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The effects of different doses of diclofenac sodium on newborn rat hippocampus exposed during the third trimester

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Abstract: The main aim of the present study was to examine the neurotoxic effects of prenatal exposure to varying doses of diclofenac sodium (DS) on the rat hippocampus. Twenty-eight Wistar albino adult female rats weighing 280–300 g were initially used for pregnancy. When vaginal plaques were seen in female rats, it was accepted as the 0th day of pregnancy. Female rats were divided into five main groups; pure control, saline, low dose DS (3.6 mg/kg), moderate dose DS (9 mg/kg), and high dose DS (18 mg/kg). They were exposed to these treatments during their gestation. After birth, all newborn male rats were euthanized by overdose anesthesia on the 7th postnatal day. Histological and stereological techniques were used for analyzing tissue samples. The stereological analyses in this study showed that the number of neurons in the hippocampus may be reduced due to the use of DS. Histological investigation of the drug-treated groups showed significant cell loss compared to the control. Darkly stained nuclei of the neurons in the high dose group compared to the other groups were also obvious. When the results are evaluated, it can be concluded that the use of diclofenac sodium during pregnancy may have teratogenic effects on the development of the nervous system.

Key words: Diclofenac sodium, hippocampus, prenatal, stereology, rat

1. Introduction

As technology has developed, health-related technological products on the market have also expanded very considerably, as in all fields, giving rise to a free market. Commercial concerns have increased as a result, and the consumption of these products in the field of health has also grown, as in all other fields worldwide. These may be considered the main reasons underlying the increase in unnecessary medicine use. The increase in the use of antibiotics, painkillers, and antirheumatic drugs has been particularly remarkable. Some researchers stated that nonsteroidal antiinflammatory drugs (NSAIDs) are commonly prescribed in the treatment of numerous rheumatic diseases since these do not cause addiction [1,2]. Diclofenac sodium (DS), used in our study, is a derivative NSAID and one of the drugs prescribed by obstetricians, gynecologists, and orthopedists and widely used by patients [3–5].

NSAIDs generally exhibit their effects by inhibiting cyclooxygenases (COX) [6–8] (Figure 1). NSAID use has been reported to cause problematic births or to increase the risk of miscarriage in some pregnant women [9]. The results of experimental studies on animals have shown harmful effects of NSAID use on the fetus, such as the closure of the ductus arteriosus (DA), pulmonary arterial hypertension, and malformation [10–15]. NSAIDs also exhibit their effects through involvement in the inhibition of various phenomena, such as enzyme activation of phospholipase C and prostaglandin (PG) synthesis and intermembrane ion transport, mitochondrial oxidative phosphorylation, and intercellular transaction [16]. The effects of prostaglandin synthesis inhibitors on fetal development and organs are not fully understood [8,17]. In addition to the treatment of rheumatic diseases, such as rheumatoid arthritis and ankylosing spondylitis, these drugs are also widely used for their antiinflammatory and antipyretic properties and as painkillers in the treatment of fever, pain, and inflammation in nonrheumatic diseases [5,18,19].

The placenta, a strong connection between mother and fetus, is selectively permeable to a certain extent. Siu et al. [6] reported that DS could easily pass through the

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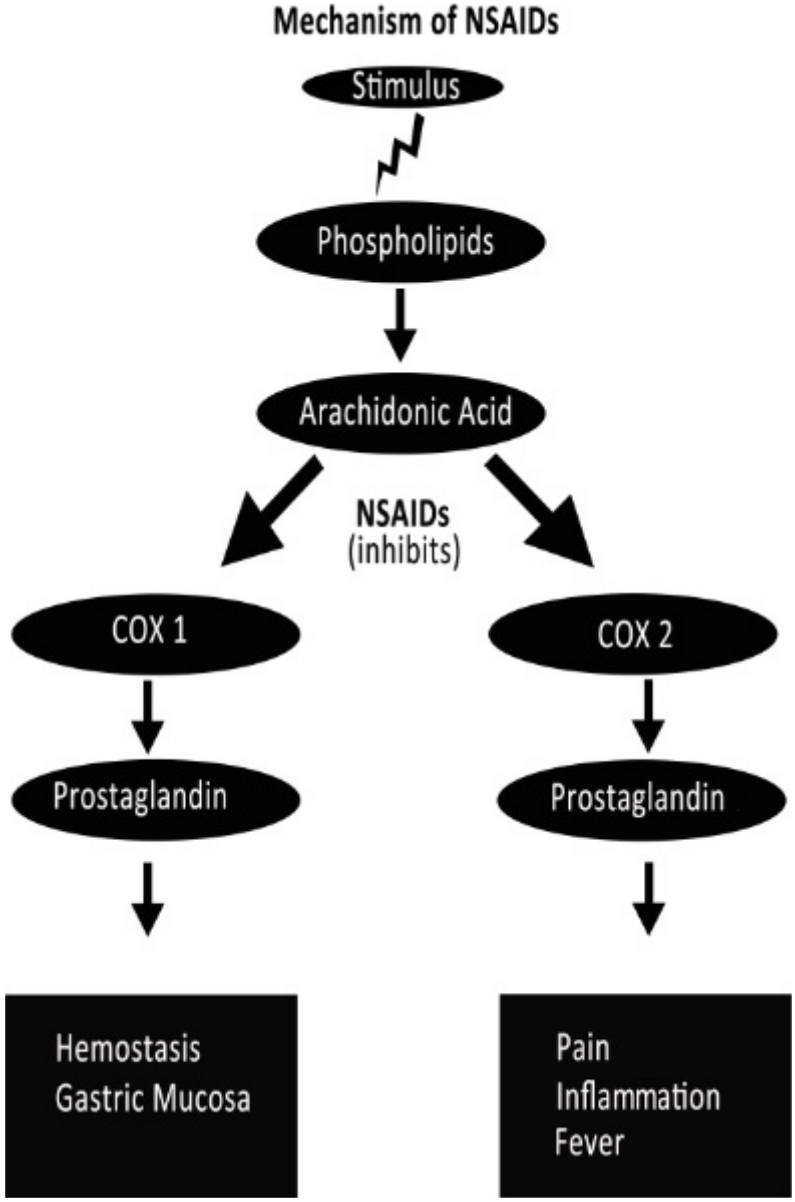

