

# Evaluation of Antiepileptogenic Effect of Atorvastatin on Development of Pentylene-tetrazole Induced Kindling in Rats: The Responsibility of Inducible Nitric Oxide Synthase and Metalloproteinase 2

## Sıçanlarda Pentilente-tetrazol ile Uyarılan Kindling Gelişimi Üzerine Atorvastatinin Antiepileptogenik Etkisinin Değerlendirilmesi; İndüklenebilir Nitrik Oksit ve Metalloproteinaz 2'nin Sorumluluğu

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**ABSTRACT Objective:** Recently, studies indicated that metalloproteinase and inducible nitric oxide synthase (iNOS), a key participant in neuronal death and neuroinflammation, are upregulated in experimental epilepsy models. Statins are inhibitors of HMG-CoA reductase that have been shown to have anti-inflammatory and neuroprotective effects. Atorvastatin (AT) was shown to possess antiepileptic effects, but the mechanism is not clear. Nitric oxide (NO) and matrix metalloproteinases (MMP) may be responsible for this effect. The purpose of this study was to assess the effect of AT against development of kindling-induced changes of endothelial nitric oxide synthase (NOS), inducible nitric oxide synthase (iNOS) and MMP2 and MMP9 expression in pentylenetetrazole (PTZ)-induced kindling. **Material and Methods:** In male Wistar rats, kindling (epileptogenesis model) was induced by repeated administration of a sub-convulsive dose (40 mg/kg, intraperitoneal) of PTZ to give rise to kindling. AT was given (5 mg/kg, intraperitoneal) every day and before 30 min from PTZ. After each PTZ injection, the convulsive behavior was observed for 60 minutes. NOS and MMP activities in hippocampus were studied using immunohistochemistry. **Results:** PTZ kindling induced the expression of iNOS and MMP2 and did not affect MMP9, but decreased eNOS expression. AT pretreatment suppressed formation of kindling, increased epithelial nitric oxide synthase, decreased iNOS and MMP2 immune reaction. **Conclusion:** iNOS and MMP2 may be regarded as proconvulsant substances while MMP9 has no effect in development of PTZ kindling. Antiepileptogenic effect of AT may result from a reduction in the activity of MMP2, iNOS with its anti-inflammatory effect. These findings can shed light on future studies, which aim to investigate therapeutic interventions for preventing epileptogenesis.

**Key Words:** Matrix metalloproteinase 2; matrix metalloproteinase 9; pentylenetetrazole; atorvastatin; nitric oxide synthase

**ÖZET Amaç:** Son yıllarda deneysel epilepsi modellerinde metalloproteinazlar ve indüklenebilir nitrik oksit sentazın (iNOS) upregüle oldukları, nöroinflamasyon ve nöron ölümünde anahtar rol oynadıkları gösterilmiştir. HMG-CoA redüktaz inhibitörü olan statinlerin anti-inflamatuar ve nöron koruyucu oldukları gösterilmiştir. Atorvastatin (AT)'in antiepileptik etkisi gösterilmiştir ancak mekanizması açık değildir. Bu etkiden nitrik oksit ve matris metalloproteinazlar (MMP) sorumlu olabilir. Bu çalışmanın amacı pentilente-tetrazol (PTZ) indüklenebilir kindlingde, kindling gelişimi ve hipokampusda endotelial NOS, iNOS ve MMP2 ve MMP9 ekspresyonu üzerine AT'nin etkilerini araştırmaktır. **Gereç ve Yöntemler:** Wistar türü erkek sıçanlarda PTZ'nin subkonvulzif dozu (40 mg/kg, intraperitoneal) gün aşırı kindling oluşana kadar verildi. AT (5 mg/kg, intraperitoneal) her gün ve PTZ verilmeden 30 dakika önce verildi. Her PTZ enjeksiyonundan sonra 60 dakika süreyle nöbet davranışı izlendi. Deney sonunda beyinler çıkarıldı ve hipokampus bölgesinde metalloproteinaz ve NOS aktivitesi immünhistokimyasal olarak incelendi. **Bulgular:** PTZ kindling, beyin hipokampus bölgesinde iNOS ve MMP2 ekspresyonunu uyarırken, epitelyal nitrik oksit sentaz (eNOS) ekspresyonunu azalttı ancak, MMP 9 üzerine bir etkisi yoktu. AT'nin kronik uygulanması kindling oluşumunu baskıladı, eNOS aktivitesini artırdı, iNOS ve MMP2 immünreaksiyonunu azalttı. **Sonuç:** PTZ kindling gelişiminde MMP9 aktivitesi değişmezken, proinflamatuar mediyatör olan iNOS ve MMP2'nin ekspresyonlarının artmış olması, bunların prokonvulzif olduklarını düşündürdü. AT'nin kindling oluşumunu baskılaması ve iNOS ve MMP2 ekspresyonunu engellemesi, anti-inflamatuar etki ile antiepileptogenik etki göstermiş olduğunu düşündürdü. Bu bulgular özgün olup epileptogenesisi engellemeye yönelik tedavi amaçlı çalışmalara ışık tutacaktır.

**Anahtar Kelimeler:** Matris metalloproteinaz 2; matris metalloproteinaz 9; pentilente-tetrazol; atorvastatin; nitrik oksit sentaz

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Epilepsy is a neurological disorder which results from an abnormal electrical activity in some parts of the brain and affects about 1-2% of the world population. Despite the significant progress in the drug treatment on epileptic seizures, about 40% of patients with epilepsy are resistant to current pharmacotherapy. Therefore, the use of animal seizure models are needed for the discovery of new antiepileptic drugs.<sup>1</sup> The pentylenetetrazole (PTZ) kindling is widely accepted as an experimental animal model for epileptogenesis. PTZ is thought to act as a noncompetitive antagonist of gamma aminobutyric acid type A (GABA-A) receptors, and repeated injection of PTZ causes a gradual, long lasting seizure development.<sup>2,3</sup> Epileptogenesis is the process by which a "normal" brain becomes epileptic. During epileptogenesis, brain undergoes neuropathologic changes, including hippocampal neurodegeneration and extensive reorganization of hippocampal circuits which lead to the occurrence of spontaneous seizures. Neuronal death, aberrant synaptic plasticity and neuroinflammation play essential roles in epileptogenesis.<sup>4</sup> However, the precise pathophysiological mechanisms responsible for epilepsy are not yet understood completely. In general, known antiepileptic drugs can only suppress seizures without antiepileptogenic effects. Thus, understanding the molecular mechanisms of epilepsy may lead to therapeutic interventions for limiting epileptogenesis.

