T.R.
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF HISTOLOGY AND EMBRYOLOGY

AN INVESTIGATION OF EFFECT OF GUM ARABIC IN HEALING OF NERVOUS TISSUE INJURY INDUCED BY DICLOFENAC SODIUM IN A RAT MODEL: A STEREOLOGICAL STUDY

Doctoral Thesis

Hosam Eldeen Elsadig Gasmalla MOHAMMED

Supervisor
Prof. Dr. Süleyman KAPLAN

SAMSUN
2020

T.R.

ONDOKUZ MAYIS UNIVERSITY

INSTITUTE OF GRADUATE EDUCATION

The thesis prepared by Hosam Eldeen Elsadig Gasmalla Mohammed entitled "An investigation of effect of Gum Arabic in healing of nervous tissue injury induced by diclofenac sodium in a rat model: a stereological study" has been supervised by Prof. Dr. Süleyman Kaplan and accepted by the committee members as PhD thesis of the Department of Histology and Embryology, following the examination on the date ----/----/2020.

Chairman: Prof. Süleyman Kaplan, Ondokuz Mayis University

Member: Prof. Dr. Mustafa Ayyıldız ,Ondokuz Mayis University

Member: Prof. Dr. Abdurrahman Aksoy, Ondokuz Mayis University

Member: Prof. Dr. Aydin Him, Bolu Abant İzet Baysal Üniversitesi

Member: Prof. .Dr. Berrin Zuhal Altunkaynak, Okan Üniversitesi

CONFIRMATION

This thesis has been approved by the committee members that already stated above and determined by the Institute Executive Board.

…. / …. /.....

Prof. Ali BOLAT

Head of Institute of Graduate Studies

ÖZET

RAT MODELİNDE DİKLOFENAK SODYUM İLE OLUŞTURULAN SİNİR DOKUSU HASARINDA ARAP ZAMKININ İYİLEŞTİRİCİ ETKİSİNİN ARAŞTIRILMASI: STEREOLOJİK BİR ÇALIŞMA

Hosam Eldeen Elsadig Gasmalla MOHAMMED

Ondokuz Mayıs Üniversitesi

Lisansüstü Eğitim Enstitüsü

Histoloji ve Embriyoloji Ana Bilim Dalı,

Doktora, Temmuz/2020

Danışman: Prof. Dr. Süleyman KAPLAN

Bu çalışmanın amacı, bir sıçan modelinde diklofenak sodyumun neden olduğu sinir dokusu hasarının iyileşmesinde Arap Zamkının etkisini stereolojik teknikler kullanılarak araştırmaktır. 40 adet 12 haftalık erkek Wistar albino sıçan kullanıldı ve her biri on fareden oluşan dört gruba ayrıldı. Kontrol grubu haricinde diğer gruplara 3.6 mg / kg diklofenak sodyum (DS grubu), Arap Zamkı (GA grubu, 7.5 g / kg) ve diklofenak sodyum + Arap Zamkı (DS + GA), 15 gün boyunca oral gavaj yoluyla verilmiştir. Beyinler histolojik ve stereolojik analizler için kafatasından çıkarıldı. Biyokimyasal analiz için kan örnekleri alındı. DS'ye ve GA maruz kalan hipokampustaki piramidal hücre sayımları, kontrol grubu ve diğer gruplarla kıyaslandı ($p < 0.05$).

Çalışmada DS'nin oral gavaj ile uygulandığında erişkin hipokampus yapısı ve fonksiyonu üzerinde zararlı bir etkisinin olmadığı gözlendi. İki davranış testi gerçekleştirildi: özel hafıza ve öğrenme için Morris Water Maze (MWM) testi, akılda tutma için pasif kaçınma testi. Sonuçlar anlamlı bir fark göstermemekle birlikte, kontrol grubunda, DS grubuna göre daha iyi performans eğilimi gözlendi. Biyokimyasal araştırmalar. Tüm grupların antioksidan seviyeleri karşılaştırıldığında gruplar arasında bir farklılık olmadı bulundu. Bilindiği üzere GA'nın hipokampusun piramidal hücreleri üzerindeki etkisi, stereolojik teknikler kullanılarak incelemesi daha önce yapılmamıştır. Çalışmamızda kantitatif ve kalitatif teknikler hipokampus morfolojisini değerlendirmek için kullanıldı. Kantitatif sonuçlarımız gruplar arasında bir farkın olmadığını, kalitatif sonuçlarımız ise DS'nin ve GA'nın hipokampusun piramidal hücreleri üzerinde toksik etki yaptığını; GA, DS ile birlikte kullanıldığında piramidal hücrelerin morfolojisini koruduğu gözlendi.

Anahtar Sözcükler: hipokampus, diklofenak sodyum, arap zamkı, stereoloji, oksidatif stress.

ABSTRACT

AN INVESTIGATION OF EFFECT OF GUM ARABIC IN HEALING OF NERVOUS TISSUE INJURY INDUCED BY DICLOFENAC SODIUM IN A RAT MODEL: A STEREOLOGICAL STUDY

Hosam Eldeen Elsadig Gasmalla MOHAMMED

Ondokuz Mayıs University

Institute of Graduate Studies

Department of Histology and Embryology

Doctorate, July/2020

Supervisor: Prof. Dr. Süleyman KAPLAN

The aim of this study is to investigate the effect of Gum Arabic in the healing of nervous tissue injury induced by diclofenac sodium in a rat model, using stereological techniques. Forty male 12-week old Wistar albino rats were used, they were divided into four groups (each consisted of ten rats), in addition to the control group, the other groups received diclofenac sodium 3.6 mg/kg (DS group), Gum Arabic (GA group, 7.5 g/kg), and diclofenac sodium + Gum Arabic (DS+GA), all by oral gavage for 15 days, the brains were removed from the cranium for histological and stereological analyses. Blood samples were taken for biochemical analysis, pyramidal cell count in the hippocampus exposed to DS and GA were compared to the Cont and other groups ($p < 0.05$).

The study found that DS has no harmful effects on the adult hippocampal structure and function when administered by oral gavage. Two behavioural tests were performed, Morris Water Maze (MWM) test for spatial memory and learning and passive avoidance test for memory retention. The results show no significant difference. However, there was a tendency for better performance in the control group than the DS group. Biochemical investigations showed no change in antioxidants levels between the groups. To our knowledge, there is no study using stereological techniques to investigate the effect of GA on the pyramidal cells of the hippocampus. In this study, we used quantitative and qualitative techniques for the evaluation of hippocampal morphology. There is no significant difference between groups in terms of pyramidal cell counting. However, our qualitative analysis showed a toxic effect of DS and GA on the pyramidal cells of the hippocampus; if GA is simultaneously used with DS, it shows a protective effect on the pyramidal cells.

Keywords: hippocampus, diclofenac sodium, gum Arabic, stereology, oxidative stress.

ACKNOWLEDGMENT

First and foremost, I would like to thank Allah Almighty for providing the strength and support to undertake this research project and to persevere and complete.

I would like to express my sincere gratitude and appreciation to my supervisor Professor Dr. Süleyman Kaplan, who encouraged me throughout my PhD journey with his continuous and everlasting support and advice. I have gained from him the attitude and the scientific behaviour as well as the scientific mind of searching and finding the answers to our questions in life.

I express my most honest and robust gratitude to my colleague Dr. Ömür Gülsüm Deniz, Dr. Kıymet Kübra Yurt, and Dr. Elfide Gizem Kivrak, for their full cooperation and support. Many thanks to everybody in the Department of Histology and Embryology for their help and support.

A special thank is due to Res. Assist. Gamze Altun, for her constant support, precious advice, and kind cooperation and unlimited support, she had helped and guided me from the beginning to the end of my thesis.

I would like to express gratitude faculty members of Histology and Embryology department for their help and contribution to my PhD education. My appreciation also extends to the department staff: secretary Ayşegül Sakallı and technician Hüseyin Sarıcaoğlu.

An honest thanks and appreciation for Res. Assist. Bahar Akyüz for her help in the behavioural tests performed to the experimental rats, and for Sercan Ince for his great help in dealing with the experimental rats.

Sincere gratitude to my friend Fathelrahman Idris Gasmelseed Ali, for his support and continuous advice throughout the course of my PhD experiment and thesis writing.

I would like to thank with profound gratitude my mother, Dr. Hind and my father, Dr. Elsadig, my brothers, and my sister, for their unquestionable and unlimited encouragement and support.

CONTENTS

ÖZET iii

ABSTRACT iv

CONTENTS vi

ABBREVIATIONS x

1. INTRODUCTION 1

2. GENERAL INFORMATION 11

2.1. Nonsteroidal Anti-inflammatory Drugs 11

2.1.1. Overview 11

2.1.2. Epidemiology 11

2.1.3. Mechanism of Action 11

2.1.4. The Use of Non-steroidal Anti-inflammatory Drugs in Neurological Diseases 12

2.2. Diclofenac 13

2.2.1. Neurological Side Effects 13

2.2.2. Diclofenac and The Nervous System 13

2.3. Hippocampus 14

2.3.1. Hippocampal Formation (HF) 14

2.3.2. The Structure of the Hippocampus Proper 15

2.3.3. Functions and Connections 17

2.3.4. Adult Hippocampal Neurogenesis 18

2.4. Diclofenac and Hippocampus 19

2.4.1. Histological Investigations 19

2.4.2. Studies Related to Pain Processing 20

2.4.3. Possible Neuroprotective Effects of Diclofenac on Hippocampus 21

2.4.4. Other Studies 22

2.5. Gum Arabic 24

2.5.1. General Uses of Gum Arabic 24

2.5.2. Gum Arabic in Medicine 24

2.6. Oxidative Stress and Antioxidants 25

2.6.1. Overview 25

2.6.2. Oxidative Stress: The Hippocampus is Vulnerable 26

2.6.3. Toxicity of Diclofenac is Associated/Induced by Oxidative Stress 27

2.6.4. Antioxidants Protect the Hippocampus....28
2.6.5. Antioxidants Reduce Toxicity of Diclofenac....29
2.6.6. Gum Arabic as an Antioxidant....30
2.7. Stereology....32
2.7.1. Range of total number of pyramidal cells in hippocampus obtained by stereological methods....32
2.7.2. Detecting changes in the total number of pyramidal cells in hippocampus obtained by stereological methods....34
2.8. Justification....34
2.8.1. Why Choosing Diclofenac?....34
2.8.2. Why Choosing The Central Nervous System?....35
2.8.3. Why Choosing Hippocampus?....35
2.8.4. Why Choosing Gum Arabic?....35
2.8.5. Why Administering Gum Arabic With Diclofenac Simultaneously?....36
2.8.6. Why Stereology?....36
2.9. Objectives of The Study....36
2.9.1. General Objective....36
2.9.2. Specific Objectives....36
3. MATERIALS AND METHODS....37
3.1. Experimental Animals....37
3.2. Formation of Experimental Animal Groups....37
3.3. Materials....39
3.3.1. Diclofenac Sodium....39
3.3.2. Gum Arabic....39
3.4. Tissue Preparation and Histological Procedures....39
3.4.1. Perfusion....39
3.4.2. Routine Histological Tissue Processing....40
3.4.3. Sectioning Methods....41
3.4.4. Staining Process....43
3.4.5. Tissue Preparation for Electron Microscopy....44
3.5. Stereological Analysis....45
3.5.1. Defining Anatomical Area....45

3.5.2. The Numerical Density of The Pyramidal Cells 46
3.5.3. The Volume of The Hippocampus 49
3.5.4. The Estimation of The Total Number of the Pyramidal Cells 49
3.5.5. The Coefficient of Variance and The Coefficient of Error 49
3.6. Biochemical Methods 51
3.6.1. Blood Sample Collection 51
3.6.2. Analysis of Superoxide Dismutase Enzyme Activity 52
3.6.3. Analysis of Catalase Enzyme Activity 53
3.7. Determination of Total Phenolic Content and Antioxidant Power 54
3.7.1. Preparation of Sample Extract 54
3.7.2. Method to Assess the Total Phenolic Content 54
3.7.3. Methods to Assess the Antioxidant Power 55
3.8. Behavioural Examinations 55
3.8.1. Morris Water Maze test 55
3.8.2. Passive Avoidance Test 57
3.9. Statistical Analysis 58
4. RESULTS 59
4.1. Behavioural tests 59
4.1.1. Morris water maze test 59
4.1.2. Passive avoidance learning test 61
4.2. Stereological Findings 62
4.2.1. The Total Number of Pyramidal Cells in Hippocampus 62
4.2.2. The Total Volume of the Hippocampus 66
4.3. Analysis of Enzyme's Activities 68
4.3.1. Analysis of Catalase Enzyme Activity 68
4.3.2. Analysis of Superoxide Dismutase Enzyme Activity 69
4.4. Light Microscopic Findings 70
4.4.1. Light Microscopic Findings of Cresyl Violet Stained Sections 70
4.4.2. Light Microscopic Findings by Toluidine Blue Stained Sections 74
4.5. Electron Microscopic Findings 114
5. DISCUSSION 143
6. CONCLUSIONS AND RECOMMENDATIONS 150

6.1. Conclusions.....150
6.2. Recommendations.....151
REFERENCES.....152
APPENDICES.....164
Ethical Committee Approval Certificate.....164
CURRICULUM VITAE.....165

ABBREVIATIONS

AD : Alzheimer's diseases

BDNF : Brain-derived neurotrophic factor

COX-1 : Cyclooxygenase 1 enzyme

COX-2 : Cyclooxygenase 2 enzyme

CA : Cornu Ammonis

CA1 : Cornu Ammonis1

CA2 : Cornu Ammonis2

CA3 : Cornu Ammonis3

CAT : Catalase

CE : Coefficient of error

CNS : Central nervous system

CV : Coefficient of variance

DS : Diclofenac sodium

GA : Gum Arabic

GPx : Glutathione peroxidase

GSH : Glutathione

H_2O_2 : Hydrogen peroxide

MWM : Morris water maze

NGF : Nerve growth factor

NSAIDs : Non-steroidal anti-inflammatory drugs

OS : Oxidative stress

ROS : Reactive oxygen species

SOD : Superoxide dismutase

1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are synthetic products of salicylic and acetylsalicylic acids. Diclofenac belongs explicitly to the phenylacetic acid derivatives (2-[2,6-dichloranilino] phenylacetic acid). It is an anti-inflammatory, analgesic, and antipyretic drug. Voltaren, DS is a slow-release tablet that was developed in 1974 in Japan for management of the pain of rheumatoid arthritis and osteoarthritis (Gan, 2010).

It is an accepted suggestion now that the anti-inflammatory action of NSAIDs is due to the inhibition of cyclooxygenase enzymes (COX-1) and (COX-2). These enzymes are derived from arachidonic acid that presents in the cell membrane. They convert arachidonic acid into endoperoxides (prostaglandin, prostacyclin, and thromboxane). In turn, endoperoxides play essential roles in mediating pain, fever, and inflammation. COX-1 is involved in physiological processes. It is expressed in platelets and involved in platelet aggregation, regulation of renal blood flow, and protection of gastric mucosa while COX-2 is involved in the inflammatory process (Atzeni et al., 2018).

COX-2 is present in joints and muscles. Thus, NSAIDs are used extensively for conditions like osteoarthritis, rheumatoid arthritis, back pain, and ankylosing spondylitis. Unlike COX-1 (which is found constitutively), COX-2 is inducible. However, it is known to be found constitutively in parts of the CNS, namely: the neurons of the cerebral cortex, the pyramidal and granular cells of the hippocampus, the granule cells of the dentate gyrus (DG) and cerebellum, the amygdaloid nucleus and the spinal cord and at lower levels in the thalamus and hypothalamus, the constitutive presence of COX-2 strongly suggests that it is not involved only in inflammation, but it plays a physiological role in normal synaptic functions. (Minghetti, 2004; Strauss, 2008).

NSAIDs are categorised into selective to COX-2 and non-selective. Non-selective NSAIDs are known to cause serious side effects on the gastrointestinal tract derived from inhibiting COX-1, that led to the development of selective COX-2 inhibitors, were they also showed a toxic effect on the cardiovascular system (Ong et al., 2007). Although diclofenac is non-selective, it is reported that it has 4-fold higher selectivity for COX-2 than for COX-1 (Warner et al., 1999; Gan, 2010).

Diclofenac has many effects on the nervous system; it inhibits both growth and differentiation of the neural stem cells (Kudo et al., 2003). In another study, it was found that it has led to growth inhibition, but not differentiation of neurons. In a study conducted using PC12 cells derived from rat pheochromocytoma which is used as a model for neuronal differentiation, cell proliferation was evaluated by using XTT assay, the toxicity of the drug is presented (Rajabalian et al., 2009). The development of neurons and the volume of the cervical spinal cord were negatively affected following the prenatal administration of DS (Ozyurt et al., 2011). Also, the spinal cord suffered degeneration following intrathecal administration of DS (Ozdogan et al., 2011). There were morphological and histopathological changes on the optic nerve following prenatal administration of diclofenac (Kaplan et al., 2013). The number and the diameter of sciatic nerve axons were reduced in rats that were prenatally exposed to DS (Keskin et al., 2015). In the cerebellum, prenatal administration of DS has no side effect on the granule cells in the male rats (EkİCİ et al., 2012). However, Purkinje cells were reduced after DS exposure (Ragbetli et al., 2007). On the other hand, it was found that the number of Purkinje cells in the cerebellum were increased after prenatal exposure of DS at the 20-week-old rats (Odaci et al., 2010).

The hippocampal formation (HF) is a part of the limbic system. It consists of the hippocampus in addition to the DG, the subicular complex, and the entorhinal cortex. The division of hippocampal regions is based on microscopic structure (in neuroanatomical studies) or in macroscopic features (in MRI studies) (Adler et al., 2014). It is essential for memory, spatial navigation, emotions, and pain processing (Sadeghi et al., 2019).

The hippocampus (hippocampus proper, Cornu Ammonis (CA) or Ammon's horn, also means seahorse) is located in the temporal lobe and bounds the lateral ventricle by forming the floor of its inferior horn. It expands anteriorly, posteriorly it is curved upwards to continue with the crus of the fornix and ends inferior to the splenium of the corpus callosum (Snell, 2010). The arrangement of the layers from the cavity of the lateral ventricle and onwards is as following the alveus, oriens, pyramidale, radiatum, lacunosum, and moleculare (Duvernoy, 2013).

The alveus lies on the intraventricular surface of the hippocampus. It is composed of efferent axons of the neurons of the hippocampus and subiculum. Its axons run into the fimbria. The stratum oriens consists of few scattered basket cells, the axons of the pyramidal cells cross this layer on their way to the alveus, the stratum pyramidale contains the pyramidal cells, this layer is further divided into CA1, CA2, and CA3. In the CA1 region, the cells are medium in size while cells of the CA3 region are large. Concerning density, cells in CA1 are crowded densely more than those of CA3 (Bayer, 1985; El Falougy et al., 2008). Pyramidal cells in CA1 are more vulnerable than those of CA3, in terms of damage due to oxidative stress (OS), superoxide anion is produced in CA1 at higher levels than its production in CA3 (Wang and Michaelis, 2010). Furthermore, CA1 cells have a lower amount of mRNA as well as protein expression of the gene for SOD than CA3 (Lister et al., 2005), in diabetes mellitus, CA1 is also sensitive more than CA3 to neuronal damage following induced diabetes mellites (Zhao et al., 2016), the same goes for prenatal malnutrition (Lister et al., 2005).

