

1 **Effects of Luteolin on liver, kidney and brain in PTZ-induced Seizures: Involvement of**  
2 **metalloproteinases and NOS activities**

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4 **Hüsniye Birman<sup>1</sup>, Kadriye Akgün-Dar<sup>2,\*</sup>, Ayşegül Kapucu<sup>2</sup>, Samet Acar<sup>2</sup> Gülay Üzüm<sup>1</sup>**

5 <sup>1</sup> Istanbul University, Istanbul Faculty of Medicine, Department of Physiology, 34390  
6 Istanbul, Turkey

7 <sup>2</sup> Istanbul University, Faculty of Science, Department of Biology, 34390 Istanbul, Turkey

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9 **Short title:** Effects of luteolin on MMPs and NOS activities

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12 **Corresponding author:** \*Assoc. Prof Dr. Kadriye Akgün-Dar,

13 Istanbul University, Faculty of Science, Department of Biology, 34390 Istanbul, Turkey

14 **Tlf:** +90 212 455 57 00 /15101

15 **Fax:**+90 212 528 05 27

16 **E-mail address:** [kakgun@istanbul.edu.tr](mailto:kakgun@istanbul.edu.tr)

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23

24 **Abstract**

25

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

31 **Material and Methods:** LUT (10 mg/kg) was given intraperitoneally during two weeks prior  
32 to seizure induction. Single dose PTZ 80 mg/kg i.p. administered and seizure were observed  
33 and evaluated regard to latency, frequency and stage for one hour.

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

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

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45 **Keywords:** Luteolin; Pentylentetrazol; Seizure; Metalloproteinases; NOS

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## 49 **Introduction**

50 Flavonoids are also common constituents of plants used in traditional medicine to treat a  
51 wide range of diseases. LUT and its glycosides are widely distributed in the plant kingdom.  
52 Flavonoids have many biological and pharmacological activities that may play a role in  
53 antioxidative properties (1) cancer prevention (2, 3) neuroprotection (4), antihypertensive  
54 effects (5). In vitro studies have been shown to reduce the expression of proinflammatory  
55 molecules (6). Epilepsy is a common neurological condition associated with some alterations.  
56 Patients with epilepsy may suffer from hepatic or renal dysfunctions that interfere with their  
57 antiepileptic drug treatment. (7). PTZ induced seizures are still the most widely used animal  
58 seizure model employed in the research for epilepsy and new antiepileptic drugs (8). It is  
59 reported that free radical generation plays a crucial role in neuronal cell death in the PTZ  
60 induced seizures in rats (9). Some studies suggested that proinflammatory molecules  
61 (e.g. proteolytic enzymes, reactive oxygen species or nitric oxide) may potentiate the damage  
62 to brain and peripheral tissues in epilepsy (10-12). Nitric oxide (NO) has been suggested to  
63 exert both anticonvulsant and proconvulsant effects. Recent studies demonstrated a strong  
64 correlation between the upregulation of MMP9 and epilepsy, and showed that kainate induced  
65 seizures result in elevated MMP9 expression (13). Flavonoids are compounds occurring  
66 naturally in food, which scavenge oxygen radicals and have anti-inflammatory properties.  
67 Recent investigations have reported that oral administration of LUT reduced clinical  
68 symptoms of experimental allergic encephalitis (14) and could protect the mice from the  
69 hepatotoxicity caused by carbon tetrachloride (15). MMPs are expressed as inactive  
70 zymogens in which the cysteine residue in the propeptide binds to  $Zn^{2+}$  present at the active  
71 site of the enzyme. MMPs which are locally inhibited by endogenous tissue inhibitors of  
72 metalloproteinases operate in extracellular matrix. These enzymes are critical for maintaining  
73 tissue homeostasis (16). It was observed that morin (a natural flavonoid) could lead to decreased

74 enzyme activities of MMP2 and MMP9 and was found to inhibit inflammation and tumor  
75 promotion (17). Co-administration of bioactive flavonoids in preoperative nutrition,  
76 attenuated ischemia-reperfusion injury and decreased apoptosis in the intestine (18). In  
77 another study, it has been reported that LUT treatment prevented ischemia reperfusion-  
78 induced renal injury and LUT exerted renoprotective effects probably by antioxidant activity  
79 (19). Studies have demonstrated that quercetin a natural flavonoid, reduced global ischemia-  
80 induced neuronal damage through inhibition of MMP9 activity (20). However, studies on  
81 MMPs inhibition of some flavonoids have not yet been analyzed in seizures.