A role for matrixmetalloproteinases (MMPs) in the pathogenesis of epilepsy has been suggested by several studies. MMPs are zinc-dependent proteases with multifactorial actions in central nervous system (CNS) physiology and pathology and they are able to degrade or modify the components of the extracellular matrix. Two members of the MMP family, MMP2 and MMP9, degrade the extracellular matrix (ECM) components of the basement membrane.<sup>5</sup> Dramatic changes in the expression of MMPs occur during excitotoxic/neuro-inflammatory processes. Experimental work has shown that the overactivity of MMPs is involved in disease processes of the CNS, such as cerebral ischemia and edema, stroke, trauma and epilepsy.<sup>6,7</sup> Cumulative

evidence indicates that MMP9, a key participant in neuronal death, aberrant synaptic plasticity and neuroinflammation, is upregulated in experimental epilepsy models.<sup>8</sup> Besides, MMPs (especially MMP9) play a physiological role in structure and function of dendritic spines in the hippocampus synaptic plasticity.<sup>9</sup> However recently, the role of MMP2 in pathogenesis of neurological diseases has been pointed out. It has been shown that the activities of MMP2 and MMP9 are increased in nerve tissues injured by brain ischemia and head trauma, where reactive astrocytes are the main sources of the increased MMPs.<sup>10</sup> In addition, nitric oxide (NO) is a known mediator in seizure susceptibility modulation. The role of NO in epileptogenesis has been examined in a number of studies, however the current data are still controversial. Its anti and/or proconvulsive effects have been revealed.<sup>11-13</sup> NO has been pointed out as potential neurotransmitter or retrograde messenger linked to synaptic plasticity and regulation of brain excitability, including the triggering of seizure activity. NO is synthesized from L-Arginine by activation of the nitric oxide synthase (NOS). Three different forms of NOS have been identified including one inducible (iNOS) and two constitutively endothelial nitric oxide synthase (eNOS) or neuronal nitric oxide synthase (nNOS) expressed forms.<sup>14</sup> Excessive NO production is linked to the activation of nNOS in PTZ kindled model, while it is showed that iNOS is associated with kindling responses.<sup>15,16</sup> However, inhibition of iNOS with aminoguanidine has discarded the involvement of iNOS in PTZ-induced seizures.<sup>17</sup> Recently, it has been demonstrated that iNOS-knockout mice reach the kindled status induced by PTZ more slowly compared to control mice.<sup>16,18</sup> It is demonstrated that NO and products of free radicals are significantly increased in the brain of rats in PTZ-induced seizures. Researchers suggest that mexidol, which is an antioxidant drug, ameliorates clonic tonic convulsions by reducing iNOS level.<sup>19</sup> Thus, we investigated in our study the iNOS immune reaction. iNOS expression is induced by some toxins and cytokines which may contribute to seizure-associated neuronal death.<sup>20</sup> In addition, it is reported that iNOS-catalyzed NO production en-

hanced MMP activation, since NO-induced oxidant is known to be a potent activator of MMPs.<sup>21</sup> In addition, MMP-2 expression correlates with increased inducible nitric oxide synthase (iNOS) production. The cholesterol lowering statins have been reported to act as neuroprotectives by showing antioxidant and anti-inflammatory effect and modulating nitric oxide.<sup>22,23</sup> Additionally, in recent years it is reported that statins can modulate MMP expression.<sup>24</sup> It has been well documented that statins have anti-inflammatory and immunomodulatory mechanisms, among which MMPs were recently found to play an important role in the immunomodulatory effects of statins in cerebral ischemic stroke. Statins are closely related to the NO production. Several lines of evidence have shown that statins increase beneficial NO production by upregulating eNOS or reduce NO overproduction by downregulating iNOS and nNOS.<sup>25,26</sup> It has been shown that atorvastatin modulated or improved seizures and other neurological diseases associated with excitotoxicity.<sup>3,27</sup> However, recent experimental findings showed that AT treatment has no beneficial effect on the brain inflammation, neuronal death or synaptic reorganization in the temporal lobe epilepsy model.<sup>28</sup>

Effects of AT on hippocampal MMPs and NOS activities in PTZ-kindling seizures has not been studied yet. Thus, in the present study we examined whether the role of AT in suppression of PTZ-kindling seizures was potentially mediated with the change of iNOS, eNOS, MMP2 or MMP9 level in the hippocampus.

## MATERIAL AND METHODS

### EXPERIMENTAL DESIGN

Adult male Wistar rats weighing 250-280 g were used in this study. The rats were housed in a controlled environment (23°C; humidity 60%; fixed 12h light-dark cycle). Food and water were provided *ad libitum*. The animals' rights were protected and ethical permission for the study was granted by Istanbul University, DETAE (123-2008). Animals were randomly divided into four groups of five animals each. Control group (0.09% NaCl was administered); PTZ-kindling group; AT group

[5 mg/kg per day intraperitoneally (i.p) was given for four weeks]; AT+PTZ kindling group (5 mg/kg/day AT i.p was given to PTZ treated rats for four weeks, AT was administered one hour before PTZ).

