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ORIGINAL RESEARCH

Benefits of Combining Antiepileptic Drugs with Vitamin B12 on Redox Balance: Penicillin-Induced Experimental Epilepsy Model

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Purpose: A combination of antiepileptic drugs and antioxidants may be an effective treatment by restoring the disrupted redox balance and reducing oxidative stress exposure to neurons. This study aims to evaluate the effects of valproate and vitamin B12 on oxidative stress in an experimental epilepsy model induced by penicillin when administered alone or in combination.

Patients and Methods: 35 male Wistar rats were used in this study. The rats were divided into five groups, which were saline group, 1 mg/kg, 2 mg/kg Vit B12 groups and Sodium valproate group Sodium valproate + Vit B12 group. The epileptic activity was induced by 500 IU of penicillin injection. Sodium valproate and Vitamin B12 were administered 30 min after penicillin administration. Electrocorticogram recordings were taken for 2 hours post-treatment and serum parameters were assessed for oxidative stress markers using spectrophotometric method.

Results: There is statistically significant difference between the groups in total antioxidant status, total oxidant status, and oxidative stress index value ($p=0.013$; $p<0.001$; $p<0.001$, respectively). The valproate+vitamin B12 group showed elevated total thiol and native thiol levels, along with reduced disulphide levels, resulting in the lowest OSI value.

Conclusion: These findings suggest the combined treatment effectively reduces oxidative stress. This study provides valuable insights into the antioxidant properties of valproate and vitamin B12, positioning them as potential agents for managing epilepsy. Understanding the efficacy and reliability of antioxidant strategies in epilepsy management could contribute significantly to advancements in epilepsy therapeutics.

Keywords: epilepsy, animal model, antiepileptic drug, Vitamin B12, oxidative stress

Introduction

Epilepsy is a neurological condition that affects millions of people worldwide, regardless of age or race.[1] As a chronic neurological state, it is characterized by repetitive and unprovoked seizures.[2] The disease is associated with abnormal neuronal activity in the brain, causing an imbalance between inhibitory GABA-mediated and excitatory glutamate-mediated neurotransmission.[3] While acute brain injuries, gene mutations, central nervous system infections, and autoimmune conditions have been identified as potential causes of epilepsy, the cause remains unknown in many cases.[4] The pathophysiological mechanisms responsible for the onset and recurrence of epileptic seizures are yet to be fully resolved.[5]

Seizure activity in epilepsy affects many chemical and biophysical processes in the brain. Studies show that increased neuronal excitability is accompanied by neuroinflammation and excessive reactive oxygen species (ROS) production.[6] Prolonged neuronal stimulation and increased mitochondrial activity with high energy demand of neurons cause an increase in ROS production. This process induces oxidative stress, and increased oxidative stress contributes to seizure-induced brain damage.[7] Current evidence points to the role of oxidative stress in the pathophysiology of epilepsy.[3] It has been stated that oxidative stress, which occurs due to the deterioration in the

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homeostatic balance between oxidants and antioxidants, plays a role in the pathophysiology of many diseases.[3,8] There are many biochemical methods used to analyze the level of oxidative stress in biological systems.[9] Thiol disulfide balance, total oxidant status (TOS), and total antioxidant status (TAS) are widely recognized as key markers of oxidative stress. These tests are frequently employed by researchers to assess oxidative stress at the systemic level. They are fast, reliable, and provide valuable information on the redox status of biological systems.[10,11]

Epilepsy is typically treated using pharmacotherapy, but around one-third of cases do not respond to available antiseizure medications.[12] To address this issue, combination therapies may be a potential strategy due to the limitations and side effects of individual drugs. Vitamins, specifically Vitamin B12, have been identified as a potential treatment option due to their antioxidant, anti-inflammatory, and immunomodulatory properties.[13] Vitamin B12 is involved in cellular metabolism and offers a range of functions including neuroprotection, immunomodulation, and antinociception.[14,15] As a result, Vitamin B12 may be able to prevent neuronal damage in the brain with its antioxidant functions.

