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Effects of Luteolin on Liver, Kidney and Brain in Pentylentetrazol-Induced Seizures: Involvement of Metalloproteinases and NOS Activities

Hüsniye Birman[1], Kadriye Akgün Dar[2], Ayşegül Kapucu[2], Samet Acar[2], Gülay Üzüm[1]

[1]Department of Physiology, İstanbul Faculty of Medicine, İstanbul University, İstanbul, Turkey
[2]Department of Biology, Faculty of Science, İstanbul University, İstanbul, Turkey

ABSTRACT

Objective: Flavonoids are an important group of recognized antioxidants in plants. Luteolin (LUT) is a natural flavonoid in the plant kingdom. This study was aimed to investigate the effects of the LUT in the liver, kidney and brain of pentylentetrazol (PTZ)-induced seizure and the relationship between nitric oxide synthases (iNOS, eNOS) and matrix metalloproteinases (MMP2, MMP9).

Materials and Methods: LUT (10 mg/kg) was given intraperitoneally during two weeks prior to seizure induction. A single dose PTZ 80 mg/kg i.p. was administered and seizures were observed and evaluated with regard to latency, frequency and stage for one hour.

Results: Seizure frequen cy after PTZ administration was significantly decreased in LUT pretreated rats ($p<0.05$). An increase of immunhistochemical reactions of iNOS and MMP2, but a decrease of eNOS activity, were observed in rat hippocampus and peripheral tissues during the PTZ induced seizures. LUT pretreatment reversed the iNOS and MMP2 activity to the control levels and significantly increased the eNOS activity ($p<0.001$).

Conclusion: LUT seems to have an effective role in reducing the seizure frequency and a protective role on peripheral organ injury in animal models of seizure. The protective effect of LUT in seizures and the seizure induced peripheral tissue damage warrant further investigations.

Key Words: Luteolin, pentylentetrazol, seizure, metalloproteinases, NOS

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Introduction

Flavonoids are also common constituents of plants used in traditional medicine to treat a wide range of diseases. LUT and its glycosides are widely distributed in the plant kingdom. Flavonoids have many biological and pharmacological activities that may play a role in antioxidative properties, (1) cancer prevention, (2, 3) neuroprotection (4) and antihypertensive effects (5). *In vitro* studies have shown reduction of the expression of proinflammatory molecules (6). Epilepsy is a common neurological condition associated with some alterations. Patients with epilepsy may suffer from hepatic or renal dysfunctions that interfere with their antiepileptic drug treatment (7). PTZ induced seizures are still the most widely used animal seizure models employed in the research for epilepsy and new antiepileptic drugs (8). It is reported that free radical generation plays a crucial role in neuronal cell death in the PTZ induced seizures in rats (9). Some studies suggested that proinflammatory molecules (e.g.proteolytic enzymes, reactive oxygen species or nitric oxide) may potentiate the damage to brain and peripheral tissues in epilepsy (10-12). Nitric oxide (NO) has been suggested to exert both anticonvulsant and proconvulsant effects. Recent studies demonstrated a strong correlation between the upregulation of MMP9 and epilepsy, and showed that kainate induced seizures result in elevated MMP9 expression (13). Flavonoids are compounds occurring naturally in food, which scavenge oxygen radicals and have anti-inflammatory properties. Recent investigations have reported that oral administration of LUT reduced clinical symptoms of experimental allergic encephalitis (14) and could protect mice from the hepatotoxicity caused by carbon tetrachloride (15). MMPs are expressed as inactive zymogens in which the cysteine residue in the propeptide binds to Zn^{2+} present at the active site of the enzyme. MMPs, which are locally inhibited by endogenous tissue inhibitors of metalloproteinases operate in extracellular matrix. These enzymes are critical for maintaining tissue allostasis (16). It was observed that morin (a natural flavonoid) could lead to decreased enzyme activities of MMP2 and MMP9 and was found to inhibit inflammation and tumor promotion (17). Co-administration of bioactive flavonoids in preoperative nutrition attenuated ischemia-reperfusion injury and decreased apoptosis in the intestine (18). In another study, it has been reported that LUT treatment prevented ischemia reperfusion-induced renal injury and LUT exerted renoprotective effects, probably by antioxidant activity (19). Studies have demonstrated that quercetin, a natural flavonoid, reduced global ischemia-induced neuronal damage through inhibition of MMP9 activity (20). However, studies on MMPs inhibition of some flavonoids have not yet been analyzed in seizures.

Address for Correspondence: *Dr. Kadriye Akgün Dar, Department of Biology, Faculty of Science, İstanbul University, 34134 İstanbul, Turkey*
Phone: +90 212 455 57 00 /15101 Fax: +90 212 528 05 27 E-mail: kakgun@istanbul.edu.tr

Hippocampal region is the most damaged part of the brain in epilepsy. Moreover, it is reported that epileptic patients have liver and kidney damages both because of the epilepsy itself and antiepileptic drugs. In pathogenesis of epilepsy, the role of MMPs and NO is known. However, their role in the damage of the liver and kidney is not studied. Hence, we studied the effects of LUT in the liver, kidney and hippocampus on MMP2, MMP9, eNOS, iNOS, which are most important in epilepsy, in PTZ- induced seizures.

Material and Methods

Animals

Wistar albino male rats (200-250g) were housed in cages and maintained on a 12h light-dark cycle with free access to water and food. Procedures involving the experimentation on animals were done in accordance with the guidelines of our institution (Istanbul University, DETAE).