### KINDLING PROCEDURE

For PTZ kindling, a subconvulsant dose of PTZ 40 mg/kg body weight was injected intraperitoneally three times per week (Monday, Wednesday, Friday). The PTZ injections were stopped when the animals showed adequate kindling, i.e. when seizure stage was 5 in both of two consecutive injections. Kindling developed after four weeks. After each PTZ injection, the convulsive behavior was observed for 30 min. *Seizure stages*; Stage 0: no response; stage 1: ear and facial twitching; stage 2: myoclonic body jerks without upright position; stage 3: myoclonic jerks, upright position with clonic forelimb convulsions; stage 4: generalized clonic convulsion, with a turn onto the side; stage 5: generalized convulsions, tonic extension episode, and status epilepticus. Seizure stage for each animal was calculated as mean of the phases. *Seizure latency* was defined as from injection of PTZ to the appearance of the first stage 1 or 2 seizure. *Seizure frequency* was defined as the number of seizures observed over the 30 minutes after PTZ injection, regardless of seizure stage.<sup>3,12</sup>

### PTZ CHALLENGE

To evaluate seizure susceptibility in each group or to show that kindling developed, a challenge dose of PTZ (40 mg/kg) was administered to the rats one week after the last PTZ injection. Animals with stage 4 or 5 seizures were considered to be fully kindled.

### DETERMINATION OF iNOS, eNOS, MMP2 AND MMP9 EXPRESSIONS

After 13<sup>th</sup> dose, rats were anesthetized with an intramuscular injection of ketamine (60 mg/kg Pfizer, Eczacıbaşı, Turkey) and xylazine (7 mg/kg Bayer, Germany) and brains were removed and hippocampus was separated. The hippocampus was fixed with 10% neutral formalin and then a routine paraffin embedding method was used.<sup>29</sup> The

sections were incubated with rabbit polyclonal anti-eNOS (NeoMarkers), rabbit polyclonal anti-iNOS (NeoMarkers) and mouse monoclonal MMP2 (SantaCruz), and goat polyclonal MMP9 (SantaCruz) was applied by diluted 1:100 and reacted with tissue specimens at room temperature for one hour. The peroxidase activity was demonstrated by AEC (3-amino-9-ethyl carbazole) substrate kit (ThermoScientific) and the sections were counterstained with hematoxylin. Primary antisera were diluted in antibody diluent (ThermoScientific). The specificity of the immunohistochemical staining was tested using PBS in the same dilutions. Control tissue sections were used as the positive control. The semiquantitative evaluation of the iNOS, eNOS, MMP2 and MMP9 immunohistochemical staining was done using H-score.<sup>30</sup> Slides were photographed and examined by using Kameran 390CU Imaging system (MikroSistem Ltd. Turkey).

#### STATISTICAL ANALYSIS

All results were expressed as medians, means±standard deviation (SD), and  $p < 0.05$  was regarded as significant. Results were evaluated with the Graphad Prism statistical program (version 5.0). Experimental groups were compared with respect

to seizure stage, seizure frequency and seizure latency using a Friedman test and Mann-Whitney test. For H-score, Kruskal-Wallis test was used.

## RESULTS

#### BEHAVIOR CHANGES

The epileptic seizures in rats were observed as follows: s ear, mouth and facial movements in first day; following days revealed myoclonic jerks, head nodding, running, clonic seizures with loss of righting reflex and generalized clonic-tonic seizures with bilateral forelimb clonus and tail up. The PTZ-induced generalized convulsions lasted intermittently 30 min. Chronic pretreatment with AT showed minor myoclonic jerks only. AT significantly suppressed the seizure stage ( $p < 0.001$ ) (Table 1), increased seizure latency ( $p < 0.001$ ) (Table 2) and decreased seizure frequency ( $p < 0.001$ ) (Table 3). No seizure activity was observed in control and AT group.

#### MMP2 AND MMP9 IMMUNE REACTION IN HIPPOCAMPUS

In PTZ-kindled rats, a significant increase of MMP2 activity was detected in the hippocampus as compared with controls ( $p < 0.001$ ) (Figure 1),

**TABLE 1:** Effect of chronic atorvastatin treatment on seizure stage in the pentylenetetrazole (PTZ)-kindling epilepsy model in rats.

Seizure Stage	N	PTZ Kindling			Atorvastatin+PTZ Kindling				Mann-Whitney p
		Median	Min	Max	N	Median	Min	Max	
Day_1	5	1 <sup>(6*10**11**12**13**)</sup>	1	1	5	1	1	1	1.000
Day_2	5	1 <sup>(11**12**13**)</sup>	1	2	5	1	1	2	1.000
Day_3	5	1 <sup>(8*9)</sup>	1	2	5	1	1	2	0.513
Day_4	5	2	1	3	5	2	1	2	0.502
Day_5	5	3 <sup>(13)</sup>	2	3	5	1	1	3	0.212
Day_6	5	3 <sup>(1)</sup>	3	4	5	2	1	3	0.033*
Day_7	5	4	2	4	5	2	2	3	0.077
Day_8	5	4	3	5	5	2	2	2	0.005**
Day_9	5	4 <sup>(3)</sup>	3	5	5	2	1	2	0.008**
Day_10	5	5 <sup>(1**)</sup>	4	5	5	2	1	3	0.008**
Day_11	5	5 <sup>(1**2**)</sup>	4	5	5	2	1	3	0.007**
Day_12	5	5 <sup>(1**2**)</sup>	4	5	5	2	1	2	0.005**
Day_13	5	5 <sup>(2**3**5*)</sup>	5	5	5	1	1	3	0.005**
p (Friedman)		0.000***			0.096				

**Note:** In the last column and the last row of the table, the significance level of Mann-Whitney U test and Friedman test are seen respectively. The multiple comparisons of post-hoc Dunn test for significant Friedman result are shown in median column with section numbers and significance levels (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.0001$ ).

**TABLE 2:** Effect of chronic atorvastatin treatment on seizure latency in the pentylenetetrazole (PTZ)-kindling epilepsy model in rats.