Animal models are widely used for the study of epilepsy, research on the pathogenesis of epilepsy, or the effectiveness of new antiepileptic agents.[16] Studies suggest that protection against oxidative damage may be effective in reducing neuronal degeneration in epilepsy. Therefore, therapeutic approaches in many neurological diseases, including epilepsy, have been suggested to also target oxidative stress.[7]

The objective of our research was to assess the impact of administering valproate and vitamin B12 either alone or in combination on oxidative stress in an experimental epilepsy model induced by penicillin. We evaluated the thiol disulfide balance as well as the total oxidant/antioxidant status to investigate the effects.

Materials and Methods

Animal

35 male Wistar rats (200–250 g) were used in this study. All animals were housed in standard transparent cages in conditions that 12 h light:12h dark cycle with food and water *ad libitum* at 22 ± 1°C. The study protocol was approved by the Gaziosmanpasa University Animal Experiments Local Ethics Committee (No: 51879863–60) and conformed to the Guide for the Care and Use of Laboratory Animals. The rats were randomly assigned into five groups each with 7 rats as follows:

Group 1 (n=7): Penicillin (500 IU, 2.5 µL, i.c.) plus saline solution 1 mL intraperitoneally (i.p.)
Group 2 (n=7): Vitamin B12 (1 mg/kg, i.p.) plus penicillin (500 IU, 2.5 µL, i.c.)
Group 3 (n=7): Vitamin B12 (2 mg/kg, i.p.) plus penicillin (500 IU, 2.5 µL, i.c.)
Group 4 (n=7): Sodium valproate (500 mg/kg i.p.) plus penicillin (500 IU, 2.5 µL, i.c.)
Group 5 (n=7): Vitamin B12 (2 mg/kg i.p.) plus sodium valproate (500 mg/kg i.p.) plus penicillin (500 IU, 2.5 µL, i.c.).

Experimental Protocol

The animals in this study were anesthetized with urethane at a dose of 1.25 mg/kg, administered intraperitoneally (i.p.). Once the animals were fully under anesthesia, they were placed in a stereotaxic frame, and their heads were shaved. Epileptic activity was induced by injecting 500 international units (IU) of penicillin G potassium. The injections were made in a position about 2 mm posterior to the bregma, 2 mm lateral to the midline, and 3.2 mm ventral to the surface of the skull with reference to the bregma point using a stereotaxic tool. Chemical agents (sodium valproate and Vitamin B12) were administered 30 minutes after the penicillin injection. Cortical electrical activity was then recorded on the cortex for 180 minutes using electrocorticography. After observing the epileptic activity of penicillin injection, all rats were decapitated, and their blood sample was collected for further evaluation of biochemical parameters. The collected samples were centrifuged at 2000–2500 rpm for 10 minutes, and the serum was immediately separated and stored at −20°C in a deep freezer.

Thiol Disulfide Homeostasis Assay

The thiol disulfide homeostasis parameters in the serum samples were identified by using the spectrophotometric method of Erel and Neselioglu.[11] This method is based on the reduction of dynamic disulphide bonds to reactive thiol groups in the presence of sodium borohydride. Total thiol and native thiol levels were directly measured. Dynamic disulfide bond is equal to half of the difference between total and native thiols. Other parameters were calculated and expressed as previously described.[17]

Measurement of the Total Antioxidant Status (TAS) and Total Oxidant Status (TOS)

Total oxidant status (TOS) levels of the serum were analyzed using Erel's method.[10] As stated in the working principle of the test, oxidants convert ferrous ions to ferric ions in the presence of o-dianisidine. The oxidation reaction that causes ferric ion formation is colored with xylenol orange compound in acidic environment. The density of the color is proportional to the level of oxidants. Hydrogen peroxide (H_2O_2) was used as the calibrator. The results were reported in μmol H_2O_2 equivalent/L.

