the intensity of current required to produce a threshold seizure and significantly decreased the dose of PTZ required to produce a threshold seizure. Han et al reported that L-arginine increased seizure severity in response to subconvulsive dose of NMDA, suggesting that NO is a proconvulsant mediator.

Epileptic rats pretreated with L-NOARG, showed significant reduction of both cortical NOx content and duration of clonic seizures. Akula et al [29] examined the effect of systemic injection of L-NOARG on PTZ induced seizures reaching the conclusion that L-NOARG preferentially suppressed tonic generalized extension and prolonged its onset latency. PTZ induced seizures are related to the activation of NMDA receptors and to competitive inhibition of GABA neurotransmission. GABA and NMDA increase intracellular Ca²⁺ which activate NO synthase [30]. Thus L-NOARG inhibition of NOS could help to suppress NO and seizures. NO is formed from L-arginine by the enzyme NO synthase and the involvement of NO in epileptic disorder has been shown in experiments with systemic injection of NOS inhibitors [31].

However, NOS inhibitor treatment has been reported either to augment or inhibit experimentally induced seizures. Acute effects of NO might involve an influence of NO on NMDA-mediated neurotransmission. NO has a complex influence on this mediated neurotransmission. NO has a complex influence on this neurotransmission. It may mediate that NMDA-induced increase in cGMP but simultaneously inhibit the NMDA-induced increase in intracellular Ca²⁺ and NOS activity, and block NMDA receptor. The influence of NO on NMDA neurotransmission may vary widely according to epilepsy model, drug concentration, and site of injection which might explain the range of conflicting results on the

role of NO in NMDA-mediated events such as epilepsy [32] demonstrated that exogenously applied NO or its precursors can enhance seizure triggering activity.

Bashkatova et al [33] found dramatic elevation of NO production was found during PTZ-induced epileptiform seizures. These results suggesting a potential role for NO in mechanisms regulating seizures induction and propagation. Rajasekaran et al [34] reported evidence for a trigger role of neuronally produced NO in epileptogenesis induced by picrotoxin.

Result of the present study also, showed that resveratrol significantly reduced cortical NOx and duration of clonic seizures. These results are in line with other studies showing the positive effects of resveratrol and other polyphenols [35] on nitric oxide synthase. A study by Virgili and Contestabile [36] was the first to suggest a neuroprotective property for resveratrol against excitotoxic brain injury. Another study demonstrated the protective effect of resveratrol pretreatment on kainic acid induced seizures and oxidative stress [37]. They suggested that cGMP/NO system may participate in neuroprotective effects of resveratrol. Also, Bastianetto et al [38] reported participation of NO in neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. Furthermore, a study has shown neuroprotection when resveratrol was administered prior to intra-cortical placement of FeCl₃ [39]. In the absence of resveratrol treatment, FeCl₃ treated animals exhibited significant epileptiform electroencephalogram (EEG) discharges and increased levels of the oxidative stress marker malondialdehyde in the brain tissue. However, in animals that received resveratrol (20 or 40 mg/kg i.p.) 30 min prior to FeCl₃ treatment, the onset of the epileptiform EEG discharges was delayed and malondialdehyde levels were reduced.

In order to prove the involvement of NO in the antiepileptic effect of resveratrol. Results of the present work showed that combined administration of L-arginine and resveratrol resulted in significant elevation of cortical NOx and increased the duration of clonic seizures. Gupta et al [40] found that the antiepileptic activity of resveratrol against PTZ induced clonic and tonic seizures was reversed by L-arginine. Moreover, combined administration of L-NOARG and resveratrol resulted in significant reduction of cortical NOx and duration of clonic seizures, but this effect was completely reversed by combined administration of L-arginine to both resveratrol and L-NORAG. Similar results were obtained by Padin et al [41].

In conclusion, results of the present work revealed that NO may be involved in seizures induced by PTZ model and suggest that NO may be involved in anticonvulsant effect of resveratrol against PTZ induced seizures. It appears that resveratrol has a promise as a therapeutic drug for acute seizure-induced chronic epilepsy development.

