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Effect of minocycline on total oxidant-antioxidant levels and penicillin induced epileptiform activity in rats

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Involvement of minocycline in stages of infection, neuroprotection, and inflammation is well known. However, the effect of minocycline on various rat brain regions and plasma TAS (total antioxidant status), TOS (total oxidant status) values, and electrophysiology with penicillin-induced epileptiform activity have not been investigated. Here, we examined the electrophysiological effects of different minocycline doses in an experimental epilepsy model that started locally and became generalised. We compared the tissue and plasma TAS-TOS values of the antiepileptic dose of the drug with the control, sham, and drug free epileptic groups. Electrophysiological demonstrations revealed that minocycline has an anticonvulsant effect on penicillin induced epilepsy rats (P<0.05, 0.01 and 0.001) and its effective dose. With biochemical components of the study, it was found that minocycline had an antioxidant effect on serum and brain tissues of rats with penicillin induced epilepsy (P<0.05, 0.01).

Keywords: Anticonvulsant, Electrocorticograhy, Epilepsy, Oxidative stress, Reactive oxygen species (ROS), Seizure, Tetracycline

Although epilepsy affects people of all ages, it manifests mostly in children and seniors, it is one of the most common neurological diseases characterised by recurrent seizures, affecting the nervous system, and can lead to mental and physical dysfunction[1]. The mechanisms underlying epilepsy have not yet been fully elucidated. The breakdown of systems that regulate neuronal activity is a frequent characteristic of seizures[2]. In a healthy condition, the antioxidant and oxidant systems are in balance; nevertheless, oxidative stress occurs when free oxygen radicals accumulate more than the antioxidant system's balance capacity[3]. As a result of free radical reactions, oxidative damage is observed[4]. Since the brain tissue metabolises 20% of the total body's oxygen, the formation of reactive oxygen radicals is also high[5]. Brain tissue, which supplies its energy from oxygen dependent mitochondrial oxidative phosphorylation, carries more oxidative risk than other tissues[6]. A high oxidative metabolic rate combined with low antioxidant defences and richness in polyunsaturated fatty acids makes the brain highly vulnerable to free radical damage[7]. Neuronal death and seizures are related to oxidative stress and mitochondrial dysfunction[8]. Reactive oxygen and nitrogen radicals are believed to have a role in epileptogenesis[9]. However, whether it is a cause of epilepsy or a result of epilepsy is still controversial. Status epilepticus also causes oxidative damage to sensitive structures such as proteins, lipids, and DNA. An increase in both mitochondrial oxidative & nitrosative stress and cell damage has been demonstrated after intractable seizures[10]. During epilepsy episodes, the release of cytokines, chemokines, and prostaglandins increases, resulting in an inflammatory response[11].

Minocycline is a semi-synthetic second generation tetracycline derivative with broad spectrum antimicrobial activity. The dimethylamino group at position seven structurally distinguishes it from tetracycline. It has significantly greater lipophilicity than other tetracyclines, allowing it to quickly penetrate the blood-brain barrier[12]. Minocycline shows anti-inflammatory and neuroprotective effects by limiting inflammation and oxidative stress[13]. Studies have revealed that it prevents neuronal and oligodendroglial cell death in a variety of neurodegenerative disorders[14,15]. Minocycline has shown neuroprotective effects[16]. While there are a limited number of studies including reactive oxygen radicals and enzyme levels, there is currently no study

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describing the total oxidant-antioxidant status and electrophysiological analysis.

In a healthy cell, there is an appropriate balance of pro-oxidant antioxidants; if this balance is disturbed and the pro-oxidant status increases, oxidative stress will occur[17]. It remains to be determined whether oxidative stress markers are a consequence of the observed excitations in seizure and EEG activity or whether they may play an appropriate active epileptogenic role by causing neuronal death. In this study, we analysed the effect of minocycline in penicillin induced epilepsy model electrophysiologically and biochemically by total oxide antioxidant capacity measurements.