Experimental design

Animals were divided into four groups each containing five rats; Group I; Control group (%0.09 NaCl administered). Group II; PTZ group (single dose of 80 mg/kg i.p. administered). Group III; LUT group (10 mg/kg i.p. LUT given each day for two weeks). Group IV; LUT+PTZ group (rats treated with 10 mg/kg i.p. LUT for two weeks and 80 mg/kg PTZ administered 30 minutes after the last LUT injection).

Drugs and doses

Luteolin (Department of Pharmacognosy, Faculty of Pharmacy, Istanbul University) was administered i.p. 10 mg/kg. The effective dose for flavonoids administered in experimental studies was between 5mg/kg/day and to 10 mg/kg/day (21, 22). Thus, it is reasonable to use a dose of 10mg/kg LUT in this experiment. Also, this dose has previously been tested in animal studies (23) and researchers have also been reported that quercetin has no treatment-related clinical signs of toxicity. PTZ was dissolved in saline and seizures were induced with a single dose of 80mg/kg i.p. PTZ (SIGMA, USA). This dose was selected as it achieves the most successful convulsive response with the lowest mortality (8).

Pentylentetrazol-Induced Seizures

The behavioral characteristics; stage, latency and frequency of seizures were observed for 60 min in individual animals after PTZ injections. Convulsion stage: Stage was scored using the following scale (24, 25); Unresponsiveness=0, ear and facial twitching=1, myoclonic body jerks=2, clonic forelimb convulsions=3, generalized clonic convulsions, turn over into side position=4, generalized clonic-tonic convulsions=5. Seizure stage for each animal was calculated as a mean of the phases. Convulsion Latency: Latency was measured as the time between injection of PTZ and appearance of the first clonic convulsion, which was indicated by a sudden twitching of the head or jerky movement of the body (26). Convulsion Frequency: Number of seizures during 60 min after PTZ injection, regardless of seizure stage.

Matrixmetalloproteinases and NOS immunohistochemistry

At the end of the experiment, animals were decapitated. Liver, kidney and brain tissues were removed, formalin fixed and, following routine laboratory methods, they were embedded in paraffin. Four-micrometer paraffin tissue sections were mounted on poly-L-lysine slides. The slides were air-dried and the tissue deparaffinized. Mounted specimens were washed in 0.01mol/L phosphate-buffered saline (PBS). After three washes with PBS, an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) was given for 10 minutes at 100ºC in a microwave oven, endogenous peroxidase was eliminated by incubation in 3% H_2O_2 in pH 7.4 in phosphate-buffered saline (PBS; 0.01 M) for 10 minutes. After washing, the specimens were treated with a blocking serum (Labvision, TR-060-UB) at room temperature for 10 minutes. The sections were incubated with rabbit polyclonal anti-eNOS (Neo Markers, dilution 1: 100), rabbit polyclonal anti-iNOS (inducible nitric oxide synthase, Neo Markers, dilution 1: 100) and mouse monoclonal MMP2 (Santa Cruz, dilution 1: 100) and goat polyclonal MMP-9 (1: 100) was applied and reacted with tissue specimens at room temperature for one hour. The sections were washed three times with PBS and incubated with biotinylated secondary antibody (Ultra Vision Detection System-HRP kit, Lab Vision, Fremont, USA) and then streptavidin peroxidase (Ultra Vision Detection System-HRP kit, Lab Vision, Fremont, USA) was given at room temperature for 30 minutes. Diaminobenzidine (DAB) was used as a chromogen, and the sections were counterstained with hematoxylin. The specificity of the immunohistochemical staining was tested using PBS in the same dilutions. Control tissue sections were used as positive controls. The semiquantitative evaluation of the iNOS, eNOS, MMP-2 and MMP-9 immunohistochemical staining was done using the H-score (27, 28). Briefly, the tissues stained with antibodies against eNOS, iNOS, MMP-2 or MMP-9 were evaluated using an Olympus microscope with a special ocular grid on 10 different fields at x400 magnification by 2 blind observers. Positive stained cells were counted and graded according to the staining intensity: 0=no staining, 1=weak, 2=mild, 3=intense, 4=high intense. For each tissue, the H-score value was given by the following formula: H-score=S Pi (i+1) where "i" is the intensity score and "Pi" is the corresponding percentage of cells presenting a given staining. Slides were examinated by using the Kameram 390CU Imaging system (Mikro Sistem) and photographed.

Statistical analysis

All results were expressed as means±SD and $p \leq 0.05$ was regarded as significant. Results were evaluated with the Graphad Prism statistical program (version 5.0). Values were tested, groups were compared according to to seizure stage, seizure latency and seizure frequency with nonparametric Mann-Whitney U test and also with the H-score non parametric one way ANOVA.