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

88

## 89 **Methods**

### 90 *Animals*

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

95

### 96 *Experimental design*

97 Animals were divided into four groups each containing five rats; Group I; Control group  
98 (%0.09 NaCl administered). Group II; PTZ group (single dose of 80 mg/kg i.p. administered).  
99 Group III; LUT group (10 mg/kg i.p. LUT was given each day for two weeks). Group IV;  
100 LUT+PTZ group (rats were treated with 10 mg/kg i.p. LUT for two weeks and 80 mg/kg PTZ  
101 administered 30 minutes after the last LUT injection).

## 102 ***Drugs and Doses***

103 Luteolin (Istanbul University, Faculty of Pharmacy, Department of Pharmacognosy) was  
104 administered i.p. 10 mg/kg. The effective dose for flavonoids administered in experimentally  
105 studies were between 5mg/kg/day and to 10 mg/kg /day (21, 22). Thus, it is reasonable to use  
106 a dose of 10mg/kg LUT in this experiment. Also this dose is previously tested in animal  
107 studies (23) and researchers have also been reported that quercetin doesn't have treatment-  
108 related clinical signs of toxicity. PTZ dissolved in saline and seizures were induced with a  
109 single dose of 80mg/kg i.p. PTZ (SIGMA, USA). This dose was selected as it achieves the  
110 most successful convulsive response and with least mortality (8).

## 111 ***Pentylentetrazol- induced seizures***

112 The behavioral characteristics; stage, latency and frequency of seizures were observed for  
113 60 min in individual animals after PTZ injections. Convulsion stage: Stage was scored using  
114 the following scale (24, 25). Unresponsiveness=0, ear and facial twitching=1, myoclonic body  
115 jerks=2, clonic forelimb convulsions=3, generalized clonic convulsions, turn over into side  
116 position=4, generalized clonic-tonic convulsions=5. Seizure stage for each animal was  
117 calculated as mean of the phases. Convulsion Latency: Latency was measured as the time  
118 between injection of PTZ and appearance of the first clonic convulsion, which was indicated  
119 by a sudden twitching of head or jerky movement of body (26). Convulsion Frequency:  
120 Number of seizures during 60 min after PTZ injection, regardless of seizure stage.

121 ***Matrixmetalloproteinases and NOS Immunohistochemistry***

122 At the end of the experiment, animals were decapitated. Liver, kidney and brain tissues  
123 were removed, formalin fixed and after routine laboratory methods they were embedded in  
124 paraffin. Four-micrometer paraffin tissue sections were mounted on poly-L-lysine slides. The  
125 slides were air-dried and the tissue deparaffinized. Mounted specimens were washed in  
126 0.01mol/L phosphate-buffered saline (PBS). After three washes with PBS, an antigen retrieval  
127 solution (0.01 M citrate buffer, pH 6.0) was given for 10 at 100°C in a microwave oven,  
128 endogenous peroxidase was eliminated by incubation in 3% H<sub>2</sub>O<sub>2</sub> in pH 7.4 in phosphate-  
129 buffered saline (PBS; 0.01 M) for 10 minutes. After washing, the specimens were treated with  
130 a blocking serum (Labvision, TR-060-UB) at room temperature for 10 minutes. The sections  
131 were incubated with rabbit polyclonal anti-eNOS (Neo Markers, dilution 1: 100), rabbit  
132 polyclonal anti-iNOS (inducible nitric oxide synthase, Neo Markers, dilution 1: 100) and  
133 mouse monoclonal MMP2 (Santa Cruz, dilution 1: 100) and goat polyclonal MMP-9 (1: 100)  
134 was applied and reacted with tissue specimens at room temperature for one hour. The sections  
135 were washed three times with PBS, and incubated with biotinylated secondary antibody (Ultra  
136 Vision Detection System-HRP kit, Lab Vision, Fremont, USA) and then streptavidin  
137 peroxidase (Ultra Vision Detection System-HRP kit, Lab Vision, Fremont, USA) was given at  
138 room temperature for 30 minutes. Diaminobenzidine (DAB) was used as a chromogen, and  
139 the sections were counterstained with hematoxylin. The specificity of the  
140 immunohistochemical staining was tested using PBS in the same dilutions. Control tissue  
141 sections were used as positive control. The semiquantitative evaluation of the iNOS, eNOS,  
142 MMP-2 and MMP-9 immunohistochemical staining was done using H-score (27, 28). Briefly,  
143 the tissues stained with antibodies against eNOS, iNOS, MMP-2 or MMP-9 were evaluated  
144 using an Olympus microscope with a special ocular grid on 10 different fields at x400  
145 magnification by 2 blinder observers. Positive stained cells were counted and graded

146 according to the staining intensity: 0= no staining, 1= weak, 2= mild, 3= intense, 4= high  
147 intense. For each tissue, the H-score value was given by the following formula:  $H\text{-score} = \sum P_i (i + 1)$   
148 where “i” is the intensity score and “ $P_i$ ” is the corresponding percentage of cells  
149 presenting a given staining. Slides were examined by using Kameran 390CU Imaging  
150 system (Mikro Sistem) and photographed.