Materials and Methods

Animals

In the experiments, sixty male Wistar albino rats weighing between 180-220 g were used. Animals were purchased from The Animal House of Ondokuz Mayis University and all animal experiments were performed following the European Union Directive (2010/63/EU) and Turkish animal experimentation legislation. These animals were randomly divided into thirteen groups and each animal group was composed of six rats as follows: Electrophysiology groups: Gr. I: No treatment control group; Gr. II: Sham (0.9% NaCl) group intracortical (ic); Gr. III: Dimethylsulfoxide (DMSO)(Solvent) group (1µL,intracerebroventricular (icv); Gr. IV: 500 IU penicillin (1 µL, ic); Gr. V-VIII: 500 IU penicillin (1 µL, ic) + Minocycline 25, 50, 100 or 200 µg (icv), respectively; and Gr. IX: Minocycline 100 µg (icv). The biochemical analysis groups were: Gr. X: no-treatment control group; Gr. XI: Sham (0.9% NaCl) group (ic); Gr. XII: 500 units penicillin (1 µL, ic); and Gr. XIII: 500 units penicillin (1 µL, ic) + Minocycline 100 µg (icv).

Placement of electrodes for electrocorticography (ECoG) recordings

The rats were anaesthetised with urethane (25% solution) and administered intraperitoneally (i.p.) at a dosage of 1.25 g/kg before the experiment after a period where they were starved for 24 h. The degree of anaesthesia was then examined using corneal and paw reflexes. The animals' skulls were then shaved, and fixation rods from the ear openings and a fixation ring from the anterior teeth were used to fix their heads to the stereotaxy apparatus. A homeothermic blanket was used to keep body temperatures stable. An incision of approximately 2 cm on the midline was made in the rat's scalp in the rostrocaudal direction. Bone wax was used to avoid tiny bleeding foci by removing tendons and fascia from the skull bone (W810, ETHICON). Then, bregma was calculated, which is the reference point for calculating the coordinates in the stereotaxy apparatus. With the use of a hand drill, two holes with a diameter of 1 mm were drilled for the electrode screws. The positive electrode was 3 mm lateral and 4 mm rostral to the bregma, whereas the negative electrode was 3 mm lateral and 4 mm caudal to the bregma, which served as the reference point. In these holes, stainless steel custom made screws were placed to a vertical depth of 1 mm. A ground electrode was placed on the earlobe. Copper wires attached to these electrodes were used to send data from brain waves to the ECoG recording device (PowerLab, 16/SP, AD Instruments, Australia). The LabChart interface was used to transform 180 min electrophysiological recordings into numerical data.

Drug and drug administration

Minocycline (Sigma Aldrich) was used in the experiment. Minocycline was dissolved in DMSO. Various research papers were reviewed to estimate minocycline dosages[18-20]. Holes were made using a hand drill at the following locations according to the rat brain atlas[21]. Intracerebroventricular injections were 1.1 mm lateral, 1.5 mm rostral, and 4.2 mm deep from the bregma, while intracortical injections were 2 mm lateral, 2 mm rostral, and 2.5 mm deep. A Hamilton microsyringe type 701N was used to inject the animals with minocycline (iv) thirty minutes after the penicillin (ic) injection. To prevent drug backflow, an additional minute was allowed to pass before the needle was removed. The experimental diagram shows EcoG activity before and after penicillin and minocycline injection at Fig. 1.

ECoG recordings

Electrodes attached to the PowerLab data acquisition equipment with copper wires were used to generate ECoG recordings from the brain, which were then analysed offline with LabChart 7 Pro (AD Instruments, Australia).