Histological investigations on the hippocampus revealed a decrease in the number of neurons associated with prenatal administration of diclofenac. Offspring of Wistar albino rats were arranged into two groups 4-weeks-old and 20-weeks-old after prenatal administration of DS (1mg/kg for intraperitoneal administration 15 days) to their mothers. The 20-weeks-old group showed a decrease in the number of pyramidal cells. Also, there was a decrease in the number of granule cells in the DG in the 4-weeks-old group. At the same time, there was an increase of granular cells in the 20-weeks-old group; the later results is because granular cells in the DG proliferate mostly postnatally, which indicates the possible role of diclofenac in increasing their rate of proliferation (Gokcimen et al., 2007). In a study to investigate the effect of the dose of diclofenac on the hippocampus, different doses were administered prenatally (3.9, 9 and 18 mg/kg, intraperitoneal administration for 7 days) in the first trimester. The decrease in the number of neurons was found to be dose-dependent (Elibol et al., 2014). The same results were obtained when diclofenac was administered to rats postoperatively. The administration was through intramuscular injection for 7 days (15 mg/kg). The experiment was conducted on rats with acute subdural hematoma (ASDH) (Turkmen et al., 2016).

Diclofenac was administered to Wistar rats (12.5 mg/kg intraperitoneal administration for 3 days), the results on the nervous system were manifested in the cerebral cortex as well as the cerebellum as the presence of balloonized (damaged) red neurons, the phenomena was also (but less observed) detected in the hippocampus (Ilic et al., 2011).

Massive bowel resection was performed to Wistar albino rats; the purpose was to investigate the protective role of an anti-ulcer agent (stable gastric pentadecapeptide BPC 157) against diclofenac toxicity after major surgeries. Thus, diclofenac was administered intraperitoneally after surgery (once, 12 mg/kg). In the group treated with diclofenac, lesions were worse than the group of surgery alone or the group that is treated by stable gastric pentadecapeptide BPC 157. Among other organs, lesions were reported in the hippocampal neurons (most affected), cerebellar nuclei and the Purkinje cells (least affected). Histological investigation of hippocampal neurons showed ballooned oedematous cells (Lojo et al., 2016).

Gum Arabic (GA) is an adhesive exudate from the stems and branches of a tropical plant called *Acacia senegal*. Sudan has been a leading country that produces GA in the 1960s, civil war and political instability changed this for the current time (Williams and Phillips, 2009). Medicinal uses of GA are many, in the renal system; it was administered orally for three months to patients with chronic renal failure in Sudan (50 g/day). There was a significant decrease in levels of concentration of serum creatinine, urea, phosphate and uric acid concentrations (Ali et al., 2008). Also, serum urea nitrogen was significantly decreased in patients with chronic renal failure received 50g/day of GA (Bliss et al., 1996). In the intestine, adding GA to Oral Rehydration Solutions (ORS) enhanced sodium absorption in rats guts (Wapnir et al., 1996). Absorption of sodium, potassium, and water was increased in rats with diarrhoea treated with ORS and GA (Wapnir et al., 1997; Teichberg et al., 1999), GA also enhanced calcium absorption in the intestine in an experiment conducted on rats (Kawase et al., 2007). In dental medicine, as an in vitro study showed, GA improved the remineralisation process of caries-like enamel lesions (Onishi et al., 2008).

Considering that diclofenac can harm on the nervous system through OS, the underlying molecular basis in which diclofenac can induce apoptosis is not fully understood and yet to be studied (Yurt et al., 2018a). The hippocampus, in particular, is vulnerable to OS in many ways. First of all, it is well known that neurons in the central/peripheral nervous system have consumed oxygen more than other tissues, ten folds more. Some regions of the brain (including the hippocampus) produce free oxygen radicals in higher rates than other regions. Thus, making them susceptible to suffer more from the OS. This is attributable to the high amount of polyunsaturated fatty acids (PUFAs) in the nervous tissue, such as arachidonic acid (AA). The AA is the main constituent of the cell membranes of neurons, and it is released upon tissue injuries. Metabolism of AA by cyclooxygenases produces proinflammatory mediators which in turn lead to OS and damage (Huang et al., 2012).

On the other hand, the expression of antioxidant enzymes in the brain is lower in the mitochondria than that of the cytosol, although the mitochondria are more vulnerable to OS. Some receptors of proinflammatory cytokines are present in pyramidal cell layer CA1, and CA3 of the hippocampus; in the interleukin-1 beta (IL-1β) is involved in memory functions by reducing the release of the necessary neurotransmitters (Matsumoto et al., 2004). OS induced by sleep deprivation in mice has led to lower performance in the passive avoidance test. However, antioxidants such as N-tert-butyl-a-phenylnitrone, vitamin E, and melatonin successfully yielded better performance in mice treated by those substances (Silva et al., 2004). OS associated with induced status epilepticus can lead to the death of neurons in the hippocampus as shown histologically in rats especially in the CA3 region of the hippocampus (Liu et al., 2010).

The hippocampus is responsive to the protective roles of substances with antioxidant properties. In a study on the mice with docetaxel-induced neurotoxicity, melatonin and selenium as antioxidants play an essential role in minimising hippocampal injury following OS. The proposed mechanism is the suppression of the production of reactive oxygen species (ROS) in the mitochondria (Ataizi et al., 2019). Grape seed polyphenols were proven as an antioxidant of benefit against age-related OS and the decrease of the number of neurons in the hippocampus (CA1 region) of male Wistar rats.

This positive effect was also shown using the optical fractionator technique, which revealed the effectiveness of this antioxidant against neuronal loss (Abhijit et al., 2018). Green tea mega EGCG^{TM} as an antioxidant was also beneficial against OS produced by electromagnetic radiation in the hippocampus of Wistar albino rats. In this study, the rats were exposed to radiation for three months. The administration of green tea was at the same time during their exposure (Ahmed et al., 2017).

Also, Boswellia sacra and folic acid as antioxidants were beneficial against OS produced by electromagnetic radiation in the hippocampus. The animals were exposed to 900-MHz radiation for an hour daily for a period of 21 days. This histological study reported a reduction in the number of cells in the hippocampus of rats that were exposed to radiation; antioxidant substances attenuated this damage (Kivrak et al., 2017). In another study, the effect of melatonin against OS in the hippocampus of Wistar rats exposed to the electromagnetic field was investigated; the results show a protective effect of melatonin (Erdem Koc et al., 2016). Dendropanax morbifera Léveille leaf extract has proven as an antioxidant of benefit to the hippocampus against OS produced by mercury, as concluded in a study on Sprague–Dawley rats. A dose of 5 μg/kg administered intraperitoneally with dendropanax morbifera Léveille leaf extract administered orally (100 mg/kg), both administered daily for four weeks (Kim et al., 2015). In a study to examine the antioxidant properties of vitamin E, the study was conducted on the hippocampus of Sprague-Dawley diabetic rats, it was found to be effective against OS associated with diabetes mellitus, although the study indicated that grape seed extracts (which are used along with vitamin E in the study) are more effective (Yonguc et al., 2015). An investigation of the antioxidant effect of black garlic extract against the OS of monosodium glutamate was done. The Morris Water Maze (MWM) test results revealed that spatial memory was affected harmfully by monosodium glutamate, in contrast, black garlic extract showed promising results considering prevention of these harmful effects (Hermawati et al., 2015). The effect of ascorbic acid (as an antioxidant) on hippocampus was investigated. Spectrophotometric methods were applied to measure the enzymatic activities in Wistar rats treated with ascorbic acid. The purpose was to assess the antioxidant activity against OS associated with pilocarpine-induced seizers. The antioxidant activity has decreased lipid peroxidation and

elevated the activities of both superoxide dismutase (SOD) and catalase (CAT) in the hippocampus (Santos et al., 2009). A study using in-vitro hippocampal cells (both from human and rats) concluded that dehydroepiandrosterone (having antioxidant properties) had protected effect on the hippocampus against OS induced by hydrogen peroxide (H_2O_2) and sodium nitroprusside (Bastianetto et al., 1999).

It has been suggested that the antioxidant properties of exogenous melatonin could act as a protector with regards to DS neurotoxicity (Aygun et al., 2012). Melatonin has a protective effect on the kidney in rats prenatally administered diclofenac (Khoshvakhti et al., 2015). B27 supplement as an antioxidant is shown to reduce neurotoxicity induced by OS derived by diclofenac (Rajabalian et al., 2009). The latter study was explicitly about the hippocampus, investigating the effect of OS induced by diclofenac and minimising it by administering an antioxidant substance.

GA is considered as an antioxidant (Trommer and Neubert, 2005; Marwah et al., 2007). It is suggested that it acts mainly as ROS neutraliser (Al-Yahya et al., 2009). The antioxidant capacity of GA is attributed to its components of amino acids tyrosine, histidine, and methionine (Rodrigues et al., 2012). In the kidneys, rats with adenine-induced chronic renal failure were treated with GA 15%, w/v, for 4 weeks, which led to improvement due to the anti-inflammatory and antioxidant properties of GA (Ali et al., 2014). The antioxidant properties of GA were shown to reduce adenine–induced chronic renal failure (Ali et al., 2013), and mercuric chloride-induced nephrotoxicity (Gado and Aldahmash, 2013). Oral administration of GA (7.5 g 100 ml for eight days) significantly reduced gentamicin-induced lipid peroxidation (Al-Majed et al., 2002). The dose of (7.5 mg/kg for five days) significantly reduced cisplatin-induced nephrotoxicity (Al-Majed et al., 2003). Urinary bladder cytotoxicity was induced by cyclophosphamide in male Swiss albino rats, in an experiment to investigate the antioxidant properties of GA, the dose was (7.5 gm/kg/day) in drinking water. GA treated group showed marked improvement, bladder inflammation and oedema were reduced, and bladder histology was almost restored to normal, GSH bladder contents were restored as well (Al-Yahya et al., 2009). In the liver, oral administration of GA was shown to attenuate acetaminophen-induced hepatotoxicity in mice by reducing OS (Gamal el-din et al., 2003). In diabetic rats, hepatic lipid peroxidation

was attenuated, and the levels of SOD, GSH peroxidase and CAT were elevated (Ahmed et al., 2015). In the heart, GA antioxidant properties were considered as a possible mechanism of protection against doxorubicin-induced cardiotoxicity in mice (Abd-Allah et al., 2002). In the gonads, GA significantly reduced lipid peroxidation and enhanced testicular antioxidant enzymes activities in diabetic rats (Fedail et al., 2016). This substance reduces lipid peroxidation and significantly increased the activities of ovarian SOD, GSH peroxidase, and CAT in mice (Ahmed et al., 2016). Administration of GA at a dose of 30g orally, daily for 12 weeks, for 46 patients with sickle cell anaemia has shown to be beneficial in minimising OS (Kaddam et al., 2017). However, it is reported that oral administration of GA in the drinking water for rats at a concentration of 2.5, 5.0 or 10.0% w/v for eight days did not lead to any significant change in the concentrations of antioxidants like GSH and superoxide dismutase in the kidney or liver (Ali, 2004).

To our knowledge, stereological studies investigating the effect of GA on the histology of the nervous tissues are scanty. This study aimed to apply stereological techniques for quantifying the role of GA in the healing of nervous tissue injury induced by diclofenac, in a rat model.

Obtaining quantitative data with statistical reliability is one of the skeletons of research. The term "stereology" describes a variety of methods that provide a three-dimensional description from two-dimensional images. It was first presented in 1961 by Hans Elias at a scientific meeting held in Germany (Miki et al., 2005). The disector principle, one of the efficient stereological techniques was introduced by (Sterio, 1984), it is a probe that provides a defined three-dimension volume for particle counting (Gundersen, 1986), while the fractionator is a sampling strategy that can be based on systematic sampling and the disector counting technique (Gundersen et al., 1988). Thus, stereology is a science of estimating three-dimensional information from two-dimensional images based on mathematical and statistical approaches. It is the finest tool owing to its characteristics: its unbiasedness is achieved by the systematic random sampling, its independence of shape, size or orientation, of particles is achieved by its three-dimensional designs of the probes (West et al., 1991). The data obtained by these techniques could be volume, surface area, or quantity of particles (Yurt et al., 2018b).

Investigating the effect of antioxidants on the hippocampus that was exposed to some toxic substance or stressful condition was evaluated by stereological techniques. Physical disector counting technique was used to estimate the total number of pyramidal cells in the hippocampus of rats exposed to monosodium glutamate, which leads to OS and black garlic extract, which has antioxidant properties. This stereological study revealed that the CA1 region of the hippocampus is more affected by the combined administration of monosodium glutamate and black garlic extract. The MWM test results revealed that spatial memory was affected harmfully by monosodium glutamate exposure. In contrast, black garlic extract showed promising results considering the prevention of these harmful effects (Hermawati et al., 2015). In another study, the optical fractionator counting technique was used to investigate the effect of melatonin against OS in the hippocampus of Wistar rats exposed to the electromagnetic field. The results showed a protective effect of melatonin (Erdem Koc et al., 2016). Optical fractionator counting technique was used in another study that related to the hippocampus. In this study, it was shown that polyphenols of grape seed have an antioxidant effect against age-related OS and neuronal loss in the CA1 region of the hippocampus of male Wistar rats (Abhijit et al., 2018).

We have to justify why such a study was selected. The reasons for this study are given below: first, NSAIDs are one of the most common medications used worldwide and diclofenac is also one of the most common NSAIDs used. And it is also found to be four times more selective for COX-2 than COX-1, and COX 2 is found constitutively in the hippocampus, that is why diclofenac is selected in this study.

Second, OS is a strongly suggested mechanism of diclofenac toxicity. The level of antioxidant enzymes in nervous tissues is relatively lower in comparison to other tissue (Yurt and Kaplan, 2018). This low level makes nervous tissue more vulnerable to OS. OS mediates neuroinflammation, which underlies the pathophysiological mechanism of diseases like schizophrenia and Alzheimer's diseases (AD) (Popa-Wagner et al., 2013). This relationship between diclofenac, OS and central nervous system (CNS) looks essential to be studied.

Third, this study is a histological and stereological study. There are few studies investigating the histological effects of diclofenac on nervous tissue.

Fourth, COX-2 is found constitutively in the hippocampus, some regions of the brain (including the hippocampus) contain relatively more oxidisable lipids and produce free oxygen radicals in higher rates than other regions, thus making them more likely to suffer from OS (Bellissimo et al., 2001; Aygun et al., 2012), which is an acceptable mechanism by diclofenac. After prenatal exposure of DS, it was found a substantial neurons loss in the hippocampus by means of stereological techniques.

Fifth, GA is known as an antioxidant; antioxidants are now considered significant in therapeutic strategy (Popa-Wagner et al., 2013). Previous studies have shown that neurotoxicity of diclofenac leads OS, the hippocampus is more vulnerable and affected by OS, it is also responsive to the protective roles of substances having antioxidant properties like GA that is herbal, natural and cheap. No studies found to investigate the effect of GA in reducing the neurotoxicity of diclofenac.

This study aims to investigate the protective role of GA in minimising damage in hippocampal tissue damage induced by diclofenac in adult Wistar rats by means of stereological, light and electron microscopic techniques, as well as biochemical analysis, and behavioural tests.

2. GENERAL INFORMATION

2.1. Nonsteroidal Anti-inflammatory Drugs

2.1.1. Overview

NSAIDs are synthetic products of salicylic and acetylsalicylic acids. Diclofenac belongs to phenylacetic acid derivatives (2-[2,6-dichloranilino] phenylacetic acid). It is used as an anti-inflammatory, analgesic, and antipyretic drug. Voltaren (DS) is a slow-release tablet that was developed in 1974 in Japan for the management of the pain of rheumatoid arthritis and osteoarthritis (Gan, 2010).

2.1.2. Epidemiology

Generally, NSAIDs are among the most commonly used drugs; more than 30 million people worldwide take NSAIDs daily (Singh, 2000). Diclofenac and ibuprofen comprise about 40% of global NSAIDs sales. Approximately 10–40% of people aged over 65 years are using NSAIDs daily (Conaghan, 2012; Cullen et al., 2000).

2.1.3. Mechanism of Action

It is an accepted suggestion now that the anti-inflammatory action of NSAIDs is due to the inhibition of COX-1 and COX-2.

COX-1 and COX-2 enzymes are derived from arachidonic acid that presents in the cell membrane. These enzymes convert arachidonic acid into endoperoxides (prostaglandin, prostacyclin, and thromboxane). Endoperoxides, in turn, play essential roles in mediating pain, fever, and inflammation. COX-1 is involved in physiological processes. It is expressed in platelets and involved in platelet aggregation, regulation of renal blood flow, and protection of gastric mucosa while COX-2 is involved in the inflammatory process (Atzeni et al., 2018).

COX-2 is found in joints and muscle (and thus used extensively for conditions like osteoarthritis, rheumatoid arthritis, back pain, and ankylosing spondylitis).

Unlike COX-1 (which is found constitutively), COX-2 is inducible, but it is known to be found constitutively in parts of the CNS, namely: the neurons of the cerebral cortex, the pyramidal and granular cells of the hippocampus, the granule cells of the dentate gyrus

and cerebellum, the Amygdaloid nucleus and the spinal cord and at lower levels in the thalamus and hypothalamus, the constitutive presence of COX-2 strongly suggests that it is not involved only in inflammation, but it plays a physiological role in normal synaptic functions (Minghetti, 2004; Strauss, 2008).

NSAIDs are categorized into selective to COX-2 and non-selective (**Figure 1**). Nonselective NSAIDs cause side effects on the gastrointestinal tract due to inhibition of COX-1. The selective COX-2 inhibitors, on the other hand, show a toxic effect on the cardiovascular system (Ong et al., 2007).

Although diclofenac is non-selective, it is reported that it has 4-folds higher selectivity for COX-2 than for COX-1 (Warner et al., 1999; Gan, 2010).

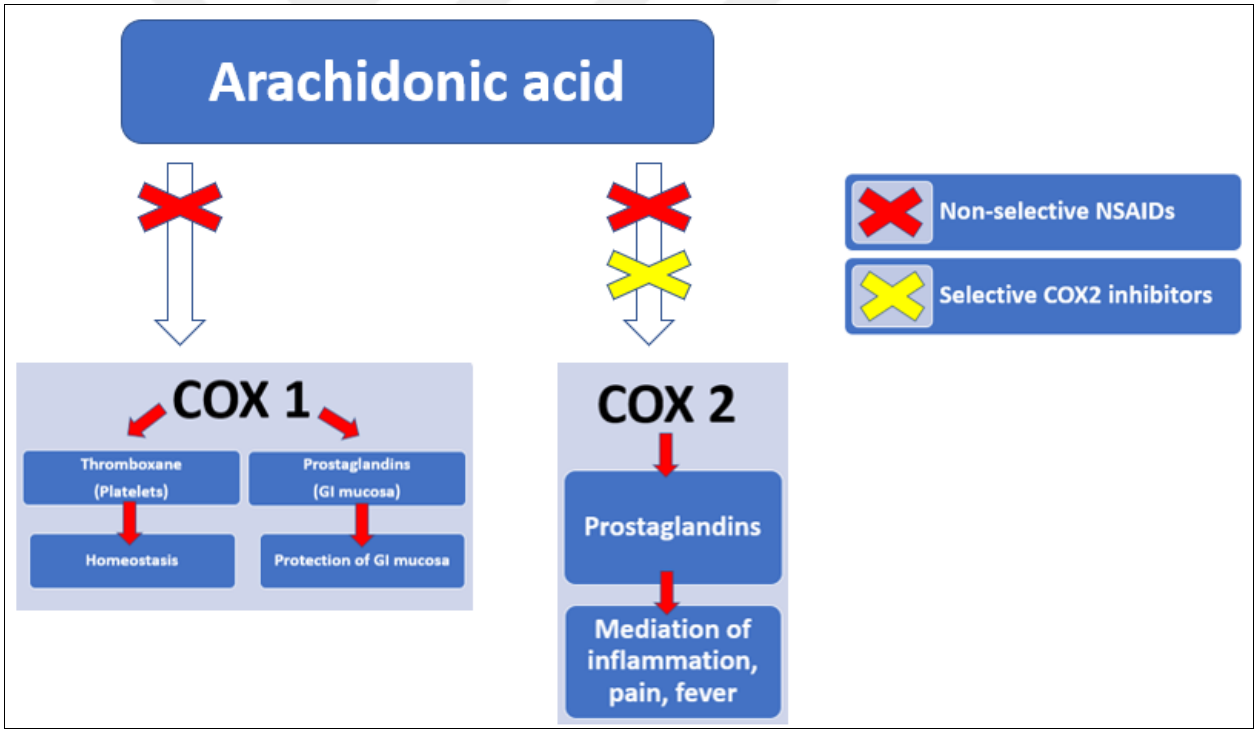


Figure 1. Mechanisms of action of NSAIDs

2.1.4. The Use of Non-steroidal Anti-inflammatory Drugs in Neurological Diseases

Reports are not conclusive concerning the use of NSAIDs in neurodegenerative and demyelinating diseases. Although few reports support the neuroprotective effects of

NSAIDs against AD, many reports conclude that they are not effective in delaying disease progression of the disease. Regarding Parkinson's disease, results were also not conclusive; some reports support that NSAIDs delay the progress of the disease. In contrast, others conclude no effect, for multiple sclerosis, indomethacin can increase endogenous remyelination (Terzi et al., 2018).