Figure 1. Effects of nonsteroidal antiinflammatory drugs on prostaglandin biosynthesis.

human placenta during the first trimester of pregnancy. By inhibiting the biosynthesis of prostanoids, NSAIDs pass through the placental barrier and enter the fetal circulation and may thus have teratogenic effects on the fetus [4,20]. Prolonged pregnancy and labor pain are other adverse effects of NSAID use observed in the mother and fetus during pregnancy [21]. Gastroschisis has been found to be a possible teratogenic effect in the early period of pregnancy as a result of using drugs such as aspirin and ibuprofen [22]. Siu and Lee [23] reported severe pulmonary hypertension and temporary hypertrophic cardiomyopathy on the right side of the heart in newborn rats as a result of early closure of the DA following short-term maternal exposure to DS. Some studies have reported that NSAIDs cause teratogenic side effects in the fetus, such as contraction of the DA, ischemia of the extremities, oligohydramnios, hydrops fetalis, cystic brain lesions, and ileal perforation [24–27].

Drug use causes an increase in the level of ROS and these may produce free radicals that lead to oxidative stress [28,29]. ROS, which form in cells, are destroyed by mechanisms known as antioxidant defense systems [30]. It is known that oxidative stress is caused by ROS [31]. Oxidative stress is seen in many chronic diseases,

but it causes cell damage caused by free oxygen radicals. It is also implicated in the pathogenesis of diseases such as preeclampsia, emphysema/bronchitis, atherogenesis, Parkinson's disease, Duchenne type muscular dystrophy, cervix cancer, alcoholic liver disease, retrolental fibroplasia, cerebrovascular disorders and ischemia/reperfusion injury, diabetes mellitus, acute renal failure, aging, and Down syndrome [32,33].

The toxic effects of NSAIDs on different organs and systems have also been investigated. In this regard, medical research on the neurodegenerative effects of DS on the central nervous system (CNS) has increased recently. In a study regarding the prenatal effects of DS on the peripheral nervous system (PNS) of 4-week-old rats, degeneration in the myelin sheath and a decrease in the numbers of axons were observed in the DS-exposed group compared to the control group [34].

Studies researching the effects of NSAIDs on the CNS have reported that DS affects the CNS and inhibits the differentiation and proliferation of neural stem cells [18,35]. In another stereological and electron microscopic study, a significant decrease was observed following DS use in the prenatal period in neuron numbers in the spinal cords of 20-week-old rats compared with a control group [36].

This literature review reveals an insufficient number of experimental studies concerning the effects on the brain during postnatal life of exposure to DS during the fetal period. It was observed that the most rapid growth rate of the hippocampus occurred between E16 and E17 (embryonic development days). However, the postnatal growth rate remains high: daily volumetric increase is seen between P1 and P7. Almost all layers of the hippocampus were developed on the seventh postnatal day [37]. In particular, there have been no stereological studies concerning changes in rats receiving varying doses of DS in the third trimester in postnatal brain tissue. Based on this information, the main purpose of the present study was to examine the effects of prenatal exposure to various doses of DS in the third trimester of pregnancy on the pyramidal neuron number in the hippocampus of 1-week-old male offspring rats using unbiased stereological methods.

2. Materials and methods

2.1. Animals and experimental design

Twenty-eight Wistar albino adult female rats weighing 280–300 g were initially used for pregnancy. During the study, the rats were housed at room temperature (22 ± 2 °C) at 40%–50% relative humidity under a 12/12-h light/dark cycle and were fed with tap water and standard rat chow. The animals were obtained from the Experimental Animals Research and Application Center of Ondokuz Mayıs University (Samsun, Turkey).

In the present study, approval (2011/11) was received from the Animal Ethics Committee of Ondokuz Mayıs University for the experimental protocol for the animals. The rats were allowed to mate in separate plastic cages. When a vaginal plaque was seen in female rats, it was accepted as the 0th day of pregnancy. Pregnant rats were divided into the three groups as control, saline, and DS-exposed groups. A lethal dose (LD_{50}) of 90 mg/kg was adopted for DS-exposed rats [38,39]. No drugs were injected into the pure control group. Daily doses of 3.6 mg/kg ($LD_{50/25}$, low dose), 9 mg/kg ($LD_{50/10}$, moderate dose) and 18 mg/kg ($LD_{50/5}$, high dose) were injected intraperitoneally in the drug-exposed pregnant rat groups. The saline group received 1 mL/kg intraperitoneal saline injections during the same period.

2.2. Animal groups

The gestation (G) periods of the rats were separated into three trimesters (G1–G7, G8–G14, and G15–G21). DS injections were performed in the third trimester in this study, namely from the 15th to the 21st gestational days (G15–G21). The developmental stages of the hippocampus were taken into account for the selection of this period [40–42]. Each treatment group was also divided into three subgroups: low dose, moderate dose, and high dose. The groups established in the present study may thus be summarized as follows:

A group: Subjected to low dose (3.6 mg/kg) DS injection during the third trimester (G15–G21).

B group: Subjected to moderate dose (9 mg/kg) DS injection during the third trimester (G15–G21).

C group: Subjected to high dose (18 mg/kg) DS injection during the third trimester (G15–G21).

D group: The pure control group was not subjected to any injection protocol during the gestation period.

E group: The saline group received 1 mL/kg saline injection during the gestation period (G15–G21).

After birth, 6 male offspring rats from each group were randomly selected to form each experimental group.

2.3. Anesthesia procedure and routine tissue process

At the end of the experimental phase of this study, i.e. on the 7th day of postnatal life, male offspring rats were euthanized by overdose of anesthesia (50 mg/kg ketamine i.p. and 10 mg/kg xylazine i.p.). After application of anesthesia, brain tissues were taken from offspring animals in a short time. Excised brain tissues were subjected to routine tissue processing. First, alcohol series from 70% to 100% were used for dehydration of the tissues. Clearing of the tissues was then performed using xylene. Finally, the tissues were embedded in paraffin blocks for light microscopic sectioning. Paraffin sections containing the hippocampal regions were taken using a rotary microtome (Leica RM 2125, Leica Instruments, Nussloch, Germany). Serial sections of 20-µm-thick paraffin were obtained in

the sagittal plane based on systematic random sampling (1/6 sampling) for stereological and histopathological analyses (Figures 2A and 2B). Sections were stained with cresyl violet.