Tissue removal and storage

At the 60th min, when the maximum antiepileptic effect was apparent, the rats in the determined groups were beheaded using a guillotine and their brains were taken. The right hemisphere, left hemisphere, cerebellum, and brain stem were packaged separately, enumerated, and deposited in a nitrogen tank. Rat

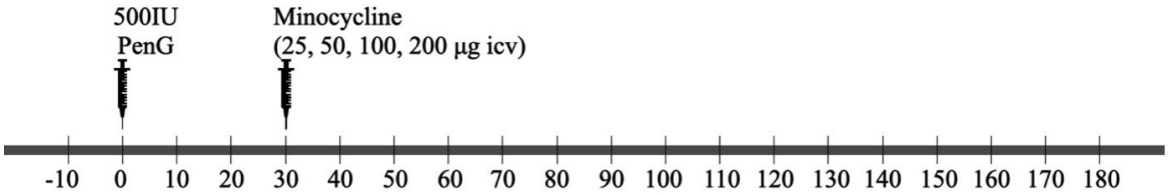

Fig. 1 — Experimental diagram depicting EcoG activity before and after penicillin (500 IU) and minocycline (25, 50, 100 & 200 µg icv) injection.

blood samples were centrifuged. All samples were kept at –80°C in a deep freezer.

Biochemical stages

The samples were brought to room temperature (an average of 23°C) when they were to be studied. Each sample was mixed one by one by vortexing homogeneously. Samples were placed in the centrifuge and rotated for 2 min at 3000 rpm. An automated pipette was used to transfer the samples from sample tube to godets. The Gode samples were processed and analysed using Mindray brand BS300 model completely automated biochemistry apparatus and "Rel Assay" brand kits.

Total antioxidant activity may be measured in two ways: radical-forming and non-radical-forming. There are two types of radical generators: calorimetric and non-calorimetric. The calorimetric TAS measuring method used in this work is based on the elimination of the ABTS radical by antioxidants. The most widely used colourimetric methods are those based on 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS•+), in which a colourless molecule, reduced ABTS, is oxidised to a distinctive blue-green ABTS•+. When coloured ABTS•+ is combined with an oxidisable material, it is reduced to its original colourless ABTS form, oxidising the reactant. This is the basic principle of methods using ABTS[22].

The TAS level indicates the antioxidant defence status of an organism and is used as a well-known and reliable marker of oxidative stress[23,24]. TAS and TOS in this study were measured using kits developed by Erel (Rel Assay Diagnostics kit; Turkey).

The TOS measurement method is performed by measuring the ferric ion with xylenol orange by oxidation of ferrous ions to ferric ions in the presence of various oxidant species in an acidic environment. This method is easy, stable, reliable, precise, inexpensive, and fully automatic[25].

The Oxidative Stress Index (OSI) was developed to integrate the information provided into a single value to simplify the assessment of oxidative stress status in plasma samples. The oxidative stress index value indicates a departure from the normal oxidative equilibrium state (zero value), which is the perfect balance between the pro-oxidant and antioxidant components of the oxidative balance. OSI, which can be expressed as the ratio of TOS to TAS, has been reported to help determine the net oxidative stress effect. OSI was calculated using the formula OSI=TOS/(TAS100).

Statistical analysis

The same program examined and translated electrophysiological data recorded on the computer with the LabChart-Life Science Data Acquisition and Analysis Software program into numerical values. GraphPad Instat (v3.06) software (GraphPad Software, San Diego, CA, USA) was used to statistically examine the numerical data. To evaluate if the given data fits the normal distribution, the Kolmogorov-Smirnov test was used first. One-way analysis of variance (ANOVA) and the Post-Hoc Tukey test was used after verifying that the data fit the normal distribution (One-Way Anova Post-Hoc Tukey) for comparison. The experimental groups' values were expressed as mean ± standard error of the mean (SEM) in the images and text. Differences with a p value less than 0.05 were determined to be significant, according to the results of statistical analysis.

Results

After penicillin (500 IU in a 2.5 µL volume) was injected into the brain via an intracortical route, epileptiform activity was observed within 2-5 min. Spike activity reached a steady state within 25–30 min and continued for 3 h. All these processes were recorded electrophysiologically (Fig. 2). 30 min after penicillin administration, minocycline 25, 50, 100 and 200 µg icv doses were administered and epileptiform activity was recorded online until the end of the experiment (Fig. 3). As a result of the analysis, when compared with the penicillin group, 100 µg icv minocycline decreased the spike number of epileptiform activity statistically from the 50th minute

