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# Presentation of the change in the number of hippocampal neurons by stereological method in surviving cases of mechanical asphyxia:
## *An experimental rat study*

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**Abstract:** Asphyxia is the most common death cause in Forensic Medicine. Strangulations do not always result in death. Cases recovering from asphyxia have many disturbances in different organs because of ischemia.

In this study, using stereological methods, after asphyxia was performed by strangulation, the mean number of pyramidal neurons in hippocampus was aimed to evaluate. Rats are divided into two groups. The first one was the control group and the other one was asphyxiated by strangulation. The brains of strangulated group rats were extracted after seven days. Sections were taken by cryostat with the method of "Systematic Randomised Sample Strategy" and stained with hematoxylen-eosin. In these sections, total pyramidal neuronal numbers in hippocampus were determined by the "Optical Fractioning Method".

In our study, the mean cell number of asphyxiated group (158985,2±17439,8) was significantly less in the control group (265689,4±39009,5) (p=0.009). In conclusion, strangulation-induced asphyxia is resulted in hippocampal neuronal lost.

**Key Words:** strangulation, mechanical asphyxia, hippocampus, neuron loss, stereology, rat.

Asphyxia is defined as the interruption in the exchange of blood, oxygen and carbon dioxide in the body [1,2]. Mechanical asphyxia involves the mechanical interruption of oxygen and carbon dioxide exchange in the case of strangulation which has three forms as hanging, ligature and manual.

Death occurs as a result of reflex cardiac arrest caused by the closure of air passages, compression of neck vessels or stimulation of glomus caroticus and baroreceptors in the neck [1,3]. However, not all strangulations result in death [4,5]. For example, 30% of the hanging cases survive after hanging [5].

Several systems, primarily the central nervous system, is affected by systemic hypoxia and decrease of cerebral blood flow caused by strangulation-induced interruption of 02–C02 exchange [6]. While amnesia is the most common finding seen in people who survive strangulation, psychic disorders are also observed [3].

Strangulation-induced brain damage may be presented in hippocampus, an area which is typically affected the most from hypoxia and ischemia. Previous studies reported a relationship between hippocampus and amnesia [7]. Memory is reported to be affected in patients with a lesion affecting the hippocampus [8,9].

Total neuron number may best be determined by stereological count which is essentially an unbiased and effective method [10]. Stereology is a science that tries to obtain numeric data about the geometrical features of a three dimensional object, such as volume, surface area, number and length, by using two dimensional section planes. It is effective because it gives the most reliable results in the shortest time [11,12,13].

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Our study aimed to determine the mean number of hippocampus pyramidal neurons by using stereological methods after strangulation in rats and to examine the effect of post-strangulation hippocampal damage on the number of hippocampus pyramidal neuron.

**Material and Method**

The study was initiated after the ethical committee approval was received from the Committee on Animal Research Ethics of the Faculty of Medicine at Pamukkale University. The study was conducted at the Experimental Research Laboratory of the Faculty of Medicine of Pamukkale University, Department of Forensic Medicine of the Faculty of Medicine of Pamukkale University, and the Department of Pathology of the Faculty of Medicine of Pamukkale University. Hippocampus total pyramidal neuron number was evaluated by systematic random sampling strategy, the fractionator and optical dissector.

*Animals*

In our study, we used 4-5 months old 10 Winstar Albino females weighing 165-290 grams. The rats were placed into special cages with plastic at the bottom and wire at the top. Throughout the study, all rats were kept in room temperature (22 ± 2 oC), at 50±5% humidity, and in an environment with 12-hour light-darkness cycle and easily accessed food and water.

Rats were selected by random sampling method and divided into control (n=5) and experiment (n=5) groups.

Formation of the strangulation model

Five rats in the experiment group were put under deep anesthesia by intramuscular administration of 5 mg/kg xylazine hydrochloride and 90 mg/kg ketamine hydrochloride combination. After deep anesthesia, rats were placed on the operation board. Blood oxygen levels of rats were monitored during the operation by using pulse oximeter. 2-mm thick string was placed around the neck in a way to wrap around the neck once, and free ends were passed through the holes of the operation board.

