Aim: Since ethyl pyruvate (EP) is a known neuroprotective agent, this study was intended to investigate the effects of EP on pyramidal cell numbers and morphology in the hippocampus cornu ammonis after brain ischemia.

Materials and methods: Female rats were divided into 5 groups: control, ischemia, ischemia + EP, incision + EP, and incision + Ringer’s lactate (groups I, II, III, IV, and V, respectively). No intervention was performed in group I. Ischemia was established in groups II and III, and neck incisions were performed in groups IV and V. EP was intraperitoneally injected in groups III and IV. Ringer’s lactate was also administered to group V. Brains were evaluated stereologically and histopathologically.

Results: The total number of pyramidal cells in group II was significantly lower than those in groups I, IV, and V. The total number of pyramidal cells in group III was significantly lower than those in groups I and IV. The total number of pyramidal cells in group III was significantly lower than those in groups I and IV.

Conclusion: Ischemia reduces the number of pyramidal cells in the cornu ammonis, and EP has no effect on the number of pyramidal cells in the 16-week-old female rat cornu ammonis after brain ischemia.

Key words: Ethyl pyruvate, hippocampus, ischemia, stereology, rat

1. Introduction
Ischemia develops with reduced or total cessation of blood flow to tissue as a result of the obstruction or tearing and hemorrhage of a blood vessel. Every tissue has a different sensitivity to ischemia. Because the central nervous system (CNS) is metabolically active, it has considerable sensitivity to ischemia (1). Significant pathologies involving vital functions in the region in which it develops therefore appear in ischemic brain injury. Studies employing experimental ischemic models have to a large extent revealed how ischemic injury arises at the cellular level (2,3). However, it has not yet been fully established how such injury can be avoided.

Because of the sensitivity of the hippocampus to ischemia, and the fact that it is one of the regions in which postnatal neurogenesis takes place, it is a frequent subject of research focus and a region of the brain concerned with memory (4–10). The hippocampus cornu ammonis (CA) regions CA3 and CA1 are known to be involved in memory, and the relation between pyramidal and granular cells is important in order for the hippocampus to be able to perform this function (11,12). Whatever the mechanism, verbal or symbolic long-term memories cannot be permanent in the absence of the right or left hippocampus. In addition, the right hippocampus exhibits greater activity in visual functions and the left hippocampus in memory-associated ones. Various studies have shown that a pathological event arising in the hippocampus (such as ischemia) affects memory (13).

Ethyl pyruvate (EP) is a therapeutic agent with a powerful protective effect. Studies have maintained that in addition to antioxidant and antiinflammatory properties, EP also exhibits neuroprotective effects (14). Its protective effect is related to its antiinflammatory property (15), and it has been suggested that thanks to this effect it reduces neuron death in the hippocampus (16). Experimental ischemia studies have shown that EP has a neuroprotective effect in the brain and that it can be used as a neuroprotective, even if administered 24 h after the induction of ischemia (15,17). Postischemic inflammation leading to ischemic injury is attributed to multiple mechanisms. EP’s neuroprotective mechanism may also therefore be attributed to various mechanisms. However, no matter the mechanism to which it is attributed, it is
important to be able to say that EP may have a protective effect on CNS tissues and can therefore prevent brain ischemia or significantly reduce its effects (15,17).

Stereological techniques are an ensemble of techniques that allow reliable results to be obtained quickly with no systematic deviation from true values (18–21). Data regarding 3-dimensional characteristics are obtained on the basis of 2-dimensional images produced with these techniques (21). Studies producing data such as synapse and neuron numbers have frequently employed the optical fractionator, a stereological technique (22,23). The aim in this study was to investigate the probable effects on pyramidal cell numbers in the hippocampus of the administration of EP following brain ischemia experimentally induced in rats. The optical fractionator technique was used to obtain cell numbers (19,20). We also examined the samples histopathologically at the light microscopic level.

2. Materials and methods

2.1. Animals and experimental groups

All experiments were performed with 30 female, 16-week-old Sprague Dawley rats, each weighing 270–300 g. These were obtained from the Karadeniz Technical University (KTU) Surgery Research Center (SRC). The animal experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (24) and were approved by the KTU Medical Faculty Local Ethics Committee. Rats were housed in the KTU SRC in standard plastic cages on sawdust bedding in an air-conditioned room at 22 ± 1 °C under controlled lighting (12 h light/12 h dark cycle). Standard rat chow and tap water were given ad libitum. The groups were kept in different cages in the same room during the experiment.