2.2. Diclofenac

2.2.1. Neurological Side Effects

The neurological side effects include headache, vertigo, insomnia, drowsiness, agitation, depression, irritability, anxiety, and dizziness (The European Agency for the Evaluation of Medicinal Products, 2003). Dizziness and headache are common side effects (about less than 10%). The side effects in some cases are the same as the complaints, yet the doctors used to prescribe it more often, and patients tend to use it without prescriptions.

2.2.2. Diclofenac and The Nervous System

Intraperitoneal administration of diclofenac decreases the concentration of pituitary β-endorphin and increases plasma β-endorphin concentrations (Sacerdote et al., 1985).

Diclofenac inhibits both growth and differentiation of the neural stem cells (Kudo et al., 2003). In another study, it has led to growth inhibition; however, not growth differentiation. In a study conducted using PC12 cells derived from rat pheochromocytoma which is used as a model for neuronal differentiation, cell proliferation was evaluated by using XTT assay, the toxicity of the drug is presented as suppression of growth (Rajabalian et al., 2009).

The development of neurons and the volume of the cervical spinal cord were negatively affected following the prenatal administration of DS to rats (Ozyurt et al., 2011). Also, the spinal cord suffered degeneration following intrathecal administration of diclofenac (Ozdogan et al., 2011)

In the cerebellum, prenatal administration of diclofenac has not affected granule cells in the male rats (EkİCİ et al., 2012). However, Purkinje cells were reduced (Ragbetli

et al., 2007). The number of Purkinje may increase after 20 weeks following postnatal life in female rats (Odaci et al., 2010).

The effect of prenatal administration of diclofenac on peripheral nerves is controversial, studies showed different results for different nerves (Yurt and Kaplan, 2018), in the optic nerve, there was no significant effect on the thickness of myelin sheath or the number of axons (Kaplan et al., 2013). However, other study showed that there was a defect in the structure of myelin sheath an a decrease in its thickness, but an increase in the number of axons in the same nerve (Colakoglu et al., 2014), in the sciatic nerve there was a decrease in the number of axons (Canan et al., 2008), in another study, the number and the diameter of sciatic nerve axons were reduced in rats by prenatal administration of diclofenac (Keskin et al., 2015). Moreover, there was no significant effect on the median nerve's number of axons, although the morphology was changed (Ayranci et al., 2013).

2.3. Hippocampus

2.3.1. Hippocampal Formation (HF)

The HF is part of the limbic system. It consists of the hippocampus in addition to the DG, the subicular complex, and the entorhinal cortex. The division of hippocampal regions can be based on microscopic structure (in neuroanatomical studies) or in macroscopic features (in MRI studies) (Adler et al., 2014), the HF is important for memory, spatial navigation, emotions, and pain processing (Sadeghi et al., 2019).

During the development of the brain, the formation of the neural tube and plate takes place in the third week. The prosencephalon (a primary brain vesicle) develops during the fourth week while the telencephalon (a secondary brain vesicle) develops during the fifth week. The hippocampal fissure on the medial surface of the temporal lobe appears by the 13^{th} week and folding takes place in the 15^{th} and 16^{th} week, the folding of the dentate gyrus and CA region into the temporal lobe continues, and by the 20^{th} week, the foetal hippocampus takes its final form (Moore et al., 2016; Sadler, 2011).

2.3.2. The Structure of the Hippocampus Proper

The hippocampus (hippocampus proper, Cornu Ammonis of Ammon's horn, also means seahorse) is located in the temporal lobe and bounds the lateral ventricle by forming the floor of its inferior horn. It is expanded anteriorly, posteriorly it is curved upwards to continue with the crus of the fornix and ends inferior to the splenium of the corpus callosum (Snell, 2010).

The arrangement of the layers from the cavity of the lateral ventricle and onwards is as following the alveus, oriens, pyramidale, radiatum, lacunosum, and moleculare (Duvernoy, 2013).

The alveus lies on the intraventricular surface of the hippocampus, it is composed of efferent axons of the neurons of the hippocampus and subiculum, the axons run into the fimbria, the stratum oriens consists of few scattered basket cells, the axons of the pyramidal cells cross this layer on their way to the alveus, the stratum pyramidale contains the pyramidal cells, this layer is further divided into CA1, CA2, and CA3 (**Figure 2**), in CA1 the cells are small to medium in size while those of CA3 are large, with regard to density, cells in CA1 are crowded densely more than those of CA3 (Bayer, 1985; El Falougy et al., 2008), (**Figures 3 and 4**), pyramidal cells in CA1 are more vulnerable than those of CA3, in terms of damage due to OS, superoxide anion is produced in CA1 at higher levels than its production in CA3 (Wang and Michaelis, 2010), furthermore, CA1 have lower amount of mRNA as well as protein expression of the gene for SOD than CA3 (Lister et al., 2005), in diabetes mellitus, CA1 is also sensitive more than CA3 to neuronal damage following induced diabetes mellites (Zhao et al., 2016), the same goes for prenatal malnutrition (Lister et al., 2005)

Authors consider CA4 as the part that lies within the curve of the DG. Cells there are few and scattered, it is functionally similar to CA3, the stratum radiatum consists of axons from pyramidal cells in addition to other connecting and commissural nerve fibers, those axons and fibers give the straight appearance of this layer.

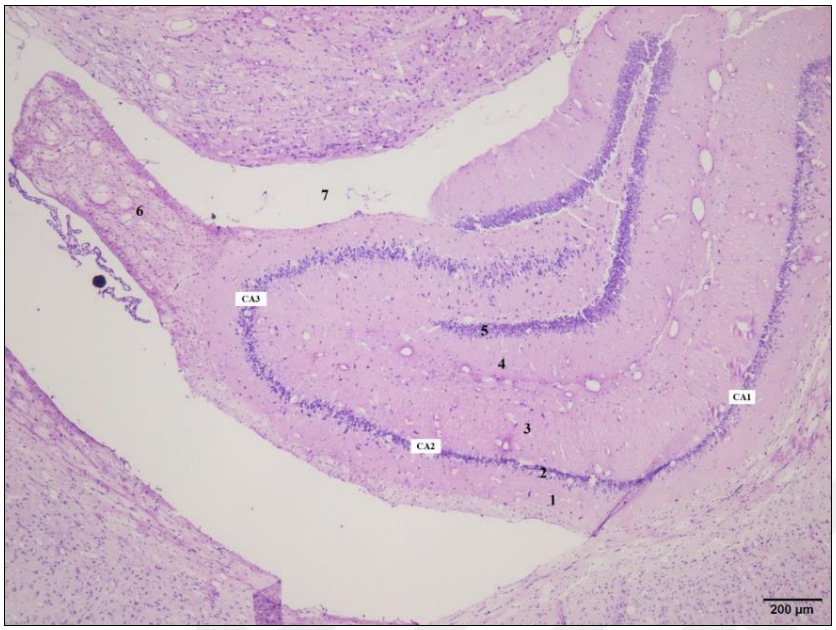


Figure 2. Representative low-magnification (X4) photomicrographs of hippocampal section taken from the rat brain that is processed for cresyl violet staining.

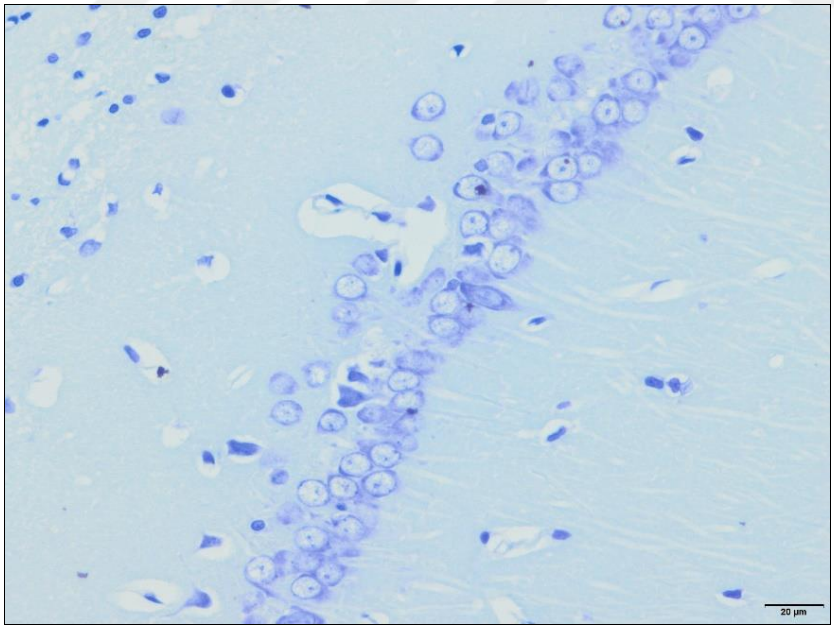


Figure 3. Representative medium-magnification (X40) photomicrograph of a hippocampal section taken from the rat brain shows pyramidal cells in the CA1 region

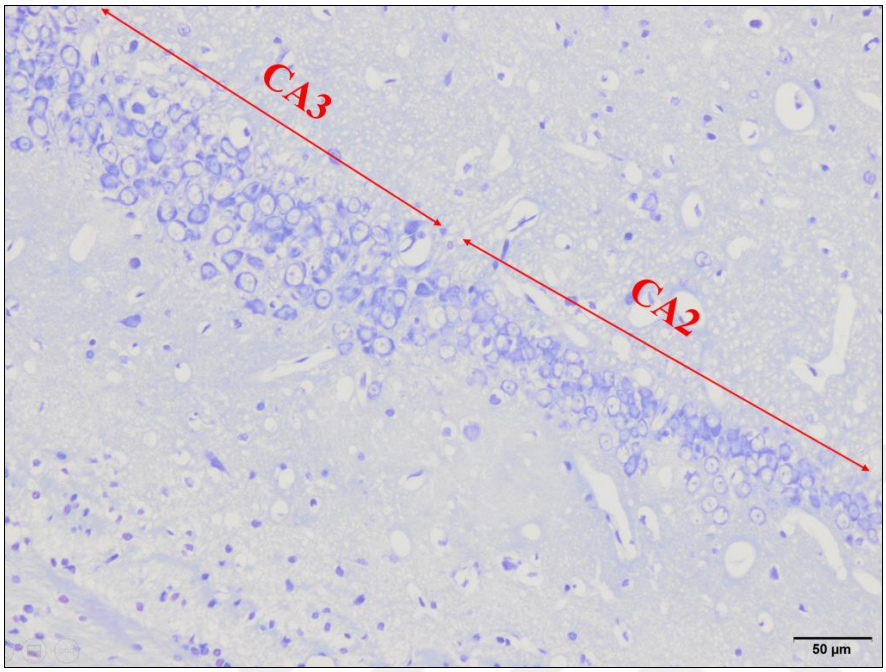


Figure 4. Representative low-magnification (X20) photomicrograph of a hippocampal section taken from the rat brain shows pyramidal cells in the CA2 and CA3

2.3.3. Functions and Connections

The HF is involved in functions related to memory, spatial learning, emotions, and pain processing. In 1957 the relation between HF and the memory was established, while the involvement of HF in navigation was described in1971 (Stella et al., 2012).

With regard to memory, the HF is involved in declarative memory: semantic, episodic and spatial memories, the semantic memory is the memory of concepts and facts, the episodic memory involves the memory of the events and the relations between them, the spatial memory is the memory of the location (Duvernoy, 2013).

Regarding spatial learning/memory, it was linked to the dorsal hippocampus (in rats) according to (Bannerman et al., 2004). In the hippocampus proper, pyramidal cells in CA1 are involved in spatial learning, this part of the hippocampus (rather than other parts as CA3) is involved in the acquisition of memories of new locations (Dupret et al., 2010).

The HF is also involved in functions related to emotions such as anxiety (Eichenbaum, 2000). The role of ventral hippocampus was linked to anxiety. However,

although the hippocampus and the amygdala are interconnected, studies suggest that the role of hippocampus on anxiety is distinctive than that of the amygdala on fear (Bannerman et al., 2004).

Evidence suggests an association between pain processing and the HF, furthermore, chronic pain can make structural and functional effects on the HF (Liu and Chen, 2009).

Regarding the connections of HF, the main afferent pathways come from the entorhinal area, after that the stored memories pass from the hippocampus to other association areas. In general, hippocampal pathways can be divided into two categories: polysynaptic and direct pathways.

The polysynaptic pathway connects the different parts of the hippocampus. It originates from the entorhinal cortex (second layer). This pathway then passes to the stratum moleculare layer of the DG. From there, the fibres pass to CA3 and CA4, and they send collateral fibres to synapse with the dendrites of CA1, enter the alveus. Then the strata radiatum and lacunosum. Fibres from CA1, CA3 and CA4 run through the alveus into the fimbria, fornix, anterior thalamic nucleus, and other thalamic nuclei. From there, the impulses run to the following cortical areas: area 23 (the posterior cingulate cortex), area 24 (the anterior cingulate cortex) and areas 29 and 30 (the retrosplenial cortex) (Duvernoy, 2013).

The direct pathway originates from the entorhinal cortex (third layer) and runs directly into CA1, from there, the impulses go to subiculum, the entorhinal cortex, the inferior temporal association, and prefrontal cortices, and the temporal pole (Duvernoy, 2013).

The polysynaptic and direct pathways are involved in different types of memories, episodic and spatial memories are associated with the former while the semantic memory is associated with the later pathway.

2.3.4. Adult Hippocampal Neurogenesis

Most cells are formed prenatally, but granular cells show postnatal neurogenesis as well (Standring, 2015). Adult hippocampal neurogenesis is essential for improving the functions related to emotional regulation, learning, and memory.

Neurons of the HF those are capable of regenerating (as well as those in the olfactory bulb and DG). These neurons are known as neural stem cells. They are identified through histochemical investigation by the presence of nestin, an intermediate filament protein (Ross and Pawlina, 2011). They are and found in the subgranular layer of the DG. On the other hand, postnatal neurogenesis is a keystone to the learning-dependant hippocampal functions. It is reported that the HF is affected by diseases like AD, temporal lobe epilepsy, schizophrenia and mental disorders like depression. The effect is mostly manifested as reduced volume, enhancing neurogenesis could be helpful in such conditions (Dokter and von Bohlen und Halbach, 2012).

Neurogenesis can be enhanced in the hippocampus (and so cognitive functions as well), by the intake of diet rich in vitamins (like B-9 and E), ω-3 fatty acids, and polyphenols (Poulose et al., 2017). It can also be negatively affected by hyperglycemia (Sadeghi et al., 2019).

2.4. Diclofenac and Hippocampus

2.4.1. Histological Investigations

Histological investigations on the hippocampus showed a decreasing number of neurons after prenatal administration of diclofenac. Offspring of Wistar albino rats were arranged into two groups 4weeks-old and 20weeks-old after prenatal administration of DS (1mg/kg for intraperitoneal administration 15 days) to their mothers. The 20-weeks-old group showed a decrease in the number of pyramidal cells. Also, there was a decrease in the number of granule cells in the DG in the 4weeks-old group while they were increased in the 20weeks-old group, the later results are because granular cells in the DG proliferate mostly postnatally, which indicates the possible role of diclofenac in increasing their rate of proliferation (Gokcimen et al., 2007). In a study to investigate the effect of the dose of diclofenac on the hippocampus, different doses were administered prenatally (3.9, 9 and 18 mg/kg, intraperitoneal administration for seven days) in the first trimester, the decrease in the number of neurons was dose-dependent (Elibol et al., 2014; Yurt et al., 2018b),

Postnatal studies on the effect of diclofenac on hippocampus were also conducted. There was a decrease in the number of neurons in rat hippocampus after diclofenac

administration. The dose was administered intramuscularly for seven days postoperatively (15 mg/kg). It was done for experimental rats with acute subdural hematoma (ASDH) (Turkmen et al., 2016).

Diclofenac was also administered to Wistar rats (12.5 mg/kg intraperitoneal administration for three days). The results on the nervous system were manifested in the cerebral cortex as well as the cerebellum as the presence of balloonized (damaged) red neurons. This phenomenon was also (but less observed) detected in the hippocampus (Ilic et al., 2011).

In another study, massive bowel resection was performed to Wistar albino rats. The purpose was to investigate the protective role of an anti-ulcer agent (stable gastric pentadecapeptide BPC 157) against diclofenac toxicity after major surgeries. Diclofenac was administered intraperitoneally after surgery (once, 12 mg/kg). In the group treated with diclofenac, lesions were worse than the group of surgery alone or the group that is treated by stable gastric pentadecapeptide BPC 157. Among other organs, lesions were reported in the hippocampal neurons (most affected), cerebellar nuclei and the Purkinje cells (least affected). Histological investigation of hippocampal neurons showed ballooned edematous cells (Lojo et al., 2016).

2.4.2. Studies Related to Pain Processing

HF has a significant role in pain processing, on the other hand, pain itself (when it is chronic) can exert an effect on the structure and functions of the hippocampus (Liu and Chen, 2009).

A study was conducted to examine the role of diclofenac in inducing antinociception. Microinjection cannula was used to inject 0.5 µl of diclofenac into the dorsal hippocampus of male Wistar rats. The study results suggest that the antinociception effect is achieved by the inhibition of GABA-ergic interneurons (Gurtskaia et al., 2014).

There was an antinociception effect following the injection of diclofenac into the dorsal hippocampus. However, the dorsal hippocampus developed tolerance with repetitive microinjection (Tsiklauri et al., 2018).

2.4.3. Possible Neuroprotective Effects of Diclofenac on Hippocampus

Many studies have highlighted the probable neuroprotective effects of diclofenac and its anti-depression effect. In an assay investigation of metabolites in rat's hippocampus, diclofenac was administered (20 mg/kg, subcutaneously). It was is reported that diclofenac prevented IFN-a induced depression by minimizing the neurochemical changes induced by IFN-a (De La Garza II and Asnis, 2003).

