The effect of piracetam on brain damage and serum nitric oxide levels in dogs submitted to hemorrhagic shock

Hemorajik şok oluşturulan köpeklerde beyin hasarı ve serum nitrik oksit seviyelerine pirasetamın etkisi

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BACKGROUND
To demonstrate the effect of piracetam on changes in brain tissue and serum nitric oxide levels in dogs submitted to hemorrhagic shock.

METHODS
The subjects were randomized into four subgroups each consisting of 10 dogs. Hemorrhagic shock was induced in Group I for 1 hour and no treatment was given to this group. Blood and saline solutions were administered to Group II following 1 hour hemorrhagic shock. Blood and piracetam were given to Group III following 1 hour shock. No shock was induced and no treatment was applied to Group IV. Blood samples were obtained at the onset of the experiment and at 60, 120 and 180 minutes for nitric oxide analysis. For histopathological examination, brain tissue samples were obtained at the end of the experiment.

RESULTS
The observed improvement in blood pressure and pulse rates in Group III was more than in Group II. Nitric oxide levels were increased in Group I; however, no correlation between piracetam and nitric oxide levels was determined. It was seen that recovery in brain damage in Group III was greater than in the control group.

CONCLUSION
Piracetam, added to the treatment, may decrease ischemic damage in hemorrhagic shock.

Key Words: Brain damage; dogs; experimental model; hemorrhagic; nitric oxide; piracetam; shock; treatment.

AMAÇ
Hemorajik şok oluşturulan köpeklerde, pirasetamın şoka bağlı nitrik oksit düzeylerinde değişikliğe ve beyin dokusunda oluşan hipoksiye etkisinin olup olmadığı araştırıldı.

GEREÇ VE YÖNTEM
Denekler randomize olarak 10'ar köpekten oluşan dört gruba ayrıldı. Grup I, bir saatlik hemorajik şoka uğratıldı ve hemorajik şok sonrası tedavi verilmedi. Grup II, bir saatlik hemorajik şoka uğratıldı ve şok sonrası kan ile birlikte serum fizyolojik verildi. Grup III, bir saat hemorajik şoka uğratıldı ve şok sonrası kan ile birlikte intravenöz pirasetam verildi. Grup IV'e ise şok ve tedavi uygulanmadı. Deneyin başlangıcında, 60., 120. ve 180. dakikalarda nitrik oksit analizi için kan örnekleri alındı. Deneyin sonunda, histopatolojik inceleme için beyin doku örnekleri çıkarıldı.

BULGULAR
Kan basıncı ve nabız değerlerindeki düzelmenin Grup III'deki deneklerde Grup II'deki deneklere göre daha iyi olduğu saptandı. Grup I'de nitrik oksit seviyelerinde yükselme olmasına rağmen, pirasetamla nitrik oksit seviyeleri arasında herhangi bir ilişki saptanmadı. Deneyin sonunda, Grup III'de hemorajik şoka bağlı gelişen beyin hasarındaki düzelmenin kontrol grubuna göre daha iyi olduğu saptandı.

SONUÇ
Tedaviye eklenen pirasetam hemorajik şokta iskemik hasarı azaltabilir.

Anahtar Sözcükler: Beyin hasarı; köpek; deneysel model; hemorajik; nitrik oksit; pirasetam; şok; tedavi.

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Decrease in blood flow to the brain in hemorrhagic shock, depending on the type and duration of ischemic event, can cause metabolic and cellular changes. The most common changes are brain edema, apoptosis, necrosis and disturbance of blood-brain barrier.[1]

Shock is a physiopathological condition in which circulation is inadequate to maintain tissue perfusion and is unable to meet the oxygen demand.[2] Increased production of free oxygen radicals due to ischemia in hemorrhagic shock and nitric oxide (NO) play an important role in the pathophysiology of hemorrhagic shock. Serum NO level decreases and therefore cellular damage occurs.[3,4]

