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The Effects of Methylene Blue and Tadalafil in Pentylenetetrazole Induced Convulsion Model

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ÖZET

Pentilentetrazol ile oluşturulan konvülziyon modelinde metilen mavisi ve tadalafilin etkileri

L-Arjinin (L-ARG) -nitrik oksid (NO) -cGMP (siklik guanozin monofosfat) döngüsünün epilepsideki rolünü gösteren bazı çalışmalar vardır. Tadalafil, fosfodiesteraz-5 (PDE-5) 'in güçlü bir inhibitörüdür ve cGMP'nin etkisini artırır. Metilen mavisi, hücre içi elektron akışını düzenleyen ve mitokondriyal etkinliğini artıran lipofilik bir ajandır. Aynı zamanda guanilat siklazı doğrudan inhibe ederek cGMP düzeylerini azaltır.Bu çalışmanın amacı metilen mavisi (MM) ve tadalafilin, pentilentetrazolle oluşturulan nöbetlere olan etkilerinin araştırılmasıdır. MM ve tadalafil, PTZ (70 mg/kg, i.p.) enjeksiyonundan 30 dk önce uygulanmıştır ve doz cevap oranları gösterilmiştir. Racine Konvülziyon Ölçeği (RKÖ) ve ilk myoklonik kasılmanın başlama zamanı (MKBZ), nöbetleri değerlendirmek için kullanılmıştır. Ayrıca ilaçlar pentilentetrazol (35 mg/kg, i.p.) enjeksiyonundan 30 dk önce uygulanarak korteksten EEG kayıtları alınmıştır ve EEG'deki diken yüzde değerleri ölçülmüştür. Tüm gruplarda plazma cGMP düzeyleri ölçülmüştür. 5 and 10 mg/kg MM verilen grupta salin grubuyla karşılaştırıldığında RKÖ değerleri ve cGMP düzeyleri azalırken , MKBZ artmıştır. 10 mg/kg tadalafil verilen grupta salin grubuna göre cGMP ve RKÖ değerleri artarken MKBZ azalmıştır. Diken dalga oranları salin grubuyla karşılaştırıldığında, 5 ve 10 mg/kg MM verilen grupta azalırken 10 mg/kg tadalafil grupta artmıştır. Özellikle PDE 5 inhibitörleri kullanılırken bu ajanların prokonvülzan özellikleri gözönünde bulundurulmalıdır.

Anahtar Kelimeler: *Epilepsi, Metilen mavisi, Tadalafil, Pentilentetrazol*

SUMMARY

L-arginine (L-ARG)-nitric oxide (NO)–cGMP (cyclic guanosine monophosphate) cycle has been determined in various studies to be associated with epilepsy. Tadalafil is a potent inhibitor of phosphodiesterase 5 (PDE5) and increases the level of cGMP. Methylene blue (MB) is a lipophilic agent regulating intracellular electron flow as well as increasing the mitochondrial effectiveness. It also decreases cGMP levels by directly inhibiting guanylate cyclase.The aim of the present study was to evaluate the effects of MB and tadalafil on pentylenetetrazol-induced seizures. MB and tadalafil were administered 30 min prior to PTZ (70 mg/kg, i.p.) injection and the dose-response ratio was determined. Racine's Convulsion Scale and first myoclonic jerk (FMJ) onset time was used to evaluate seizures. Besides, drugs were administered 30 min prior to pentylenetetrazol (35 mg/kg, i.p.) injection and EEG was recorded from cortex and the spike wave discharges was determined in EEG. Plasma cGMP levels were measured in all groups. 5 and 10 mg/kg MB decreased Racine convulsion stage, increased FMJ onset time and decreased cGMP levels when compared with saline. 10 mg/kg tadalafil increased cGMP levels and Racine convulsion stage but decreased FMJ onset time compared with saline. Spike wave discharges were decreased in 5 and 10 mg/kg MB groups and increased in 10 mg/kg tadalafil group when compared with saline group.The proconvulsant properties of all the agents may be taken into consideration, especially when prescribing PDE5 inhibitors.