Another study was conducted to investigate the possibility of involvement of arachidonic acid cascade activation on the diminishing of the working memory. The dorsal hippocampus in both hemispheres in Wistar rats was injected by IL-1β; the resulted impairment was reduced by subcutaneous administration of 10 mg/kg of diclofenac(Matsumoto et al., 2004).

In another study, an assay investigation for IL-1β and IL-6 mRNA in the hippocampus was conducted. Diclofenac was administered subcutaneously at a dose of 2.5 mg/kg. It was is reported that diclofenac may diminish lipopolysaccharide (LPS)-induced alterations, which make it a possible candidate for minimizing depressive-like behaviour (De La Garza et al., 2005).

The rise in serum levels of pro-inflammatory cytokines like IL-1β, IL-6 and TNFα have been reportedly associated with some neurological conditions like cerebral ischemia, AD, multiple sclerosis and epilepsy. It has been found that diclofenac (5 and 10 mg/kg, intraperitoneally administered for 15 days) decreased the level of serum IL-6 and TNFα levels in the hippocampus. It also minimised seizure in kindling seizure model rats (Vieira et al., 2016). However, high levels of inflammatory cytokines may weaken the functions of the blood-brain barrier, which lead to an increase of COX inhibitors concentration in the brain with subsequent possible damage (Fukuda et al., 2005). It is also suggested that diclofenac is associated with the development and/or increased severity of influenza-associated encephalitis/ encephalopathy (Morishima et al., 2002). This would be rather contradicting findings.

It is reported that cannabinoids can induce changes in behaviour (behavioural suppression) through the activation of the arachidonic acid cascade. This behavioural suppression has been attenuated by administering diclofenac intraperitoneally to Wistar/ST

strain rats 15 minutes before administration of Δ8- tetrahydrocannabinol (which activates the arachidonic acid cascade). In this study, COX1 and COX2 were reported to be found constitutively in the cerebral cortex and the hippocampus (Yamaguchi et al., 2004).

Diclofenac is found to be neuroprotective by decreasing the acidity through inhibition of acid- sensing ionic channels (ASIC) expressed in the hippocampus. This inhibition of proton-induced currents is suggested to be protective for neurons from acidification- associated damage (Dorofeeva et al., 2008).

In order to investigate the possible protecting role of diclofenac against memory dysfunction induced by scopolamine, DS was administered to a group of rats (5mg/kg, oral gavage for two weeks). In this study, glutamic content was reported to be reduced significantly in the hippocampus in rats while GABA and glycine hippocampal content were significantly increased in male rats (Ali and Arafa, 2011).

2.4.4. Other Studies

The interaction between quinolone antimicrobials and some NSAIDs regarding γ-aminobutyric acid (GABA) was studied on pyramidal cells of the hippocampus. The study focused on the GABA-induced chloride current, and it reported that some NSAIDs as fenbufen, indomethacin, and naproxen, mefenamic acid (all listed in order from the most potent) inhibit the GABA response when administered combined with quinolone antimicrobials which may lead to neurotoxic adverse effects, diclofenac was mentioned to have the same effect; however, its potency was ranked the fourth (Shirasaki et al., 1991).

The effect of diclofenac on neuronal potassium channels was investigated. A motoneuron-like cell line NSC-34 and dorsal root ganglion neurons were used as well as a simulation of hippocampal pyramidal cells (CA1). The researchers reported that diclofenac could affect the delayed-rectifier K^+ current directly by blocking it and accelerating its decay; this effect was reported to be dose-dependent and time-dependent (Huang et al., 2013).

Table 1. Doses and routes of administration of diclofenac in some hippocampus-related studies

Diclofenac and Hippocampus						
No.	Dose	Route	Duration	Study details	Result	Citation
1.	1 mg/Kg	Intraperitoneal	15 days	Prenatal. Wistar albino rats 4wks and 20wks	Pyramidal cells in CA decreased. The granular cell in DG increased in 20w	(Gokcimen et al., 2007)
2.	1 mg/kg	Subcutaneously	15 days from day 5 to day 20	Prenatal. Wistar albino rats	Long memory was not affected	(Elibol et al., 2019)
3.	3.6 mg/kg	Intraperitoneal	10 days	Prenatal. Wistar albino rats	The number of cells in the hippocampus and DG decreased	(Yurt et al., 2018 a)
4.	3.9 mg/kg 9 mg/kg 18 mg/kg	Intraperitoneal	7 days	Prenatal. Wistar albino rats	The decrease in the number of neurons was dose-dependent	(Elibol et al., 2014)
5.	5 mg/kg	Intraperitoneal	5 days	male Sprague Dawley rats	Long memory was not affected	(Emad et al., 2017)
6.	5 mg/kg	Oral gavage	14 days	Wistar albino rats	Long memory was not affected	(Ali and Arafa, 2011)
7.	5 mg/kg	Oral gavage	15 days	Wistar rats	glutamic content, GABA and glycine were reduced in the hippocampus	(Ali and Arafa, 2011)
8.	10 mg/kg	Subcutaneously	Before the experiment	IL-1β was injected to the hippocampus of Wistar rats	The produced memory impairment was reduced by diclofenac	(Matsumoto et al., 2004).
9.	12 mg/kg	Intraperitoneal	Once after surgery	Wistar rats Massive bowel resection	Histological lesions	(Lojo et al., 2016)
10.	12.5 mg/kg	Intraperitoneal	3 days	Wistar rats	Red (damaged) neurons were detected in the cerebral cortex and cerebellum; this was less obvious in the hippocampus	(Ilic et al., 2011)
11.	15 mg/kg	Intramuscular	7 days	Wistar rats	A decrease in the number of neurons	(Turkmen et al., 2016)
12.	20 mg/kg	Subcutaneously	One day	Wistar rats	DS prevents IFN-a induced depression by minimising the neurochemical changes	(De La Garza II and Asnis, 2003)

2.5. Gum Arabic

GA is the adhesive exudate from the stems and branches of a tropical plant called *Acacia Senegal*. Sudan has been the leading country that produces GA in the 1960s, civil war and political instability changed this for the current time (Williams and Phillips, 2009).

GA is a branched-chain, complex polysaccharide (a mixture of oligosaccharides and heavy glycoproteins), composed of mixed calcium, magnesium and potassium salts of the polysaccharide GA acid (Arabic acid), it is composed of mainchain (backbone) and side chains, the backbone chain consists of 1,3-linked b-D-galactopyranosyl units while the side chains consist of two to five 1,3-linked b-D galactopyranosyl units, joined to the main chain by 1,6-linkages (Al-Yahya et al., 2009). It includes 39–42% galactose, 24–27% arabinose, 12–16% rhamnose, 15–16% glucuronic acid, 1.5–2.6% protein, 0.22–0.39% nitrogen, and 12.5–16.0% moisture (Ali et al., 2009b).

2.5.1. General Uses of Gum Arabic

It has been used widely as an emulsifier and stabiliser in food industries. It is used in chewing gum, candies, toffees, caramels and soft drinks. It also used in beers due to its foam stabilisation properties, it makes wines clear, due to its ability to inhibit the formation of ice crystals and its high melting point it is used in ice-cream industries (Williams and Phillips, 2009).

In pharmaceutical industries, it is used as a suspending agent, emulsifier, and adhesive in syrups and tablets (Verbeken et al., 2003). GA is added to Ampicillin to prolong periods of sustained release of the drug (Nishi et al., 2007). GA is used in lotions and creams due to its stabilisation properties (Phillips and Williams, 2001).

2.5.2. Gum Arabic in Medicine

In the renal system, GA was administered orally for three months to patients with chronic renal failure in Sudan (50 g/day), there was a significant decrease in levels of concentration of serum creatinine, urea, phosphate and uric acid concentrations (Ali et al., 2008). Also, Serum urea nitrogen was significantly decreased in patients with chronic renal failure received 50g/day of GA (Bliss et al., 1996).

In the intestine, adding GA to Oral Rehydration Solutions (ORS) enhanced sodium absorption in the rats' guts (Wapnir et al., 1996). Absorption of sodium, potassium, and water was increased in rats with diarrhoea treated with ORS and GA (Wapnir et al., 1997; Teichberg et al., 1999), GA also enhanced calcium absorption in the intestine in an experiment conducted on rats (Kawase et al., 2007). In dental medicine, as an in vitro study showed, GA improved the remineralisation process of caries-like enamel lesions (Onishi et al., 2008).

There were no reported significant toxic effects associated or linked to the use of GA (Ali et al., 2009a).

2.6. Oxidative Stress and Antioxidants

2.6.1. Overview

In order to explore OS as a mechanism by which diclofenac can affect the hippocampus, the vulnerability of the hippocampus to OS, the responsiveness of both diclofenac and hippocampus to antioxidants, and the possible role of GA as an antioxidant, the following paragraphs highlight the relation between OS, antioxidants, hippocampus, diclofenac, and GA.

The production of the oxidants as ROS and the body's antioxidants are balanced. OS arises when there is a loss of this balance in a way that makes ROS more than antioxidants, the oxidative process can develop following ROS production either through NADPH Oxidase or mitochondrial dysfunction (Sies, 1997). ROS comprise both oxygen-derived radicals and non-radical species. The radicals comprise superoxide, peroxyl, and hydroxyl while the nonradical are hydrogen peroxide, singlet oxygen, and hypochlorous acid (Rodrigues et al., 2012), ROS are produced mainly by the mitochondria (mitochondrial energy production pathway). However, they are also produced in the endoplasmic reticulum, cytoplasm and plasma membranes, in the mitochondria the production of Adenosine Triphosphate (ATP) may produce free radicals during the reduction of oxygen into water, some electrons skip the reactions in electron transport chain, thus some free radicals are formed like superoxide anion and hydroxyl radicals and H_2O_2. Phagocytic cells

such as neutrophils produce free radicals to use in destroying invaders as a means to defend the body (Valko et al., 2007; Ighodaro and Akinloye, 2018).

The effects of free radicals on cells are many. They reduce the production of ATPs leading to changes in the permeability of the mitochondria, which allow the release of cytochrome c, this, in turn, activates the intrinsic apoptotic pathway. ROS can cause DNA mutations, through inducing crosslinks, modifications in purine and pyridine bases, and strand's breaks (Valko et al., 2007).

The body's antioxidant mechanisms include enzymatic and non-enzymatic antioxidants; the enzymes are SOD, CAT and glutathione peroxidases (GPx), while examples of the non-enzymatic antioxidants include glutathione (GSH), vitamin E, ascorbic acid, β-carotene, and melatonin. They can also be categorized into four lines of defence. The first line prevents the formation of free radicals. Examples are SOD, CAT, and GPx, CAT is found in peroxisomes and not in the mitochondria (while GPx is present in mitochondria), the second line scavenges free radicals, examples are GSH, vitamin E and ascorbic acid (Ighodaro and Akinloye, 2018). SOD is the major antioxidant, while GSH is the main antioxidant in the CNS (Gandhi and Abramov, 2012). However, the hippocampus relays mainly on CAT and GSH as scavengers more than SOD (Freitas et al., 2005).

OS is related to some neurodegenerative conditions, like Parkinson's disease (PD) and AD (Mattson et al., 1997). However, the affected brain regions are not the same, in PD, the dopaminergic neurons in the substantia nigra are affected while in AD the affected regions are the hippocampus, the entorhinal and frontal cortices, and the amygdala (Gandhi and Abramov, 2012).

2.6.2. Oxidative Stress: The Hippocampus is Vulnerable

Considering that diclofenac can harm the nervous tissue (including the hippocampus) through OS, the underlying molecular basis in which diclofenac can induce apoptosis is not fully understood and yet to be studied (Yurt et al., 2018a).

The hippocampus is vulnerable to OS in many ways. First of all, neurons consume oxygen more than other tissues, ten folds more. Some regions of the brain (including the hippocampus) produce free oxygen radicals in higher rates than others. Thus, making them

susceptible to suffer from OS, this is attributable to the high amount of polyunsaturated fatty acids (PUFAs) in the nervous tissue, such as arachidonic acid (AA). It is the main constituent of the cell membranes of neurons, and it is released upon tissue injuries. Metabolism of AA by cyclooxygenases produces proinflammatory mediators which in turn lead to OS and damage (Huang et al., 2012). On the other hand, the expression of antioxidant enzymes in the brain is very low in the mitochondria than that of the cytosol, although the mitochondria are more vulnerable to OS.

Some proinflammatory cytokines' receptors are present in the hippocampus in the pyramidal cell layer CA1, and CA3, interleukin-1 beta (IL-1β) is involved in memory functions by reducing the release of the necessary neurotransmitters (Matsumoto et al., 2004).

OS induced by sleep deprivation in mice led to lower performance in the passive avoidance test. However, antioxidants such as N-tert-butyl-a-phenylnitrone, vitamin E and melatonin successfully yielded better performance in mice treated by those substances (Silva et al., 2004).

OS can lead to the death of neurons in the hippocampus, as shown histologically. That was evident in the CA3 region. The OS was associated with induced status epilepticus (Liu et al., 2010).

2.6.3. Toxicity of Diclofenac is Associated/Induced by Oxidative Stress

OS as a mechanism of diclofenac neurotoxicity has been suggested. It affected the brains of Wistar albino rats (Motawi et al., 2019) as well as brains of *Cyprinus Carpio* (Islas-Flores et al., 2013).

In the liver, reports suggest that diclofenac impairs the balance in a way that makes ROS more than antioxidants leading to hepatotoxicity-induced OS. This was indicated by heme oxygenase-1 being induced in diclofenac treated mice (Cantoni et al., 2003). Diclofenac-induced OS was indicated in hepatotoxicity (Bort et al., 1999; Gómez-Lechón et al., 2003), it is also reported that the inhibition of oxidative phosphorylation in the mitochondria leads to OS in the liver (Hickey et al., 2001).

Diclofenac also induced acute kidney injury through OS (Fattori et al., 2017), and nephrotoxicity (Hickey et al., 2001). HL-60 cells' growth was inhibited following the administration of diclofenac that induced apoptosis by generating ROS (Inoue et al., 2004).

The release of ROS was linked to diclofenac-induced apoptosis in gastric epithelial cells (Kusuhara et al., 1999). However, as mentioned earlier, studies show that the toxicity of diclofenac may not be confined to OS. Diclofenac may affect the transport systems in the cell membrane, leading to an accumulation of neurotoxins as 1-methyl-4-phenylpyridinium. OS is necessarily not the sole reason of cell death, as indicated by the inefficiency of administration of an antioxidant and a radical scavenger (N-acetyl-L-cysteine and phenyl-N-butyl-nitrone, respectively) in minimising the effect of diclofenac (Morioka et al., 2004).

2.6.4. Antioxidants Protect the Hippocampus

The hippocampus is vulnerable and affected by OS. However, it is responsive to the protective roles of substances having antioxidant properties. In a study among mice with Docetaxel-induced neurotoxicity, melatonin and selenium as antioxidants play an essential role in minimising hippocampal injury following OS. The proposed mechanism is the suppression of the production of ROS in the mitochondria (Ataizi et al., 2019).

Grape seed polyphenols were proven as an antioxidant of benefit against age-related OS and the decrease of the number of neurons in the hippocampus (CA1 region) of male Wistar rats. This decrease was also supported using the optical fractionator technique, which revealed the effectiveness of this antioxidant against neuronal loss (Abhijit et al., 2018).

Green tea mega EGCG^{TM} as an antioxidant was also beneficial against OS produced by electromagnetic radiation in the hippocampus of Wistar albino rats. The rats were affected two months, and for three months to radiation, administration of green tea was at the same time during their exposure (Ahmed et al., 2017). Also, Boswellia sacra and folic acid as antioxidants were beneficial against OS produced by electromagnetic radiation in the hippocampus. The animals were exposed to 900-MHz radiation for an hour daily for 21 days. This histological study reported a reduction in the number of cells in the hippocampus

of rats exposed to radiation, and antioxidant substances attenuated the damage (Kivrak et al., 2017).

In another study, the effect of melatonin against OS in the hippocampus of Wistar rats exposed to the electromagnetic field was investigated; the results show a protective effect of melatonin (Erdem Koc et al., 2016). Dendropanax morbifera Léveille leaf extract has been proven as an antioxidant of benefit to the hippocampus against OS produced by mercury. As concluded in a study on Sprague–Dawley rats, in which a dose of 5 μg/kg administered intraperitoneally with dendropanax morbifera Léveille leaf extract administered orally (100 mg/kg), both administered daily for four weeks (Kim et al., 2015).

In a study to examine the antioxidant properties of vitamin E, the study was conducted on the hippocampus of Sprague-Dawley diabetic rats. It was proven to be effective against OS associated with diabetes mellitus, although the study indicated that grape seed extracts (which are used along with vitamin E in the study) are more effective (Yonguc et al., 2015).

An investigation of the antioxidant effect of black garlic extract against the OS of monosodium glutamate was done, the MWM test results revealed that spatial memory was affected harmfully by monosodium glutamate while black garlic extract showed promising results considering prevention of these harmful effects (Hermawati et al., 2015).

The effect of ascorbic acid (as an antioxidant) on hippocampus was investigated, spectrophotometric methods were applied to measure the enzymatic activities in Wistar rats treated with ascorbic acid, to assess the antioxidant activity against OS associated with pilocarpine-induced seizers, the antioxidant activity has decreased lipid peroxidation and elevated the activities of both SOD and CAT in the hippocampus (Santos et al., 2009).

A study using in-vitro hippocampal cells (both human and from rats) concluded that dehydroepiandrosterone (having antioxidant properties) had protected the hippocampus against OS induced by H_2O_2 and sodium nitroprusside (Bastianetto et al., 1999).

2.6.5. Antioxidants Reduce Toxicity of Diclofenac

It has been suggested that the antioxidant properties of exogenous Melatonin could act as a neuroprotector against DS neurotoxicity (Aygun et al., 2012). Melatonin is proven

to prevent pathological effects on the kidney in rats prenatally administered diclofenac (Khoshvakhti et al., 2015).

B27 supplement as an antioxidant is shown to reduce neurotoxicity induced by OS derived by diclofenac (Rajabalian et al., 2009). This study investigated the direct link between the neurotoxicity of diclofenac through OS and minimising this effect by administering an antioxidant substance (**Figure 5**).