Results

Evaluation of PTZ-induced seizures

Pentylentetrazol induced generalized clonic-tonic seizures in all animals. Results of behavioral characteristics in PTZ-in-

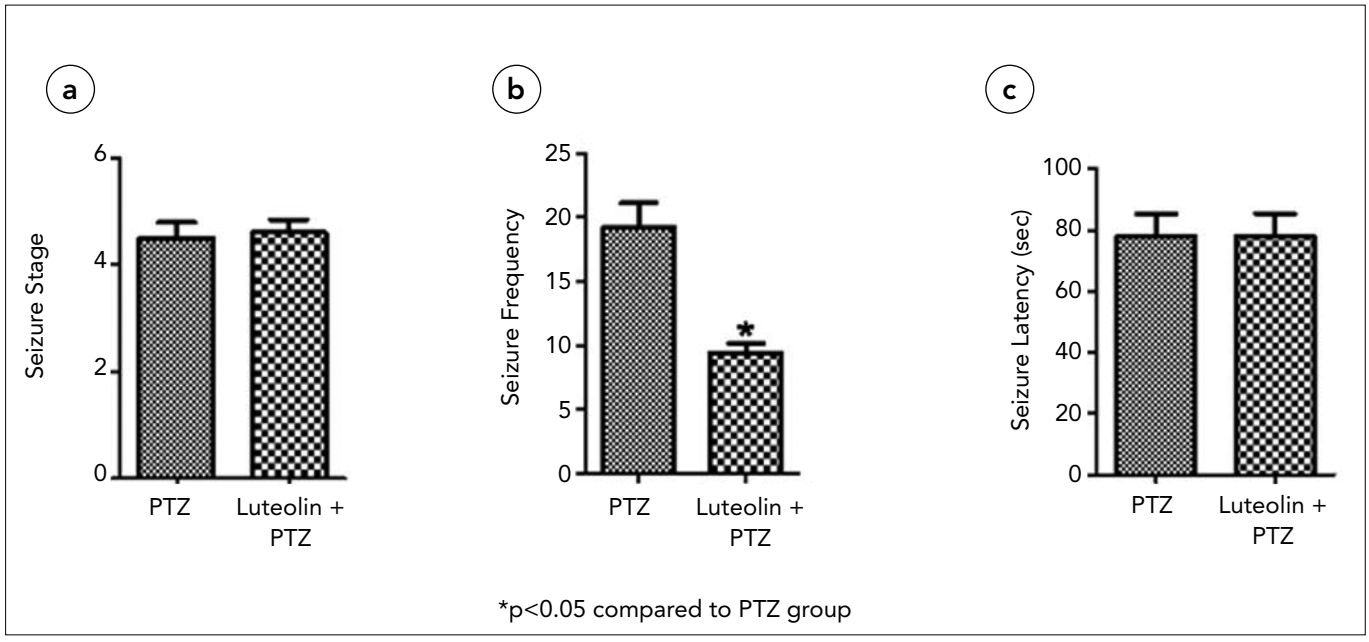

Figure 1. The effect of luteolin treatment on the development of PTZ induced seizure stage (a), seizure frequency (b) and seizure latency (c)

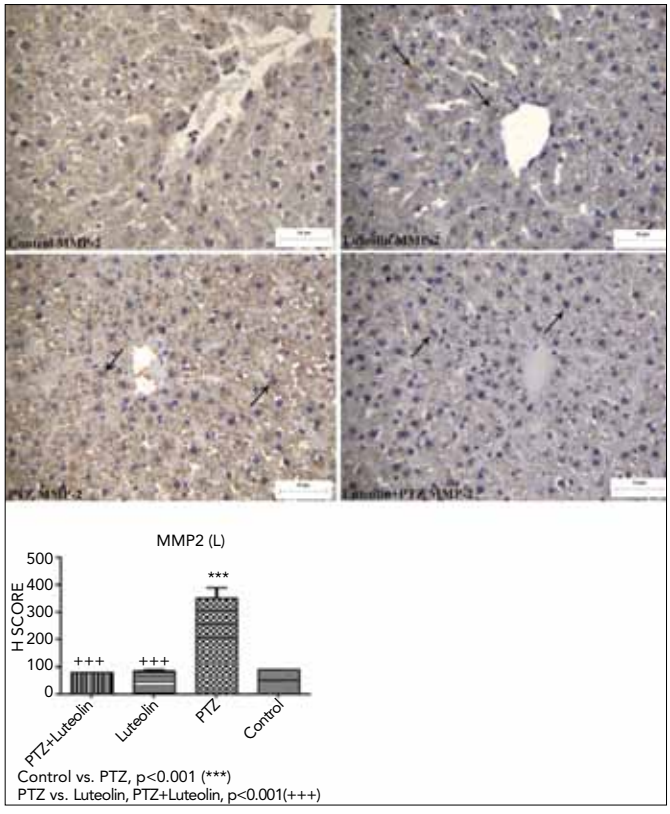

Figure 2. Immunohistochemical detection of MMP2 staining (arrows), in liver sections in control and experimental groups (Bar: 50 µm) and semiquantitave evaluation (H-score) in liver (L) of all groups. Immunostaining intensity was assessed by semiquantation of MMP2 on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