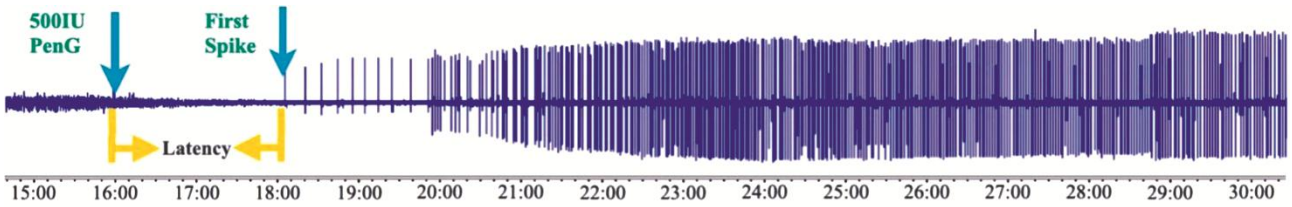

Fig. 2 — Epileptiform activity after intracortical injection of 500 IU of penicillin

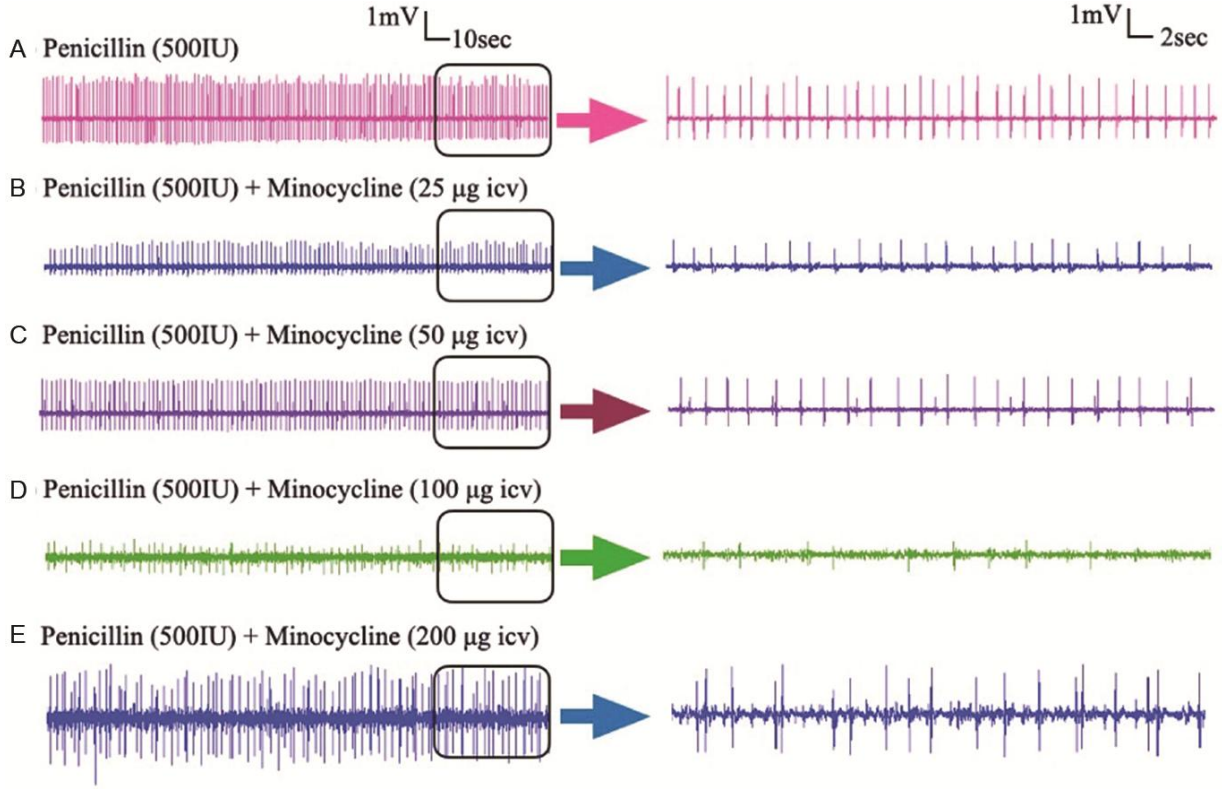

Fig. 3 — Comparison of electrophysiological recordings.

onwards (P<0.05–0.001) and this decrease continued until the end of the experiment (Fig. 4). There was no statistically significant difference between the minocycline dose groups and the penicillin group in terms of amplitude values (P>0.05) (Fig. 5). According to these results, it was determined that a 100 µg dose of minocycline was the effective anticonvulsive dose.