Piracetam is the first clinically used nootropic agent. Its cytoprotective, antihypoxic, antioxidant and microcirculation protective effects were proven in some studies.[5] With both central and peripheral effects, it decreases thrombocyte aggregation and morphologic erythrocyte anomalies, and improves peripheric microcirculation.[6]

Blood and fluid resuscitation is performed in the treatment of hemorrhagic shock in emergency departments. Blood is the best resuscitative fluid in the treatment of hemorrhagic shock.[7]

We aimed to investigate whether administration of piracetam, with its proven cytoprotective and antioxidant properties, as an add-on therapy to early blood replacement in hemorrhagic shock is effective on brain tissue damage and serum NO levels.

MATERIALS AND METHODS

This experimental study was performed in Erciyes University Faculty of Medicine, Hakan Cetinsaya Experimental and Clinic Research Center (DEKAM), Emergency Department, Biochemistry and Pathology Laboratory, with the permission of the Ethics Board, and it was supported by Erciyes University Research Fund (Project no: TT-03-17). Forty male Mongrel dogs weighing 17 to 32 kg were used in the study. Before the experiment, dogs were fasted for 12 hours. General anesthesia was applied to all groups. Anesthesia was maintained with intravenous (I.V.) 5 mg/kg ketamine (Ketalar®, Pfizer) + 1 mg/kg xylazine hydrochloride (Rompun®, Bayer) by catheter placed in the dogs' left front leg vein. The subjects were managed to spontaneous respiration without intubation. Surgical sterilization procedures were followed during the experiment. Two polyethylene catheters with diameters of 3 mm were placed in both vessel lumens after determining femoral arteries and veins of the subjects. A three-way shunt was placed at the tip of the catheter. One tip of the triple shunt placed in the artery was connected to the monitor for continuous measurement of arterial pressure via pressure transducer during the experiment. The other routes were used to maintain hemorrhage in dogs and collect blood samples for NO analysis. Blood, saline and piracetam were administered through the catheter placed into the vein.

According to modified Wiggers technique, via catheter placed in artery, bleeding was maintained by 50 cc/min hemorrhage rate until 40 mmHg mean arterial pressure was achieved in subjects exposed to shock. During exposure to shock, in order to maintain 40 mmHg mean arterial pressure, re-hemorrhage or redistribution of withdrawn blood was applied to subjects. Following hemorrhage, blood withdrawn from subjects was preserved in phlebotomy bags at room temperature until redistribution.

The subjects were randomized into 4 subgroups consisting of 10 dogs each:

Shock group (Group I, n=10)

The subjects were exposed to shock for 1 hour and no treatment was given after shock.

Control group (Group II, n=10)

Subjects were perfused with their withdrawn blood and placebo 4 cc/kg saline I.V. following 1-hour shock.

Piracetam treatment group (Group III, n=10)

Subjects were perfused with their withdrawn blood and 4 cc/kg (800 mg/kg) piracetam (Nootropil®, UCB) I.V. bolus infusion following 1-hour shock.

Sham group (Group IV, n=10)

No shock or treatment was applied to the subjects. However, other attempts were performed.

For NO analysis, 10 cc blood samples were obtained from the subjects at the outset of the experiment and at 60, 120 and 180 minutes.

Table 1. Histopathologic evaluation of the brain

Grade	Findings
0	No injury
1	Mild injury, scarce necrotic cells (<10%)
2	Moderate injury, moderate necrotic cells (10-50%)
3	Severe injury, many necrotic cells (>50%)

For histopathological analysis, at the end of the 3rd hour, brain tissues were obtained from all subjects by incision of cranium from temporal area.

All subjects were sacrificed by bleeding at the end of the experiment.

Lactate analysis

Heparinized blood samples were taken from the subjects at the beginning of the experiment, and at 60, 120 and 180 minutes. These blood samples were analyzed by Rapid Lab 865 auto-analyzer device in the emergency biochemistry laboratory.