A weight of 1,226 Newton was attached to each free end of the string. Strangulation was applied for five minutes and asphyxia (hypoxia-ischemia) was created [14]. In rats with blood oxygen level of 40-50%, strangulation was removed and reperfusion was applied. 5 mg/kg xylazine hydrochloride and 90 mg/kg ketamine hydrochloride combination was administered intramuscularly to the rats in the control group in order to exclude the effect of deep anesthesia on hippocampus. The rats in experimental and control groups were monitored for seven days under the same environment conditions, but in different cages.

Preparing the tissue and obtaining the sections

At the end of day seven, ten rats including the control group were put under deep anesthesia and sacrificed by cervical dislocation. The brains were removed and placed into cryostat (Leica CM3050) adjusted beforehand to -50ºC, and tissue fixation was achieved by fast freezing. Frozen brains were then cut into pieces of 150 µm in thickness on a horizontal plane by cryostat at -15ºC. In accordance with systematic random sampling method, section sampling rate was determined by throwing away two successive sections after the first one and taking the third section (SeSR=1/3). The obtained sections were stained with hematoxylin-eosin (HE).

*Stereologic analysis*

Microscopic images obtained from pyramidal cell layers in the hippocampus using x100 oil-immersion lens (N.A. 1.25) with a microscope (Nicon Eclipe E 600) were transferred to a monitor (Sony Trinitron Color Video Monitor PVM- 14N1MDE) using a video camera (Hitachi OSP Color Video Camera VK- C220E).

Sampling rate, which is one of the parameters to be used in calculating total cell number according to optic dissection method, was measured as 1/162 (unbiased counting frame area was 144 µm2, and x, y stepping area was 23328 µm2). x, y stepping area was measured by the method defined by Adiguzel et al. [15].

Section thickness and dissector height was measured by micro-screw calibration method developed by Korkmaz and Tumkaya [16]. Thickness sampling fraction was calculated by dividing dissector height (h) by mean section thickness (t) for each rat (tsf = h/t). Hippocampus total pyramidal neuron number (Ntotal) for each rat was calculated by using the following formula [17]:

$$N_{total} = (\Sigma Q^{-}) \times (1/ssf) \times (1/asf) \times (1/tsf)$$

$\Sigma Q^{-}$: The total number of neurons counted in the dissectors on the sampled sections, Ssf: The section sampling fraction or the fraction of the sections sampled, Asf: The area sampling fraction, Tsf: The section thickness sampling fraction (h/tort), t: The mean thickness of the sections (µm), h: The height of the dissector (µm).

As each pyramidal cell has a single nucleus, nucleus numbers were taken as cell numbers in the calculations. In the pyramidal layer, there are basket cells with a low rate of approximately 1% besides pyramidal cells. Since basket cell nuclei are similar to pyramidal cell nuclei, total neuron number was calculated in a way to include these cells as well. Inclusion of these cells to the count does not affect the study [17].

*The coefficient of error (CE)*

Adequacy of the sampling plan was controlled by calculating the coefficient of error for each rat. Number of sections counted for each rat and number of dissector particles counted for each section (Q-) were used in the calculation of CE.

Error coefficient was found to be below 10%, showing the adequacy of the sampling plan and the reliability of the study [17].

*Statistical method*

Statistical analyses were performed by using SPSS 13.0 for Windows statistical method. Mann-Whitney U test was used basically in statistical analysis.

## Results

The mean number of neurons in the control group was calculated as 265689,4±39009,5. The mean number of neurons in the group exposed to asphyxia due to strangulation was calculated as 158985,2±17439,8, and this value was lower than that of the control group to a statistically significant extent (p=0,009). This study showed that there was a statistically significant difference between the mean total numbers of both groups (Mann-Whitney U test; p=0,009).