In biochemical analysis, the left hemisphere TAS value of the penicillin group was significantly lower than the control and sham groups (P<0.05), and a statistically significant difference was observed between the penicillin group and the penicillin + minocycline (100 µg) group (P<0.05) (Table 1). The left hemisphere TOS value was significantly higher in the penicillin administered group than in the control and sham groups (P<0.05), and a statistically significant difference was found between the penicillin group and the penicillin + minocycline (100 µg) group (P<0.01). When the left hemisphere OSI values were examined, the penicillin group increased significantly (P<0.01) compared to the sham and control groups, and minocycline treatment brought this value closer to normal at a highly significant level (P<0.01). Plasma TAS values in the penicillin group were lower than those in the sham and control groups (P<0.01), and the TAS value in the minocycline-treated group was significantly closer to the control group values (P<0.05). The serum OSI value of the penicillin group was significantly higher than that of the control and sham groups (P<0.05), and a statistically significant difference was observed between the penicillin group and the penicillin + minocycline (100 µg) group (P<0.05) (Fig. 6).

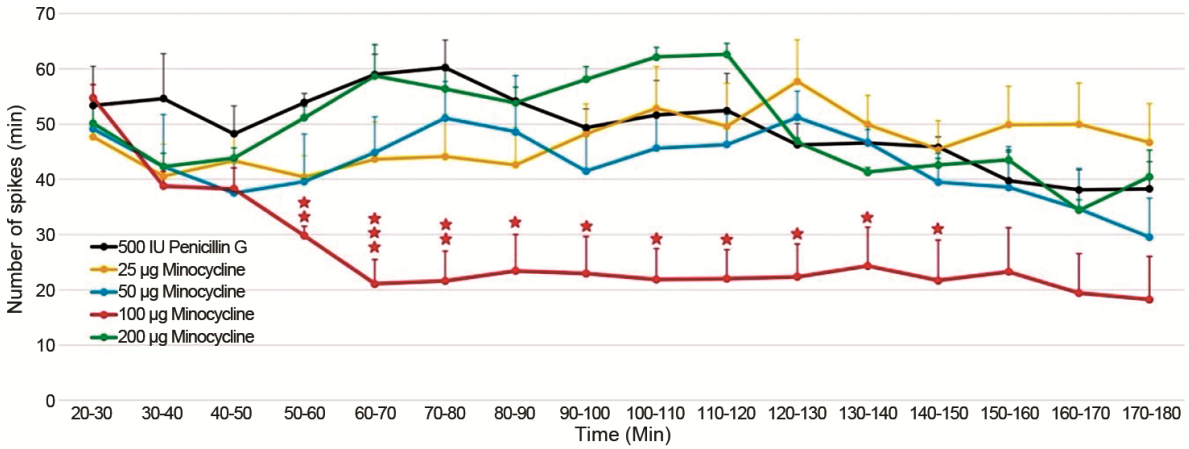

Fig. 4 — Impact of 25, 50, 100 & 200 µg minocycline dosages on the number of spikes of penicillin-induced epileptiform activity. Ten minute averages and SEM values (Mean ± SEM). Comparison of groups with the Penicillin G group. (★ P<0.05, ★★P<0.01, ★★★ P<0.001)