Nitrite and nitrate analysis

Serum was isolated from blood samples taken from dogs for analysis of nitrite and nitrate, and stored at -20 °C until the study day. As products of the reaction between NO and oxygen, nitrite and nitrate levels were determined for permanent assessment of serum NO level. Nitrate/Nitrite Calorimetric Measurement Kit (Calbiochem®) was used for measurement.

Histopathological analysis

Brain tissue obtained for histopathological analysis was fixed in 10% formalin. All tissues were embedded in paraffin following routine tissue procedures, and paraffin sections of 5-8 microns were prepared.

Preparations stained with hematoxylin-eosin were observed under light microscope. Histopathological grading recommendations by Warner et al.[8] were used to evaluate the histopathological changes (Table 1).

Statistical analysis

All data were analyzed using the SPSS software package. One-way ANOVA test was used to compare blood pressure, pulse, and serum nitrite and nitrate levels among groups. Scheffe procedure was preferred in post hoc evaluation. Kruskal-Wallis variant analysis was used to compare the histopathological results among groups. Mann-Whitney U rank test was performed in the determination of differences between groups. Values of $p<0.05$ were considered as significant. Values were expressed as mean ± SD.

RESULTS

Blood pressure values

Median blood pressure was determined as 40 mmHg in the 15th minute of the experiment in all groups exposed to hemorrhagic shock. To achieve this blood pressure, test subjects were bled 852.2±119.6 cc. There was no difference between the groups with respect to blood loss. Blood pressure was maintained at this value during shock. Blood pressure values were increased in Groups II and III in which hemorrhagic shock was treated. At the end of the experiment, whereas a statistically significant difference was observed between Groups II and IV ($p<0.05$), no differences were determined between Groups III and IV ($p>0.05$) (Fig. 1).

Pulse values

It was determined that pulse started to increase at the 15th minute in all groups subjected to hemorrhagic shock and was decreased in the treated groups. No statistically significant difference was observed between Groups II and III at the end of the experiment ($p>0.05$). No significant difference was found between Groups III and IV ($p>0.05$), whereas a statistically significant difference was determined between Groups II and IV at the end of the experiment ($p<0.05$) (Fig. 2).

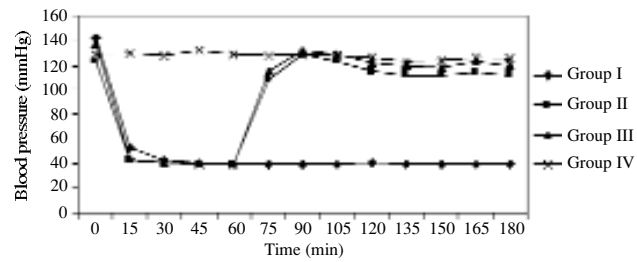

Fig. 1. Blood pressure values among groups. *(Median blood pressure value was determined as 40 mmHg in the 15th minute of the experiment in all groups exposed to hemorrhagic shock. Blood pressure was maintained at this value during shock. In treated groups, blood pressure increased to the normal ranges).*

Lactate values

Serum lactate levels were increased in hemorrhagic shock-applied groups and then decreased in the treatment groups. There was no statistically significant difference between Groups II and III (p>0.05). The difference between Groups II and IV was statistically significant (p<0.05), whereas it was not significant between Groups III and IV (p>0.05) (Table 2).

Nitrite and nitrate values

A significant difference with respect to nitrite and nitrate values was determined among groups at the 60th minute of the experiment. The differences between Groups I and III and between Groups II and IV were statistically significant (p<0.05). However, the differences between Groups II and III and between Groups I and IV were not statistically significant (p>0.05) (Table 3).