2.4. Stereological analysis

Stereology is the name for series methods that use two-dimensional images from three-dimensional (3-D) samples to interpret their 3-D geometric features. This technique includes many rules developed to estimate the values closest to reality with methods that involve fewer errors in a short time and do not cause systematic deviation from actual values [43]. We therefore used stereological techniques to give neutral values reflecting results as close as possible to reality.

The optical fractionator technique were performed for estimation of the total numbers of pyramidal neurons in the hippocampus using a Stereo Investigator analysis system (Stereo Investigator 9.0, MicroBrieldField; Colchester, USA) and a light microscope (Leica M 4000 B, Germany) with a digital color camera attachment (MicroBrightField; Colchester, USA). A counting frame of 30 × 30 µm in size and a sampling grid area of 200 × 200 µm were used to count the numbers of pyramidal neurons in the hippocampus (Figures 3A–3D).

2.5. Statistical analysis

One-way ANOVA tests were performed for comparison of each group according to the normality of distribution of variables. When there was a significant difference among groups, post hoc comparisons were made using the Tukey test. Results were expressed as mean ± SEM. $P \leq 0.05$ was considered significantly different. Statistical analyses were performed on IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA)

3. Results

In the group injected with high dose DS, a few days before delivery, bleeding and stillbirths were observed. However, no birth problems were observed in the low and moderate dose groups. In the stereological and light microscopic examination of the groups, changes in the number of neurons in the CA1, CA2, and CA3 regions of the hippocampus and neurodegeneration in the DS exposure groups were observed.

3.1. Stereological results for pyramidal neuron numbers in the hippocampal regions

Pyramidal neuron numbers in the different groups' CA1, CA2, and CA3 regions were estimated using the optical fractionator method [44]. No significant difference was observed between the pure control and saline groups in terms of the cell numbers in the regions evaluated ($P > 0.05$). However, cell numbers highly significantly decreased in the DS-exposed groups in comparison with the pure control and saline groups ($P \leq 0.01$). A highly significant decrease was determined in cell numbers in the A, B, and C groups, i.e. low, moderate, and high doses of DS, in terms of DS doses received ($P \leq 0.01$). Additionally, there was a highly significant difference between the D and E groups compared to other groups ($P \leq 0.01$) (Table; Figure 4). The average neuron numbers of the CA1, CA2, and CA3 regions were within acceptable ranges [44]. These values are shown in the Table.

3.2. Light microscopic results from group D (pure control)

The general structure of the hippocampus and neurons had a normal appearance in the sections from group

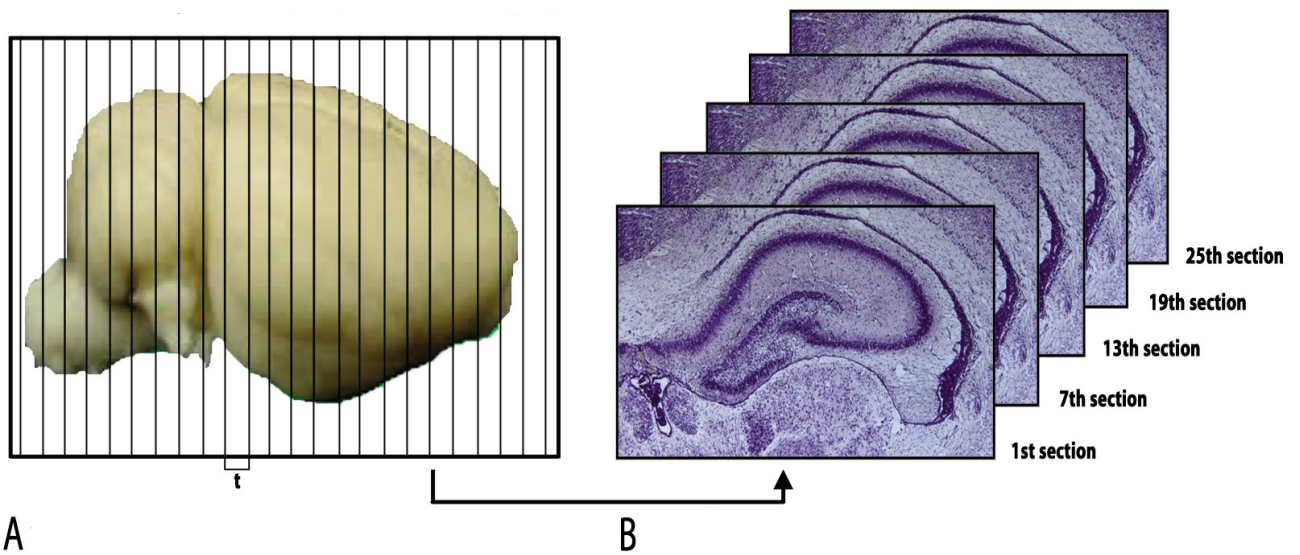

Figure 2. Section sampling fraction for stereological analysis: A) the whole rat brain; sections that are systematic randomly sampled.