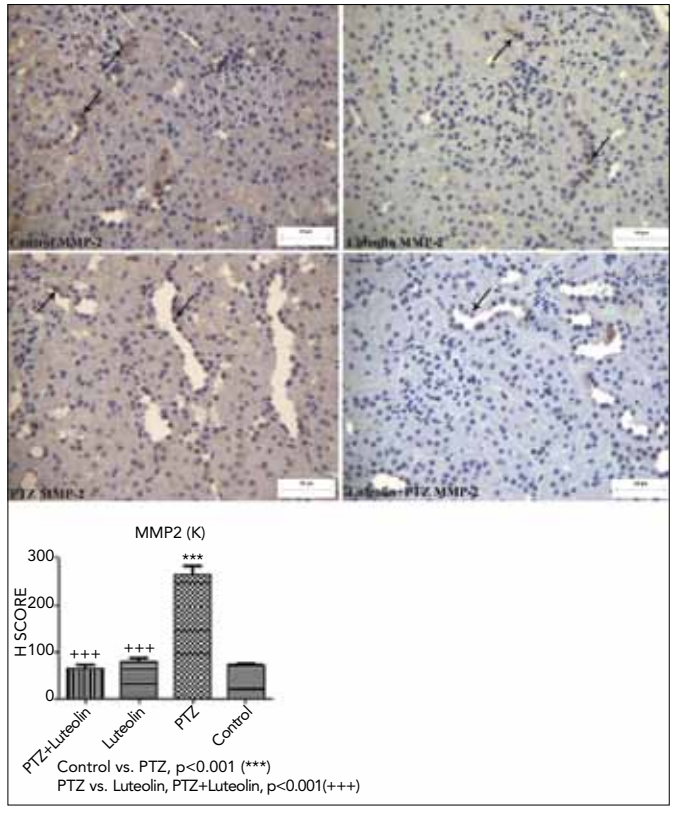

Figure 3. Immunohistochemical detection of MMP2 staining (arrows), in kidney sections in control and experimental groups (Bar: 50 µm) and semiquantitative evaluation (H-score) in kidneys (K) of all groups. Immunostaining intensity was assessed by semiquantation of MMP2 on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

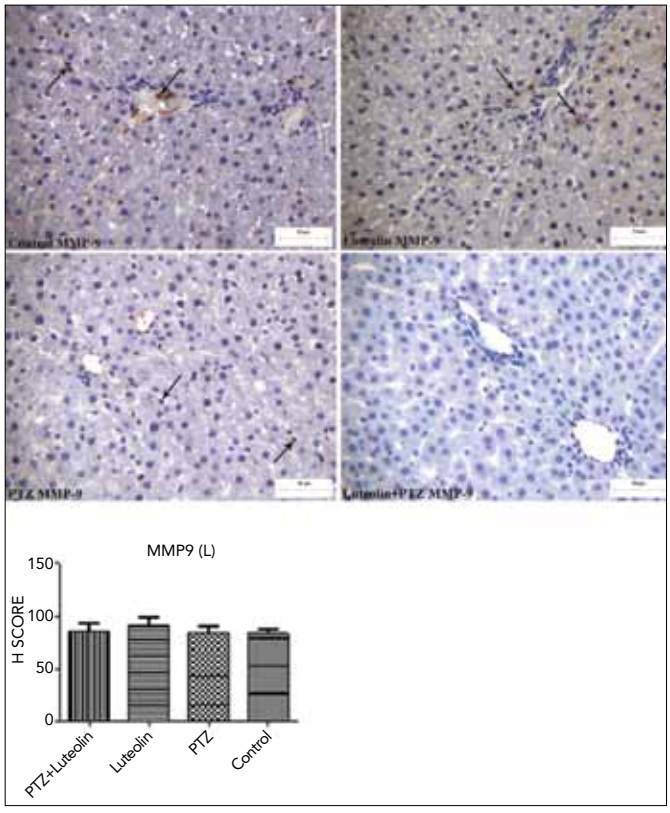

Figure 4. Immunohistochemical detection of MMP9 staining (arrows), in liver sections in control and experimental groups (Bar: 50 µm) and H-score values in liver (L) of all groups. Immunostaining intensity was assessed by semiquantation of MMP9 on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

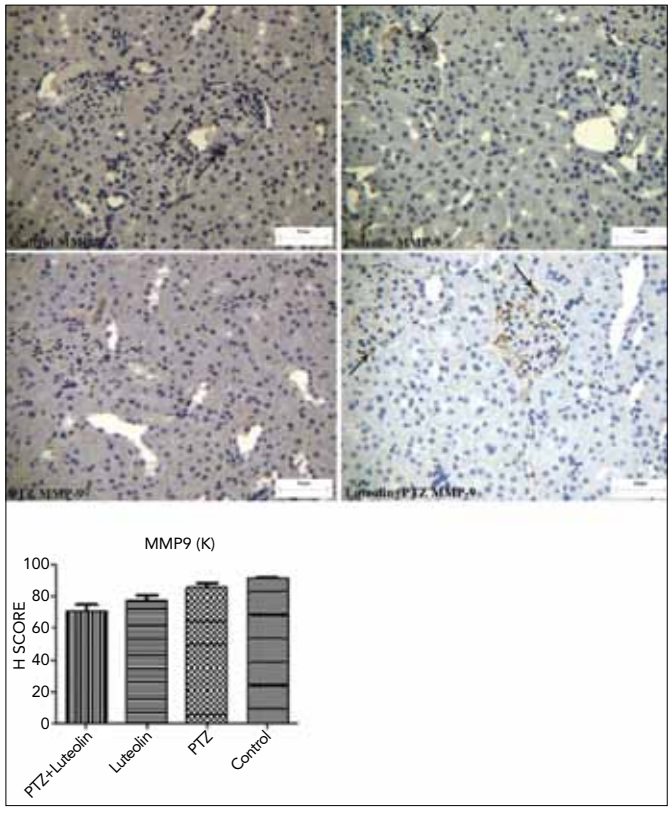

Figure 5. Immunohistochemical detection of MMP9 staining (arrows), in kidney sections in control and experimental groups (Bar: 50 µm) and H-score values in kidneys (K) of all groups. Immunostaining intensity was assessed by semiquantation of MMP9 on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