Seizure Stage	N	PTZ Kindling			Atorvastatin+PTZ Kindling				Mann-Whitney p
		Median	Min	Max	N	Median	Min	Max	
Day_1	5	3 <sup>(6.8-13<sup>**</sup>)</sup>	2	7	5	2	2	3	0.118
Day_2	5	4 <sup>(6.8-13<sup>**</sup>)</sup>	2	7	5	2	1	3	0.159
Day_3	5	2	1	5	5	3	2	3	0.656
Day_4	5	2	1	3	5	2	1	2	0.192
Day_5	5	2	2	3	5	2	1	2	0.018*
Day_6	5	1 <sup>(1.2<sup>**</sup>)</sup>	1	3	5	2	1	3	0.514
Day_7	5	2	1	2	5	2	1	3	0.214
Day_8	5	1 <sup>(1.2<sup>**</sup>)</sup>	1	2	5	2	1	3	0.166
Day_9	5	1 <sup>(1.2<sup>**</sup>)</sup>	1	1	5	3	2	3	0.005**
Day_10	5	1 <sup>(1.2<sup>**</sup>)</sup>	1	1	5	1	1	3	0.134
Day_11	5	1 <sup>(1.2<sup>**</sup>)</sup>	0.5	1.0	5	3	1.0	4.0	0.033*
Day_12	5	1 <sup>(1.2<sup>**</sup>)</sup>	0.5	1.0	5	2	1.0	3.0	0.013*
Day_13	5	1 <sup>(1.2<sup>**</sup>)</sup>	0.5	.5	5	2	1.0	2.5	0.005**
p (Friedman)		0.000***			0.218				

**Note:** In the last column and the last row of the table, the significance level of Mann-Whitney U test and Friedman test are seen respectively. The multiple comparisons of post-hoc Dunn test for significant Friedman result are shown in median column with section numbers and significance levels (\*p<0.05, \*\*p<0.01 and \*\*\*p<0.0001).

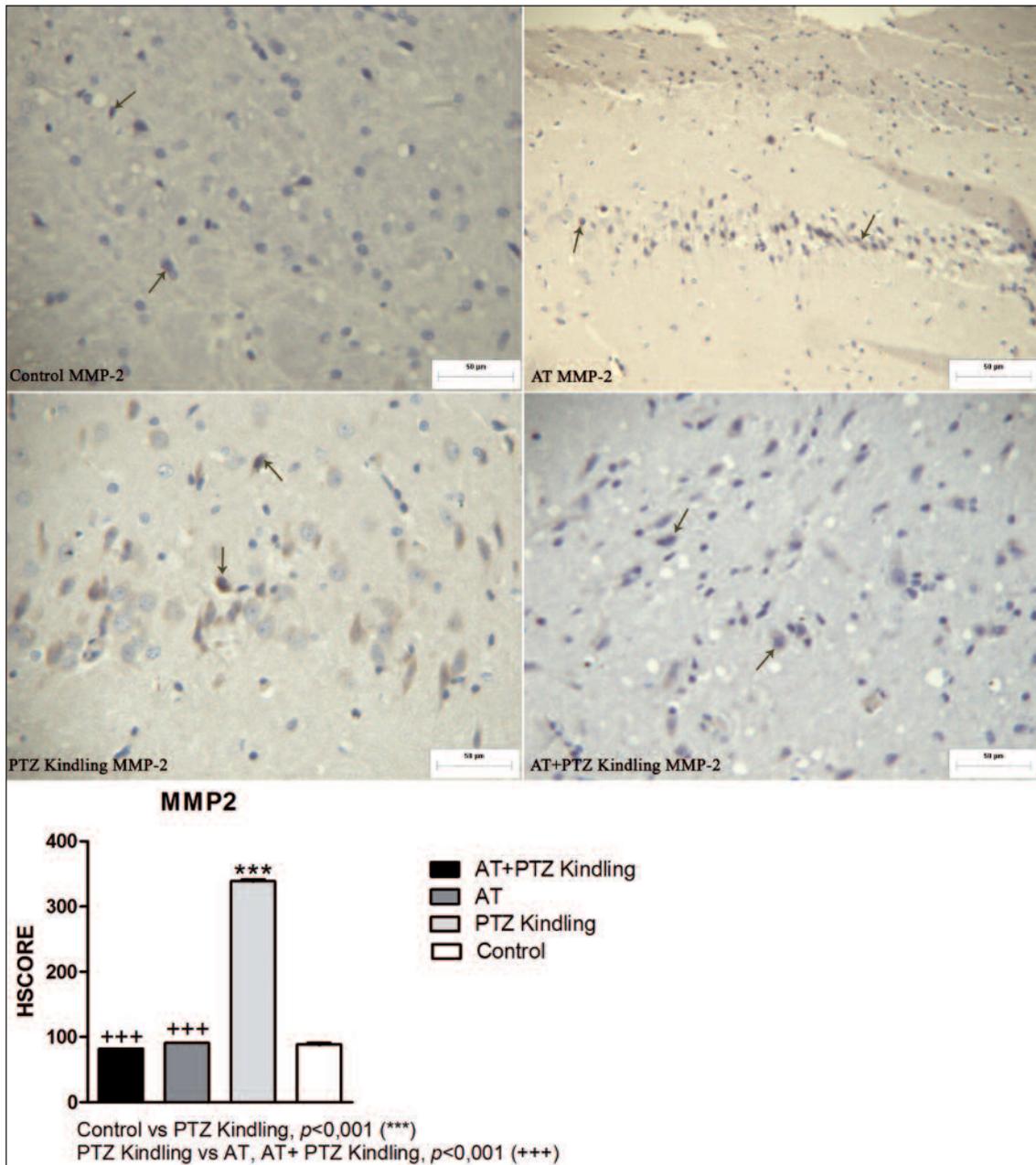
**TABLE 3:** Effect of chronic atorvastatin treatment on seizure frequency in the pentylenetetrazole (PTZ)-kindling epilepsy model in rats.