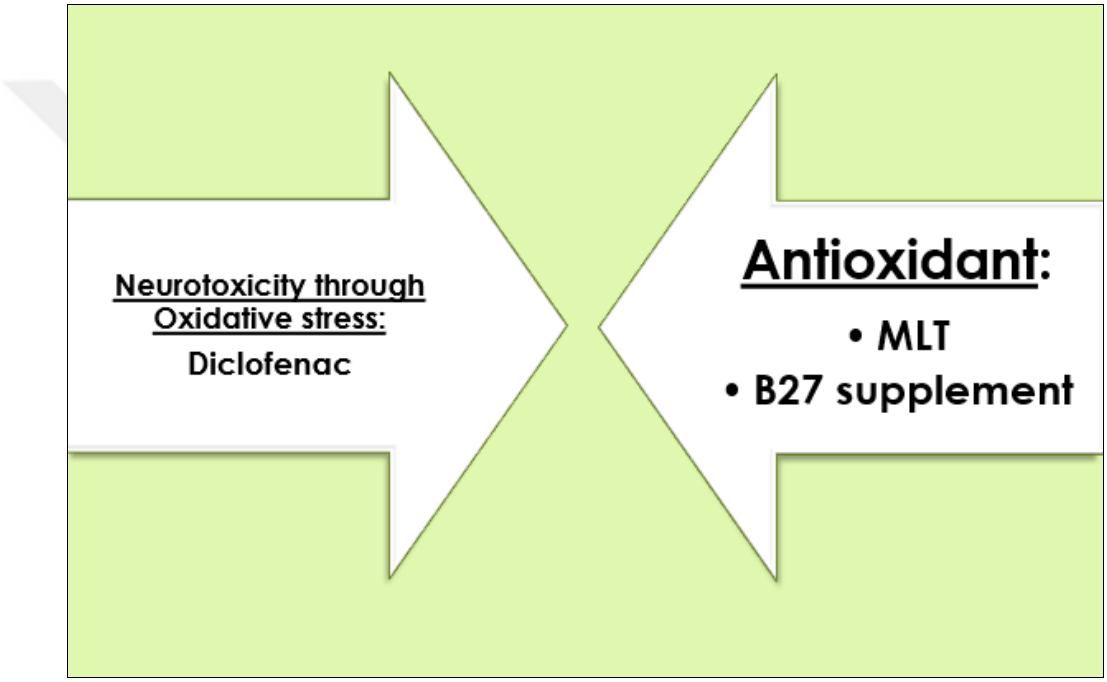


Figure 5. The relation between OS as a mechanism by which diclofenac causes neurotoxicity and its responsiveness to antioxidants. MLT: melatonin

2.6.6. Gum Arabic as an Antioxidant

GA is considered as an antioxidant (Trommer and Neubert, 2005; Marwah et al., 2007). It is suggested that it acts mainly as ROS neutraliser (Al-Yahya et al., 2009). The antioxidant capacity of GA is attributed to its components of amino acids tyrosine, histidine, and methionine (Rodrigues et al., 2012).

In the kidneys, rats with adenine-induced chronic renal failure were treated with GA 15%, w/v. for 4 weeks, which led to improvement due to anti-inflammatory and antioxidant

properties of GA (Ali et al., 2014), the antioxidant properties of GA are shown to reduce adenine–induced chronic renal failure (Ali et al., 2013) and mercuric chloride-induced nephrotoxicity (Gado and Aldahmash, 2013), oral administration of GA (7.5 g 100 ml for eight days) significantly reduced gentamicin-induced lipid peroxidation (Al-Majed et al., 2002). The dose of (7.5 mg/kg for five days) significantly reduced cisplatin-induced nephrotoxicity (Al-Majed et al., 2003).

Urinary bladder cytotoxicity was induced by cyclophosphamide in Swiss albino rats (males) to investigate the antioxidant properties of GA. The dose was (7.5 gm/kg/day) for six days in drinking water. GA treated group showed marked improvement, bladder inflammation and oedema were reduced, and bladder histology was almost restored to normal, GSH bladder contents were restored as well (Al-Yahya et al., 2009).

In the liver, oral administration of GA was shown to attenuate acetaminophen-induced hepatotoxicity in mice by reducing OS (Gamal el-din et al., 2003). In diabetic rats, hepatic lipid peroxidation was attenuated, and levels of SOD and GSH peroxidase, and CAT was elevated (Ahmed et al., 2015). In the heart, GA antioxidant properties were considered as a possible mechanism of protection against Doxorubicin-induced cardiotoxicity in mice (Abd-Allah et al., 2002). In the gonads, GA significantly reduced lipid peroxidation and enhanced testicular antioxidant enzymes activities in diabetic rats (Fedail et al., 2016). GA reduced lipid peroxidation and significantly increased the activities of ovarian SOD, GSH peroxidase, and CAT in mice (Ahmed et al., 2016). Administration of GA at a dose of 30g orally, daily for 12 weeks, for 46 patients with sickle cell anaemia has shown to be beneficial in minimising OS (Kaddam et al., 2017). However, it is reported that oral administration of GA in the drinking water for rats at a concentration of 2.5, 5.0 or 10.0% $^{w/v}$ for eight days did not lead to any significant change in the concentrations of antioxidants like GSH and Super-oxide dismutase in the kidney or liver (Ali, 2004).

To our knowledge, stereological studies investigating the effect of GA on the histology of the nervous tissues are scanty. This study aimed to apply stereology in quantifying the role of GA in the healing of nervous tissue injury induced by diclofenac, in a rat model.

2.7. Stereology

Obtaining quantitative data with statistical reliability is the skeleton of research, the term “stereology” describes a variety of methods that provide three-dimensional description from a two-dimensional image based on mathematical and statistical approaches, the earliest concepts of stereology was described in the 1930s by William Thompson (Kaplan et al., 2012), the term “stereology” itself was presented in 1961 by Hans Elias at a scientific meeting held in Germany (Miki et al., 2005), the disector principle, a probe that provides a defined three-dimension volume for particle counting (numerical density), was first introduced by (Sterio, 1984) by which then stereology, as we know, was established, the optical disector as a probe was described by (Gundersen, 1986) in which shape, size or orientation of particles can’t affect the estimation due to the three-dimensional designs of the probes owing to its mathematical basis, the fractionator is a sampling strategy that adopts systematic random sampling approach to achieve unbiased results (Gundersen et al., 1988). Stereology is the finest tool owing to its characteristics: it is unbiasedness (the fractionator – as a sampling strategy), and its independence of shape, size or orientation, of particles (the optical disector – as a probe), the combination of both creates the optical fractionator (West et al., 1991). The data obtained by these techniques could be volume, surface area, or quantity of particles (Yurt et al., 2018b).

2.7.1. Range of total number of pyramidal cells in hippocampus obtained by stereological methods

The total number of pyramidal cells in the hippocampus is ranged between 632,000 for one-month aged Wistar rats, 930,000 for two-months, and 687,000 for 14-months aged rats (Schmitz and Hof, 2005), in (**Table 2 and 3**) is a summary of the total count of neurons in the hippocampus in the control groups in different studies.

Table 2. The total number of neurons in the hippocampus of Wistar rats. [1](Miki et al., 2005), [2](Miki et al., 2003), [3](Miki et al., 2004), [4](West et al., 1991), [5](Gokcimen et al., 2007), [6](Hosseini-Sharifabad and Nyengaard, 2007), [7](Cardoso et al., 2011), [8](Olesen et al., 2015), [9](Bas et al., 2009), [10](Yurt et al., 2018a)

No. of cells / Age	Total number of pyramidal cells	CA1	CA3	Granular cells in DG
2 weeks[1]	-	176 800 ± 8 900	169 300 ± 10 600	441 400 ± 31 100
16 days[2]	-	-	-	424 700 ± 21 600
16 days[3]	-	168 800 ± 6 600	96 200 ± 4 400	-
4 weeks[1]	-	152 700 ± 6 000	149 600 ± 10 800	524 500 ± 25 000
30 days[2]	-	-	-	515 700 ± 16 700
30 days[3]	-	182 300 ± 11 200	85 900 ± 3 700	-
4 weeks[4]	-	382 000	250 000	1 200 000
4 weeks[5]	<700 000	-	-	>1 200 000
2 months[6]	-	324 000	188 000	1 080 000
10 weeks[7]	-	358 513 ± 11 918	238 055 ± 17 654	1 160 128 ± 95 189
8 weeks[8]	1 680 000	-	-	-
12 weeks[9]	595,209±25,111	-	-	-
12 weeks[10]	~700 000	~430 000	~220 000	~1 300 000
20 weeks[5]	~750 000	-	-	~1 000 000

Table 3. The total number of neurons in the hippocampus of Sprague-Dawley rats.[1](Lister et al., 2005), [2](Zhao et al., 2016), [3](Fitting et al., 2010),[4](Fitting et al., 2008) [5](Grady et al., 2003) *CA2 included

No. of cells / Age	Total number of pyramidal cells	CA1	CA3	Granular cells in DG
90 days[1]	330 998	330 998*	181 570*	-
4 months[2]	-	410 000± 50 000	-	$7.2 \times 10^5 \pm 0.4 \times 10^5$
~5 months[3]	-	262 181	416 498*	1 172 290
~$7^1/_2$ months[4]	-	233 042	252 504*	970 029
Adult[5]	-	387 800	262 200*	1 150 600

2.7.2. Detecting changes in the total number of pyramidal cells in hippocampus obtained by stereological methods

Investigating the antioxidants' effect on hippocampus was performed using stereology. The physical disector was used to estimate the total number of pyramidal cells in the hippocampus of rats exposed to monosodium glutamate, which leads to OS and black garlic extract, which have antioxidant properties. Stereological studies revealed that the CA1 area is more affected one by the combined administration of monosodium glutamate and black garlic extract. The MWM test results revealed that spatial memory was affected harmfully by monosodium glutamate, while black garlic extract showed promising results considering the prevention of these harmful effects (Hermawati et al., 2015). In another study, the optical fractionator was used to investigate the effect of melatonin against OS in the hippocampus of Wistar rats exposed to the electromagnetic field. The results show a protective effect of melatonin (Erdem Koc et al., 2016). Optical fractionator technique was involved in another study regarding the hippocampus. Grape seed polyphenols were proven as an antioxidant of benefit against age-related OS and neuronal loss in the CA1 region of the hippocampus of male Wistar rats. This was supported using the optical fractionator technique, which revealed the effectiveness of this antioxidant against neuronal loss (Abhijit et al., 2018).

2.8. Justification

2.8.1. Why Choosing Diclofenac?

NSAIDs are one of the most common medications used worldwide; diclofenac is one of the most common NSAIDs used, yet, it has some harmful effects on the nervous tissues, including the hippocampus. Diclofenac, although nonselective NSAID, is found to be four times more selective for COX-2 than COX-1. COX 2 is found constitutively in the hippocampus. The constitutive presence of COX-2 strongly suggests that it is not involved only in inflammation. However, it plays a physiological role in normal synaptic functions.

2.8.2. Why Choosing the Central Nervous System?

It is acceptable now OS is a mechanism of diclofenac toxicity. Antioxidant enzymes' level in nervous tissues is relatively lower in comparison to other tissue (Yurt and Kaplan, 2018), making nervous tissue more vulnerable to OS. OS mediates neuroinflammation, which underlies the pathophysiological mechanism of diseases like schizophrenia and AD (Popa-Wagner et al., 2013). Few studies are investigating histological effects of diclofenac on nervous tissue in comparison to those considering other systems, especially the digestive, cardiovascular and renal systems, even those studies are with controversial results.

2.8.3. Why Choosing Hippocampus?

COX-2 is found constitutively in the hippocampus. Some regions of the brain (including the hippocampus) contain relatively more oxidisable lipids and produce free oxygen radicals in higher rates than other regions. Thus, making them more likely to suffer from OS (Bellissimo et al., 2001; Aygun et al., 2012). OS is the acceptable mechanism by which diclofenac can harm the hippocampus. Stereological studies showed a reduction in the number of cells in hippocampus after prenatal administration of diclofenac. OS is related to some neurological disorders such as AD, Parkinson's disease, and amyotrophic lateral sclerosis. The hippocampus is shown to be responsive to the protective roles of substances having antioxidant properties.

2.8.4. Why Choosing Gum Arabic?

OS plays an important role in neurodegeneration. Antioxidants are now considered significant in therapeutic strategy (Popa-Wagner et al., 2013). The hippocampus is vulnerable and affected by OS. It is also responsive to the protective roles of substances having antioxidant properties, such as dehydroepiandrosterone (Bastianetto et al., 1999), ascorbic acids (Santos et al., 2009), dendropanax morbifera Léveille leaf extract (Kim et al., 2015), vitamin E (Yonguc et al., 2015), black garlic extract (Hermawati et al., 2015), green tea mega EGCG^{TM} (Ahmed et al., 2017), Grapeseed polyphenols (Abhijit et al., 2018) and

melatonin (Yurt et al., 2018a). Nevertheless, no study has investigated the antioxidant properties of GA.

Studies have shown that OS induces the neurotoxicity of diclofenac. GA is an antioxidant and is shown to reduce toxicity as a result of OS. İt is also herbal, natural, and cheap. There are no studies found to investigate the capability of GA to reduce the neurotoxicity of diclofenac.

2.8.5. Why Administering Gum Arabic With Diclofenac Simultaneously?

According to the definition of antioxidants: 'any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell, 1995). Thus, the optimum timing is during the administration of diclofenac.

2.8.6. Why Stereology?

It is a combination of accurate, unbiased and efficient techniques.

2.9. Objectives of The Study

2.9.1. General Objective

To investigate the role of *GA* in minimising hippocampal tissue damage induced by diclofenac. By using stereological analysis on rat models.

2.9.2. Specific Objectives

To detect the histological effects of DS, GA, and DS plus GA on the pyramidal cells of the hippocampus, using stereological analysis.

To detect the levels of antioxidants in rats treated with DS, GA, and DS plus GA.

3. MATERIALS AND METHODS

3.1. Experimental Animals

Forty Wistar albino male rats, with 12-week-old and with weights of 280±30g were used for this experiment. Experimental animals were obtained from the Experimental Animal Research Center and Surgical applications of Ondokuz Mayis University, Samsun, Turkey. The study was initiated after the approval of the Ethical Committee and Animal Care of the Ondokuz Mayis University, Samsun, Turkey (approval number 2015/46). All experimental procedures were carried out according to the protocol guidelines. The rats were kept in stainless-steel cages. During the whole period of the experiment, the rats were maintained under 12h light/12h dark cycle in room temperature (22±2 °C) with humidity between 40-50 per cent in the Animal house. The rats were free to obtain food and water and were maintained under standard laboratory conditions and fed with standard feed (**Figures 6 and 7**).

Figure 6. Housing conditions of the animals

3.2. Formation of Experimental Animal Groups

Rats were randomly divided into four groups (10 rats for each group), all the experimental procedures were done blindly (i.e. the investigator had no clue about the

animals' allocation to groups, all animals were coded and the transcription of the codes was kept until the experiments and the stereological procedures were done).

1. Control group (Cont.): This group was not subjected to the administration of any substances.
2. Diclofenac sodium group (DS): This group was subjected to 3.6 mg/kg/day of DS by oral gavage (Voltaren from Novartis Health, Food and Agricultural Products Industry and Trade Inc. Kurtköy - Pendik, Istanbul, Turkey)
3. Gum Arabic group (GA): This group was subjected to 7.5 g/kg/day of GA by oral gavage (Gum Arabic Company, Khartoum, Sudan).
4. Diclofenac + Gum Arabic group (DS+GA): This group was subjected to both 3.6 mg/kg/day of DS by oral gavage (Voltaren, Novartis) and 7.5 g/kg/day of GA by oral gavage (Gum Arabic Company, Khartoum, Sudan).

Figure 7. Wistar albino rats in a cage

All animals were coded by means of numbers thus; all analyses were performed blindly for the experimental group to which any rat belonged.

3.3. Materials

3.3.1. Diclofenac Sodium

Tabs (Voltaren from Novartis Health, Food and Agricultural Products Industry and Trade Inc. Kurtköy - Pendik, Istanbul), dose: 3.6 mg/kg/day administered by oral gavage daily for 15 days. The lethal dose (LD) for DS has been determined at 90 mg/kg (Hirose et al., 1984). We used 1/25 of the LD 50 dose (3.6 mg/kg).

3.3.2. Gum Arabic

Powder from the GA Company Khartoum, Sudan. 7.5 gm/kg/day administered by oral gavage daily for 15 days.

3.4. Tissue Preparation and Histological Procedures

The experimental parts of the study that includes histological and stereological analysis were done in the Department of Histology and Embryology at the Faculty of Medicine, Ondokuz Mayis University, Samsun, Turkey.

3.4.1. Perfusion

Animals were weighed before perfusion. The average weight of the rats is about 280±30 g. At the end of the experimental period, all rats have received intraperitoneal 80 mg/kg ketamine (Ketalar®, Eczacibasi, Istanbul, Turkey) and 10 mg/kg xylazine (Xylazinbio®, Bayer, Istanbul, Turkey) for general anaesthesia (Fish et al., 2011). After general anaesthesia, cardiac perfusion was carried out first using normal saline solution and then a 10% formalin solution. All used solutions and materials required for perfusion was prepared one day before the operation; the solutions were kept at room temperature.

The deeply anesthetized rats were placed on the operating table with it's back down, and then the chest was opened with the help of a pair of scissors and scalpel from the bottom of the diaphragm to the top of the chest to reveal the heart. When the heart was in operation, the tissue perfusion performed by inserting a plastic cannula in the apex of the left ventricle. Simultaneously with this procedure, a small incision was made in the right atrium with the aid of the scalpel to make sure the solution is flowing freely. Throughout

the perfusion process, tetanic contractions were observed in the rat extremities, which are a sign of formalin fixation. All the rats used in our study showed irregular contractions (formalin dance) during the perfusion process. Craniotomy was performed via bone scissors, and the whole brain was removed.

Both hemispheres of the brains were separated. The right hemispheres were used for light microscopic and stereological analysis. One left hemisphere from each group was spared for electron microscopy.

3.4.2. Routine Histological Tissue Processing

After the brain was removed entirely, it was entirely placed in a plastic tissue container containing 10% formalin and was left in the fixation solution for two weeks. During the fixation period, the fixative solution has been changed every three days. The purpose of this perfusion technique was to minimise the artefacts during fixation.

Table 3. Chemical solutions and duration of follow-up of hippocampus tissues

Chemical Solution	Duration
70% of Alcohol (Ethyl Alcohol-Tekkim Chemicals -Turkey)	1 hour
80% Alcohol	1 night
96% Alcohol	1 hour
96% Alcohol	1 hour
100% Alcohol	2 hours
100% Alcohol	2 hours
Xylene (Sigma-Aldrich)	1 hour
Xylene	1 hour
Xylene	1 hour
Paraplast (Tekkim Chemicals -Turkey)	$1^{1}/_{2}$ hour
Paraplast	$1^{1}/_{2}$ hour

The brain of each rat was processed by the routine histological procedures: it was taken from the 10% formalin fixative solution and washed in the running water for one

night. Following the washing process, the tissue follow-up procedures shown in (**Table 3**) were performed for dehydration and subsequent removal of alcohol.

Tissue processes were done by means of the Thermo Scientific TM Citadel 2000 Tissue Processor (Fisher Scientific UK Ltd Bishop Meadow Road Loughborough LE11 5RG) (**Figure 8**). Brains of the rats were embedded into the paraplast at 60° C. After routine histological processes of brains. They are embedded in the paraffin wax to prepare tissue blocks for the cutting process.

Figure 8. Automated dehydration.

3.4.3. Sectioning Methods

The strategy of the hippocampal tissue sectioning was determined by conducting a pilot study using a single hippocampus tissue block before obtaining the coronal sections of hippocampus tissues for this study. After a random selection of the first section, systematic random sampling (SRS) was applied for selecting sampled sections for stereological analysis using the physical disector technique. Sectioning continued until the whole hippocampus was cut exhaustively; thus, for each hippocampus, approximately 20 ± 2 consecutive sampled sections were taken. The 4-µm-thick serial sections were obtained in coronal plane from the tissue blocks with section interval 1/30. Tissue sections of 4 µm thickness were also taken for histopathological examination.

Figure 9. A rotary microtome (Leica RM2125RT, Nussloch, Germany)

.

Paraffin sections were taken from the tissue blocks using disposable metal microtome blades (Type N35, Feather Company, Osaka, Japan) and rotary microtome (Leica RM2125RT, Nussloch, Germany) (**Figure 9**). The sampled sections were collected on the slides that were covered by 5% of powdered gelatine (in the form of dissolving 5 g of gelatin in 100 ml of distilled water). This was achieved by means of adding to the distilled water heated to 45°C in the flotation bath (MH8517 Paraffin Section Flotation Baths). As a result, the sections stick firmly to the slide and do not drop from the glass slide during the staining process. The slides with sampled sections were put in an oven at 58° C for one night (**Figure 10**) to remove the paraffin (deparaffinization) from the tissues before the staining process was started. As a result of SRS, an average of 20 pairs (±2) of sections were obtained from each animal, and these sections were stained with cresyl violet.