The rats were divided into 5 groups: control (group I, n = 6), ischemia (group II, n = 6), ischemia + EP (group III, n = 6), incision + EP (group IV, n = 6), and incision + Ringer’s lactate (group V, n = 6). The rats of these groups were selected at random and monitored in separate cages throughout the study. No procedure was performed on the rats in group I, the control group. Thirty minutes of ischemia was applied to the rats in groups II and III. No further procedure was performed in group II, and these rats were monitored for 10 days. EP (40 mg/kg body weight) dissolved in Ringer’s lactate was administered intraperitoneally to the rats in group III once a day at the same time for 10 days, beginning on the day of the surgical procedure. Ringer’s lactate alone was administered to group V, once a day and at the same time every day, in the same dose as that given to groups III and IV, beginning on the day of surgery.

2.2. Drug and drug administration

EP (Sigma Chemical Co., 98% pure) is a balanced salt solution containing calcium and potassium (15). The literature has shown that EP prepared in a solution containing calcium and potassium, such as Ringer’s lactate, can be administered to experimental animals without spoiling or losing its efficacy (14,17). Therefore, we used EP diluted in Ringer’s lactate. Studies in the literature also report a therapeutic dose of EP in experimental animals of 30 mg/kg body weight. Therefore, EP was administered intraperitoneally (at 40 mg/kg body weight), the solutions being diluted to give an injection volume of 1 mL per dose (14,17).

2.3. Ischemia procedures

Rats were anesthetized with urethane (1.25 g/kg), administered intraperitoneally. Rats were placed on a surgical table, on their backs with their heads extended and feet fixed to the table. The anterior neck regions of the rats were shaved and cleaned with Isosol antiseptic solution (polyvinylpyrrolidone iodine 10%, Merkez Lab., Turkey). A 2-cm vertical incision was made down the center of the anterior neck region. The incision was opened laterally with the help of a retractor, in such a way that did not damage the skin tissues. The right and left carotid arteries were accessed, again without damaging the tissues and vessels in the incision region. In order to clamp the internal carotid arteries effectively, the neck skin fascia in the dorsal parts was isolated from the prevertebral neck muscles.

In the rats in groups II and III, blood flow was halted by clamping both arteries simultaneously with Yaşargil aneurism clamps (BA045). Brain ischemia was thus induced by interrupting blood flow to the brain for 30 min. During this time the incision and ischemia sites were prevented from drying out by moistening the exposed surgical site with an isotonic solution. Vital findings such as respiration and heartbeat were monitored constantly. Clamps were removed after 30 min. The incision region was then sutured, with care being taken over the circulation and tissue positions. In groups IV and V, following anesthesia, the incision alone was performed in the manner described above. No procedure was performed on the rats in group I. Rats were monitored for 10 days following surgery. EP was administered intraperitoneally to the rats in groups III and IV at the same time every day, between 0800 and 1000 hours. Ringer’s lactate was administered to the rats in group V, again at the same time every day (0800–1000 hours) and in the same volume given to groups III and IV.
2.4. Histological procedures

All rats in all groups were decapitated in the same way on day 11 postoperatively and their brain tissues were removed. The brains were dissected and transferred to a 10% formaldehyde solution for stereological analysis and histopathological examination. After the brains were processed through graded alcohols and xylene, they were embedded in paraffin blocks. Hippocampus sections were taken using a rotary microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany) with disposable metal microtome blades (Leica 819, Leica Instruments) to obtain 30-µm-thick serial sections in a coronal plane from the paraffin block of tissues. Each sampled section of brain hemisphere that included the hippocampus was collected on slides coated with a gelatin–formaldehyde mixture and stained with cresyl fast violet for stereological and histopathological evaluations (25–28).

2.5. Stereological analyses

Stereological analyses were conducted using the Stereology Analyses System (SAS) at the Stereology Laboratory of the Histology and Embryology Department, Medical Faculty, KTU (Trabzon, Turkey). The SAS consisted of a light microscope (Leica DM4000 B, Germany), a personal computer and a computer-controlled motorized specimen stage (Prior ProScan, USA), a CCD digital camera (JVC, Japan), and an electronic microratoc (Heidenhain, Germany). The entire system was controlled by Stereoinvestigator software (Version 9, MicroBrightField Inc., USA). Analysis was carried out at a final magnification of 5139× (i.e. using a 100× Leica HCX Plan Apo objective; NA = 1.135).