The mean of hippocampus total pyramidal neuron numbers of rats in both groups are given in Figure 1 and Figure 2 presents the hippocampus images of rats in the control group and in the strangulated group, with magnifications of x40 and x100.

The CE for the sampling scheme in this study was between 0.010 and 0.016.

## Discussion

Primary articles on strangulation in forensic medicine literature are about these postmortem findings [1,2,3]. However, not all these strangulation cases result in death [4,5]. It affects many systems in the body, primarily the central nervous system [6].

Cerebral injury occurs in as a result of hypoxia-ischemia (asphyxia) caused by the occlusion of the vessels leading to the brain due to the compression in the neck and the closure of air way passages during strangulation [1].

In a study presenting the physical findings of victims who survived after strangulation; early and late mental state changes secondary to hypoxia, amnesia, psychosis, epilepsy, choreic movements, focus signs, progressive irreversible encephalopathy were reported [18]. Symptoms like hyperventilation, memory loss and uncontrolled shivering were reported in 7% of the cases exposed to strangulation [18].

Studies have demonstrated a relationship between hippocampus and amnesia [7]. In a patient with a lesion affecting the hippocampus, it was observed that short-term memory loss did not develop into long-term memory loss (anterograde amnesia) [8,9].

Anterograde amnesia induced by hanging and similar to Korsakoff's syndrome was identified. Two cases with hanging-related anterograde amnesia and without nuerological finding except mild bradykinesia and tremor were presented. Neurological and neuropsychological symptoms in these two cases developed after the hanging episode.

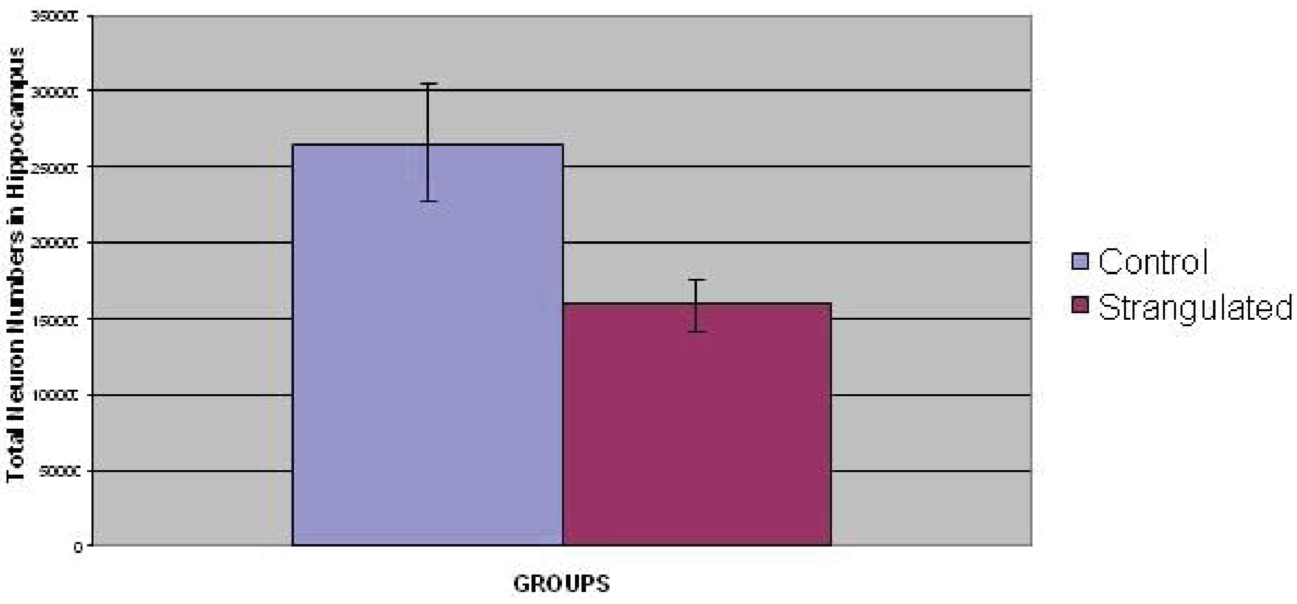


**Figure 1.** The total pyramidal neuron numbers of hippocampus in control and strangulated groups. Values are mean ± SD. The total number of hippocampus pyramidal neurons in rats for strangulated group were significantly decreased according to control group (Mann-Whitney U test, p< 0,05).