### 151 *Statistical analysis*

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

156

## 157 **Results**

### 158 *Evaluation of PTZ-induced seizures*

159 Pentylentetrazol induced generalized clonic-tonic seizures in all animals. Results of  
160 behavioral characteristics in PTZ-induced seizures were shown in Fig 1. In our control and  
161 LUT administered groups, there was no seizure activity. For this reason, these groups were  
162 not shown in the figures. Following intraperitoneal PTZ injection, generalized seizures started  
163 in the first minute with facial clonus (stage 1). After the forelimb muscles' contraction added  
164 with neck and tail extensions (stage 2), wild running and usually with extended clonic  
165 activities has been observed (stage 3, 4) then we see loss of straightening reflex with tonic  
166 flexion-extension (stage 5) and the seizures intermittently lasted in 60 minutes. Seizure stage,  
167 frequency and latency in PTZ group were measured as  $4.5 \pm 0.57$  (Fig.1A),  $19.2 \pm 4.26$   
168 (Fig.1B), and  $79 \pm 15.16$  sec (Fig. 1C) respectively. Seizure stage, frequency and latency in  
169 PTZ+LUT group were measured as  $4.6 \pm 0.54$  (Fig.1A),  $9.4 \pm 1.67$  (Fig.1B) and  $78 \pm 16.43$  sec  
170 (Fig.1C) respectively. No effect of LUT was observed on seizure duration (not shown in

171 figure). LUT pretreatment showed significant attenuation in the seizure frequency ( $p < 0.05$ )  
172 (Fig.1 B).

### 173 ***Morphological Findings***

174 Pentylentetrazol receiving rats showed sinusoidal enlargement, bleeding areas, many red  
175 blood cells in the liver and markedly renal injury was including distal tubules and glomerular  
176 atrophy as compared with the control group. LUT +PTZ group showed an increase in  
177 connective tissue and slightly glomerular injury and invagination in distal tubules as  
178 compared with the PTZ group. Animals receiving LUT+PTZ showed reduced number of  
179 bleeding areas and fewer erythrocytes in liver. Only LUT treated groups exhibited similar  
180 morphological features to the control group.

### 181 ***Qualitative and semiquantitative evaluation of MMP2, MMP9***

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

### 191 ***Endothelial nitric oxide and iNOS immunohistochemical reactions***

192 Endothelial nitric oxide activity decreased dramatically in liver (Fig.6), kidney (Fig. 7) and  
193 hippocampus of rats with single dose PTZ administration (Fig. 8) while iNOS activity was  
194 markedly increased in the same tissues (Fig. 9, 10, 11) respectively. LUT pretreatment  
195 significantly increased eNOS activity in the liver, kidney and hippocampus as compared to

196 PTZ administered rats. LUT also prevented the increase of iNOS activity in the same tissues  
197 (Fig.9, 10, 11) respectively. iNOS activity was higher in PTZ received rats but the lowest  
198 amount of eNOS was detected. This result indicated that chronic LUT pretreatment might  
199 restore eNOS and iNOS activity in hippocampus and peripheral tissues of PTZ administered  
200 rats. The results of these findings are supported by H-score.

201

## 202 **Discussion**

203 Although there are many studies related to epilepsy, its effects on peripheral tissues are not  
204 deeply investigated yet. Patient with epilepsy may suffer from renal or hepatic dysfunction  
205 that interfere with their antiepileptic drug treatment as well as from their seizures. Recently  
206 there has been an increasing interest in the biochemical effects of medical plants with  
207 antioxidant properties as they could be the candidates for the prevention of oxidative damage.  
208 LUT and other natural flavonoids have recently been reported to have an antioxidative, anti-  
209 cancerogen, antihypertensive, proinflammatory effect and neuroprotective activities (1, 2, 4,  
210 21, 6). Therefore we investigated the protective effect of LUT on brain, liver and kidney  
211 which are damaged by PTZ as well as the other effects on the seizure characteristics. In the  
212 present study, it was observed that the administration of LUT 10 mg/kg i.p. for two weeks  
213 decreased the seizure frequency. This result may be at least partly due to the antioxidant effect  
214 of LUT as described by some studies (19). Recently one study revealed that flavones exerted  
215 their neuroprotective effects via direct interaction with the apoptotic caspase pathway  
216 independent of their antioxidant activity (29).