In line with previously published papers, we used the optical fractionator technique for pyramidal cell counting (19,20,26,27). The total number of pyramidal cells of the hippocampus (N) was estimated via the following formula:

\[
N = \sum Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}
\]

where \(\sum Q\) represents the total disector number of pyramidal cells counted in all optically sampled fields of the hippocampus, ssf represents the section-sampling fraction, asf represents the area sampling fraction, and tsf represents the thickness sampling fraction (Figure 1). The coefficient of error (CE) of the sampling schedule of the hippocampus was validated from the pilot study. As stated previously, the CE should be ≤10% (19,20). It was also possible to estimate the coefficient of variation (CV) within the hippocampus in each group. These are valuable data for determining whether the number of subjects in each group is sufficient (26,27), and the mean CE for the stereological estimation of neuron numbers and other stereological parameters are given in Tables 1 and 2, respectively.

On the basis of the pilot study, the first section in the series to be analyzed was chosen at random from the first 7 sections, and every successive seventh section was collected from the series, resulting in a section-sampling fraction (ssf) of 1/7. Some 15 to 18 sections were sampled from each hippocampus in a systematic, random manner. The unbiased counting frame size and step size were 596 µm² and 40,000 µm² for pyramidal cells in the CA, respectively. This means that the area sampling fraction (asf) is 596/40,000. The disector height was 10 µm, and a 5-µm zone at the uppermost part of the section was excluded from the analysis at every step as the upper guard zone. Therefore, a thickness sampling fraction (tsf) of 10 µm/t was used, where t represents the mean section thickness (Figure 1; Table 2).

2.6. Determination of pyramidal cells

The neurons in the hippocampus vary by region. For example, pyramidal neurons are intensively present in the CA1 region while they are compressed into a narrow area in the CA2 region. Additionally, neurons in CA2 and CA3 are larger than those in CA1 (29). Therefore, during the counting of the pyramidal neurons in areas CA1 to CA3, these neurons were not considered different parts of the
region because it is difficult to define exact boundaries between the hippocampal areas (25–27). Stereological studies on the subject have frequently been based on the neuron nucleus in counting neurons (30). In our study, too, pyramidal cell nuclei that can easily be distinguished at the light microscopy level were counted, and a pyramidal cell nucleus was taken as a pyramidal cell. Therefore, the nucleus of the pyramidal cell was regarded as a disector particle and counted if its largest nuclear profile came into focus within the unbiased virtual counting frames (Figure 1) systematically and randomly spaced throughout the delineated regions.

An expert (A. İkinci) on the type of cells in the hippocampus performed the cell counting in the CA region. The pyramidal neurons in the CA of the hippocampus can be easily distinguished from other cell types such as neuroglia in the pyramidal cell layer because the size of the nuclei of pyramidal cells is larger than the nuclei of neuroglia and the cytoplasm of a neuron in this layer is stained darkly with cresyl violet, while the neuroglia do not have such features. Furthermore, the percentage of neuroglia in the pyramidal cell layer is as low as 2%–3% (26,27). This amount would not significantly affect our pyramidal cell count. The estimated total number of neurons was calculated from the number of counted neurons and the sampling probability (20,25,31).

2.7. Statistical analysis
The means of the groups were compared using the ANOVA post-hoc Bonferroni test. Results are expressed as mean ± SEM. P-values of less than 0.001 and 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 13.1 (SPSS Inc., Chicago, IL, USA).

3. Results
3.1. Histopathological results
Hippocampus sections from groups I, II, III, IV, and V were examined histopathologically. At the light microscopic level, the morphology of the pyramidal cells was normal in groups I (Figures 2A–2C), IV, and V. There were not any damaged neurons (i.e. black or shrunken perikaryon) in the ischemia groups’ (group II and III) hippocampi after the brain ischemia (D–F) and III (G–I) in comparison with the control group (A–C). Substantial cell loss is apparent in groups II (F) and III (I) in comparison with group I (C). It should be noted that substantial neuron loss may not always be seen in such images since the orientation of cutting may result in a very differently sectioned surface area for each sampled area. CA, cornu ammonis; DG, dentate gyrus; V, ventricle (Bar = 100 µm for A, B, D, E, G, and H; 200 µm for C, F and I).