Figure 10. Laboratory drying oven

3.4.4. Staining Process

The tissue sections were stained with cresyl violet. Cresyl violet staining solution was prepared by dissolving 0.1 g cresyl violet into 100 ml distilled water. The dyes and solutions used during the staining of the hippocampus sections are shown in (**Table 4**). The duration of penetration of the dye into the hippocampal sections was determined by a pilot study applied in a single section. Then the same process was implemented to all sections based on the pilot study. Before the staining process, the cresyl violet solution was heated in the oven at about 55°C to get better penetration of the dye into the tissues and to enhance staining. The following procedures were carried out for the staining of the sections (**Table 4**). The light microscope (Olympus, BX51, Japan) was used to investigate the quality of the cresyl violet-stained sections.

Table 4. Chemicals and duration used for staining of the hippocampus sections

Chemicals	**Duration**
Xylene (x4) (Sigma-Aldrich)	2 hours
100% alcohol (x2)	10 minutes
96% alcohol	10 minutes
80% of Alcohol (Ethyl Alcohol-Tekkim Chemicals - Turkey)	10 minutes
70% alcohol	10 minutes
Distilled water	5 minutes
Cresyl violet (Sigma C5042-10G)	5 minutes
Distilled water	One minute
Acetic acid	Rinse once
96% alcohol	Rinse once
%100 alcohol	Rinse once
Xylene (x2)	2 hours
Entellan (Merck KGaA Darmstadt Germany)	For mounting

3.4.5. Tissue Preparation for Electron Microscopy

One animal from each group was perfused "intra-cardiac perfusion" through the left ventricle with 4% glutaraldehyde + 4% paraformaldehyde solutions for EM analysis. Then the samples were immersed in 4% glutaraldehyde solution for many weeks. Then the brain samples were washed with Milloning buffer. After washing with buffer, then they were put into the 1% osmium tetroxide for 1.5-2 hours in the dark. The samples were rewashed with Milloning buffer for 4x15 minutes, and the process continues with dehydration.

For dehydration:

- 50% acetone for 15 minutes,
- 75% acetone for 15 minutes,
- 95% acetone for 15 minutes,
- 100% acetone for 20 minutes,

Subsequently:

- Propylene oxide for 20 minutes,

- Propylene oxide for 20 minutes,
- 50% of propylene oxide + 50% Araldite mixture for 1 hour,
- Araldite 100% for 1 hour was subjected to the infiltration process.

Then, hippocampal tissues were blocked in the silicone embedding mould at 45^{o} C. The temperature was increased 5^{o} C every 30 minutes, and lastly, it reached 62^{o} C. They were incubated at 62^{o} C for 48 hours to ensure polymerization. At the end of this process, 500 nm thick and 70 nm sections were taken using a glass knife. 500 nm thickness sections were stained with 1% toluidine blue.

3.5. Stereological Analysis

The estimation of the total number of pyramidal cells was performed using the physical disector method, which is an unbiased stereological method of particle counting. A pilot study was done to determine the suitable sectioning interval and the number of sampled sections. The sectioning interval ratio for this study was 1/30. Serial two consecutive sections of 4-µm-thick were taken from each tissue block. The transition between the CA1 and the subiculum was defined where the cell bodies of CA1 pyramidal neurons lose their packing and not stuffed anymore, the transition between CA2 and CA3 were defined by the point where the small area of CA2 changed into the large area of CA3.

3.5.1. Defining Anatomical Area

In CA1 area the cells are medium in size while those of CA3 are large, with regard to density, cells in CA1 are crowded densely more than those of CA3 (Bayer, 1985; El Falougy et al., 2008) since the area of CA2 is small, it was included with the CA1 region (**Figure 11**).

Figure 11. Anatomical identification of CA1 and CA3 and closed polygon measurement for volume estimation

3.5.2. The Numerical Density of The Pyramidal Cells

Several coupled images at a magnification of x100 from the two consecutive sections were taken using a light microscope (Olympus BX43F, Tokyo, Japan) coupled with a microscope camera (Olympus SC50, Olympus Soft Imaging Solutions GmbH, Münster, Germany) connected to a desktop computer (**Figure 12**). The imaging software used was Olympus CellSens Entry 1.14.

Each pair of images that were taken from adjacent sections is used as a *reference* and *lookup* images for counting the number of particles using an unbiased counting frame and disector counting technique (**Figure 13**). The pyramidal cells profiles found entirely within the counting frame and do superimpose with inclusion line but not intersecting with any of the borders of the forbidden line and their extension was counted.

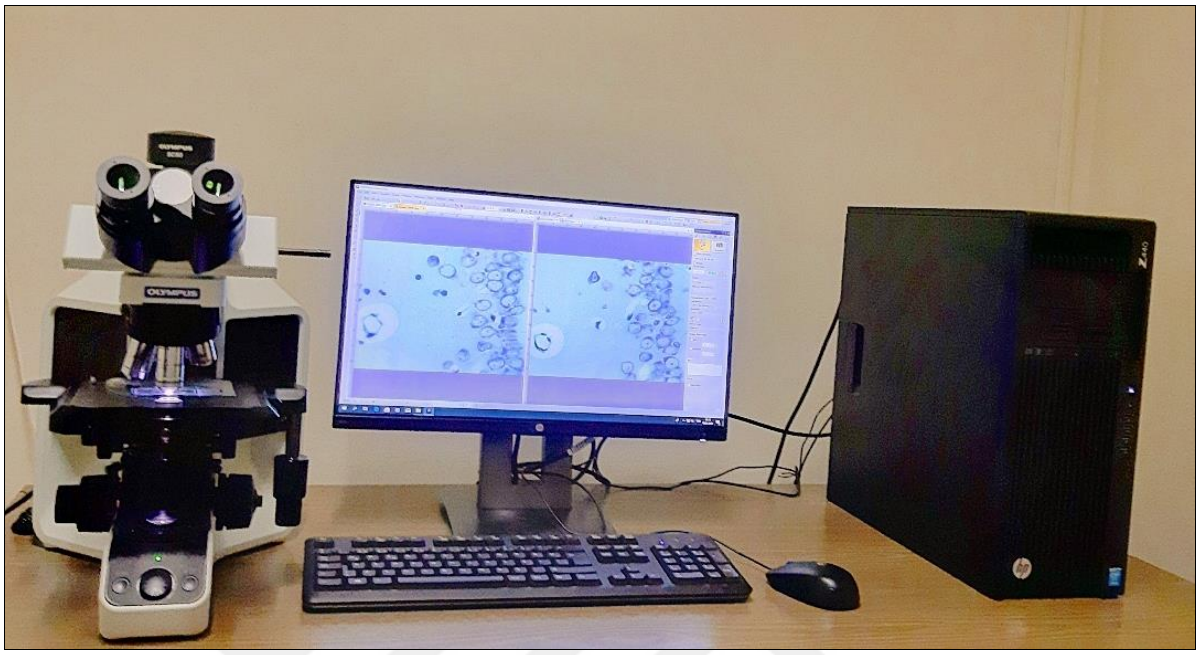


Figure 12. Stereological analysis system

The pyramidal cell profiles contacting the inclusion line and totally inside of the frame area were counted, while pyramidal cell profiles contacting the exclusion (red) border, or its extensions were not counted. The *reference* and *look up* images are shown and compared in the *ImageJ* program (**Figure 14**). Each pyramidal cell profile fulfilling all the unbiased counting frame rules and found in the *reference* section but not in the *lookup* section was counted, while others were excluded, counting was based on nucleoli. The counting was also performed after reversing the *reference* and the *lookup* images; the *reference* used as *lookup* and the *lookup* as a *reference*. The disector particle number, the counting frame area, and the section thickness were used in a specific formula to estimate the numerical density that is given below for each rat.

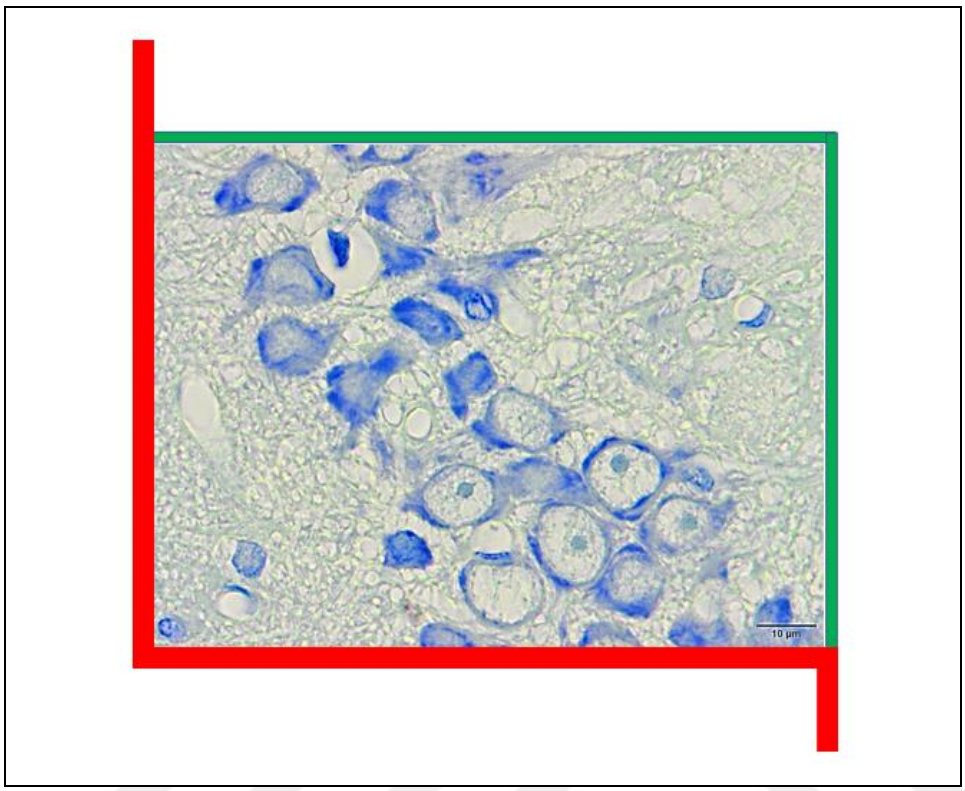


Figure 13. Unbiased counting frame

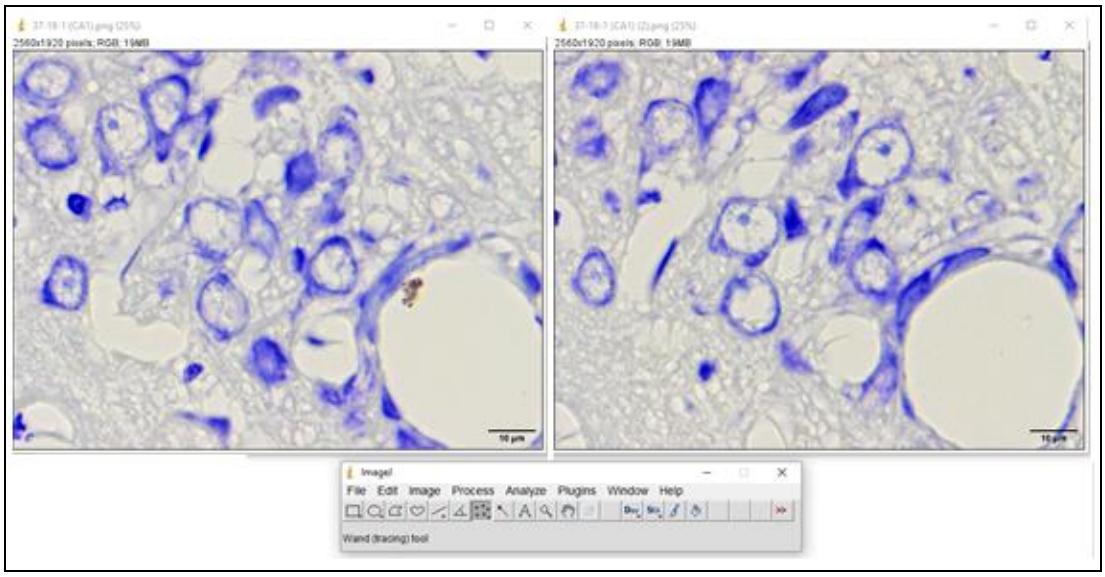


Figure 14. A pair of disector section is seen in the ImageJ program

3.5.3. The Volume of The Hippocampus

The reference volume of the hippocampus of each rat was estimated by using Cavalieri's principle. We estimated the total area of the hippocampus from the sampled sections for each rat by using the (closed polygon measurement) option in the software in the same system (**Figure 11**), which enables us to measure the hippocampus section area in (μm^2) directly. Multiplying the total area of the sampled sections ($\sum$**A**) with the distance between these sections' areas (**T**) gives us an unbiased estimate of the reference volume ($\mathbf{V_{ref}}$) of the pyramidal layer of the hippocampus. The distance (T) is equal to the sectioning interval multiplied by the section thickness. The following formula was used:

$$V_{ref} = \sum A \times T$$

3.5.4. The Estimation of The Total Number of the Pyramidal Cells

The total number of the pyramidal cells (N) in the hippocampus of each rat was estimated using ***the physical fractionator***, by applying the following formula:

N = Σ Q x (1/ssf) x (1/asf)

Σ Q: The total number of disector particle (i.e. nucleoli of pyramidal cells)

ssf: The section-sampling fraction

asf: The area sampling fraction

While:

Step size was = 59365.55 μm^2

Counting frame size was = 9498.5 μm^2

Section sampling fraction was = 1/ (1/30) = 30

Area sampling fraction= 1/ (9498.5/296827.75) = 31.25

Total number of pyramidal cells = Σ Q x (1/ (1/30)) x (1/ (9498.5/296827.75))

3.5.5. The Coefficient of Variance and The Coefficient of Error

We estimated the coefficient of variance (CV) and the coefficient of error **(**CE) values to ensure that our study has sufficient precision of design. The CV is a measure of the group

variability, whereas CE is a measure of the amount of error in the stereological estimation. The equations that we used in the calculations of CV and CE are shown below (Gundersen and Jensen, 1987; West et al., 1991; Gundersen et al., 1999).

$$CV = \frac{\text{standard deviation}}{\text{mean}} = \frac{\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}}{\bar{x}}$$

x: the number of pyramidal cells in the individual animal; $\bar{x}$: the mean number of pyramidal cells in each animal group; n: the number of animals in each group.

$CE_{(Nv)}$ for the estimate of the pyramidal cells' numerical density was calculated as follows:

$$A = \sum Q_i \cdot Q_i$$

$$B = \sum Q_i \cdot Q_{i+1}$$

$$C = \sum Q_i \cdot Q_{i+2}$$

$$Noise = \sum Q$$

$$Var_{(srs)} = (3(A\text{-}Noise) - 4B + C)/240$$

$$Total\ Var = Noise + Var_{(srs)}$$

$$CE_{(Nv)} = \frac{\sqrt{Total\ Var}}{\sum Q}$$

$\sum \mathbf{Q}$: the total number of disector pyramidal cells counted in all sampled sections of each rat; $\mathbf{Q_i \cdot Q_i}$: the square of the disector pyramidal cells in each section; $\mathbf{Q_i \cdot Q_{i+1}}$: the number of disector pyramidal cells from each section (i) multiplied by the number of the disector pyramidal cells from the next section (i+1) in the sequence; $\mathbf{Q_i \cdot Q_{i+2}}$**:** the number of disector pyramidal cells from each section (i) multiplied by the number of the disector pyramidal cells from the second next section (i+2) in the sequence.

$CE_{(V)}$ for the estimate of the total volume of the hippocampus was calculated as follows:

$$A = \sum A_i \cdot A_i$$

$$B = \sum A_i \cdot A_{i+1}$$

$$C = \sum A_i . A_{i+2}$$

$$\text{Noise} = \sum 0.0724 \times 8 \times \sqrt{\sum A}$$

$$\text{Var}_{(srs)} = (3(\text{A-Noise}) - 4B + C)/240$$

$$\text{Total Var} = \text{Noise} + \text{Var}_{(srs)}$$

$$CE_{(v)} = \frac{\sqrt{\text{Total Var}}}{\sum A}$$

$\sum \mathbf{A}$: the total area of all sampled hippocampus sections of each rat; $\mathbf{A_i} . \mathbf{A_i}$: the square of the area of each hippocampus section; $\mathbf{A_i} . \mathbf{A_{i+1}}$: the area of each hippocampus section (i) multiplied by the area of the next hippocampus section (i+1) in the sequence; $\mathbf{A_i} . \mathbf{A_{i+2}}$**:** the area of each hippocampus section (i) multiplied by the area of the second next section (i+2) in the sequence.

The upper limit for CE and CV is considered to be 0.05 and 0.10, respectively. Values below these limits indicate that the stereological design is sufficiently precise.

3.6. Biochemical Methods

Rats were perfused two days after the end of the study (to allow for the behavioural examinations of the rats), and blood samples were taken from the heart, this delay did not affect the level of antioxidant activity because diet supplementation of antioxidants will keep the antioxidants' activity high even after a week of ceasing the supplementation (Limberaki et al., 2012).

3.6.1. Blood Sample Collection

During the perfusion process and after the access of the heart, 3 ml of intracardiac blood was collected from the heart of the rat into blood collection tubes. The blood was taken before passage of perfusion solutions through the circulatory system of the rats and centrifuged at 4 °C on (HettichEB 20 centrifuge, Hettich, Tuttlingen, Germany) for 15 min at 2000 rpm (**Figure 15**). The obtained serum samples were stored at -80 ° C until biochemical analysis was done. The CAT and SOD enzyme activities in the serum samples were analysed by measuring absorbance values at the biochemistry department.

Figure 15. Blood centrifuging machine

3.6.2. Analysis of Superoxide Dismutase Enzyme Activity

Highly reactive radicals produced by enzymes, such as xanthine oxidase and mitochondrial electron chain, appear in different pathologies with the accumulation of superoxide anions (O2-) in cells and tissues (Viggiano et al., 2003). Accumulation of these molecules and other oxidant radicals should be avoided in order to resist the toxic effects of oxygen in aerobic organisms. When oxygen toxicity occurs in living tissues, enzymatic defence mechanism initiated by SOD catalyzes the dismutation of oxygen and metalloenzymes (Peltola et al., 1992; Assady et al., 2011).

In our study for assessing OS, SOD enzyme activity was measured using the SOD assay kit (Cayman Chemical Company, USA). The basic principle of the SOD assay kit used is based on the principle that xanthine-xanthine oxidase reaction results in the reaction with tetrazolium compound and purple reaction by removing superoxide radicals. The SOD in the sample removes the superoxide radicals from the medium and inhibits the reaction of the tetrazolium compound with oxygen. The absorbance values of the purple-coloured solutions obtained after the experiment were measured using a microplate absorbance reader (Sunrise^{TM}, Tecan Trading AG, Männedorf, Switzerland) (**Figure 16**) at a wavelength of 450 nm (Ukeda et al., 1999).