duced seizures were shown in Fig 1. In our control and LUT administered groups, there was no seizure activity. For this reason, these groups were not shown in the figures. Following intraperitoneal PTZ injection, generalized seizures started in the first minute with facial clonus (stage 1). Later, the forelimb muscle contraction added to neck and tail extensions (stage 2), wild running and usually with extended clonic activities has been observed (stage 3, 4) then we saw loss of straightening reflex with tonic flexion-extension (stage 5) and the seizures lasted intermittently in 60 minutes. Seizure stage, frequency and latency in the PTZ group were measured as 4.5±0.57 (Fig. 1A), 19.2±4.26 (Fig. 1B), and 79±15.16 sec (Fig. 1C) respectively. Seizure stage, frequency and latency in the PTZ+LUT group were measured as 4.6±0.54 (Fig.1A), 9.4±1.67 (Fig. 1B) and 78±16.43 sec (Fig. 1C) respectively. No effect of LUT was observed on seizure duration (not shown in figure). LUT pretreatment showed a significant attenuation in the seizure frequency ($p<0.05$) (Fig. 1 B).

Morphological findings

Pentylentetrazol administered rats showed sinusoidal enlargement, bleeding areas, many red blood cells in the liver and marked renal injury, including distal tubules and glomerular atrophy as compared with the control group. LUT+PTZ group showed an increase in connective tissue and slight glomerular injury and invagination in distal tubules as compared with the PTZ group. Animals receiving LUT+PTZ showed a reduced number of bleeding areas and fewer erythrocytes in the liver. Only LUT treated groups exhibited similar morphological features to the control group.

Qualitative and semiquantitative evaluation of MMP2, MMP9

Matrixmetalloproteinase 2 immunohistochemical reactions of liver (Fig. 2) and kidney (Fig. 3) tissues were markedly increased in the PTZ group, and this effect was found to be reduced in the LUT administered group. The MMP2 immunohistochemical reaction was observed in kidney glomeruli and distal tubules (Fig. 3). A strong MMP9 immunohistochemical reaction was seen in the central vein of the liver and connective tissue areas in PTZ administered rats (Fig. 4). The immunohistochemical reaction of MMP9 was weaker than of MMP2, which was detected in the blood vessels of the glomerulus and in the connective tissues (Fig. 5). These findings were supported by the H-score for semiquantitative evaluation. H-score results were shown together with all figures.

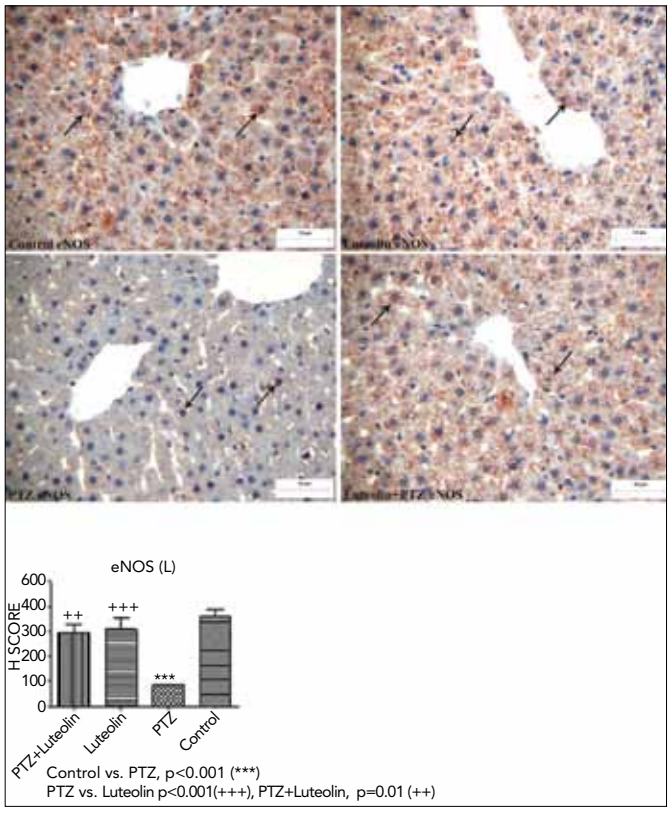

Figure 6. Immunohistochemical detection of eNOS staining (arrows) in liver sections in control and experimental groups (Bar: 50 µm) and H-score values in liver (L) of all groups. Immunostaining intensity was assessed by semiquantation of eNOS on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