Seizure Stage	N	PTZ Kindling			Atorvastatin+PTZ Kindling				Mann-Whitney p
		Median	Min	Max	N	Median	Min	Max	
Day_1	5	6(3-13)*	4	8	5	1(3-13)*	1	1	0.005**
Day_2	5	8(5.10)*	6	9	5	5	3	16	0.242
Day_3	5	9(1)*	8	12	5	9(1)*	3	10	0.395
Day_4	5	9(8.10.12.13)*	7	12	5	9(1)*	6	13	1.000
Day_5	5	14(1.2)*	11	17	5	8(1)*	4	9	0.009**
Day_6	5	15(1.2)*	9	17	5	8(1)*	7	12	0.027*
Day_7	5	18(1)*	16	20	5	8(1)*	8	10	0.008**
Day_8	5	16(1)*	14	18	5	7(1)*	6	8	0.008**
Day_9	5	17(1)*	15	18	5	7(1)*	5	8	0.009**
Day_10	5	16(1)*	15	17	5	6	5	10	0.008**
Day_11	5	18(1)*	12	18	5	6(1)*	6	9	0.007**
Day_12	5	16(1)*	14	20	5	7(1)*	5	8	0.009**
Day_13	5	16(1)*	13	18	5	9(1)*	6	10	0.009**
p (Friedman)		0.000***			0.015**				

**Note:** In the last column and the last row of the table, the significance level of Mann-Whitney U test and Friedman test are seen respectively. The multiple comparisons of post-hoc Dunn test for significant Friedman result are shown in median column with section numbers and significance levels (\*p<0.05, \*\*p<0.01 and \*\*\*p<0.0001).

whereas interestingly MMP9 activity did not change (Figure 2). Chronic pretreatment with AT significantly prevented the increase of MMP2 activity in hippocampus (p<0.001) (Figure 1), The administration of AT alone did not change MMP2

activity in hippocampus, while the AT slightly increased the MMP9 activity, but this did not show significant difference compared to saline-treated (control) group (Figure 2). The semiquantitative evaluation of MMP2 and MMP9 immunohisto-



**FIGURE 1:** Effects of atorvastatin on pentylenetetrazole (PTZ) kindling-induced changes in MMP2 level in hippocampus. Immunohistochemical detection of MMP2 staining (brown) in hippocampus in control and experimental groups and semiquantitative evaluation (H-score) in hippocampuses of all groups. Arrows indicate staining cells (Bar: 50  $\mu$ m).

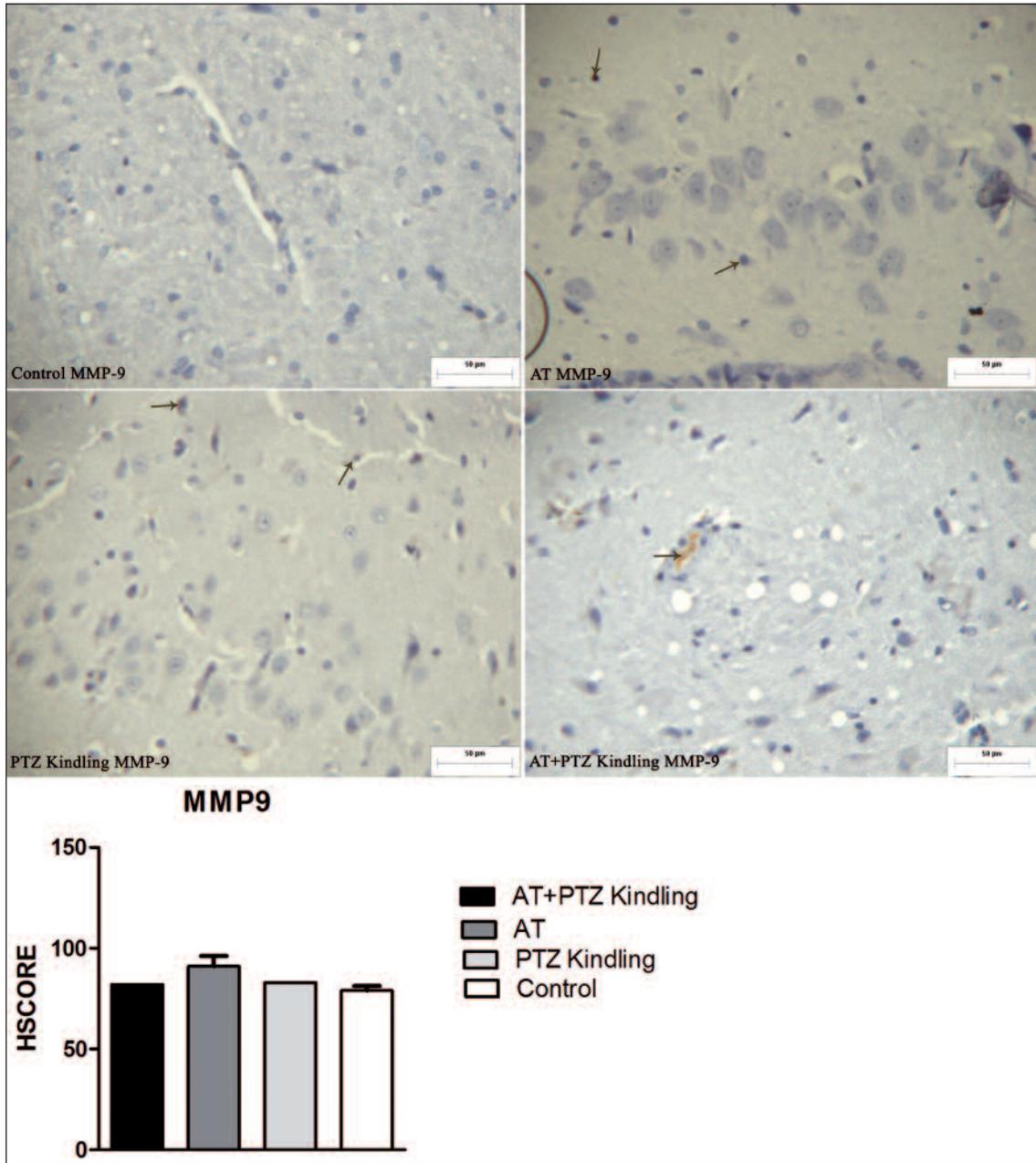
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chemical staining was done with H-score (Figure 1, 2).