Key words: *Epilepsy, Methyleneblue, Tadalafil, Pentylenetetrazole*

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Introduction:

Epilepsy is defined as a condition of recurrent seizures which occur through the hyper-discharge of neurons (1). Certain parts of epilepsy are symptomatic, while certain parts of epilepsy are idiopathic (1). Epilepsy is a disease that is very common in the general society. It is stated in a previously performed study that the prevalence of epilepsy is around 5-8/1000 in the young adult population, while the prevalence of the disease differs from society to society (2). Despite developing technology and the advanced studies that have been performed, the mechanism of the event resulting in hyper-discharge of neurons has not been completely understood as of yet. Many studies have been performed to determine the pathogenesis of epilepsy, and it is established that the disease is associated with various mechanisms. L-arginine-nitric oxide(NO)-cyclic guanosine monophosphate (cGMP) cycle has been determined in various studies to be associated with the epilepsy (3).

Tadalafil, sildenafil, vardenafil, and udenafil are potent inhibitors of phosphodiesterase 5 (PDE5). They increase the effect of cGMP. These medicines are generally used for treatment of sexual dysfunction, and the side effects caused by smooth muscle vasodilatation, such as headache, indigestion, and nasal congestion (4). Unlike those side effects, there are also case reports regarding that these medications may decrease the seizure threshold, resulting in an increase of the frequency of epileptic seizures (5,6).

Pentylenetetrazole (PTZ) is a selective GABA-A receptor blocker and is a chemical agent, which may dose-dependently cause various epileptic activities, from subconvulsion to generalized tonic-clonic seizures (7).

MB is a lipophilic agent, and it regulates intracellular electron flow as well as MB increases mitochondrial effectivenes. MB also decreases cGMP levels by directly inhibiting guanylate cyclase, and it is stated in some studies to directly inhibit nitric oxide (8). Accordingly, it is apparent that both MB and tadalafil affect the cGMP.

The aim of the present study was to evaluate the effects of MB and tadalafil on pentylenetetrazol-induced seizures in the presence of EEG and plasma cGMP levels (9).

Materials and methods:

Animal and Laboratory

All experiments, performed in this study, were carried out according to the rules in the Guide for the Care and Use of Laboratory Animals, as adopted by National Institutes of He-

alth (U.S.), and received the Gaziosmanpasa University Animal Ethics Committee's consent. Approval number of ethics Committee is 51879863-61. Sixty male Sprague–Dawley rats, weighing 200–250 g each were utilized for this study, thirty of them for EEG recording and thirty of them are for behavioral studies. The rats were housed in quiet rooms with a 12 hour –12 hour light–dark cycle (light from 07.00 to 19.00) and a 22–24 ◦C ambient temperature. The rats were given standard laboratory food and tap water ad libitum.

Drugs

Tadalafil (Cialis ® 20 mg tablets , Lilly) and Metilene Blue (Blumet 100 mg/10 ml i.v. enjection, Defarma) were dissolved in a 0,9% saline solution. Drug solutions were freshly prepared.

Experimental Procedures

First, small hole was opened with a drill under stereotaxically. The electrodes (Polyamide-coated stainless steel wires, 0,1 mm diameter and electrical resistance <1Ω/10 mm) were implanted on dura over left frontal cortex (2,0 mm lateral to the midline, 1,5 mm anterior to the bregma) and the reference electrode was implanted over cerebellum (1,5 mm posterior to the lambda, on midline) for EEG recording (10,11).

The electrodes were then fixed using a dental acrylic (numerous alloys are used in the making of dental restorations). Rats were anesthetized utilizing ketamine (60 mg/kg) (Alfamine®, Ege Vet, Alfasan International B.V. Holland) and xylazine (10 mg/kg) (Alfazyne®, Ege Vet, Alfasan International B.V. Holland) intraperitoneally (ip). Seven days after the electrodes were fixed, thirty rats were then divided into 5 groups (n = 6) for EEG recording. The first group was described to be the control and were given no medication. The second group was administered saline intraperitoneally. The third group was given 5 mg/kg tadalafil i.p. The fourth group was administered 5 mg/kg MB i.p. The fifth group was given 10 mg/kg MB i.p. All drugs were administered 30 minutes prior to PTZ (35 mg/kg, i.p.) injection administration. All groups except the first control group received 35 mg/kg PTZ, and EEG was recorded (12). EEG recordings were taken in rats while awake in a special container. An EEG recording was taken every 2 hours (12,13) (Figure 1). The signals were amplified 10,000 times and the signals were also filtered with a range of 1–60 Hz. System records were taken using the Biopac MP 30 differential amplifier system. Two clinical neurophysiologists scored the EEG data for spike percentage. We defined "spike percentage"as a reproducible way to quantify epileptiform activity is to quantify the percentage of 1-second bins with at least one spike-wave in them, termed spike-wave percentage (14). Subsequently, the same groups were rearranged with different thirty rats for behavioral assesment. Respectively: saline, 5 mg/kg tadalafil, 5 and 10 mg/kg MB was given 30 min prior to PTZ (70 mg/kg, i.p.) injection (12). Racine's Convulsion Scale (RCS) and onset times of 'first myoclonic jerk' (FMJ) was used to evaluate the seizures (for only PTZ 70 mg/kg) as follows: 0 = no convulsion; 1 = twitching of vibrassae and pinnae; 2 = motor arrest with more pronounced twitching; 3 = motor arrest with generalized myclonic jerks; 4 = tonic clonic seizure while the animal remained on its feed; 5 = tonic–clonic seizure with loss of the righting reflex; 6 = lethal seizure. Rats were observed for onset times of FMJ as previously described. The onset times were recorded as seconds. The observation period for PTZ-induced seizures were limited to 30 minutes duration. After this duration, the animals were euthanized.