Figure 2. Light microscopic photomicrographs of the hippocampus sections from group I (A–C), group II (D–F), and group III (G–I) (stain: cresyl violet). There were no damaged neurons (i.e. black or shrunken perikaryon) in the ischemia groups’ (group II and III) hippocampi after the brain ischemia (D–F) and III (G–I) in comparison with the control group (A–C). Substantial cell loss is apparent in groups II (F) and III (I) in comparison with group I (C). It should be noted that substantial neuron loss may not always be seen in such images since the orientation of cutting may result in a very differently sectioned surface area for each sampled area. CA, cornu ammonis; DG, dentate gyrus; V, ventricle (Bar = 100 µm for A, B, D, E, G, and H; 200 µm for C, F and I).
numbers in the groups are given in Table 1. Our analysis revealed that the total number of pyramidal cells in group II was significantly lower than those in groups I, IV, and V (P < 0.001). However, there was no significant difference between the total number of pyramidal cells in group III (P > 0.05). The total number of pyramidal cells in group III was significantly lower than those in groups I and IV (P < 0.001). However, there was no significant difference between the total number of pyramidal cells in groups III and II or between groups III and V (P > 0.05). Additionally, there was no significant difference between the total number of pyramidal cells in groups I and IV, and groups I and V (P > 0.05), nor between the total numbers of pyramidal cells in groups IV and V (P > 0.05).

4. Discussion

Studies have suggested that EP protects vital organs during pathological events such as ischemia and has a neuroprotective effect on CNS tissues (14,15,17,32). For example, Yu et al. (17) reported that EP had a powerful protective effect in ischemia that they induced with middle artery obstruction, and Shen et al. (32) reported a neuroprotective effect of EP against neonatal hypoxic-ischemic brain damage, while Choi et al. (33) reported that EP had a preventive effect on dopaminergic cell death in Parkinson disease models. In a study performed on rabbits, Wang et al. (34) suggested that EP prevented spinal cord ischemic injury. These studies particularly suggested that EP being an antiinflammatory agent and having an inhibitor effect on cell death gives rise to this neuroprotective property.

There are only a limited number of studies investigating the protective effect of EP on the hippocampus. Cho et al. (35) investigated whether or not EP has an effect on kainic acid in a study on mice. Kainic acid, which is found in the brain and spinal cord and is also an analogue of glutamic acid, which is an excitatory neuromediator, stimulates neuronal cell death. That study investigated pyramidal neuron deaths in the CA1 and CA3 regions of the hippocampus by the application of kainic acid by the intracerebroventricular route and by the administration of EP 12 h after the injection of kainic acid. At the end of the study, EP was seen to reduce cell deaths in the hippocampus (35). In a study on rats, Moro and Sutton (36) investigated the effects of sodium pyruvate (SP) and EP administered after head trauma. At the end of the study, there was a decrease in the number of dead cells in the hippocampi of the animals in the groups that were

### Table 1. Mean values for the total pyramidal cell numbers (MTPCN), the coefficient of variation (CV) and the coefficient of error (CE) of stereological analysis, the mean dissector particle numbers (MDPN), and the mean section thicknesses (MST) for the estimation of the total neuron numbers in the groups.

<table>
<thead>
<tr>
<th>Group properties</th>
<th>MTPCN (mean ± SEM)</th>
<th>MST (µm)</th>
<th>MDPN</th>
<th>CE</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>603,376 ± 8297***</td>
<td>21.27</td>
<td>1039.1</td>
<td>0.46</td>
<td>0.09</td>
</tr>
<tr>
<td>II. Ischemia</td>
<td>499,226 ± 6863</td>
<td>20.93</td>
<td>930.4</td>
<td>0.47</td>
<td>0.08</td>
</tr>
<tr>
<td>III. Ischemia + EP</td>
<td>523,021 ± 13,115</td>
<td>20.35</td>
<td>1023.27</td>
<td>0.43</td>
<td>0.12</td>
</tr>
<tr>
<td>IV. Incision + EP</td>
<td>595,447 ± 11,215**</td>
<td>21.91</td>
<td>1032.22</td>
<td>0.49</td>
<td>0.05</td>
</tr>
<tr>
<td>V. Incision + Ringer’s lactate</td>
<td>576,055 ± 21,127*</td>
<td>21.39</td>
<td>1027.16</td>
<td>0.46</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*Significantly lower in group II vs. groups I, IV, and V; **significantly lower in group III vs. groups I and IV.