Figure 16. A microplate absorbance reader (Sunrise™, TECAN)

3.6.3. Analysis of Catalase Enzyme Activity

CAT is an antioxidant enzyme involved in the detoxification of H_2O_2, which is a toxic product of normal aerobic metabolism. The H_2O_2 at a low level stimulates the expression of stress-associated proteins and antioxidant enzymes, thus providing a response against oxidants. The conversion of the H_2O_2 molecule into oxygen and water occurs by the CAT enzyme. CAT demonstrates peroxidation activity, in which low molecular weight alcohols can serve as electron donors (Madeo et al., 1999; Martins and English, 2014). To determine the oxidative effect, Cayman's CAT Assay Kit (Cayman Chemical Company, USA) utilized for the determination of enzyme activity in the serum. The basic principle in the method is based on the CAT enzyme reaction with methanol in the presence of H_2O_2. In this reaction, formaldehyde acts as a chromogen, causing a pink colour in the reaction. After the relevant reactions, the absorbance of the samples was measured at a wavelength of 540 nm using a multi-mode microplate reader (BioTek Synergy 4, BioTek Instruments Inc., Vermont, USA) (**Figure 17**).

Figure 17. A microplate absorbance reader (BioTek Synergy 4)

3.7. Determination of Total Phenolic Content and Antioxidant Power

3.7.1. Preparation of Sample Extract

In this study, extraction was made by ultrasound-assisted extraction with 80% methyl alcohol. For this, 2.5 g of the sample was added to 50 mL of 80% methyl alcohol at 20 ° C for 30 minutes in an ultrasonic water bath, then at room temperature for 2 hours. It was put in a centrifuge for 15 minutes at 3000 rpm to remove turbidity. The pre-analysis samples were filtered through a 0.45 Teflon filter.

3.7.2. Method to Assess the Total Phenolic Content

Singleton and Rossi (1965) 's method of determining the total phenolic content was modified (Singleton and Rossi, 1965). 2.5 ml of Folin-Ciocalteu was added onto 0.5 ml of sample diluted with the appropriate amount of methyl alcohol and left for 5 minutes in a dark place to add 7.5% sodium carbonate solution. At the end of the period, the total

phenolic content was calculated by means of the standard calibration curve, which was read against the witness at 760 nm wavelength and plotted with pure gallic acid.

3.7.3. Methods to Assess the Antioxidant Power

3.7.3.1. Ferric Iron Reduction Antioxidant Power (FRAP)

Samples extracted with methyl alcohol were diluted in an appropriate proportion; 10: 1: 1 (v: v: v) 300 mM acetate buffer: mixed with 20 mM FeCl3: 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (dissolved in 40 mM HCl), the absorbance at 593 nm was determined on the spectrophotometer. A calibration curve was drawn by using Trolox prepared solutions, and the result was calculated (Gao et al., 2000).

3.7.3.2. Determination of DPPH Free-Radical Scavenging Effect

The method described by Tural and Koca (2008) was used to determine the reduction effect of DPPH radical (2,2-diphenyl-1- picrylhydrazyl) (Tural and Koca, 2008). 50 L of the methyl alcohol-containing extract was mixed with 1 ml of DPPH solution and kept at room temperature in the dark until the reaction was completed, and the absorbance at 517 nm was determined.

3.8. Behavioural Examinations

3.8.1. Morris Water Maze test

The MWM test for spatial learning and memory was developed more than 30 years ago (Morris et al., 1982), it is particularly sensitive to the effects of hippocampal lesions in rats, the impairment is related to the size of the damage and the hippocampal activity (D'Hooge and De Deyn, 2001; Zhao et al., 2016). Sex differences in performance of rats in MWM are only significant with young rats, but there is no significant difference at an older age (Bucci et al., 1995) in which will be used in this study (adult 12 -16 weeks), this age is also ideal, for the older rats demonstrate declined performance in this test. Thigmotaxis is known to be more prominent in mice than rats; this study using rats (Brandeis et al., 1989; D'Hooge and De Deyn, 2001).

The apparatus (**Figure 18**) consists of a large circular pool filled with water. The small platform is kept hidden by being one centimetre below the opaque water; a review by Rudi D'Hooge mentioned that there are many different sizes for the pool ranging from 1 to 2 meters in diameter (D'Hooge and De Deyn, 2001). While another review stated, the dimensions vary from 0.80-2.20 m for the diameter by 0.40-0.60 m for the height (Brandeis et al., 1989).

The pool's diameter is 130 cm by 60 cm high. The inner surface is devoid from cues, non-reflective and painted white, the temperature of the water is 25 -26 degrees, the water was rendered opaque by adding non-toxic white colouring material, the platform is 25 cm high and 9 cm in diameter (Morris, 1984).

Procedure (spatial acquisition) (Vorhees and Williams, 2006): The procedure started after the experiment (Scheff et al., 1997; Biessels et al., 1998; Logue et al., 1997).

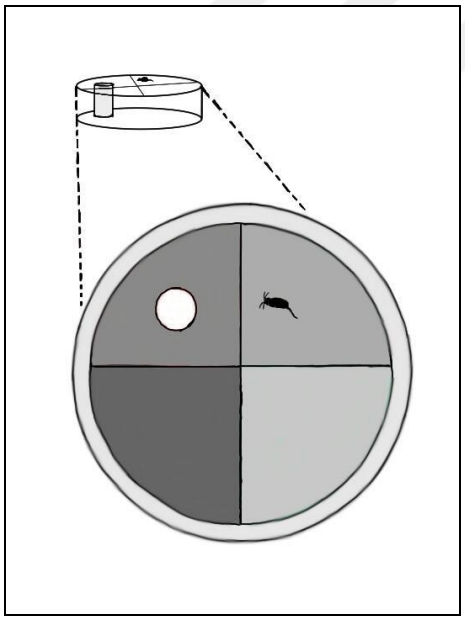


Figure 18. Morris water maze test apparatus

Visible platform training: We used the visible platform setting as a control method to exclude visual and motor deficits; the visible platform was moved from quadrant to another in every trial.

Hidden platform training: The animal was placed in three start positions in the tank (the three other quadrants in which the platform is not placed), its face into the tank's wall, placed into water with the tail first, the time it takes to reach a hidden platform (latency) was recorded.

Three trials for each animal per day for three days, each trial lasted for 90 seconds; if the rats couldn't reach the platform within 90 seconds, it was placed on the platform. The inter-trial interval (ITI) was 15 seconds.

3.8.2. Passive Avoidance Test

Passive avoidance test for memory retention (Lorenzini et al., 1996; Lorenzini et al., 1997; Sarkaki and Rezaiei, 2013) was performed. The apparatus used in this test composed of two adjacent rooms separated by a door, one of the rooms was white and brightened by a light on its ceiling, the other room was dark (**Figure 19**), the floor of the darkroom was electrified. On the first day, the rats were placed on the lightroom and could explore the apparatus, when then rat moves to the darkroom, the door was automatically lowered, and a 1-mA of electrical shock was delivered. On the second day, the procedure was repeated, latency time was automatically recorded.

Figure 19. Passive avoidance test apparatus.

3.9. Statistical Analysis

The numerical data obtained from this study were evaluated by the SPSS software program (version 22.0). A one-way ANOVA test was used to detect significant differences among the groups. Thereafter, Tukey's test was applied to compare the differences among the groups. According to normality test results, the data of the hippocampal volume and the total pyramidal cells' number had abnormal distribution. Therefore, Tamhane's T2 test was performed to compare each of the hippocampal volume and the total pyramidal cells' number among the groups. a p-value of less than 0.05 was considered significant.

4. RESULTS

The present study was conducted to investigate the possible effects of GA on minimizing the neurodegenerative effects of DS on rat hippocampus, behavioural, stereological, biochemical, and analyses were conducted.

4.1. Behavioural tests

4.1.1. Morris water maze test

Rats were prepared by being exposed to the experiment's conditions with a visible platform, a day before the experiment itself, with three trials, we set the platform on different locations close to the centre of the pool in each trial, the purpose was to exclude any visual or motor deficits.

In the other day the experiment conducted "hidden platform protocol", the latency was measured and compared between control (Cont.), DS (DS), GA (GA) and DS and GA (DS+GA) groups (**Table 5**), the mean performance of rats in "hidden platform protocol" for each one of the three trial was calculated, and a comparison was made between those groups (**Table 6**).

Table 5. The mean performance of each rat in the group in the three rounds, for all groups, in "hidden platform protocol"

Groups	Latency (seconds)			
	Cont.	GA	DS	DS+GA
Mean performance of each rat in the three rounds	75	51	53	25
	30	33	37	21
	22	26	73	52
	48	44	60	47
	19	56	49	33
	16	34	41	28
	16	24	25	72
	20	19	10	24
	11	40	16	19
	13	31	29	-

Table 6. The mean performance of rats in "hidden platform protocol" in the three rounds (all groups)

Group	Latency (seconds) Mean performance		
	Round One	Round Two	Round Three
Control (Cont.)	38.21	21.2	21.1
Diclofenac (DS)	58.41	32.92	26.89
GA (GA)	60	27.6	20.3
Diclofenac + GA (DS+GA)	54.8	31.9	19.1

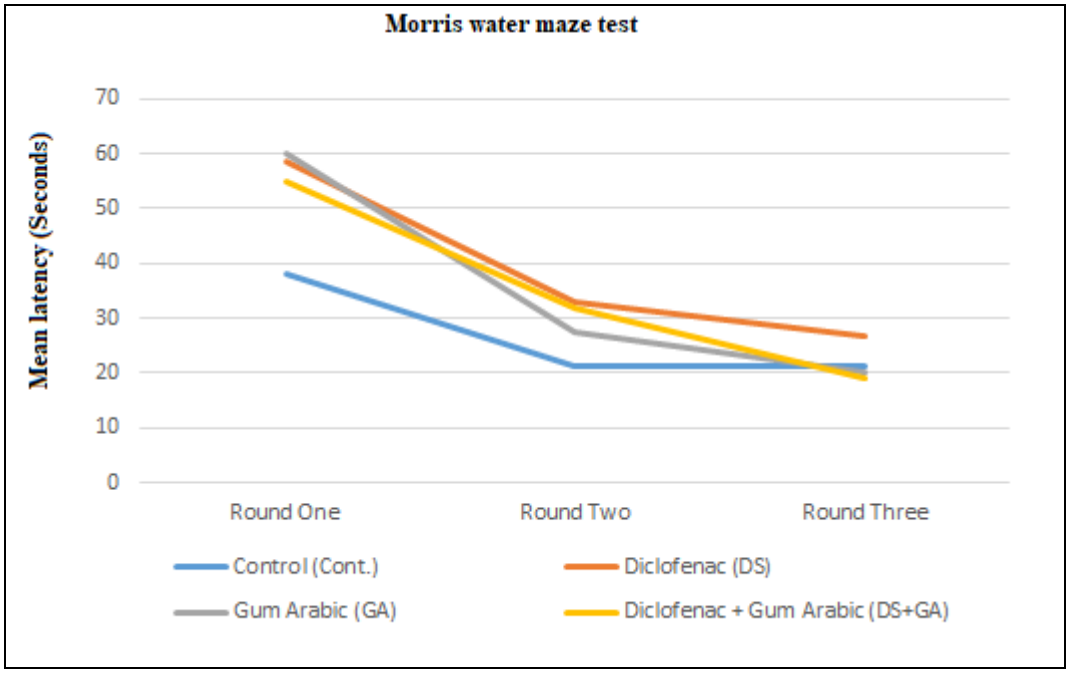


Figure 20. The mean performance of rats in "hidden platform protocol" in the three trials (all groups)

As it is shown the performance was lower in the DS group and the best performance was in the control group (**Figure 20**), the performance in the GA group is better than that of the DS group. However, no significant differences were found between the performances of the groups.

4.1.2. Passive avoidance learning test

Table 7. Latency time in passive avoidance learning test for all groups (in seconds). The standard error of the mean (SE)

Rats	Control (Cont.)		Gum Arabic (GA)		Diclofenac (DS)		Diclofenac + Gum Arabic (DS+GA)	
	T1	T2	T1	T2	T1	T2	T1	T2
1.	42.7	71.3	300.00	300.00	104.5	8.2	73.6	35.8
2.	35.7	5.9	186.00	300.00	23.9	300	300	300
3.	44.3	300	34.90	131.50	21.2	300	300	300
4.	40.7	35.7	106.00	300.00	300	300	11.2	4.4
5.	3.4	225.5	16.90	300.00	4.4	239.8	3.9	158.5
6.	54.7	161	17.10	300.00	17.3	86.1	38.7	34.4
7.	22.1	300	298.00	8.90	73.5	96.8	7.6	300
8.	3.3	14.5	27.80	300.00	2.8	133	5.8	300
9.	5.3	100.3	16.90	264.90	25.6	300	35.6	300
10.	4.2	39.3	13.40	108.90	86.8	300	-	-

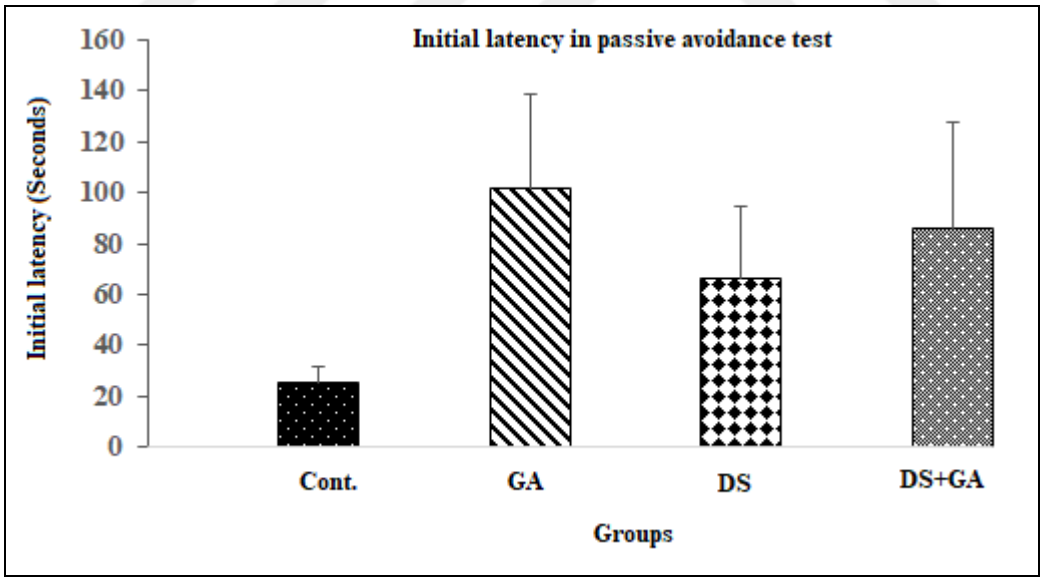


Figure 21. Initial latency in passive learning avoidance test, control (Cont.), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium, and Gum Arabic (DS+GA). Data are expressed as mean ± standard error of the mean

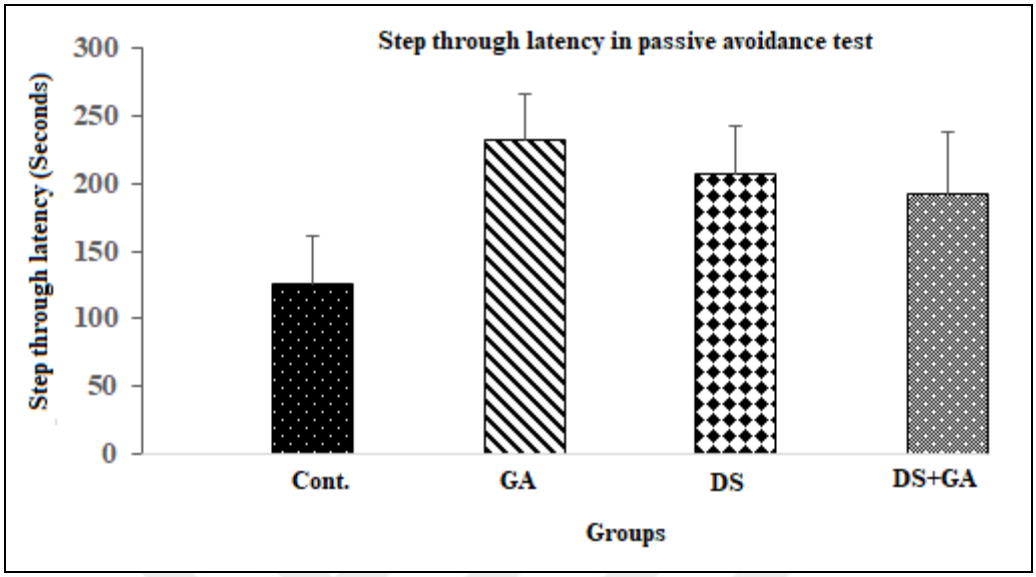


Figure 22. Step through latency in passive avoidance test, control (Cont.), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium, and Gum Arabic (DS+GA). Data are expressed as mean ± standard error of the mean

Memory retention shows no significant difference between groups in both the initial latency and the step-through latency in passive learning tests as shown in (**Figures 21 and 22**)

4.2. Stereological Findings

The total number of pyramidal cells was calculated in the whole hippocampus using the physical fractionator technique. The total volume of the hippocampus was estimated using Cavalieri's principle and calculated using the given equations in the methodology section. Those estimations were done for the four groups: Cont group, GA group (rats received GA by oral gavage), DS group (rats receive DS by oral gavage), and DS+GA group (rats received both DS and GA by oral gavage).

4.2.1. The Total Number of Pyramidal Cells in Hippocampus

The total number of pyramidal cells of the hippocampus and the coefficient of error (CE) for it in the regions of CA1, CA3, and the whole hippocampus are shown in the following tables and figures (**Tables 8, 9 and 10**; **Figure 23. 24 and 25**).