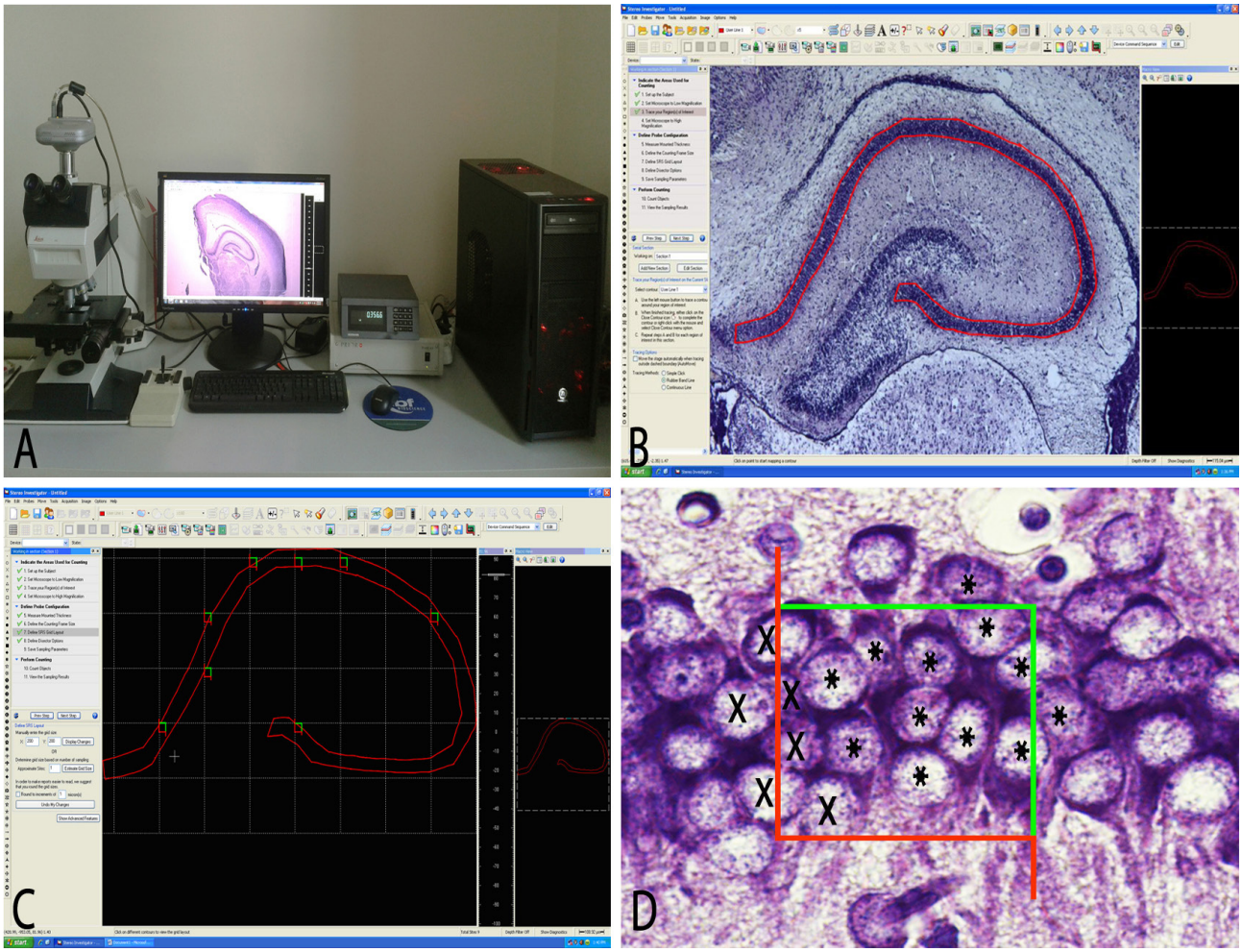

Figure 3. A) The stereological analysis system. B, C, D) The optical fractionator technique application and unbiased counting frame. Asterisks indicate the counted cells in the section of the hippocampus while an X indicates uncounted cells in the hippocampus.

Table. The mean coefficient of variation (CV) and the coefficient of error (CE) of stereological analysis for the hippocampal pyramidal neuron numbers of each group are given below.

Groups	CA1		CA2		CA3		Total	
	CE	CV	CE	CV	CE	CV	CE	CV
A (3.6 mg/kg DS)	0.04	0.04	0.05	0.06	0.05	0.10	0.04	0.05
B (9 mg/kg DS)	0.05	0.08	0.04	0.16	0.05	0.08	0.05	0.07
C (18 mg/kg DS)	0.06	0.10	0.04	0.16	0.04	0.20	0.06	0.11
D (Control)	0.03	0.06	0.03	0.08	0.06	0.06	0.04	0.05
E (1 mL/kg saline)	0.04	0.10	0.03	0.14	0.05	0.05	0.03	0.10

D. In light microscopic examination, the neurons were pyramidal in shape. Additionally, the borders of the neurons were regular and could be seen clearly. These cells also had a euchromatic nucleus with a prominent nucleolus. Neuron dendrites and axons were of normal width. Pyramidal neurons were composed of 6- to 7-row cell layers in the hippocampus. Neuroglia cells also exhibited a normal appearance (Figures 5, 6, and 7).