REFERENCES

1. Bertram E. The relevance of kindling for human epilepsy. *Epilepsies*, 48, 2007, 65-74.
2. Duncan JS, Sander JW, Sisodiya SM, Walker MC. Adult epilepsy. *Lancet*, 367, 2006, 1087-1100.
3. Luttjohann A, Fabene PF, Van Luijckelaar G. A revised Racine's scale for PTZ-induced seizures in rats. *Physiol Behav*, 98, 2009, 579-586.
4. McNamara JO, Huang YZ, Leonard AS. Molecular signaling mechanisms underlying epileptogenesis. *Science's STKE, Signal transduction knowledge environment*, 10, 2006, 356.
5. Frantseva MV, Perez Velazquez JL, Tsoraklidis G, Mendonca AJ, Adamchik Y, Mills LR, Carlen PL, Burnham MW. Oxidative stress is involved in seizure-induced neurodegeneration in the kindling model of epilepsy. *Neuroscience*, 97, 2000, 431-435.
6. Jelenkovic A, Jovanovic M, Ninkovic M, Maksimovic M, Bokonjic D, Boskovic B. Nitric oxide NO and convulsions induced by pentylentetrazol. *Ann N Y Acad Sci*, 962, 2002, 296-305.
7. Marango AH, Yildirim M, Ayyildiz M, Marangoz C. The interactions of nitric oxide and acetylcholine on penicillin-induced epilepsy in rats. *Neurochem Res*, 37, 2012, 1465-1474.
8. Waxman EA, Lynch DR. N-methyl-D-aspartate receptor subtypes, multiple roles in excitotoxicity and neurological disease. *Neuroscientist*, 11, 2005, 37-49.
9. Machado-Nils AV, de Faria LO, Vieira AS, Teixeira SA, Muscará MN, Ferrari EA Daily cycling of nitric oxide synthase NOS in the hippocampus of pigeons *C. livia*. *J Circadian Rhythms*, 1(11), 2013, 12.
10. Garthwaite J Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci*, 27, 2008, 2783-2802.
11. Han D, Yamada K, Senzaki K, Xiong H, Nawa H, Nabeshima T. Involvement of nitric oxide in pentylentetrazole-induced kindling in rats. *J Neurochem*, 74, 2000, 792-798.
12. Akula KK, Dhir A, Kulkarni SK. Nitric oxide signaling pathway in the anti-convulsant effect of adenosine against pentylentetrazole-induced seizure threshold in mice. *Eur J Pharmacol*, 587, 2008, 129-134.
13. Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system, neuroprotection versus neurotoxicity. *Nat Rev Neurosci*, 8, 2007, 766-75.
14. Bertelli AAA and Das DK. Grapes, wines, resveratrol, and heart health. *Journal of Cardiovascular Pharmacology*, 54, 2009, 468-476.
15. Ates O, Cayli SR, Yucel N. Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats. *Journal of Clinical Neuroscience*, 14, 2007, 256-260.
16. Venturini CD, Merlo S, Souto AA, Fernandes MDC, Gomez R, Rhoden CR. Resveratrol and red wine function as antioxidants in the nervous system without cellular proliferative effects during experimental diabetes. *Oxidative Medicine and Cellular Longevity*, 3, 2010, 434-441.
17. Das S, Das DK. Resveratrol, a therapeutic promise for cardiovascular diseases. *Recent Patents on Cardiovascular Drug Discovery*, 2, 2007, 133-138.
18. Bertelli AAE, Ferrara, Diana FG. Resveratrol, a natural stilbene in grapes and wine, enhances intraphagocytosis in human promonocytes, a co-factor in antiinflammatory and anticancer chemopreventive activity. *International Journal of Tissue Reactions*, 21, 1999, 93-104.
19. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer, preclinical and clinical studies. *Anticancer Research*, 24, 2004, 2783-2840.
20. Del-Bel EA, Oliveira PR, Oliveira JA, Mishra PK, Jobe PC, and Garcia-Cairasco N. Anticonvulsant and proconvulsant roles of nitric oxide in experimental epilepsy models. *Braz J Med Biol Res*, 30, 1997, 971-9.
21. Ferraro TN, Golden GT, Smith GG, St Jean P, Schork NJ, Mulholland N, Ballas C, Schill J, Buono RJ, Berrettini WH. Mapping loci for pentylentetrazol-induced seizure susceptibility in mice. *J Neurosci*, 19, 1999, 6733-9.
22. Rege SD, Kumar S, Wilson DN, Tamura L, Geetha T, Mathews ST, Huggins KW, Broderick TL, Babu JR. Resveratrol protects the brain of obese mice from oxidative damage. *Oxid Med Cell Longev*, 41, 2013, 90-92.
23. Gupta YK, Briyal S, Chaudhary G. Protective effect of trans-resveratrol against kainic acid-induced seizures and oxidative stress in rats. *Pharmacol Biochem Behav*, 71, 2002, 245-9.
24. Cortas NK, Wakid NW. Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. *Chemistry*, 36, 1990, 1440-3.
25. Hashiguchi W, Nagatomo I, Akasaki Y, Uchida M, Tominaga M, Takigawa M. Influences of caffeine to nitric oxide production and zonisamide concentration in the brain of seizure-susceptible EL mice. *Psychiatry Clin Neurosci*, 55, 2001, 319-24.
26. Shaner DM, McCurdy SA, Herring MO, Gabor AJ. Treatment of status epilepticus, A prospective comparison of diazepam and phenytoin versus phenobarbital and optional phenytoin. *Neurology*, 38, 1988, 202-207.
27. Sirven JI, Waterhouse E. Management of status epilepticus. *Am Fam Physician*, 68, 2003, 469-476.