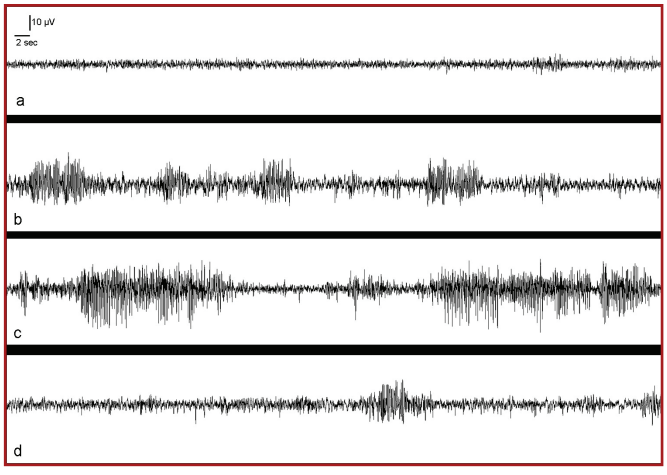

Figure 1: EEG recording (a): control, (b): PTZ (35 mg/kg) and saline, (c): PTZ (35 mg/kg) and 10 mg/kg tadalafil group, (d): PTZ (35 mg/kg) and 10 mg/kg methylene blue group

Measurement of plasma cGMP

After administering the respective treatments, rat plasma samples were collected, immediately frozen, and stored at −80°C until extraction for cyclic nucleotide assay. The concentration of cGMP was determined by enzyme-linked immunosorbent assay (Cusabio, Biotech Co. Ltd. Wuhan) .

Statistical analysis

Results were expressed as a mean ± standard error of mean (SEM). Data analyses were performed utilzing SPSS version 15.0 for Windows. The Racine convulsion score, first myoclonic jerk (FMJ) time were evaluated by one-way analysis of variance (ANOVA). Post-hoc Bonferonni test was utilized to identify differences between the experimental groups. The value of $p<0.05$ was accepted as statistically significant.

Results:

Evaluation of groups in terms of Racine convulsion stage;

When PTZ (70 mg/kg) and 5 or 10 mg/kg MB and given groups were compared with the PTZ (70 mg/kg) and saline group, the Racine convulsion stage was significantly lower ($p<0.001$); furthermore, when PTZ (70 mg/kg) and 5 or 10 mg/kg MB given groups were evaluated between themselves, the Racine convulsion stage of PTZ (70 mg/kg) and 10 mg/kg MB given group was lower than the PTZ (70 mg/kg) and 5 mg/kg MB given group ($p<0.001$) (Table 1). However, when the PTZ (70 mg/kg) and 10 mg/kg tadalafil given group and the PTZ (70 mg/kg) and saline group were compared, the scale of Racine convulsion had the similar convulsion stage and no significant difference was detected ($p>0.05$) (Table 1).

Evaluation of groups in terms of FMJ onset time;

FMJ onset time was seen to be shorter in PTZ (70 mg/kg) and 10 mg/kg tadalafil given group than the PTZ (70 mg/kg) and saline group ($p<0.05$). FMJ onset time values of PTZ (70 mg/kg) and 10 mg/kg MB given group were longer than in PTZ (70 mg/kg) and 5 mg/kg MB given group, and these findings were determined to be statistically significant ($p<0.01$). Furthermore, FMJ onset time values were determined to be signi-

ficantly longer in PTZ (70 mg/kg) and 5 or 10 mg/kg MB given groups than the PTZ (70 mg/kg) and saline group (p<0.01) (Table 1).