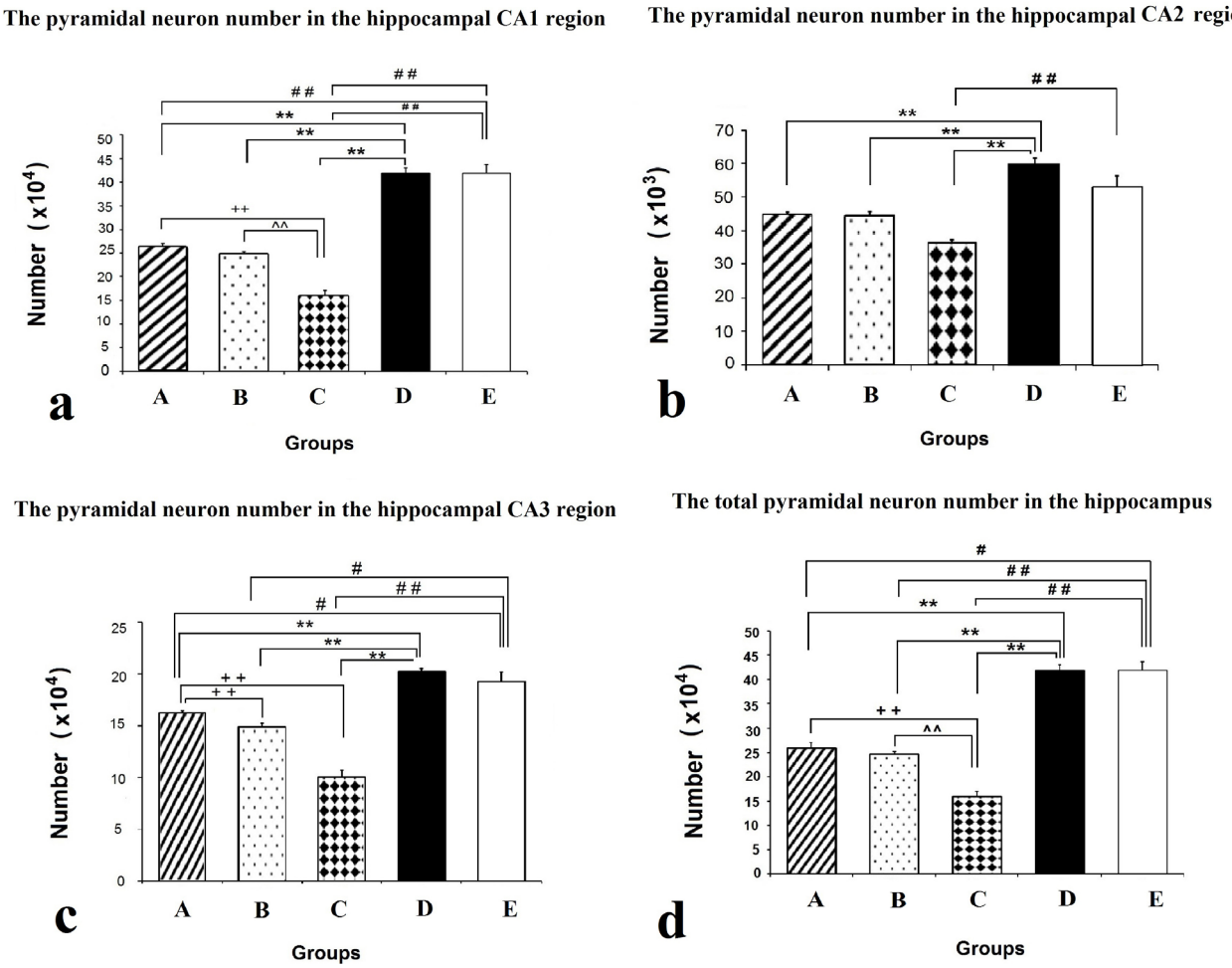

Figure 4. Graphs representing the pyramidal neuron numbers in the CA1, CA2, and CA3 regions of the hippocampus and the total pyramidal neuron numbers in hippocampus (mean ± SEM) (a–d). Significant differences among all groups (A–E) are indicated: #: $P < 0.05$; **, ^^, ##, ++: $P < 0.01$. The effects of DS on hippocampal regions of each group are shown in the third trimester according to doses of 3.6 mg/kg (group A), 9 mg/kg (group B), and 18 mg/kg (group C) (a–d) and the pure control group (D) and saline-administrated group (E).

3.3. Light microscopic results from group E (saline)

The general structure of the hippocampus was normal and healthy pyramidal neurons were observed in the slides of the group receiving saline. In light microscopic examination, the pyramidal neurons with euchromatic nuclei had a normal structure and neuron boundaries were prominent. Dendrites and axons of neurons were of normal width. A regular arrangement of pyramidal neurons and normal and healthy neuroglia cells were seen in the CA1, CA2, and CA3 regions of the hippocampus (Figure 5, 6, and 7).

3.4. Light microscopic results of group A (low dose DS)

In light microscopic examination, the neurons in the sections from the group receiving low dose DS during pregnancy exhibited more eosinophilic cytoplasm in comparison with the pure control group. These cells had euchromatic nuclei and a prominent nucleolus. The neuron dendrites and axons were extended. The borders of both cells and nuclei were irregular in appearance. In addition, the neuroglial cells in this group had large nuclei and prominent nucleoli in comparison with the pure control and saline groups (Figures 5, 6, and 7).

3.5. Light microscopic results from group B (moderate dose DS)

In light microscopic examination, neurons in the sections from the group receiving a moderate dose of DS during pregnancy had a normal histological appearance. Most neurons had a heterochromatic and pyknotic nucleus with dark cytoplasm in in comparison with the D and E groups. The number of pyramidal neuron layers was lower compared to the D, E, and A groups. More damaged neurons and large-sized neuroglia cells were observed in this group compared to the D, E, and A groups. Some necrotic areas were observed in the hippocampal regions (Figures 5, 6, and 7).