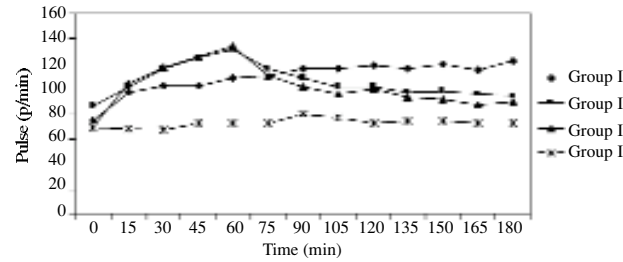

Fig. 2. Pulse rates of groups. *(It was determined that pulse started to increase at the 15th minute in all groups subjected to hemorrhagic shock and was decreased in treated groups. In treated groups, pulse rate decreased to the normal ranges).*

Statistically significant differences between Group I and Groups II, III and IV were determined at the 120th minute and at the end of the experiment (p<0.05). No significant differences between Groups II, III and IV were found (p>0.05).

Table 2. Lactate values among groups

Time (Min)	Group I X±SD (mEq/L)	Group II X±SD (mEq/L)	Group III X±SD (mEq/L)	Group IV X±SD (mEq/L)	f	p
0	1.9±0.6	2.7±1.3	2.9±1.0	1.9±0.5	3.3	>0.05
60	7.8±2.3[d]	7.8±1.5[d]	8.7±1.4[d]	2.3±1.1[abc]	31.2	<0.01
120	8.9±1.7[bcd]	5.2±1.7[ad]	4.3±0.9[ad]	1.7±0.4[abc]	49.8	<0.01
180	10.2±1.8[bcd]	3.9±1.2[ad]	2.6±0.5[a]	1.8±0.7[ab]	106.1	<0.01

*Values of p<0.05 were considered as significant; a: Demonstrates the difference compared to Group I;
b: Demonstrates the difference compared to Group II; c: Demonstrates the difference compared to Group III;
d: Demonstrates the difference compared to Group IV.

Table 3. Nitrite and nitrate values among groups

Time (Min)	Group I X±SD (mEq/L)	Group II X±SD (mEq/L)	Group III X±SD (mEq/L)	Group IV X±SD (mEq/L)	f	p
0	9.3±0.5	9.3±0.6	9.2±0.7	9.3±0.6	0.1	>0.05
60	9.4±0.9[c]	10.6±0.9	10.7±1.2[ad]	8.9±1.3[c]	6.2	<0.05
120	11.1±1.6[bcd]	9.6±0.5[a]	8.9±1.2[a]	9.1±0.5[a]	7.9	<0.01
180	14.4±1.2[bcd]	9.3±0.5[a]	8.4±0.4[a]	9.1±0.8[a]	129.9	<0.01

*Values of p<0.05 were considered as significant; a: Demonstrates the difference compared to Group I;
b: Demonstrates the difference compared to Group II; c: Demonstrates the difference compared to Group III;
d: Demonstrates the difference compared to Group IV.

Table 4. Histopathological evaluation of groups

	Group I Median (min-max)	Group II Median (min-max)	Group III Median (min-max)	Group IV Median (min-max)	χ^2	p
Brain	1 (0-2)[cd]	0 (0-2)[d]	0 (0-1)[a]	0 (0-0)[ab]	13.9	<0.05

*Values of p<0.05 were considered as significant; a: Demonstrates the difference compared to Group I;
b: Demonstrates the difference compared to Group II; c: Demonstrates the difference compared to Group III;
d : Demonstrates the difference compared to Group IV.

Table 5. Histopathological changes in brain tissue

	Grade 0	Grade I	Grade II	Grade III
Group I	3	4	3	0
Group II	6	2	2	0
Group III	9	1	0	0
Group IV	10	0	0	0

Histopathological evaluation

Difference in brain tissue between Groups II and III was found statistically insignificant ($p>0.05$). No difference was found between Groups III and IV ($p>0.05$), but there was a statistically significant difference ($p<0.05$) between Groups II and IV (Tables 4, 5, Figs. 3, 4).