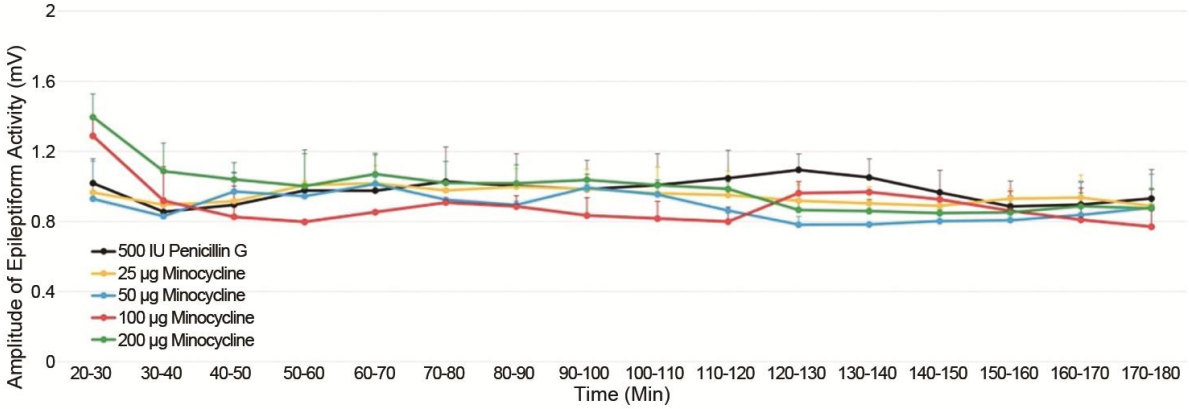

Fig. 5 — Impact of 25, 50, 100 & 200 µg minocycline dosages on penicillin-induced epileptiform activity spike amplitude values. 10 minute averages and SEM values (Mean ± SEM). The Penicillin G group was compared to the other groups.

Table 1 — Comparison of epileptiform activity among group, sham and controls

Tissue	Group	Control	Sham	PEN (500IU)	PEN +MIN (100 µg)
Plasma	TAS	1.58±0.05	1.53±0.08	1.1±0.06★●◆	1.38±0.03◆
	TOS	9.6±0.48	9.29±0.2	10.45±0.44	9.94±0.2
	OSI	0.61±0.05	0.61±0.02	0.96±0.09★●	0.69±0.03◆
Right Hemisphere	TAS	1.28±0.08	1.29±0.03	1.27±0.15	1.31±0.13
	TOS	9.08±0.56	9.2±0.24	10.2±0.11	9.32±0.46
	OSI	0.71±0.02	0.71±0.03	0.81±0.09	0.72±0.05
Left Hemisphere	TAS	1.29±0.02	1.33±0.04	1.05±0.06★●	1.27±0.02◆
	TOS	8.59±0.08	9.13±0.18	11.18±0.28★●	8.14±0.67◆◆
	OSI	0.67±0.001	0.69±0.01	1.07±0.03★★●●	0.64±0.06◆◆
Cerebelum	TAS	0.96±0.04	0.98±0.11	0.95±0.05	0.98±0.07
	TOS	14.11±0.78	14.31±0.96	14.54±0.82	13.91±0.99
	OSI	1.47±0.03	1.54±0.24	1.56±0.17	1.43±0.24
Pons	TAS	1.01±0.06	1.19±0.16	0.99±0.1	1.01±0.08
	TOS	9.71±0.48	9.56±0.49	9.8±0.16	8.71±0.35
	OSI	0.97±0.08	0.8±0.02	1.05±0.21	0.87±0.03

[PEN: Penicillin, MIN: Minocycline, ★: Comparison of the penicillin group with the control group, ●: Comparison of the penicillin group with the sham group, ◆ : Comparison of the penicillin group with the penicillin+ minocycline group (★ *P<0.05*, ● *P<0.05*, ◆ *P<0,05*, ★★ *P<0.01*, ●● *P<0.01*, ◆◆ *P<0.01*)]

Discussion

Analysis of electrophysiological recordings in this study showed that a 100 µg dose of intracerebroventricularly administered minocycline reduced penicillin induced epileptiform activity. In biochemical analysis, it was observed that the left hemisphere TAS value decreased, the TOS value increased, the plasma TAS value decreased, and all of them returned to normal values after minocycline administration in rats whose epileptiform activity was created with penicillin.