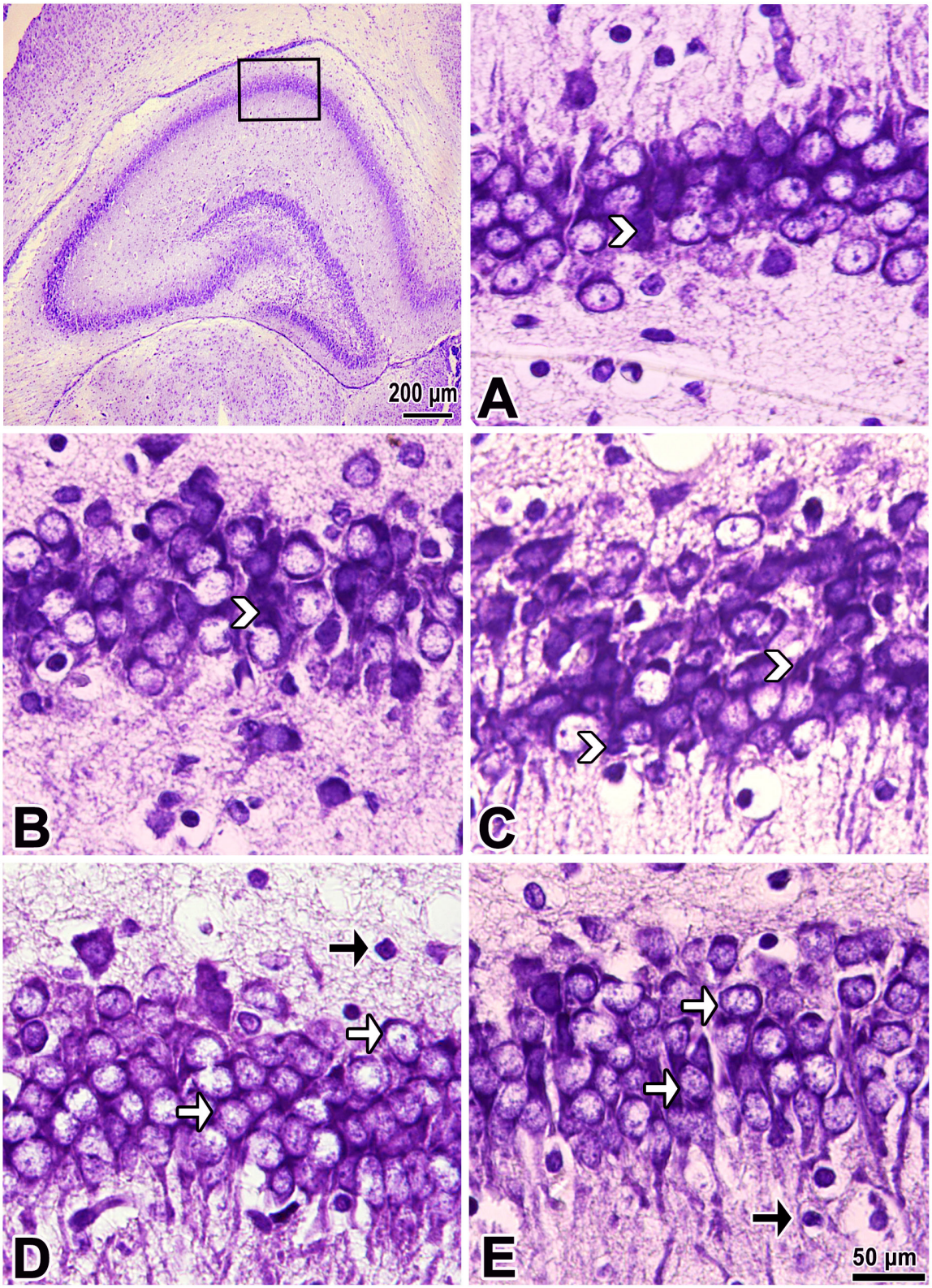

Figure 5. Representative images of hippocampal CA1 region from the pure control group, 1 mL/kg saline injection group, 3.6 mg/kg DS injection group, 9 mg/kg DS injection group, and 18 mg/kg DS injection group, respectively. White arrows indicate healthy neurons, white arrowheads show degenerated neurons, and black arrows show neuroglia cells. It should be noted that the appearance of images may be changed by tissue shrinkage or swelling and the section plane taken. Cresyl violet staining.

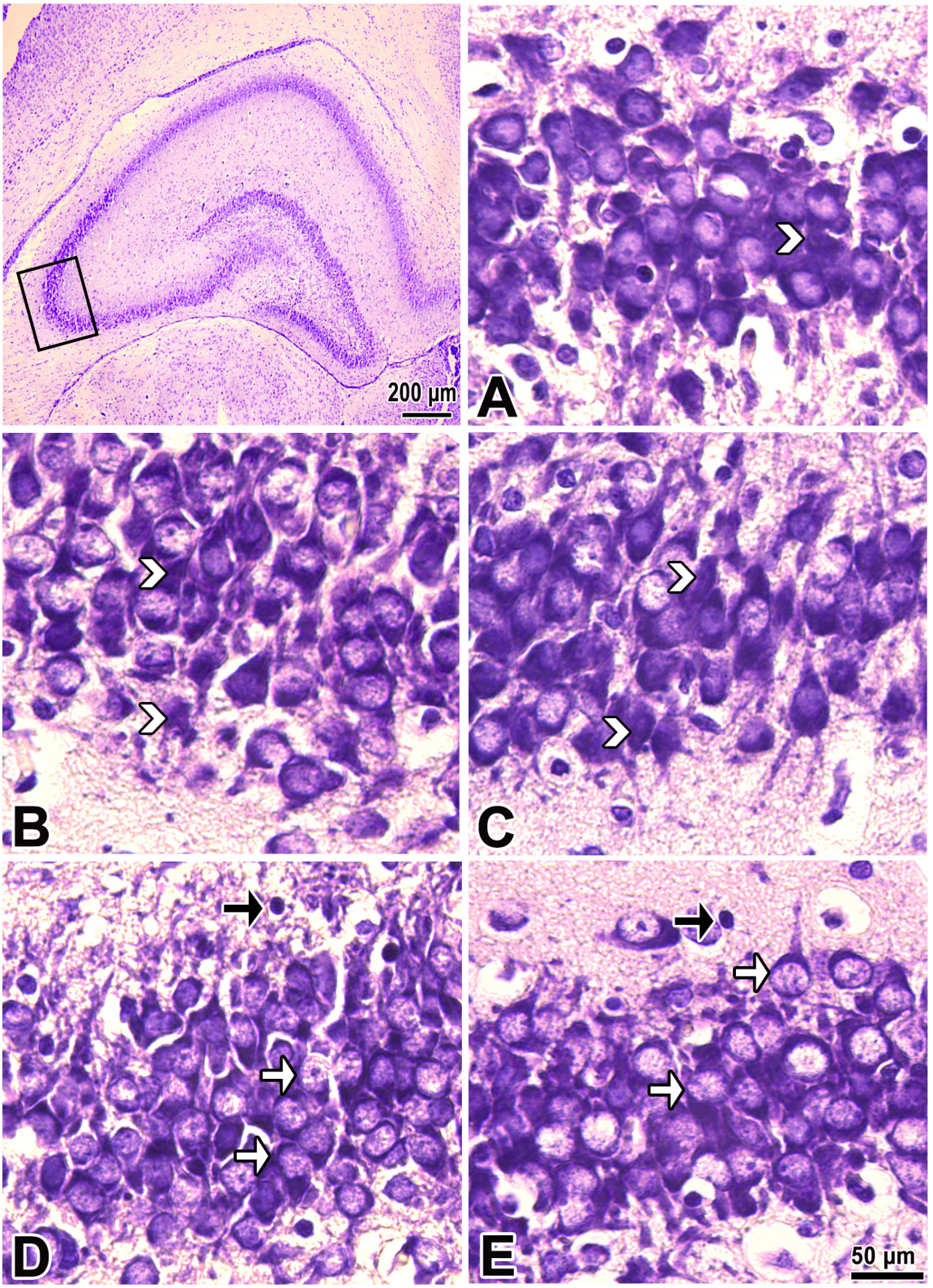

Figure 6. Representative images of hippocampal CA2 region from the pure control group, 1 mL/kg saline injection group, 3.6 mg/kg DS injection group, 9 mg/kg DS injection group, and 18 mg/kg DS injection group, respectively. White arrows indicate healthy neurons, white arrowheads show degenerated neurons, and black arrows show neuroglia cells. It should be noted that the appearance of images may be changed by tissue shrinkage or swelling and the section plane taken. Cresyl violet staining.