#### eNOS AND iNOS IMMUNE REACTION IN HIPPOCAMPUS

In PTZ-kindled rats, significant increase of iNOS activity was detected in the hippocampus as compared with controls ( $p < 0.0001$ ) (Figure 3), whereas

a very significant decrease was observed in eNOS activity ( $p < 0.001$ ) (Figure 4). Chronic pretreatment with AT significantly prevented the increase of iNOS activity in the hippocampus ( $p < 0.001$ ) (Figure 3), while the pretreatment significantly recovered the eNOS activity compared with PTZ-kindled group ( $p < 0.001$ ) (Figure 4). The ad-



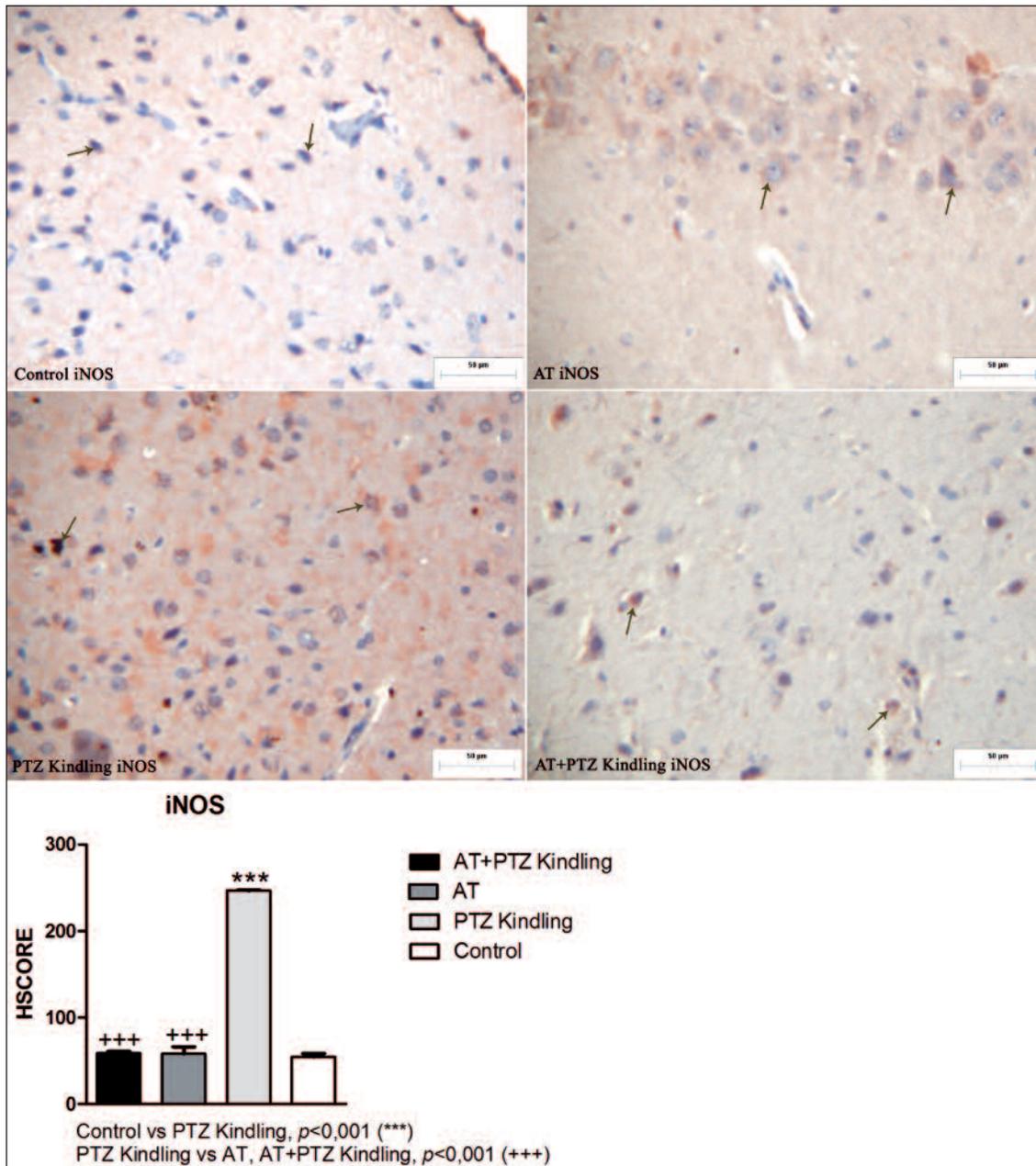
**FIGURE 2:** Effects of atorvastatin on pentylenetetrazole (PTZ) kindling-induced changes in MMP9 level in hippocampus. Immunohistochemical detection of MMP9 staining (brown) in hippocampus in control and experimental groups and semiquantitative evaluation (H-score) in hippocampi of all groups. Arrows indicate staining cells (Bar: 50 µm).

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ministration of AT alone did not change iNOS and eNOS activity in hippocampus as compared to saline-treated (control) group (Figures 3, 4). The semiquantitative evaluation of iNOS and eNOS immunohistochemical staining was done with H-score (Figures 3, 4).