Table 1. Racine convulsion stage and Evaluation of groups in terms of FMJ onset time.

Drugs Group	Convulsion Stage (Racine)	FMJ onset time (sec)
1-Control	0	0
2-PTZ (70 mg/kg) and saline	5.83 ± 0.16	68.33 ± 12.07
3-PTZ (70 mg/kg) and 10 mg/kg tadalafil	5,66 ± 0.3 #	56.5 ± 2.78 *
4- PTZ (70 mg/kg) and 5 mg/kg methylene blue	4.33 ± 0.49 **	166.6 ± 19.2 **
5- PTZ (70 mg/kg) and 10 mg/kg methylene blue	3.16 ± 0.87 ** ††	204.5 ± 32.2 ** †

Data were expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA and post- hoc Bonferroni test. *p<0.05, **p<0.01,# p> 0.05 (different from PTZ (70 mg/kg) and saline group). † p <0.01, †† p<0.001 (different from PTZ (70 mg/kg) and 5 mg/kg methylene blue given group)

Evaluation of groups in terms of Spike Percentage;

Spike percentage values were higher for the PTZ (35 mg/kg) and 10 mg/kg tadalafil given group when compared to the PTZ (35 mg/kg) and saline group (p<0.05). Spike percentage values were statistically significantly lower PTZ (35 mg/kg) and 5 or 10 mg/kg MB given groups versus the PTZ (35 mg/kg) and saline group (p<0.01). Spike percentage values were lower for the PTZ (35 mg/kg) and 10 mg/kg MB given group when compared to the PTZ (35 mg/kg) and 5 mg/kg MB given group (p<0.05) (Table 2, Figure 1).

Table 2. Spike Percentage of all groups

Drugs Group	Spike Percentage
1-Control	% 0
2-PTZ (35 mg/kg) and saline	% 63.2 ± 8.4
3-PTZ (35 mg/kg) and 10 mg/kg tadalafil	% 72.5 ± 6.3 *
4- PTZ (35 mg/kg) and 5 mg/kg methylene blue	% 42.4 ± 7.1 **
5- PTZ (35 mg/kg) and 10 mg/kg methylene blue	% 33.8 ± 4.5 ** †

Data were expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA and post- hoc Bonferroni test. *p<0.05, **p<0.01 (different from PTZ (35mg/kg) and saline group). † p <0.05, (different from PTZ (35 mg/kg) and 5 mg/kg methylene blue given group)

Evaluation of groups in terms of Plasma cGMP levels;

Plasma cGMP levels were higher for tadalafil groups when compaired to the control and saline plus PTZ (70 mg/kg) group (p<0.05). Plasma cGMP levels were lower for 5 and 10 mg/kg methylene blue groups when compaired to the control and saline plus PTZ (70 mg/kg) groups (p<0.05, p<0.01). (Table 3)

Table 3. Plasma cGMP levels of all groups

	Plasma cGMP Level (pmol/L)
1-Control	15.21 ± 3.07
2-PTZ (70 mg/kg) and saline	19.85 ± 1.67 #
3-PTZ (70 mg/kg) and 10 mg/kg tadalafil	26.08 ± 1.87 *
4- PTZ (70 mg/kg) and 5 mg/kg methylene blue	14.91 ± 1.62 *
5- PTZ (70 mg/kg) and 10 mg/kg methylene blue	11.41 ± 1.69 **

Data were expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA. # p< 0.05 (different from control); *p<0.05, **p<0.01 (different from PTZ (70 mg/kg) and saline group).

Discussion:

The basic results obtained in our study is that MB increases the epileptic threshold. therefore MB is an anticonvulsant. Tadalafil decreases the epileptic threshold, so tadalafil would be considered as a proconvulsant. It also has been determined in this study that MB given dose of 10 mg/kg, both decreases the convulsion stage and extends the FMJ onset time more than when compared to administration dose of 5 mg/kg. This observation gives rise to the thought that the anticonvulsant capacity of MB increases dose-dependently. Furthermore, it may interpreted as an indicator of proconvulsant property of tadalafil. Tadalafil has a higher spike percentage and has shorter FMJ onset time, when compared only with the saline control group. When the literature is reviewed, there are no current studies that have been performed on tadalafil, however there are publications reporting the anticonvulsant property of MB and the proconvulsant property of sildenafil (3, 15).

cGMP has a very important role in providing and erection and the continuation of the erection. Tadalafil inhibit PDE that leading to cGMP degradation and show effectiveness by increasing cGMP level. There are eleven known phosphodiesterase enzymes within the human body. Even though these medications essentially inhibit PDE 5, it is known that sildenafil inhibits the high-dose PDE 6 especially; the importance of this is associated with the presence of PDE within the retina and the brain. Central side effects of these medications were demonstrated in various studies previously performed (5, 16-18). Even though the studies show that these inhibitors only inhibit the high-dose PDE enzyme, some publications have previously reported and our results give rise to thought that PDE inhibitors have a proconvulsant property even in normal doses (5).