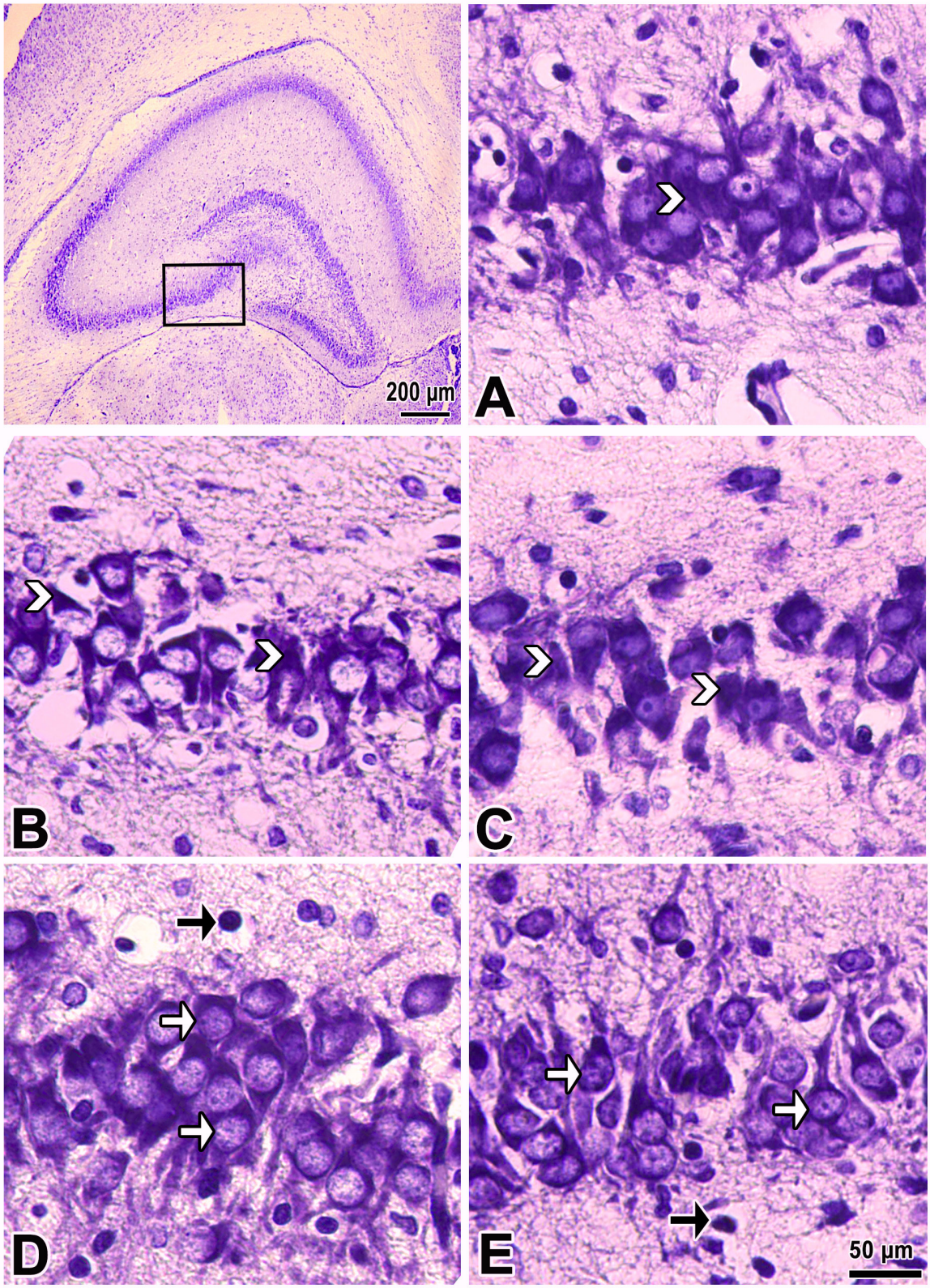

Figure 7. Representative images of hippocampal CA3 region from the pure control group, 1 mL/kg saline injection group, 3.6 mg/kg DS injection group, 9 mg/kg DS injection group, and 18 mg/kg DS injection group, respectively. White arrows indicate healthy neurons, white arrowheads show degenerated neurons, and black arrows show neuroglia cells. It should be noted that the appearance of images may be changed by tissue shrinkage or swelling and the section plane taken. Cresyl violet staining.

3.6. Light microscopic results from group C (high dose DS)

In light microscopic examination of the sections from the group receiving a high dose of DS during pregnancy, fewer neurons had a normal histological appearance than in the other groups. Dilated blood vessels were observed between neighboring neurons. In some areas, damaged neurons had unclear borders. The number of pyramidal neuron layers was lower compared to the other groups. There was also pronounced degeneration, such as pyknosis in the nucleus and an increasing of the cytoplasmic density and in the number of vacuolar and edematous structures, and high doses caused hippocampal neurodegeneration (Figures 5, 6, and 7).

4. Discussion

NSAIDs have been prescribed in the symptomatic treatment of many diseases as analgesics, antiinflammatories, and antipyretics for many years. Due to the widespread use of these drugs, DS was chosen as a pharmacological agent in our study. Studies have shown that these drugs cause early closure of the fetal DA and consequently cause adverse effects in the fetus, such as respiratory problems, kidney problems, and pulmonary hypertension dysfunction [21,45].