Both minocycline and penicillin, which were given to rats in this study, are antibiotics, with one producing epileptiform activity and the other decreasing epileptiform activity. Penicillin G is a short-acting beta-lactam antibiotic that inhibits cell wall production and is resistant to penicillinase and acidase[26]. Minocycline is a second-generation tetracycline derivative, an antimicrobial that inhibits protein synthesis. It has antibacterial activity via

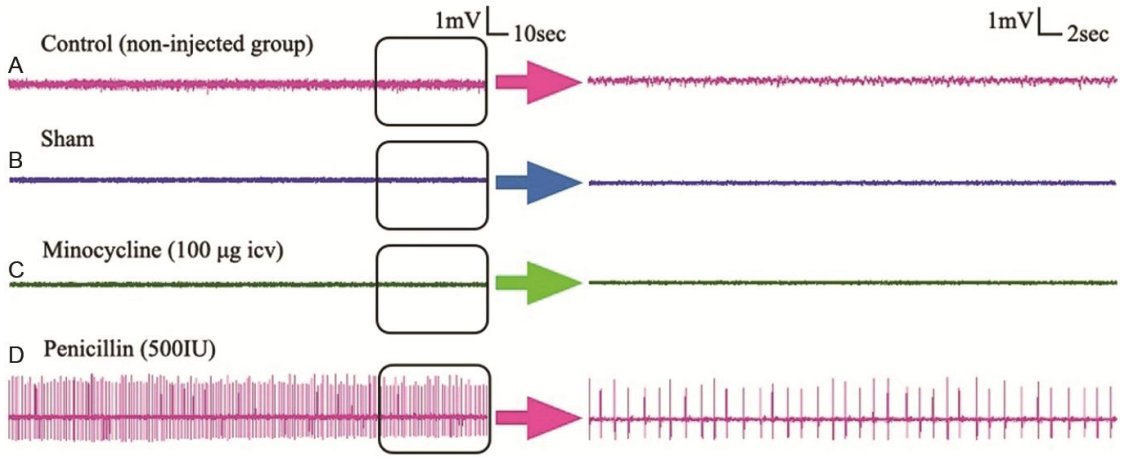

Fig. 6 — Electrophysiological activity recordings of the control, sham, penicillin (500 IU) and minocycline alone (100 g effective based on dose study) groups.

attaching to 30S subunit of ribosome[27]. Penicillin is a chemical convulsant that is commonly used as an antibiotic[28]. Penicillin injections into the neocortex result in epileptic episodes in that location[29]. Penicillin G has an epileptiform impact by inhibiting the GABAA receptor without competition[30], and electrophysiological recordings in the right and left hemispheres with unilateral intracortical injection demonstrate no significant differences[31]. Some compounds, such as penicillin, are widely used as models of acute seizures. Thus, the effects of antiepileptic drugs can be investigated quickly[32].

Patients with risk factors are more susceptible to the development of seizures that may occur with any antibiotic treatment they receive for any reason. The administration of antibiotics when needed together with the drugs used in the treatment of seizures may also lead to an increased risk of seizures due to drug interactions that predispose to changes in drug metabolism and therapeutic efficacy[33]. From this point of view, it is suggested that minocycline can be seen as an option to be used more safely in antibiotherapy for patients who are currently under treatment for epilepsy or who are in the risk group. Minocycline is thought to be useful in epilepsy, which is a chronic condition since it has a low tendency to develop antibiotic resistance and may be used long-term[12].