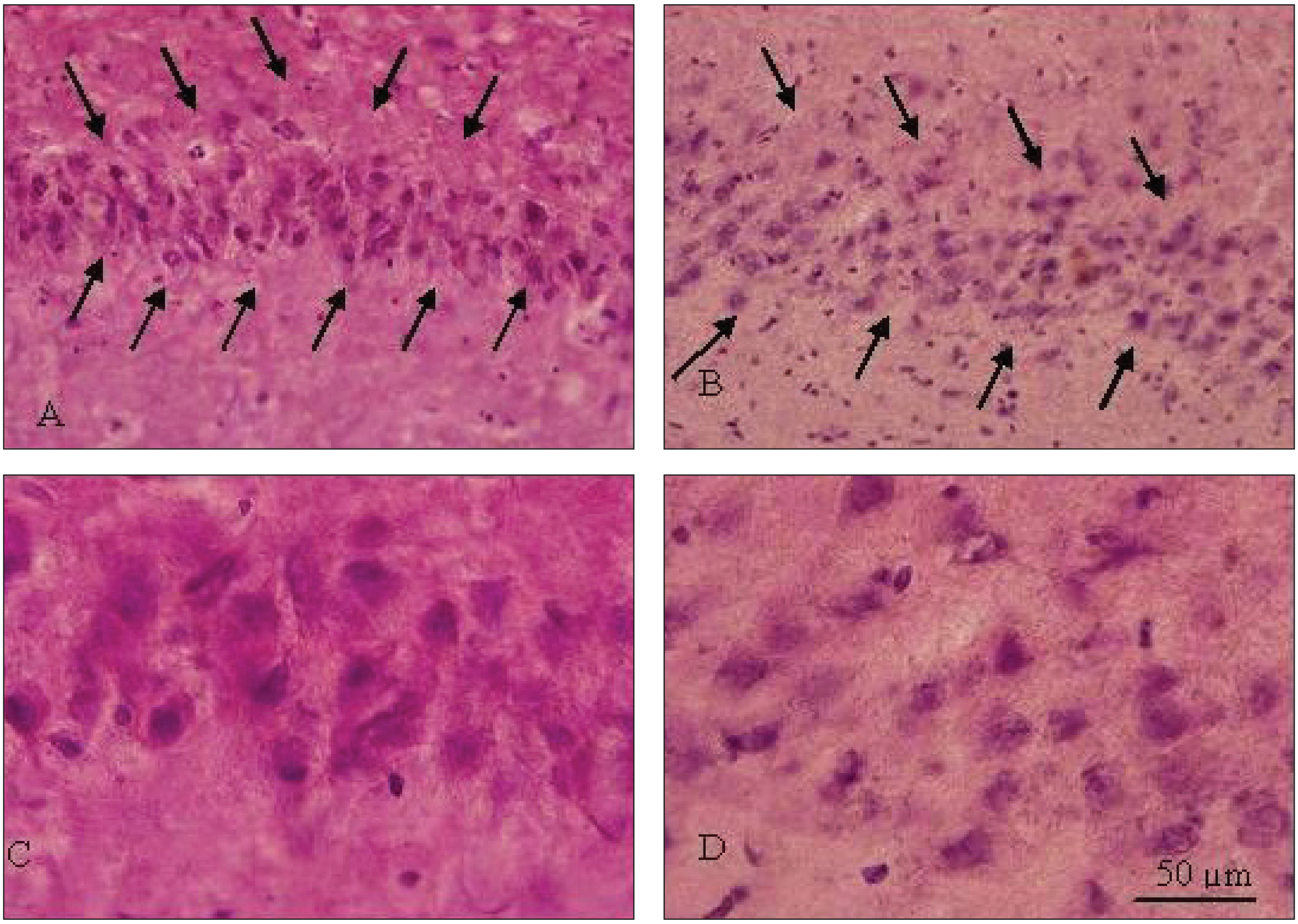


**Figure 2.** Micrographs of hippocampus stained with hematoxylen-eosin. A: the control group, x40; B: the strangulated group, x40; C: the control group, x100; D: the strangulated group, x100.