Total antioxidant status (TAS) levels, the other component of oxidative balance, were analyzed using the Erel method.[18] Erel's method is based on the bleaching-decolorizing of the characteristic color of stable ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid)) radical cation by antioxidants. The experiments were performed by spectrophotometrically at 660 nm with Microplate Reader (ThermoFisher Scientific Varioskan™ LUX multimode microplate reader, USA). The results were reported in mM Trolox equivalent/L. The ratio of TOS to TAS was defined to be OSI. The resulting unit of TAS was converted to μmol/L. Calculation of OSI value was made using the formula;

OSI (arbitrary unit) =[TOS (μmol H_2O_2 equivalent/L)/ TAS (μmol Trolox equivalent/L)] ×100

Statistical Analysis

Statistical analyses were carried out using the SPSS 25.0 package program (IBM SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was used to investigate whether the data showed normal distribution. Non-parametric tests were used to evaluate data that did not comply with normal distribution. Results are expressed as median (Q1-Q3). Differences between groups were investigated with the Kruskal Wallis *H*-test. P values of less than 0.05 were considered statistically significant.

Results

TAS, TOS and OSI, which are one of the best indicators of systemic oxidant-antioxidant balance, were analyzed in serum samples. There was a statistically significant difference between the groups in TAS, TOS and OSI values (p= 0.013; p<0.001; p<0.001, respectively). Notably, Group 5 (Valproate + vitamin B12) demonstrated the lowest TOS and OSI values, while also exhibiting the highest TAS value (Table 1). These results suggest that the combination of valproate and vitamin B12 may effectively reduce oxidative stress, as evidenced by the significant decrease in TOS and OSI levels and an increase in TAS levels.

An additional marker that can indicate oxidative stress is the disruption of Thiol disulfide homeostasis. The group administered with valproate + vitamin B12 (group 5) displayed the highest levels of native and total thiols, with a statistically significant difference observed in the total thiol value (p = 0.017) when compared to other groups.

Table 1 Total Oxidant/Antioxidant Status and Oxidative Stress Index Levels of Groups

Characteristics	Group 1 (n=7)	Group 2 (n=7)	Group 3 (n=7)	Group 4 (n=7)	Group 5 (n=7)	p value
TAS, mmol trolox Equiv. / L Median (Q1-Q3)	0.68 (0,58–0,73)	0,56 (0,52–0,66)	0,57 (0,48–0,62)	0,55 (0,39–0,66)	0,77 (0,69–0,80)	**0.013** [a]
TOS, μmol H_2O_2 Equiv. / L Median (Q1-Q3)	222 (86–354)	68 (53–77)	59 (48–95)	36 (31–42)	30 (25–40)	**<0.001** [b,c]
OSI, AU Median (Q1-Q3)	36,5 (10,9–53,0)	10,8 (9,5–14,1)	12,1 (8,9–19,2)	7,2 (5,3–8,8)	4,2 (3,2–5,7)	**0.001** [a,b,c]

Notes: Note: Bold values statistically significant at p < 0.05. [a]Group 3 vs Group 5. [b]Group 1 vs Group 4. [c]Group 1 vs Group 5.
Abbreviations: TOS, Total oxidant status; TAS, Total antioxidant status; OSI, Oxidative stress index.