A significant reduction in seizure frequency was observed during minocycline 50 mg administered daily twice to patients with severe symptomatic epilepsy due to astrocytoma[34]. Acute administration of minocycline (120 mg/kg) (ip) is an anticonvulsant in a model of pentylenetetrazole (PTZ) induced epilepsy[20]. In a study investigating the effects of tetracycline (255 mg/kg), minocycline (170 mg/kg), and doxycycline (157 mg/kg) in an acute cocaine intoxication model in BALB/c mice, minocycline and doxycycline significantly reduced cocaine-induced seizures (157 mg/kg) and minocycline reduced mortality[35]. The effects of tetracycline group antibiotics were investigated in MES (maximal electroshock), 6 Hz (minimal clonic seizure), and subcutaneous metrazol epilepsy models. It showed an anticonvulsant effect on epileptic activity induced by minimal clonic seizures. No anticonvulsant effect was observed for any of the three drugs on the other two models[36]. In Theiler's model of murine encephalomyelitis virus (TMEV) in which spontaneous recurrent seizures occur in C57Bl/6J mice, minocycline reduced seizure load but not latency when given once daily[37]. It has been found that minocycline blocks the long-term effect of early seizures on seizure susceptibility and microglia activation in the later stages of life, minimising susceptibility to seizures[38]. In a study with toluene, which produces reactive oxygen species (ROS) that activate glial cells, minocycline prevented neuronal hyperexcitability and normalised firing frequency[39].

Antibiotics from the tetracycline groups lowered glutamate, an excitatory neurotransmitter while increasing GABA, an inhibitory neurotransmitter, in the brain[35]. There are various studies indicating that minocycline reduces seizures by decreasing inflammatory mediators and increasing GABA[39]. Additionally, in an experimental model of microglial activation and neuronal death, blockade of microglial activation through chronic treatment of mice with minocycline reduced the incidence of epileptic seizures with a reduction in neuronal death but did not restore GABAergic synaptic deficits[40]. It has been shown that

minocycline has neuroprotective effects by activating caspase-dependent and independent pathways[41]. In various experimental epilepsy models, in studies on tissues of different brain regions of rats and mice, the indicators measured did not show the total status but the level of oxidation and certain antioxidant enzymes[42,43]. In our study, the measurement of the total oxidant-antioxidant capacities of biological samples and their correlation in a ratio called an index was used since it is impractical to measure different oxidants and antioxidant molecules separately because it is costly and time consuming, and the procedures are often complicated, as well as the oxidant or antioxidant effects are additive[22-25].

Although there are clinical and experimental studies on the effect of minocycline on epilepsy, as far as we know, there are no electrophysiological and biochemical studies on penicillin-induced epileptiform activity. In the present study, it was found that a 100 µg dose of minocycline had an anticonvulsive effect on the epileptiform activity induced by penicillin. To determine the molecular mechanism of minocycline in penicillin model epilepsy, biochemically total oxidant and antioxidant levels were investigated in the right hemisphere, left hemisphere, brain stem, and cerebellum tissues.

It is accepted that reactive oxygen and nitrogen species play a role in epileptogenesis. However, it is still controversial whether it is a cause of epilepsy or a consequence of epilepsy. Regulating oxidative stress with natural and chemical treatments is discussed as a way to alleviate epileptogenesis and seizure onset. Therefore, in the present study, rats were decapitated by choosing the period in which minocycline was most effective as an anticonvulsant according to electrophysiological data, and TAS and TOS values were determined using Erel's method by taking right and left brain hemispheres, brain stem, cerebellum, and blood plasma[22-25].

In the present study, minocycline brings the increase in total oxidant capacity, decrease in total antioxidant capacity, and decrease in total antioxidant capacity in plasma back to normal values caused by epileptic activity induced by penicillin in the left brain hemisphere. In this study, the anticonvulsant and antioxidant properties of minocycline on penicillin-induced epileptiform activity were found for the first time. These findings may be helpful in understanding the mechanism of epilepsy and in the development of antiepileptic drugs.

Conclusion

In this study, the anticonvulsant effect of minocycline on penicillin induced epileptiform activity was demonstrated electrophysiologically, and it was concluded that this was also supported by the results of biochemical analysis. Minocycline reduced the oxidant load and epileptiform activity elicited during experimental epilepsy. Further studies, including molecular and genetic studies, are needed to elucidate the mechanisms that reveal the role of minocycline in epilepsy.

Acknowledgment

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Conflict of interest

Authors declare no competing interests.

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