## DISCUSSION

The main finding of this study is that AT pretreatment decreased MMP2 and iNOS immune reaction, increased eNOS immune reaction in hippocampus and prevented the development of

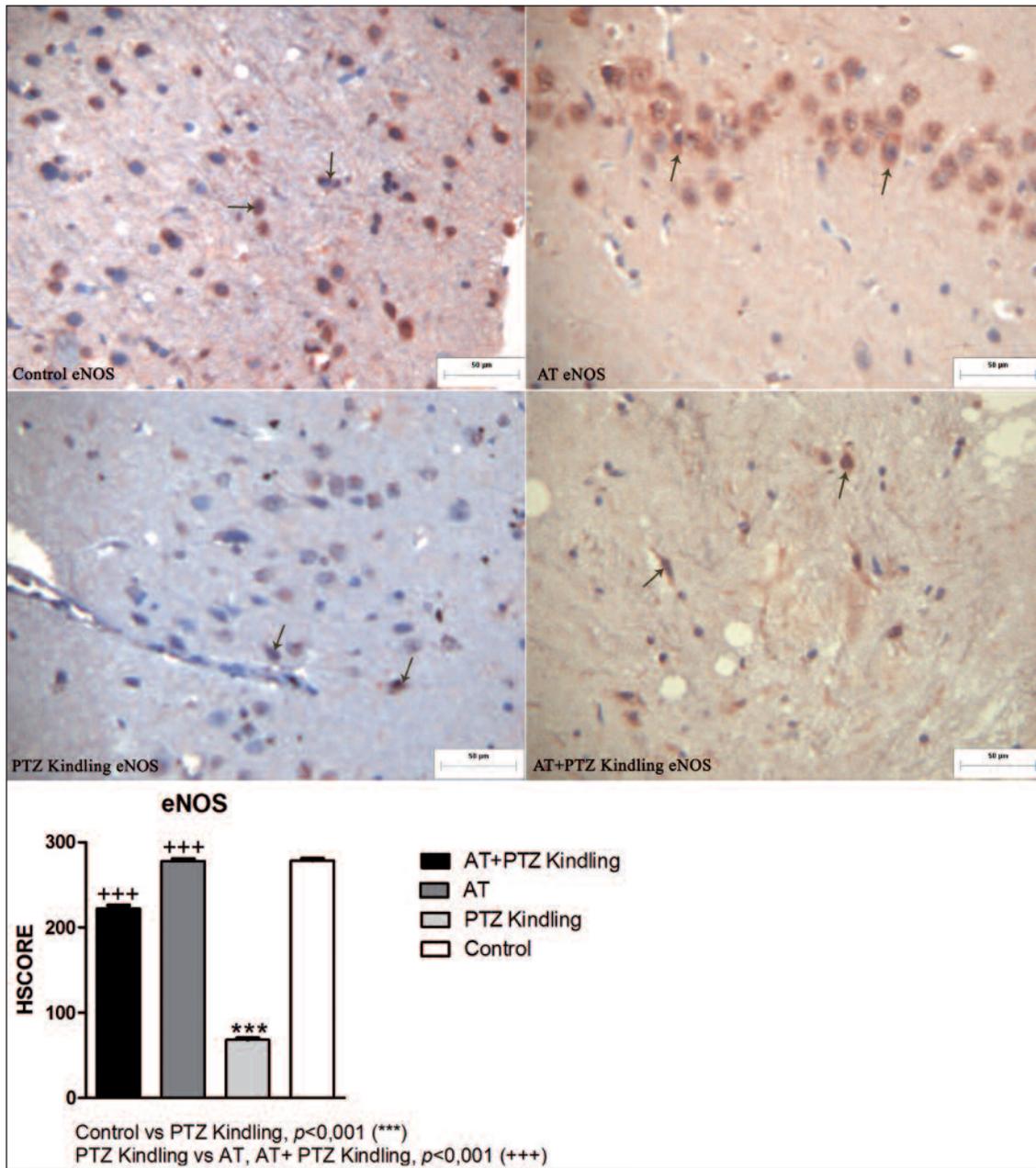


**FIGURE 3:** Effects of atorvastatin on pentylenetetrazole (PTZ) kindling-induced changes in iNOS level in hippocampus. Immunohistochemical detection of iNOS staining (brown-red) in hippocampus in control and experimental groups and semiquantitative evaluation (H-score) in hippocampi of all groups. Arrows indicate staining cells (Bar: 50 µm).

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epilepsy in the PTZ-treated rats. The studies on the effects of statins in epilepsy are limited and their results are controversial. In our previous study, we demonstrated that AT suppressed PTZ kindling development and improved the memory deficit caused by epilepsy.<sup>3</sup> Likewise, in studies which investigate the effect of AT on epilepsy, it

has been reported that statins reduce cell loss in kainate, pilocarpine, and quinolinic acid-induced epilepsy, on the contrary in another study it has been shown that 10 mg/kg AT treatment did not affect the duration of status epilepticus or the development of epilepsy in electrical stimulation-induced status epilepticus.<sup>27,28,31</sup> We suggest that



**FIGURE 4:** Effects of atorvastatin on pentylene tetrazole (PTZ) kindling-induced changes in eNOS level in hippocampus. Immunohistochemical detection of eNOS staining (brown-red) in hippocampus in control and experimental groups and semiquantitative evaluation (H-score) in hippocampi of all groups. Arrows indicate staining cells (Bar: 50 µm).