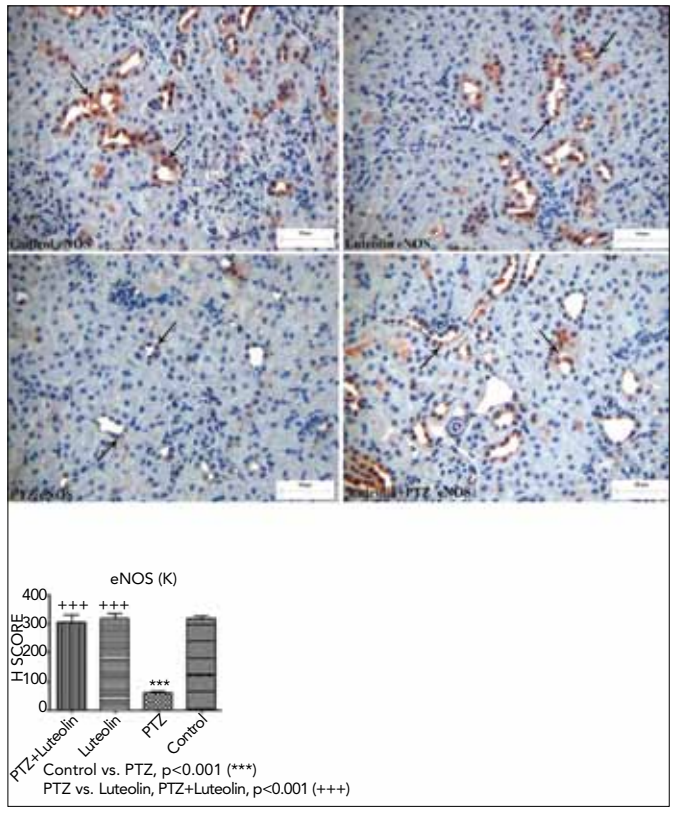

Figure 7. Immunohistochemical detection of eNOS staining (arrows) in kidney sections in control and experimental groups (Bar: 50 µm) and H-score values in kidneys (K) of all groups. Immunostaining intensity was assessed by semiquantation of eNOS on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

Endothelial Nitric Oxide and iNOS immunhistochemical reactions

Endothelial nitric oxide activity decreased dramatically in theliver (Fig. 6), kidney (Fig. 7) and hippocampus of rats with single dose PTZ administration (Fig. 8) while iNOS activity was markedly increased in the same tissues (Fig. 9-11) respectively. LUT pretreatment significantly increased eNOS activity in the liver, kidney and hippocampus as compared to PTZ administered rats. LUT also prevented the increase of iNOS activity in the same tissues (Fig. 9-11) respectively. The iNOS activity was higher in PTZ administered rats but the lowest amount of eNOS was detected. This result indicated that chronic LUT pretreatment might restore eNOS and iNOS activity in the hippocampus and peripheral tissues of PTZ administered rats. The results of these findings are supported by the H-score.

Discussion

Although there are many studies related to epilepsy, its effects on peripheral tissues have not yet been thoroughly investigated. A patient with epilepsy may suffer from renal or hepatic dysfunction that interferes with their antiepileptic drug treatment as well as their seizures. Recently there has been an increasing interest in the biochemical effects of medical plants with antioxidant properties, as they could be candidates for the prevention of oxidative damage. LUT and other natural flavonoids have recently been reported to have an antioxidative, anti-cancerogenic, antihypertensive, proinflammatory effect and neuroprotective activities (1, 2, 4, 6, 21). Therefore we investigated the protective effect of LUT on the brain, liver and kidney, which are damaged by PTZ as well as other effects on the seizure characteristics. In the present study, it was observed that the administration of LUT 10 mg/kg i.p. for two weeks decreased the seizure frequency. This result may be at least partly due to the antioxidant effect of LUT as described by some studies (19). Recently one study revealed that flavones exerted their neuroprotective effects via direct interaction with the apoptotic caspase pathway independent of their antioxidant activity (29).

Our findings have shown that LUT has no significant effect on seizure latency and seizure stage, while it significantly decreases seizure frequency. This result shows a protective effect of LUT on seizure frequency. Some studies suggested that flavonoid glycosides are easily metabolized by the organism and it could be possible that secondary metabolities may activate $GABA_A$ receptors to mediate a sedative effect (30). On the other hand,it has been reported that LUT metabolites might show a higher affinity for the benzodiazepine receptor and

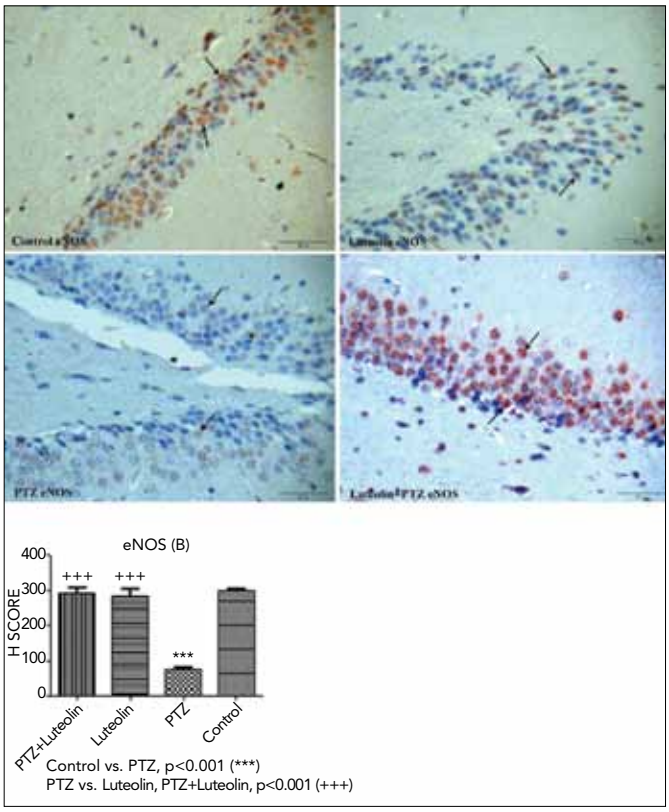

Figure 8. Immunohistochemical detection of eNOS staining (arrows) in hippocampus of brain in control and experimental groups (Bar: 50 µm) and H-score values in hippocampus (B) of all groups. Immunostaining intensity was assessed by semiquantation of eNOS on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±D (one way ANOVA)