217 Our findings have shown that, LUT has no significant effect on seizure latency and seizure  
218 stage while it significantly decreases seizure frequency. This result shows a protective effect  
219 of LUT on seizure frequency. Some studies suggested that flavonoid glycosides are easily

220 metabolized by the organism and it could be possible that secondary metabolites may activate  
221 GABA<sub>A</sub> receptors to mediate sedative effect (30). On the other hand, LUT metabolites might  
222 show a higher affinity for the benzodiazepine receptor and anxiolytic like effects through a  
223 GABAergic mechanism has been reported (31). Hence, our finding that the reducing in  
224 seizure frequency by LUT supported by these studies.

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

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

235 In recent years, it has become an increasing evident that the drugs used for epilepsy may be  
236 associated with hepatotoxicity. In our study, the liver was affected more than the kidneys in  
237 the PTZ administered group. Recently, the various types of glutamate receptors have been  
238 identified in liver, kidney, lung, heart and endocrine cells (35). In addition we suggest that the  
239 hepatotoxic effect caused by PTZ may be associated with glutamate receptors in liver. PTZ-  
240 induced convulsions have been modulated by endogenous NO production and ionotropic  
241 glutamate receptor-mediated stimulation. Our findings show that, the protective effect of LUT  
242 may elicit to nitric oxide mediation. eNOS activity was significantly increased in the liver,  
243 kidney and hippocampus tissues of rats chronically treated with LUT and LUT+PTZ as  
244 compared to PTZ group. We suggest that the protective effect of LUT against PTZ induced

245 seizures in rats is possibly via eNOS activity. This finding is congruent with the result of other  
246 researchers who also reported that some of flavonoids are potent inhibitors of NOS2 (iNOS)  
247 induction at the same time they may increase endothelial NOS3 (eNOS) activity (36).

248 Matrixmetalloproteinases are also activated during epileptic seizures. The extensive data  
249 indicate that MMP9 is a molecule of great importance for neuronal physiology and pathology.  
250 Its activation appears to be intimately linked to glutamate acting as a potent neurotoxin (37).  
251 Recent studies indicate that MMP9 is an important participant in aberrant plasticity and  
252 neuroinflammation and neuronal death and it is upregulated in experimental epilepsy models  
253 (38). Despite many studies about the pathophysiology of seizures and specific target of  
254 MMP9 in seizure related neuronal death are unclear. It is reported that, MMP9 was related to  
255 synaptic plasticity. Recent studies demonstrated that MMP9 induction might exhibit functions  
256 like homeostatic synaptic plasticity rather than neuronal death (12). Moreover, MMP9 might  
257 be a promising target as a neuroprotective agent to prevent seizure induced hippocampal  
258 damage (39).

259 Interestingly, in contrast to other studies there are no changes in MMP9 were found in  
260 tissues from different experimental groups in our study but PTZ administration caused an  
261 increase in MMP2 activity. However, LUT treatment decreased MMP2 and iNOS activity in  
262 hippocampus, liver and kidney tissues while eNOS activity was dramatically increased in the  
263 same tissues. We suggest that MMP9 doesn't seem to be responsible for PTZ induced seizures  
264 and related peripheral tissue damage. In the present study, MMP2 immunohistochemical  
265 reaction markedly increased only in PTZ administered rats. This novel and interesting finding  
266 suggests that increase in MMP2 may be responsible for seizure frequency possibly via  
267 aberrant synaptic plasticity. iNOS is induced in diseases associated with inflammation and  
268 oxidative stress. It is reported that reactive oxygen/nitrogen species regulate iNOS function  
269 (35). In our study there was an increase in the iNOS activity in the hippocampus and

270 peripheral tissues (indicator of the oxidative stress due to reactive oxygen radicals) and LUT  
271 reversed the increased iNOS activity, thus confirming the hypothesis that, the protective effect  
272 of LUT is possible via antioxidant effect. Moreover, pretreatment with LUT also reversed the  
273 PTZ induced increase in MMP2 activity. Our result congruent with the result of other  
274 researchers who also observed inhibition of MMP2 and MMP9 by LUT (40).