### Table 2. Sampling strategy used for stereological analysis.

<table>
<thead>
<tr>
<th>Analysis parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section-sampling fraction (ssf)</td>
<td>1/7</td>
</tr>
<tr>
<td>Some sampled sections from each hippocampus</td>
<td>15–18</td>
</tr>
<tr>
<td>Unbiased counting frame area (XY) (µm²)</td>
<td>40,000</td>
</tr>
<tr>
<td>Disector volume (XYZ) (µm³)</td>
<td>400,000</td>
</tr>
<tr>
<td>Disector height (Z) (µm)</td>
<td>10</td>
</tr>
<tr>
<td>Upper and lower guard zone (µm)</td>
<td>5</td>
</tr>
<tr>
<td>Area sampling fraction (asf)</td>
<td>596/40,000</td>
</tr>
<tr>
<td>Thickness sampling fraction (tsf)</td>
<td>10/t (mean section thickness)</td>
</tr>
</tbody>
</table>
administered SP and EP and an accompanying decrease in neuronal tissue loss. In addition, the neuroprotective effect of EP was reported to commence before that of SP in that study. Another study also reported that EP reduces neuron death in the hippocampus thanks to its antiinflammatory effect (16).

In terms of neuron numbers in the groups in this study, our results revealed a significant difference between groups I and II and between groups I and III. These results show that ischemia leads to a decline in neuron numbers, but that EP does not alter that decrease. Although there was a rise in neuron numbers in group III in comparison to group II, it was not significant. Another finding confirming this result is the absence of any significant difference between groups I and IV. These findings may be interpreted as EP having no effect on ischemia and neuron numbers. Additionally, there was no significant difference in neuron numbers between groups II and IV and between groups III and IV. These results show that EP has no effect on cell damage caused by ischemia. Our study, which shows that ischemia reduces neuron numbers, is also supported by other studies. For instance, Herguido et al. (6) reported a loss of neurons in the hippocampus after ischemia in a study on rats. The most important question arising here is how ischemia reduces cell numbers. It is impossible to account for this on the basis of our results. However, it has been suggested in other studies that pathologies that may initiate the cell cycle, such as brain ischemia, lead to a decline in neuron numbers by causing apoptosis in postmitotic neurons (6,37,38).

In contrast to our findings, it has also been maintained in some studies that ischemia increases neuron numbers, that neurogenesis takes place in some regions of the brain (particularly the gyrus dentatus, the subventricular zone), and that the new neurons that form can migrate to the injured regions (3,39). However, it has been noted that some of these studies were not conducted with stereological techniques that allow reliable results with no systematic deviation from true values (8,19–21,40). For example, Jiang et al. (39) reported that new neurons developed in a study in which they performed middle cerebral artery occlusion (41). In a similar study, Jin et al. (3) showed that neuroproliferation took place in the subgranular zone of the gyrus dentatus and in the rostral subventricular zone.

The literature contains very few studies investigating the effects of EP on hippocampus neuron numbers. While some of these studies support our finding that EP has no effect on ischemia, others maintain that EP has a neuroprotective effect in ischemia induced in the CNS (16,35,36). Cho et al. (35) reported in a study on mice that EP reduced cell death in the hippocampus. In a study on rats, Moro and Sutton (36) reported a decrease in dead cell numbers in the hippocampus of animals in the group administered EP and that neuronal tissue loss also decreased in line with this. Another study also reported that EP reduced cell death in the hippocampus (16).

We used Ringer’s lactate as a solvent in order to be able to administer EP intraperitoneally. Group V was added to the study in order to determine whether Ringer’s lactate had any impact on neuron numbers. The data obtained from group V show no significant difference when compared with groups I and IV. These results show that Ringer’s lactate can be safely used as an EP solvent. Compared with groups III and V, there was a slight decrease in neuron numbers in group III compared with group V, but there was no significant difference between them. We are unable to fully account for this because the absence of a significant difference between group V and groups I and IV should also require a significant difference between groups III and V. This finding was not confirmed by the literature (42,43).

In conclusion, we have demonstrated that ischemia reduced hippocampus neuron numbers in 16-week-old rats, but that EP had no effect on cell injury caused by ischemia. Although EP is regarded as a powerful therapeutic agent of potential use in the treatment of ischemia, our findings do not confirm this. Our results also show that Ringer’s lactate can be safely used as an EP solvent.

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