28. Rauca C, Zerbe R, Jantze H. Formation of free hydroxyl radicals after PTZ-induced seizure and kindling. *Brain Res*, 847, 1999, 347-351.
29. Akula KK, Dhir A, Kulkarni SK. Rofecoxib, a selective cyclooxygenase-2 COX-2 inhibitor increases pentylenetetrazol seizure threshold in mice, possible involvement of adenosinergic mechanism. *Epilepsy Res*, 78, 2008, 60-70.
30. Huang RQ, Bell-Horner CL, Diabas MI, Covey DF, Drewe JA, Dillo GH. Pentylenetetrazole-induced inhibition of recombinant gamma-aminobutyric acid type A GABAA receptors, mechanism and site of action. *J Pharmacol Exp Ther*, 298, 2001, 986-995.
31. Itoh M, Watanabe H. CGAS, Comparative genomic analysis server. *Bioinformatics*, 25, 2009, 958-9.
32. Tian J, Kim SF, Hester L, Snyder SH. S-nitrosylation/activation of COX-2 mediates NMDA neurotoxicity. *Proc Natl Acad Sci*, 105, 2008, 10537-10540.
33. Bashkatova V1, Narkevich V, Vitskova G, Vanin A The influence of anticonvulsant and antioxidant drugs on nitric oxide level and lipid peroxidation in the rat brain during pentylenetetrazole-induced epileptiform model seizures. *Prog Neuropsychopharmacol Biol Psychiatry*, 27, 2003, 487-92.
34. Rajasekaran K1, Jayakumar R, Venkatachalam K. Increased neuronal nitric oxide synthase nNOS activity triggers picrotoxin-induced seizures in rats and evidence for participation of nNOS mechanism in the action of antiepileptic drugs. *Brain Res*, 979, 2003, 85-97.
35. Petrovski G, Gurusamy N, Das DK. Resveratrol in cardiovascular health and disease. *Ann N Y Acad Sci*, 1215, 2000, 22-33.
36. Virgili A, Contestabile M. Contestabile Partial neuroprotection of in vivo excitotoxic brain damage by chronic administration of the red wine antioxidant agent, trans-resveratrol in rats. *Neurosci Lett*, 281, 2011, 123-126.
37. Gupta YK, Chaudhary G, Srivastava AK. Protective effect of resveratrol against pentylenetetrazole-induced seizures and its modulation by an adenosinergic system. *Pharmacology*, 65, 2002, 170-4.
38. Bastianetto, Stéphane, Zheng, Wen-Hua Quirion, Rémi. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *British Journal of Pharmacology*, 131, 2000, 711-720.
39. Gupta Y, Chadhary G, Sinha K, Srivastava AK protective effect of resveratrol against intracortical FeCl₃-induced model of posttraumatic seizures in rats. *Methods Find Exp Clin Pharmacol*, 23, 2001, 241-244.
40. Padin CE, de Diego AM, Fernandez-Morrales JC. Resveratrol augments nitric oxide generation and causes store calcium release in chromaffin cells. *Eur J Pharmacol*, 685, 2012, 99-107.