Table 2 Thiol Disulfide Homeostasis Parameters of Groups

Characteristics	Group 1 (n=7)	Group 2 (n=7)	Group 3 (n=7)	Group 4 (n=7)	Group 5 (n=7)	p value
Native Thiol, µmol / L Median (Q1-Q3)	82 (76–91)	77 (68–98)	80 (43–160)	73 (65–89)	121 (82–191)	0.311
Total Thiol, µmol / L Median (Q1-Q3)	208 (192–230)	246 (233–273)	243 (222–306)	218 (211–256)	270 (244–315)	**0.017**[c]
Disulphide, µmol / L Median (Q1-Q3)	64 (56–70)	81 (70–101)	86 (59–101)	78 (65–88)	53 (43–109)	0.287
Reduced Thiol, % Median (Q1-Q3)	39 (36–42)	32 (25–41)	35 (19–48)	33 (28–38)	52 (27–68)	0.507
Oxidized Thiol, % Median (Q1-Q3)	30 (28–31)	33 (29–37)	32 (25–40)	33 (30–35)	23 (15–36)	0.507
ThiolOxidation-Reduction Median (Q1-Q3)	76 (68–86)	107 (70–148)	94 (57–214)	97 (81–128)	44 (23–133)	0.507

Notes: Bold values statistically significant at $p < 0.05$. [c]Group 1 vs Group 5.

There was no significant difference in disulfide level among the groups, but the lowest disulfide level was noted in the valproate + vitamin B12 group (group 5). Compared to the epilepsy group (Group 1) and the valproate (group 4) group, the valproate + vitamin B12 group (group 5) demonstrated lower disulfide levels (Table 2). Overall, the combination of valproate + vitamin B12 was found to be effective in reducing oxidative stress based on these results.

Discussion

In this study, for the first time in the literature, we investigated the effect of valproate + vitamin B12 combination treatment on thiol disulfide homeostasis and oxidative stress in epilepsy. The major findings were that (a) Total thiol, and native thiol levels were higher in the valproate + vitamin B12 group and (b) Disulphide levels were lower in the same group (c) The group with the lowest OSI value was the valproate + vitamin B12 group. These findings have significant implications for the treatment of epilepsy and provide valuable insights into the potential therapeutic benefits of this combination therapy.

Epileptogenesis is a concept that encompasses all biological factors that cause or support the progression of epilepsy.[19] It is known that the increase in ROS contributes to the processes of neuroinflammation, neurodegeneration and invalid neurogenesis, which are considered to be the key mechanisms that cause epileptogenesis.[20] Excessive ROS production causes mitochondrial DNA damage and decreased activity of tricyclic acid cycle enzymes. Additionally, ROS-induced changes in the structure of glutamate receptors lead to a decrease in the energy-dependent effect of glutamate transporters and a loss of GABA-ergic neurons in the hippocampus, ultimately increasing neuronal excitability and seizure susceptibility. These findings strengthen the notion that oxidative stress may play an important role in epileptogenesis.[3] The findings obtained in our study support this opinion. To balance this redox balance that is disrupted in epilepsy, combination treatments of antiepileptic drugs and substances with antioxidant effects may be effective in reducing the exposure of neurons to oxidative stress.

Experimental models and results obtained from patients support the role of oxidative stress in the seizure formation process in epilepsy and the mechanisms associated with resistance to drug treatment.[7] It has been stated that oxidant agents result from excitotoxicity resulting from overstimulation of the glutamatergic system observed in epilepsy.[3] The increase in glutamate in the synaptic cleft causes excessive activation of N-methyl-D-aspartate (NMDA) receptors and subsequent intraneuronal accumulation of excessive amounts of calcium. This Ca^{2+} overload promotes the formation of reactive oxygen/nitrogen species that compromise mitochondrial function, cause metabolic impairment, and activate necrotic and apoptotic pathways.[21] This results in oxidative stress, which causes functional cellular deterioration.

Various biochemical markers are utilized to evaluate the level of oxidative stress in the body. The most commonly used indicators are thiol disulfide balance, total oxidant status (TOS), and total antioxidant status (TAS). These tests can serve as a reliable and practical technique to assess oxidative stress. Besides being a systemic marker of oxidative stress, thiol disulfide balance also has crucial functions in numerous biological activities such as signal transduction, enzymatic activities, detoxification, and programmed cell death.[22,23] Clinical studies have shown that the change in thiol-disulfide homeostasis can be used in the monitoring of many diseases or may play a role in the pathogenesis of the disease.[8,17] Therefore, it is important to determine the thiol-disulfide status in diseases.