DISCUSSION

Acute hemorrhage initiates specific cardiovascular, hormonal, and metabolic responses. Cardiac output is decreased in hemorrhagic shock and an increase in peripheral vascular resistance occurs to compensate for this decrease. Blood pressure has to be increased as long as this increase is maintained; however, it decreases because a sufficient compensation mechanism cannot be achieved in the following stages.[9,10]

It was shown with many experimental studies that blood pressure decreased and pulse rate increased during hemorrhagic shock and then returned to normal with treatment.[11-13] In all groups in which shock was applied, we determined that the mean arterial blood pressures were decreased to 45 mmHg at the 15th minute of the experiment and pulse was increased. We also observed that blood pressure was gradually increased and pulse was decreased in treated groups II and III. In spite of the statistically significant difference obtained between placebo and sham groups at the end of the experiment, these values were in normal ranges for dogs. No difference was observed between the piracetam-treated group and sham group.

Some prognostic parameters show whether or not treatment of shock is appropriate. It was shown that the amount of oxygen shortage is a value to determine the irreversible shock period.[14] Lactate has been used for a long time to evaluate the severity of shock.[15] In many studies, it was reported that blood lactate level increased in hemorrhagic shock, and then decreased in treated groups; however, it was detected in some of these studies that it did not decrease to pre-treatment level.[11,13,16]

In this study, at the end of the experiment, we determined a statistically significant difference between the group treated with blood and normal saline and the sham group ($p<0.05$), but there was no statistically significant difference between the sham group and group treated with blood and piracetam ($p>0.05$). We observed that improvement in lactate level, which is an indicator of tissue perfusion, was better with the addition of piracetam to shock treatment.

Irreversible hemorrhagic shock is characterized by progressive vasodilatation related to loss of vas-

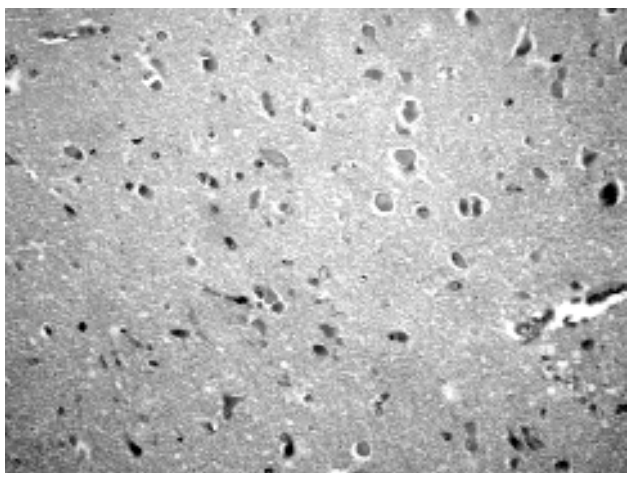

Fig. 3. Necrotic cells in brain tissue of a dog in Group II (H-E x 400).

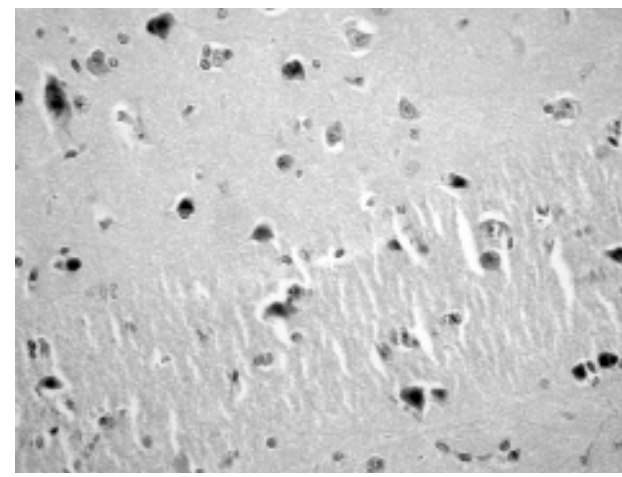

Fig. 4. Healing in the brain tissue of a dog after treatment in Group III (H-E x 400).