It was noted that the body weight effect of the string and its impairing effect on circulation may lead to ischemic hippocampal damage and amnesia [19].

In the death of neurons in the brain, caspase-dependent apoptosis is the main cause of death. In addition, death related to autophagy, morphological and mechanical changes may also be seen. In cerebral ischemia which causes excitotoxic neuron death, different cell death programs may be activated and different manifestations may occur.

Necrotic, apoptotic cascade or apoptotic-necrotic cascade may possibly be seen, depending on the affected subject's age and the affected area. Injury begins after ischemia and end on day five [20,21].

Pyramidal cells in the hippocampus are highly sensitive to hypoxia-ischemia [22]. Transient ischemia leads to damage in specific areas of the brain, such as CA1 area, resulting in neuron death. In the histological evaluation of the hippocampus of a 63-year-old female patient who died nine days after transient cardiac arrest at cardiac surgery, it was shown that almost all the neurons especially in CA1 area were dead [21].

Late neuron injury is known to occur especially in area CA1 in the hippocampus during post-ischemia reperfusion [10,21,23]. While histological changes occur for the first time 2-3 days after ischemia in the pyramidal cells in CA1 area, they become distinct on days five and seven [21,23]. In recent studies, a decrease in total neuron numbers was reported in all areas except CA2 in rat hippocampus 14 days after ischemia induced by cardiac arrest [10]. CA2 area in mammal hippocampuses is known to be resistant to ischemia and, thus, neural death is delayed [10,24].

While pyramidal cells in CA1 area degenerate 5-10 minutes after ischemia, dentate gyrus neurons and those in CA3 area are protected. CA3 area is more resistant to ischemia compared to CA1 area [24,25]. Asphyxia-related brain damage may be seen in the hippocampus which is typically the area most affected from hypoxia and ischemia [7]. In our study, the effect of strangulation-related hypoxia and ischemia on the brain was evaluated in rat hippocampus.

Zola-Morgan et al. presented the clinical and histological findings of a case who experienced memory loss after ischemic episode, and reported anterograde memory loss, bilateral damage in CA1 area, and low level of injury in other areas of the brain [7].

Consequently, the lesion in hippocampus leads to amnesia. Holdstock et al. reported a case with bilateral hippocampal damage and global anterograde amnesia [26].

Ischemia causes a damage which results in the death of neurons in brain areas [21]. Since neurons are the main unit of the nervous system, total neuron number is the most important criterion in the evaluation of the results of ischemia that affects the central nervous system [10].

In the studies conducted on rats to investigate the effect of ischemia on hippocampus, a decrease in the neuron numbers in CA1 area and post-ischemia atrophy in CA1 area were reported [27-29]. Herguido et al. determined a significant difference between the mean total neuron numbers in CA1 area in the control group and the groups exposed to ischemia [27].

Wu et al. detected a decrease in the neuron numbers in hippocampus CA3 and CA1 areas in rats [30]. Plamondon et al. observed rats for 180 days after 3-6 minutes of ischemia, and reported that pyramidal neuron numbers in CA1 area decreased compared to the controls, and ischemic rats in the first 15 minutes after ischemia were more active than the controls [29].

In this study, a decrease of total number in hippocampus pyramidal neuron occured in as a result of hypoxia-ischemia (asphyxia) caused by the occlusion of the vessels leading to the brain due to the compression in the neck and the closure of air way passages during strangulation.

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