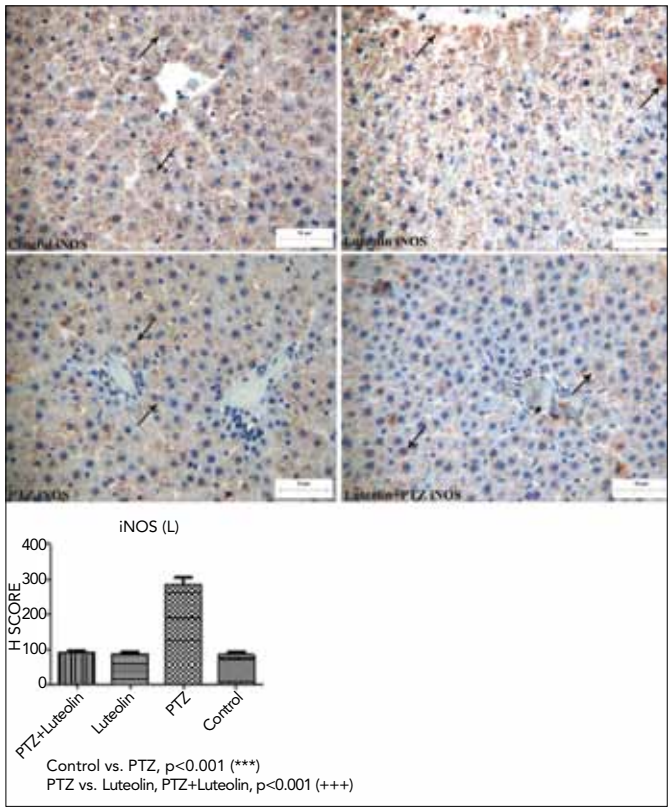

Figure 9. Immunohistochemical detection of iNOS staining (arrows) in liver sections in control and experimental groups (Bar: 50 µm) and H-score values in livers (L) of all groups. Immunostaining intensity was assessed by semiquantation of iNOS on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

anxiolytic like effects through a GABAergic mechanism (31). Hence, our finding that the reduction in seizure frequency by LUT is supported by these studies.

Our findings indicate that LUT treatment markedly decreased iNOS levels in PTZ induced seizures. This data might be explained by the antioxidant effect of LUT. In our study, the antioxidant effect of LUT has not been revealed directly, but the increase of eNOS in brain and other tissues and decrease of iNOS, shows that LUT has an indirect antioxidant effect. There are also many studies reporting that iNOS can create an oxidative stress (32, 33).

On the other hand, hippocampal damage is the most common pathology in epilepsy. High seizure frequency and duration are risk factors for hippocampal damage in epilepsy (34). We observed that the seizure frequency and iNOS activity in the hippocampus and peripheral tissues were significantly decreased in the LUT+PTZ group. Hence we suggest that there is a protective effect of LUT on hippocampal and peripheral tissue damage in PTZ-induced seizures.

In recent years, it has become increasingly evident that the drugs used for epilepsy may be associated with hepatotoxicity. In our study, the liver was affected more than the kidneys in the PTZ administered group. Recently, various types of glutamate receptors have been identified in liver, kidney, lung, heart and endocrine cells (35). In addition we suggest that the hepatotoxic effect caused by PTZ may be associated with glutamate receptors in the liver. PTZ-induced convulsions have been modulated by endogenous NO production and ionotropic glutamate receptor-mediated stimulation. Our findings show that the protective effect of LUT may be elicited by nitric oxide mediation. eNOS activity was significantly increased in the liver, kidney and hippocampus tissues of rats chronically treated with LUT and LUT+PTZ as compared to the PTZ group. We suggest that the protective effect of LUT against PTZ induced seizures in rats is possibly via eNOS activity. This finding is consistent with the result of other researchers who also reported that some flavonoids are potent inhibitors of NOS2 (iNOS) induction and, at the same time, they may increase endothelial NOS3 (eNOS) activity (36).

Matrixmetalloproteinases are also activated during epileptic seizures. The extensive data indicate that MMP9 is a molecule of great importance for neuronal physiology and pathology. Its activation appears to be intimately linked to glutamate acting as a potent neurotoxin (37). Recent studies indicate that MMP9 is an important participant in aberrant plasticity and neuroinflammation and neuronal death and it is upregulated in experimental epilepsy models (38). Despite many studies, the pathophysiology of seizures and specific