The change in redox balance in epilepsy has been investigated by several researchers.[24] In their study, Menon et al reported that antioxidant levels were significantly lower in epilepsy patients. The presence of low catalase, SH group, vitamin E and total antioxidant levels in epilepsy patients expressed this finding.[25] Güngör et al reported that antioxidant activity decreased and oxidant activity increased in patients with epilepsy, and native thiol and total thiol levels were found to be lower in epilepsy patients compared to controls.[26] Similarly, in another study, low native and total thiol levels were found in epilepsy patients compared to the control group.[27] However, there is also a study reporting that there is no significant difference in thiol-disulfide balance.[28] Kocatürk et al stated that there was no significant difference in thiol-disulfide homeostasis between well-controlled epilepsy patients and healthy controls, and the possible reason for this was that the well-controlled epilepsy group was seizure-free.[29] Seizure occurrence may be related to the homeostatic imbalance of antioxidants and oxidants.

Antiepileptic drugs used in the treatment of epilepsy mainly target ion channels and act by blocking neuronal excitability. One of the most commonly used among these drugs is valproate.[1,30] Valproate acts by increasing inhibitory GABAergic activity and inhibiting glutamatergic transmission through the modulation of sodium and potassium channels.[31] In the treatment of epilepsy, not only more effective seizure control but also regulation of disorders resulting from redox imbalance may increase potential recovery. Therefore, in this study, we investigated the effects of vitamin B12 in combination with valproate on the redox balance disrupted in epilepsy.

The main functions of vitamin B12 include methylation processes related to DNA and cell metabolism. It affects the nervous system, including its neuroprotective and neurotrophic effects in promoting the growth and repair of nerve tissues, especially during neuronal injuries.[15] Studies have provided evidence that vitamin B12 pretreatment can reduce seizures in pentylenetetrazole (PTZ)-induced rat models.[32,33] They also report that the combination of lamotrigine, an antiepileptic drug, and vitamin B12 is effective in reducing oxidative stress in the PTZ-induced epilepsy model.[32] Our study differs from studies in the literature both in terms of the method used to create epilepsy and the antiepileptic drug used. Our findings show that combined administration of valproate and vitamin B12 is effective in reducing oxidative stress in a penicillin-induced experimental epilepsy model. Vitamin B12 showed an antioxidant effect. This situation manifests itself with a significant decrease in TOS and OSI levels and an increase in TAS level in the valproate + vitamin B12 administered group. At the same time, the increase in Native thiol and total thiol in the combination group is also compatible with this effect.

Conclusion

Epilepsy is a neurological disease characterized by epileptic seizures caused by neuronal hyperexcitability. Many currently available drugs attempt to target seizures rather than the pathophysiology of epilepsy. Despite this, some patients continue to have seizures that cannot be resolved with medication. Targeting processes underlying epileptogenesis, such as oxidative stress and redox balance, may be a fruitful area of research for new antiepileptic therapies. This is a pioneering study investigating the effects of the combined application of valproate, a commonly used drug in the treatment of epilepsy, and vitamin B12 on oxidative stress, and the oxidative stress level was evaluated using multiple parameters. Our findings show that the combined application of valproate and vitamin B12 is effective in reducing oxidative stress in a penicillin-induced experimental epilepsy model. Combining antiepileptic drugs with vitamin B12 to control epileptogenesis activities and impaired redox balance in epilepsy may lead to an increase in potential improvements. The results obtained in this study may guide the development of clinical interventions used to treat epilepsy. Further studies are needed to evaluate the effectiveness of this combination prepared with different dosage regimens for different durations in various epilepsies with different etiological origins or medically resistant epilepsies.

Data Sharing Statement

The data presented in this study may be requested from the corresponding author on reasonable grounds.

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Disclosure

The authors have no potential conflicts of interest to disclose for this work.

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