When literature is reviewed, there are various studies which examine the relationship of L-arginine-NO-cGMP cycle with epilepsy. Sildenafil is a proconvulsant in both PTZ and bicuculline-induced epilepsy models. It is shown in this current study study that sildenafil is a proconvulsant in 10 mg/kg and higher doses in the PTZ model (3). This situation may be associated with the inhibition of PDE 6 enzyme at high doses that would be in line with the literature. However, the fact that the sildenafil also becomes a proconvulsant in 5 mg/kg dose in bicuculline convulsion model gives rise to the thought that the presence of the other mechanisms, in addition to PDE 6 inhibition, gives the proconvulsant property of this agent (3). This supports an idea that the tadalafil given in our study becomes a proconvulsant at a low dose (5 mg/kg). MB is lipophilic agent that acts

as both a guanylate cyclase inhibitor and direct NO inhibitor, it decreases cGMP level (8). L-NAME (nitro arginine methyl esterase) is the nitric oxide synthase inhibitor; it decreases NO, thereby decreases cGMP level. MB and L-NAME are given before sildenafil, they are stated to suppress the proconvulsant property of that agent dose-dependently. Unlike the fore mentioned, the fact that the sildenafil shows a proconvulsant property in both PTZ and bicuculline model gives rise to thought that it may directly affect the seizure regulating centers of the brain (3). In our study, MB in both 5 mg/kg and 10 mg/kg doses similarly was showed an anticonvulsant property.

When previous studies are evaluated, various studies found relationship between the NO, cGMP and convulsion. In the study by Bahremand et al in 2010, they showed an antiepileptic effect of lithium and examined the relationship of that effect with the L-arginine-NO-cGMP cycle. In addition, methylene blue, L-NAME, 7-NI (nitroindazole), aminoguanetidinin, L-ARG, sildenafil are other agents having effect upon NO-cGMP, and these agents do not have any effect upon convulsion in rats alone. L-NAME and 7-NI in the same doses are seen to increase the anticonvulsant effect of lithium. Furthermore, it has been determined that low doses of L-ARG and sildenafil suppress the anticonvulsant effect when given with lithium simultaneously, and they show a strong proconvulsant effect when given before lithium (19). In our study the changes in plasma cGMP levels suggesting that tadalafil (increased plasma cGMP levels) and methylene blue (decreased plasma cGMP levels) might have been shown opposite effect on convulsions via cGMP. There are also publications regarding NO inhibition of GABA having inhibitor effect on central nervous system, in addition to cGMP (20) and activates NMDA having excitatory effect (21). On the other hand, there are also a few select publications which report that NO is proconvulsant (22) however, Uzum et al stated in their study that this contradictory condition may change according to type of seizure, source of NO, gender, and type of animal used (23). Additionally, apart from these effects, NO is accepted to have a role in the release of neurotransmitters, such as acetylcholine (24) and adenosine (25) and may have an effect upon the convulsion pathway by this mechanism. It has been shown in previous studies that NO regulates adenosine release from the hippocampus and striatum (26, 27). Based on this in the PTZ induced convulsion model of Akula et al, the sildenafil, L-ARG and sodium nitroprusside which increased the NO effectiveness, decreased the anticonvulsant effect of adenosine (15). In our study, tadalafil in 10 mg/kg doses was showed an proconvulsant effect.

The most important limitation of this study is that only plasma cGMP levels were examined but NO levels were not examined and also the dose response of tadalafil was not studied. However, since the effect of MB and sildenafil upon L-arginine-NO-cGMP pathway was made clear through the many previous studies outlined. When the mentioned literature data and our study are evaluated together, it can be stated that the relationship of cGMP with epilepsy is apparent in all of these complex mechanisms. Accordingly, the proconvulsant capacity of all the agents have effect upon that pathway in epileptic patients may be taken into consideration, especially when prescribing PDE inhibitors, of which the frequency of using has increased gradually over the past years.

Conflict of interest: The authors have no conflicts of interest.

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