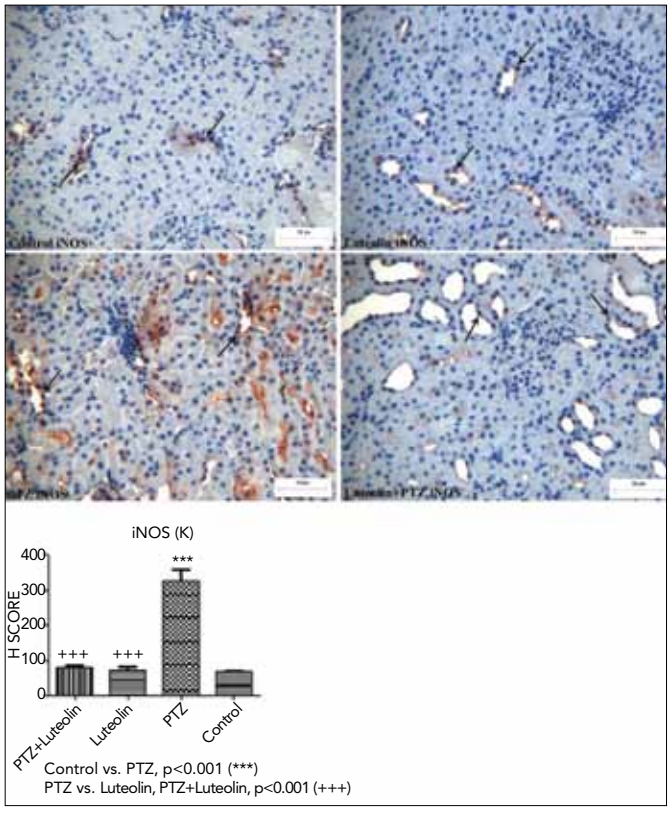

Figure 10. Immunohistochemical detection of iNOS staining (arrows) in kidney sections in control and experimental groups (Bar: 50 µm) and H-score values in kidneys (K) of all groups. Immunostaining intensity was assessed by semiquantation of iNOS on arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

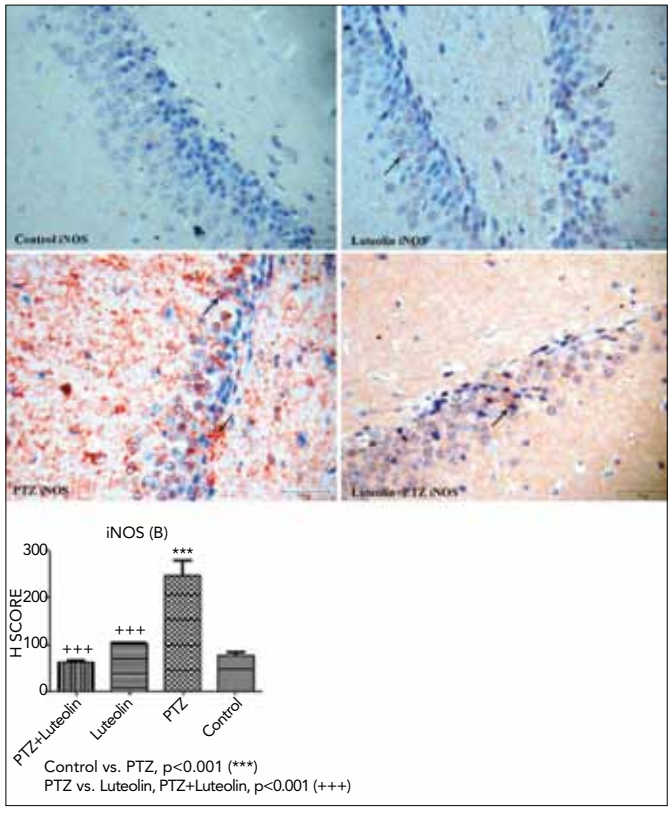

Figure 11. Immunohistochemical detection of iNOS staining (arrows) in hippocampus of brain in control and experimental groups (Bar: 50 µm) and H-score values in hippocampus (B) of all groups. Immunostaining intensity was assessed by semiquantation of iNOS on an arbitrary four-point scale (0=not detectable, 1=weak, 2=mild and 3=intense, 4=high intense). Data are reported as means±SD (one way ANOVA)

target of MMP9 in seizure related neuronal death are unclear. It is reported that MMP9 was related to synaptic plasticity. Recent studies demonstrated that MMP9 induction might exhibit functions like homeostatic synaptic plasticity rather than neuronal death (12). Moreover, MMP9 might be a promising target as a neuroprotective agent in preventing seizure induced hippocampal damage (39).

Interestingly, in contrast to other studies, no changes in MMP9 were found in tissues from different experimental groups in our study, but PTZ administration caused an increase in MMP2 activity. However, LUT treatment decreased MMP2 and iNOS activity in the hippocampus, liver and kidney tissues, while eNOS activity was dramatically increased in the same tissues. We suggest that MMP9 does not seem to be responsible for PTZ induced seizures and related peripheral tissue damage. In the present study, MMP2 immunohistochemical reaction markedly increased only in PTZ administered rats. This novel and interesting finding suggests that increase in MMP2 may be responsible for seizure frequency, possibly via aberrant synaptic plasticity. iNOS is induced in diseases associated with inflammation and oxidative stress. It is reported that reactive oxygen/nitrogen species regulate iNOS function (35). In our study, there was an increase in the iNOS activity in the hippocampus and peripheral tissues (indicator of the oxidative stress due to reactive oxygen radicals) and LUT reversed the increased iNOS activity, thus confirming the hypothesis that the protective effect of LUT is possible via an antioxidant effect. Moreover, pretreatment with LUT also reversed the PTZ induced increase in MMP2 activity. Our result is consistent with the result of other researchers who also observed inhibition of MMP2 and MMP9 by LUT (40).

Conclusion

Our results indicate that LUT not only decreases seizure frequency but also reverses the increase in MMP2 and iNOS, with no significant difference in MMP9. In addition, according to our results, interestingly MMP9 does not seem to be responsible for PTZ induced seizures. We suggest that the findings presented here underline the important roles of MMP2 and iNOS in seizure frequency and possible seizure induced tissue damage. Therefore, LUT could offer useful support to the basic drug treatment by preventing the tissue damage caused by PTZ.

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Conflict of Interest

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