In a study of the toxic effects of NSAIDs, ibuprofen and tolmetin were administered at high doses during the prenatal period, and both of these drugs caused toxic effects in the mother, inhibited intrauterine development, and resulted in developmental variations [46]. Another study found that NSAIDs and aspirin used in the prenatal period increased the risk of miscarriage [47]. In our study, hemorrhage and stillbirths were observed a few days before birth in the group injected with high doses. It is possible that the drug we used on the animals in our study, DS, has similar effects to those of the drugs described above. It has been reported by the United States Food and Drug Administration that DS is a class C drug in terms of the pregnancy risk [48]. Additionally, DS may cause some malformations in newborns due to passage through the placental barrier and then into the fetal circulation [49]. In this context, the toxicity of DS for different animal model systems has been clearly shown by many studies during embryogenesis. Gokcimen et al. [3] reported that apoptosis was seen in neurons of fetuses after DS exposure in the pregnancy period. Furthermore, numerous studies on DS-treated rodents have shown fetuses with various morphological abnormalities such as DA, limb, and palate defects [20,22,50]. Felice et al. [51] also demonstrated in DS-treated zebrafish embryos that mitochondrial dysfunctions and abnormal expression of apoptosis genes occurred via differential mRNA and transcriptome analyses [51]. These studies and our previous studies, which were performed on different animal models, do not comprehensively reflect the conditions in humans, so further research is needed to better understand the toxic effects due to DS exposure in embryogenesis. Until the toxic effects of DS in humans are made more clear by clinical studies, the application of DS, especially in high doses, to women who are pregnant should proceed carefully. Different rat models have been used to determine the effects of DS on the sciatic, median, and optical nerves, as well as the cerebellum, the spinal cord, and the hippocampus [3,29,34–36,52,53]. The hippocampus has a key role in physiological events such as nutrition intake, ion balance, pain perception, reproduction, learning and memory, blood pressure, and life and cultural development. The hippocampal formation is generally subdivided into the CA and dentate gyrus. In addition, CA2 of the CA region has been neglected in studies for many years because it is very small. CA1 and CA3 are considered as the main functional regions in hippocampal formation [54]. Additionally, the CA2 region expresses different genes than the other CA regions [55]. The CA2 region puts up resistance in the case of hypoxia/ischemia [56]. While neurons in CA2 present resistance to moderate and low dose DS exposure in the third trimester, neuronal degeneration occurs in CA2 in high dose exposure. Neuromodulation in the CA2 region is highly effective on social memory and behavior [54]. In this context, it can be concluded that high-dose DS adversely affects social memory. In addition, the loss of glutamatergic and pyramidal neurons in the CA1 region and the entorhinal cortex is also seen in Alzheimer's disease [56]. CA1 plays a selective role in memory. The CA1 and CA3 areas are important especially in spatial and contextual learning tasks [57]. In this regard, from the neuronal loss in the CA1 and CA3 regions, we can say that the specific memory in the high dose group is more impaired than in the other dose groups. The third trimester is the most critical period for the development of hippocampal formation. The neuronal changes that occur during this period may indicate significant effects, especially when synaptic development increases [58]. During pregnancy, exposure to DS can lead to significant neurotoxicity and affect neural development. In the present study DS has caused dose-dependent histopathological changes in the hippocampal region. Another study has shown that the prenatal stress caused by serum saline injection can also lead to a decrease in neuron numbers in the rat brain during the postnatal period [3]. Stress causes histopathological changes such as dendritic branching, neuronal and neuroglia cell changes in the hippocampal region, and decreased neurogenesis. Similarly, in the current study, a decrease was also observed in the average number of total hippocampal neurons in the saline group in comparison with the pure control group. However, this

decrease was not significant. The stereological results of the present study revealed a significant decrease in the neuron numbers of the CA1, CA2, and CA3 regions with varying doses of DS compared to the pure control and saline groups ($P \leq 0.01$). A significant decline was also determined in the average number of neurons as the dosages increased ($P \leq 0.01$). Gökçimen et al. [3] stated that the number of neurons in the hippocampus significantly decreased as a result of 1 mg/kg/day DS injection during pregnancy compared to the pure control group. Even a low dose of 3.6 mg/kg DS administered during the third trimester exhibiting a similar toxic effect is an easily anticipated result. Toxic effects seen with the low dose were also observed with the moderate and high doses of DS exposure. Diclofenac accumulation was reported in fetal tissue [6]. In addition, drugs in this category may cause skeletal-heart defects, a decrease in fetus numbers, and fetal growth retardation [59]. In this study, we observed a fall in the fetus numbers and also observed infant-maternal mortality during pregnancy and at the end of pregnancy. However, after birth, no morphological abnormalities were seen in the offspring.

In another study regarding DS, electron microscopic examination showed that DS exhibited neurotoxic effects on the group receiving it. DS was observed to cause extracellular edema and dose-dependent neurodegeneration and therefore to give rise to deleterious effects in cell organelles [32]. The underlying mechanism of the teratogenic effect of DS is still unclear. However, Ornoy et al. [60] suggested that this might be related to ROS and other components in the developing embryo. Another suggestion is that these drugs inhibit the synthesis of vasodilator prostaglandins and thus may cause cell death and malformation due to decreased blood support [50]. Another possible reason for increased cell loss at high doses may be decreased blood support. In the present study, in light microscopic examination of groups D and E, the neurons were pyramidal in shape and their nucleoli were distinct, and the borders of neuronal perikarya were regular. In contrast to the saline and control groups, examination of the groups receiving the drug revealed significant cell loss in the cell layers in the CA1, CA2, and CA3 regions. These groups also exhibited numerous peripherally located dark-stained basophilic neurons, neuroglial cell changes, and various structures resembling dead cell residues with unclear cell borders in the extracellular matrix. These structures were identified as necrotic areas in some sections. Chan et al. [50] reported that exposure to DS caused an increase in general embryonic malformations, including neural tube defects, craniofacial malformation, and deficient growth. The fact that no such malformations were observed in our study may be attributed to insufficient dose application or unknown reasons. On the other hand, we observed that the number of offspring from each animal of the drug-treated groups was less than in the control and saline groups. Fetuses that developed abnormally due to the high dose may have been eliminated in the mother's womb. Clancy et al. [41] showed that the duration of nervous system development varies depending on the pregnancy periods of humans, rats, and mice. Neuron numbers in the rat hippocampus reach their highest levels on days 15–17 of embryonic development. In the present study, the statistically significant difference in terms of cell number between the different dose groups (low-moderate-high) and the pure control group and also with the saline group suggested that DS has a toxic effect in the third trimester of pregnancy.

In conclusion, high, moderate, and low doses of DS exposure may create various problems in the developmental processes and cognitive activity of the brain. Hippocampal formation plays a key role in learning and memory. Information on the effects of DS in the third trimester is limited. Hippocampal development is quite critical in the third trimester of pregnancy, in which the number of synapses increases significantly. In this sense, it is likely that neuronal damage occurring during this period will lead to significant deficiencies in the formation of cognitive activity. These effects may be considered as teratogenic. They can be effective especially in the deterioration of social and spatial memory. Physiological and molecular studies involving the evaluation of brain plasticity are needed for a complete understanding of the effects in the CA1, CA2, and CA3 regions.

Conflict of interest statement

The authors state that they have no conflict of interest.

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