cular reactivity in spite of sympathetic nervous system activity. Insufficient vascular response to vasoconstrictor agents is found to be related to increased NO production and release. It is known that NO is derived from L arginine through the mediation of nitric oxide synthesis (NOS) in vascular endothelial cells and causes permanent active vasodilatation.[3,17]

In two different hemorrhagic shock models performed with rats, NO levels were increased in irreversible and untreated hemorrhagic shock.[17,18] Daughters et al.[19] established an increase in blood pressure by using NOS inhibitors in experimental hemorrhagic shock. They reported that NO is increased in hemorrhagic shock and contributes to hypotension.

Our results were similar to results of these studies. There was no difference between groups according to NO levels. In the untreated group in which hemorrhagic shock was induced, we determined no significant alteration at the 60th minute; however, at and after the 120th minute, a significant increase in NO values was observed. We observed that the NO values were equal to their baseline in the treated groups.

Piracetam's cytoprotective, antihypoxic, and microcirculation regulatory effects and its ability to inhibit lipid peroxidation were demonstrated by several studies.[5] In an experimental study with rats performed by Stockmans et al.,[6] it was demonstrated that piracetam enhanced both central and peripheral microcirculation by reducing thrombocyte aggregation and erythrocyte deformability.

Because of these effects of piracetam, we proposed in our study to demonstrate whether it has an effect on NO in hemorrhagic shock. However, we could not determine a correlation between piracetam and NO. The reason for this may be that NO level increases in advanced stages of shock and we treated the hemorrhagic shock state before the irreversible stage.

According to some studies, brain edema, apoptosis, necrosis and disturbance of blood-brain barrier due to ischemia were seen after hemorrhagic shock.[1] Normal cerebral blood circulation can be maintained by cerebrovascular autoregulation until a hemorrhage-defeated organism's compensatory mechanisms. Decrease in blood circulation in intracranial structures was demonstrated after withdrawal of 30% of the total blood volume of anesthetized rats. Loss of 35% of total blood volume may lead to intermediate ischemia and loss of 50% of total blood volume leads to severe cerebral ischemia.[20] We also detected necrotic cells due to ischemia in the brain tissues of subjects exposed to hemorrhagic shock.

In order to demonstrate piracetam's cytoprotective and apoptosis-preventing effect, Gabryel et al.[21] administrated piracetam to astrocyte cell cultures *in vitro* following hypoxia. As a result, it was shown that piracetam significantly reduced the amount of apoptosed cells.

In their experimental model, Grassler et al.[20] looked for antihypoxic effects of piracetam in rats exposed to hypoxia by creating shock. They showed that piracetam prevented the changes after hemorrhage in intermediate shock.

In an experimental model performed with rats, it was found that 800 mg/kg intraperitoneally administered piracetam had neuroprotective effects against hypoxia. When the rabbits were given 500 mg/kg of piracetam I.V., the antihypoxic effect was found to be more significant than placebo.[22] We administered high-dose piracetam (800 mg/kg) I.V. to the subjects for its antihypoxic, antioxidant, cytoprotective and microcirculation protective effects.

We also demonstrated in our study that necrotic cells were generated in brain tissue with intermediate frequency after hemorrhagic shock and that this damage can be decreased by blood replacement. We also found that the brain damage occurring in the piracetam-treatment group was less than in the control group, though statistically insignificant. A statistically significant difference was observed between the control and sham groups, whereas no statistical difference was determined between the piracetam- treatment group and sham group.

In conclusion, the main treatment in hemorrhagic shock is to stop bleeding and then replace blood. However, when added to the blood replacement, piracetam treatment may decrease the brain damage. Piracetam has no positive or negative influence on serum NO levels in hemorrhagic shock.

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