(See color figure at <http://tipbilimleri.turkiyeklinikleri.com/>)

different results could depend on the epilepsy model used and the dose of the statin. Wilczynski et al. identified MMP9 as a novel synaptic enzyme and a key pathogenic factor in these animal models of kainate-induced epilepsy and PTZ kindling epilepsy.<sup>32</sup> It demonstrates that MMP9 activity is up-regulated in hippocampal synapses upon seizures, contributing to remodeling of dendritic

spines and aberrant synaptogenesis.<sup>32</sup> In another study, it has been demonstrated that MMP9 induces apoptotic hippocampus cell death by interrupting the integrin-mediated survival signaling after status epilepticus. Thus, MMP9 may be a promising target for a neuroprotective approach to prevent seizure-induced hippocampus damage.<sup>33</sup> In another study, repeated PTZ treatment

(40 mg/kg) increased MMP-9 expression in the hippocampus. On the other hand, hippocampal MMP-9 levels were not affected in mice that showed convulsive seizures in response to a single 60 mg/kg PTZ dose. No changes in hippocampal MMP2 levels were detected following acute or repeated PTZ treatment.<sup>34</sup>

However it is reported that, especially altered regulation of MMP2 and MMP9 has been linked to several nervous system disorders.<sup>30</sup> As far as we know, there is no study showing the MMP2 increase in epileptogenesis. Interestingly, our findings show that as a result of kindling there is an increased MMP2 immune reaction in hippocampus while MMP9 immune reaction was unchanged. We observed in our study a novel finding: in PTZ-kindled rats there is an increase in the expression of MMP2 which is inhibited by AT. Although not related to epilepsy, a study showed that in myocardial ischemia/reperfusion injury, rosuvastatin administration before ischemia reduced infarct size by reducing MMP2.<sup>14</sup> AT's preventive effects in formation of kindling lead us to define that its mode of action might be through a different pathway. AT might be protective by reducing the formation of MMP2 and iNOS. A study showed increased MMP2, as well as caspase 3, in response to hypoxia in rodent endothelial cells. The researchers suggested that the increase in MMP2 might be responsible for the cerebral endothelial cell death which causes blood brain barrier (BBB) breakdown, in hypoxia-reoxygenation.<sup>35</sup> It is reported that BBB breakdown occurs during epileptogenesis and the chronic epileptic phase and it has been suggested that this can contribute to the progression of epilepsy.<sup>36</sup> With regard to reports of Lee and Lo and Van Vliet et al., our results suggest that AT might have decreased the seizure sensitivity and protected BBB by reducing MMP2.<sup>35,36</sup> There is numerous evidence to prove that NO's activation is increased in hippocampus, cerebral cortex and some other brain regions in PTZ-treated rats.<sup>37</sup> It still remains unclear whether the increase of NO production in brain after seizure is anticonvulsive or proconvulsive.<sup>38</sup> It is suggested that there can be an involvement of NO in the mechanisms related with kindling.<sup>39</sup> Itoh et

al., showed that NO was produced by the activation of nNOS in PTZ kindling model; however, De Luca et al. revealed that kindling occurred more slowly in iNOS knock-out mice when administered PTZ.<sup>15,16</sup> In contrast, the data of another investigation shows that iNOS is not expressed in the kindling model.<sup>40</sup> In parallel, another study showed that iNOS was not expressed in the hippocampus of both acutely convulsed and kindled animals.<sup>8</sup> On the contrary, our results indicated that PTZ-induced kindling increased iNOS immune reactivity in hippocampus. iNOS is expressed by macrophages. It is activated by inflammatory signals such as cytokines, and it is known that iNOS is responsible for the cytotoxicity, while eNOS and nNOS are responsible for the molecular communication.<sup>41,42</sup> In addition, brain inflammation contributes significantly to determine seizure threshold in susceptible brain regions such as hippocampus, thus playing a role in seizure precipitation and their recurrence.<sup>43</sup> In our study, AT decreased iNOS activity in hippocampus while suppressing development of kindling in PTZ-treated rats. A study supporting us showed that chronic treatment with aminoguanidine (iNOS inhibitor) decreased the seizure latency in groups chronically treated with atorvastatin in the intravenous pentylenetetrazol-induced seizure model.<sup>44</sup> According to this and our data, it appears that iNOS might be responsible for the anti-seizure activity of atorvastatin. We can suggest that the antiepileptogenic effect of AT may be related with an anti-inflammatory effect.

NO, oxidative stress and proinflammatory cytokines, which play a role in epilepsy pathogenesis, can induce expression and activation of MMPs.<sup>45</sup> Likewise it is reported that iNOS-catalyzed NO production enhanced MMP2 activation, since NO-induced oxidant is known to be a potent activator of MMPs.<sup>22</sup> In parallel, it has been shown that induction of iNOS in atheroma of high-cholesterol-fed ApoE<sup>-/-</sup>/iNOS<sup>+/+</sup> mice leads to increased production of MMP2 and MMP9.<sup>11</sup> Likewise, we found an increase in the iNOS and MMP2 immune reaction in hippocampus of PTZ kindled rats. We suggest that iNOS and MMP2 might have a major role in at least PTZ kindling induced epileptogen-

esis, and that specific drugs such as statins targeting iNOS and/or MMP2 could prove useful therapeutic regimens in preventing epileptogenesis. Further work is urgently needed to determine whether the inhibition of MMP2 and iNOS is useful as a therapeutic strategy for limiting epileptogenesis.

## CONCLUSIONS

In conclusion, our findings demonstrate that MMP2 and iNOS immune reaction are significantly increased, eNOS immune reaction is decreased and MMP9 immune reaction did not change in the hippocampus of PTZ induced kindled rats, which may be related to epileptogenesis. Anticonvulsant effects of AT have been reported, in acute seizure model, but as far as we know there is no data concerning the influence of AT on iNOS and MMP2 activity in PTZ kindling model, which is chronic epilepsy model. Our results provide the first direct evidence that AT significantly decreased iNOS and

MMP2 activity in hippocampus of PTZ treated rats. While the molecular mechanism underlying AT's effect is unclear, it is likely that its effect on MMP2 and iNOS activity is contributing to its antiepileptogenic effect. These data, although not showing directly, might support that the antiepileptogenic effect of AT may correlate with its anti-inflammatory and antioxidant ability by iNOS inhibition. Brain inflammation and the associated BBB damage is important in epileptogenesis. Therefore, we suggest that AT might have protected BBB and impaired hippocampal damage by reducing MMP2.

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