275

## 276 **Conclusions**

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

283

## 284 ***Conflict of interest Disclosure***

285 None

286

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290

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415 **Figure legends**

416

417 **Fig. 1.** The effect of luteolin treatment on the development of PTZ induced seizure stage (A),  
418 seizure frequency (B) and seizure latency (C).

419

420 **Fig. 2.** Immunohistochemical detection of MMP2 staining (arrows), in liver sections in  
421 control and experimental groups (Bar: 50  $\mu$ m) and semiquantitative evaluation (H-score) in  
422 liver (L) of all groups. Immunostaining intensity was assessed by semiquantitation of MMP2  
423 on arbitrary four-point scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high  
424 intense). Data are reported as means  $\pm$  SD (one way ANOVA).

425

426 **Fig. 3.** Immunohistochemical detection of MMP2 staining (arrows), in kidney sections in  
427 control and experimental groups (Bar: 50  $\mu$ m) and semiquantitative evaluation (H-score) in  
428 kidney (K) of all groups. Immunostaining intensity was assessed by semiquantitation of MMP2  
429 on arbitrary four-point scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high  
430 intense). Data are reported as means  $\pm$  SD (one way ANOVA).

431

432 **Fig. 4.** Immunohistochemical detection of MMP9 staining (arrows), in liver sections in  
433 control and experimental groups (Bar: 50  $\mu$ m) and H-score values in liver (L) of all groups.  
434 Immunostaining intensity was assessed by semiquantitation of MMP9 on arbitrary four-point  
435 scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense). Data are reported  
436 as means  $\pm$  SD (one way ANOVA).

437

438 **Fig. 5.** Immunohistochemical detection of MMP9 staining (arrows), in kidney sections in  
439 control and experimental groups (Bar: 50  $\mu$ m) and H-score values in kidney (K) of all groups.

440 Immunostaining intensity was assessed by semiquantation of MMP9 on arbitrary four-point  
441 scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense).Data are reported  
442 as means  $\pm$  SD (one way ANOVA).

443

444 **Fig. 6.** Immunohistochemical detection of eNOS staining (arrows) in liver sections in control  
445 and experimental groups (Bar: 50  $\mu$ m) and H-score values in liver (L) of all groups.  
446 Immunostaining intensity was assessed by semiquantation of eNOS on arbitrary four-point  
447 scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense). Data are reported  
448 as means  $\pm$  SD (one way ANOVA).

449

450 **Fig. 7.** Immunohistochemical detection of eNOS staining (arrows) in kidney sections in  
451 control and experimental groups (Bar: 50  $\mu$ m) and H-score values in kidney (K) of all groups.  
452 Immunostaining intensity was assessed by semiquantation of eNOS on arbitrary four-point  
453 scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense). Data are reported  
454 as means  $\pm$  SD (one way ANOVA).

455

456 **Fig. 8.** Immunohistochemical detection of eNOS staining (arrows) in hippocampus of brain in  
457 control and experimental groups (Bar: 50  $\mu$ m) and H-score values in hippocampus (B) of all  
458 groups. Immunostaining intensity was assessed by semiquantation of eNOS on arbitrary four-  
459 point scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense). Data are  
460 reported as means  $\pm$  SD (one way ANOVA).

461

462 **Fig. 9.** Immunohistochemical detection of iNOS staining (arrows) in liver sections in control  
463 and experimental groups (Bar: 50  $\mu$ m) and H-score values in liver (L) of all groups.  
464 Immunostaining intensity was assessed by semiquantation of iNOS on arbitrary four-point

465 scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense).Data are reported  
466 as means  $\pm$  SD(one way ANOVA).

467

468 **Fig. 10.** Immunohistochemical detection of iNOS staining (arrows) in kidney sections in  
469 control and experimental groups (Bar: 50  $\mu$ m) and H-score values in kidney (K) of all groups.  
470 Immunostaining intensity was assessed by semiquantation of iNOS on arbitrary four-point  
471 scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense).Data are reported  
472 as means  $\pm$  SD (one way ANOVA).

473

474 **Fig. 11.** Immunohistochemical detection of iNOS staining (arrows) in hippocampus of brain  
475 in control and experimental groups (Bar: 50  $\mu$ m) and H-score values in hippocampus (B) of  
476 all groups. Immunostaining intensity was assessed by semiquantation of iNOS on arbitrary  
477 four-point scale (0= not detectable, 1= weak, 2= mild and 3= intense, 4=high intense). Data  
478 are reported as means  $\pm$  SD (one way ANOVA).

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