Table 8. Total number of pyramidal cells in the hippocampus, control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Groups	Mean	Standard error of the mean
Cont	775468	188890
GA	823928	101793
DS	796874	112381
DS+GA	633437	183876

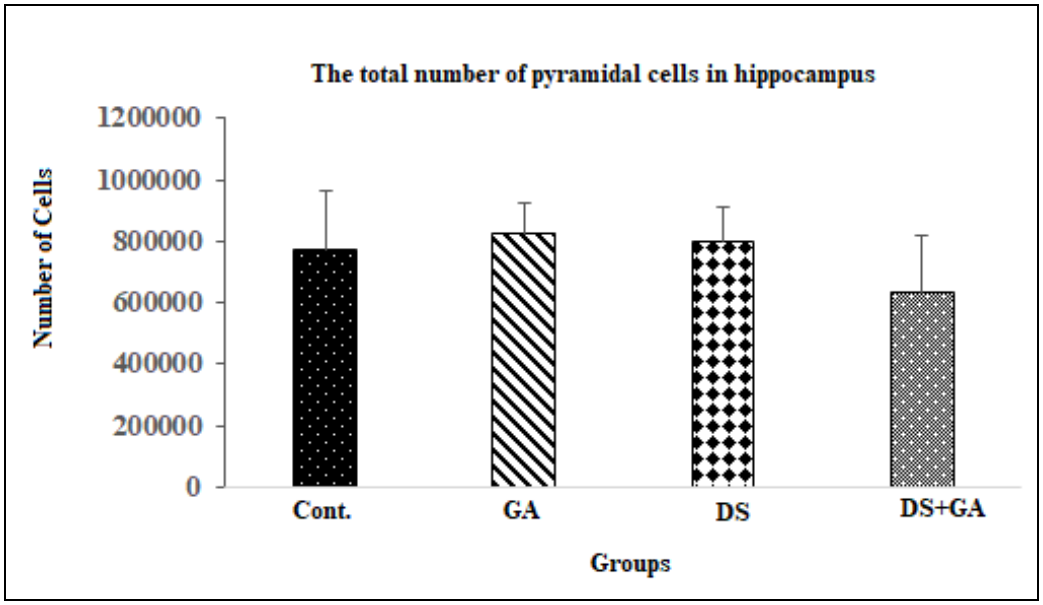


Figure 23. Total number of pyramidal cells in the hippocampus, control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Table 9. Total number of pyramidal cells in CA1 region of the hippocampus, control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Groups	Mean	Standard error of the mean
Cont	503906	121022
GA	514419	80673
DS	476249	72395
DS+GA	409062	125879

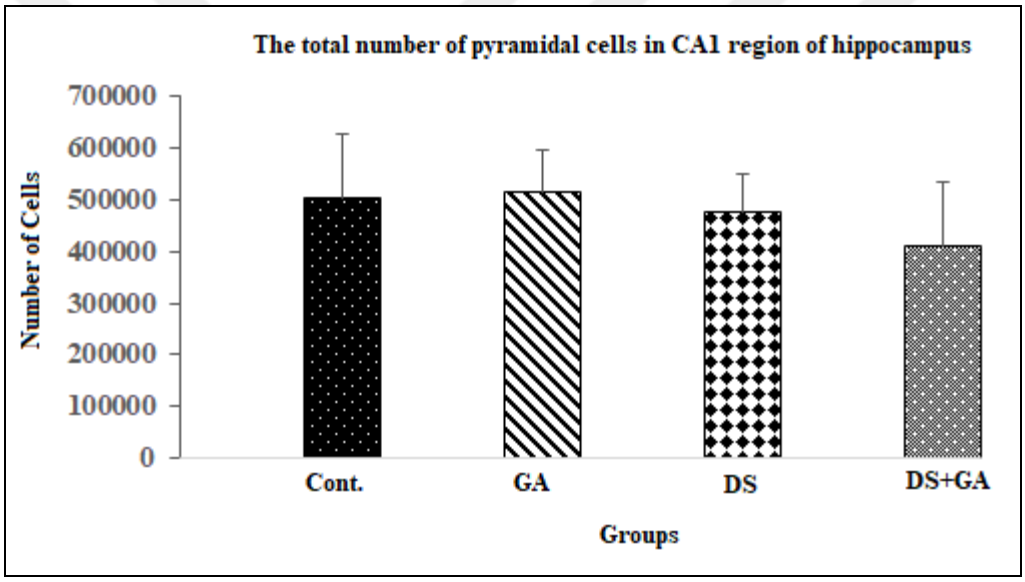


Figure 24. Total number of pyramidal cells in CA1 region of the hippocampus, control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Table 10. Total number of pyramidal cells in CA3 region of the hippocampus, control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Groups	Mean	Standard error of the mean
Cont	272343	75063
GA	302812	35625
DS	320000	49536
DS+GA	224375	61317

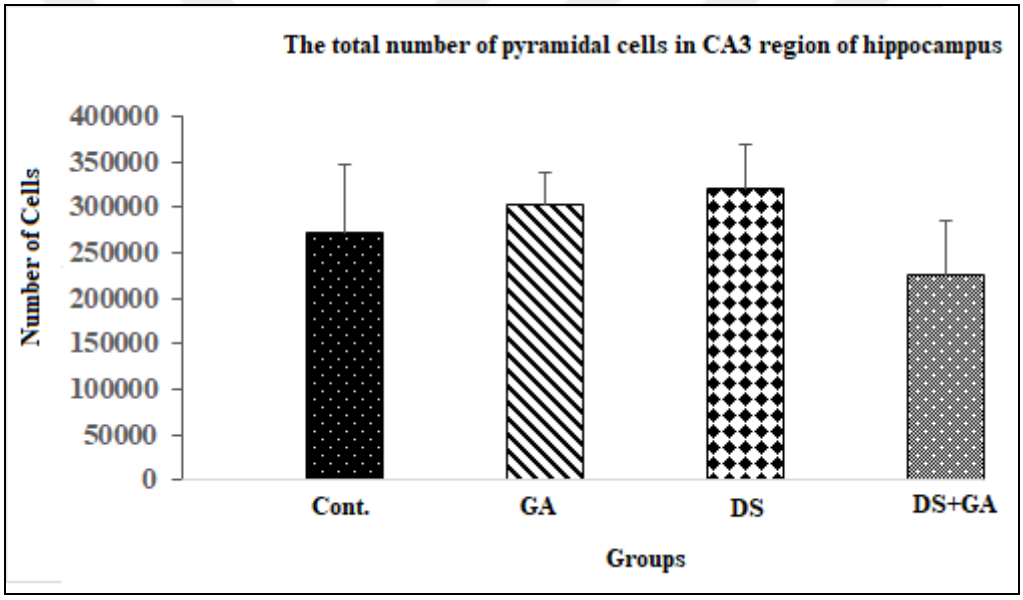


Figure 25. Total number of pyramidal cells in CA3 region of the hippocampus, control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

As a result of the statistical analysis of the data, it was found that there is no significant difference between the groups with regards to the total number of pyramidal cells in the hippocampus, as well as in its CA1 and CA3 regions.

4.2.2. The Total Volume of the Hippocampus

The mean total volumes of the hippocampus are shown in the following table and figure (**Table 11**; **Figure 26**).

Table 11. Volume estimation of the hippocampus (mm³), control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Groups	Mean	Standard error of the mean
Cont	0.854	0.172
GA	0.956	0.139
DS	0.911	0.229
DS+GA	0.963	0.287

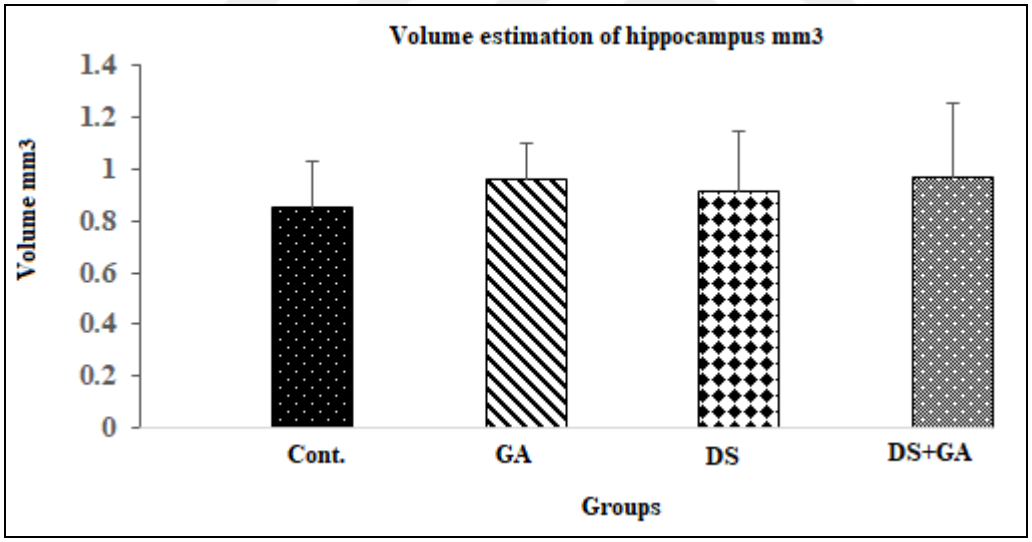


Figure 26. Volume estimation of the hippocampus (mm³), control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

The coefficient of error CE, and the coefficient of variance CV for the numerical density, the total number of cells and total volumes of the hippocampus are shown in (**Table 12**).

Table 12. The coefficient of error CE and the coefficient of variance CV for the numerical density, the total number of cells and total volumes of the control (C), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA) groups

Groups	CE values					CV values	
	CA all regions		CA1	CA3	Hippocampal volume	Total number of pyramidal cells	Hippocampal volume
	Total number of pyramidal cells	Numerical density					
Cont	0.039	0.036	0.045	0.056	0.0090	0.222	0.183
GA	0.036	0.034	0.042	0.054	0.0087	0.114	0.134
DS	0.035	0.037	0.047	0.053	0.0075	0.128	0.229
DS+GA	0.040	0.041	0.049	0.060	0.0082	0.264	0.272

As a result of the statistical analysis of the data, it was found that there is no significant difference between the groups with regards to the volume of the hippocampus.

4.3. Analysis of Enzyme's Activities

4.3.1. Analysis of Catalase Enzyme Activity

Table 13. CAT enzyme activity (nmol/min/ml (540 nm)), control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Groups	Mean	Standard error of the mean
Cont	216.000	34.811
GA	322.794	16.743
DS	245.370	18.610
DS+GA	279.866	41.281

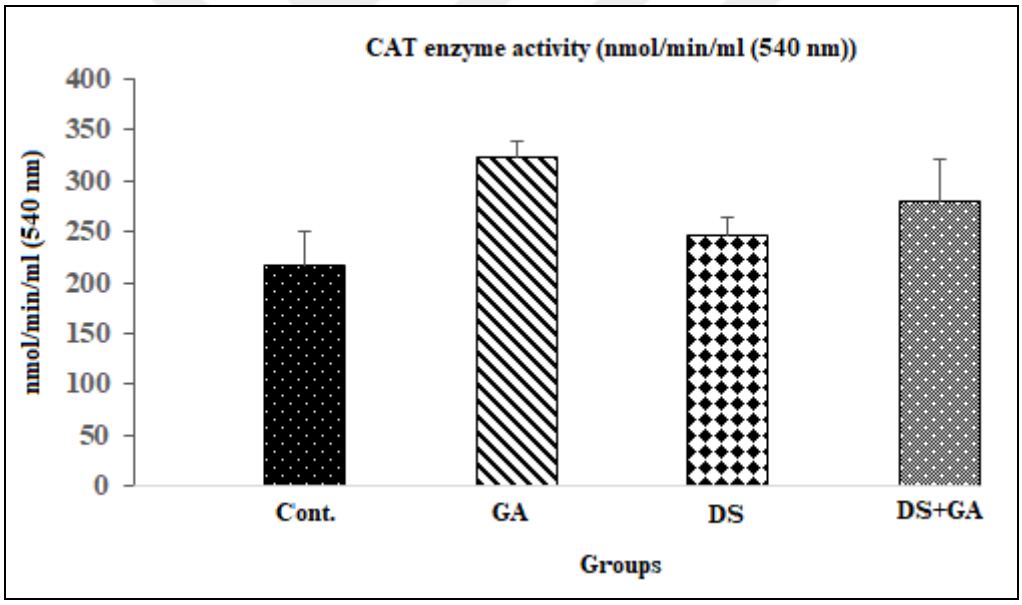


Figure 27. CAT enzyme activity (nmol/min/ml (540 nm)), control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

4.3.2. Analysis of Superoxide Dismutase Enzyme Activity

Table 14. SOD enzyme activity (U/ml (450 nm)), control (Cont), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

Groups	Mean	Standard error of the mean
Cont	27.2	0.275
GA	26.6	0.407
DS	27.0	0.292
DS+GA	26.8	0.483

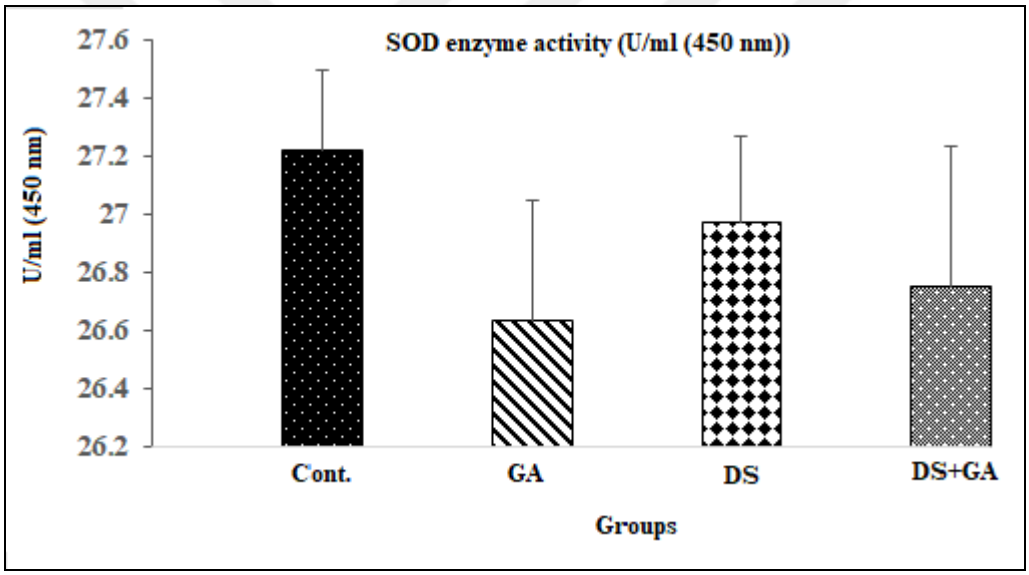


Figure 28. SOD enzyme activity (U/ml (450 nm)), control (Cont.), Gum Arabic (GA), diclofenac sodium (DS), diclofenac sodium + Gum Arabic (DS+GA)

As a result of the analysis of the enzyme's activities, statistical analysis of the data shows no significant difference between the groups (**Figures 27 and 28**).

4.4. Light Microscopic Findings

4.4.1. Light Microscopic Findings of Cresyl Violet Stained Sections

Tissue sections were taken from the hippocampus of all experimental animals. These sections were stained with cresyl violet staining, and the CA layer of hippocampus was examined by the light microscope (**Figure 29, 30, 31, 32, 33, 34, 35, 36**).

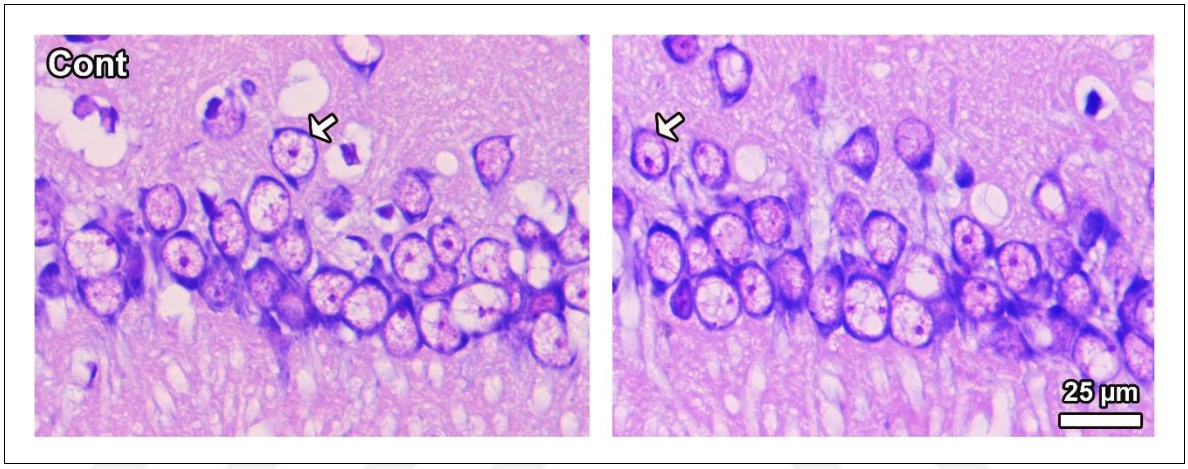


Figure 29. Histological images of the CA1 layer of the hippocampus stained with cresyl violet staining are seen. The general structure of the hippocampus is fine. The arrows indicate healthy pyramidal cells. Original magnification: X100

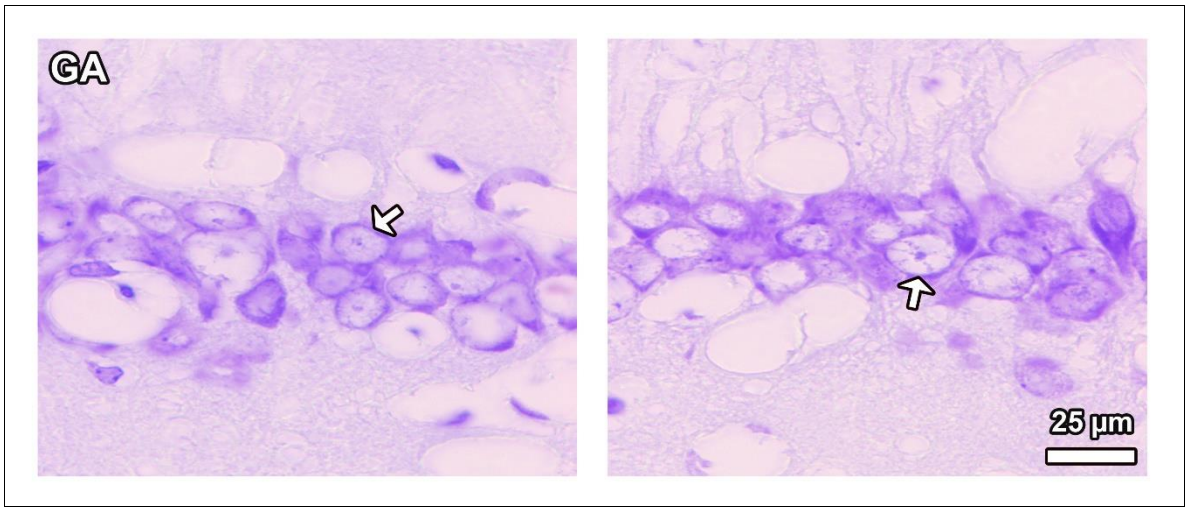


Figure 30. Histological images of the CA1 layer of the hippocampus stained with cresyl violet staining are seen. The arrows indicate healthy pyramidal cells. Original magnification: X100

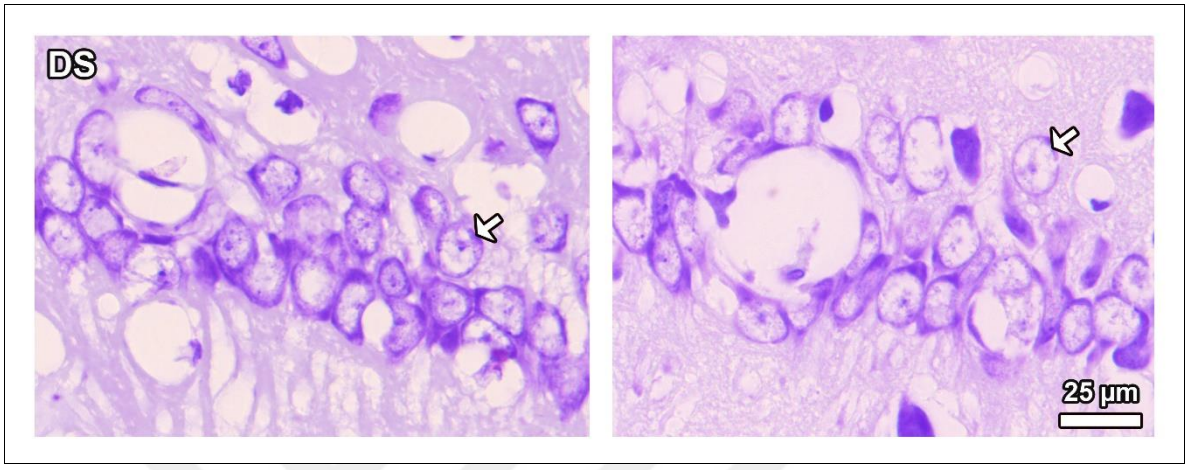


Figure 31. Histological images of the CA1 layer of the hippocampus stained with cresyl violet staining are seen. The arrows indicate healthy pyramidal cells. Original magnification: X100

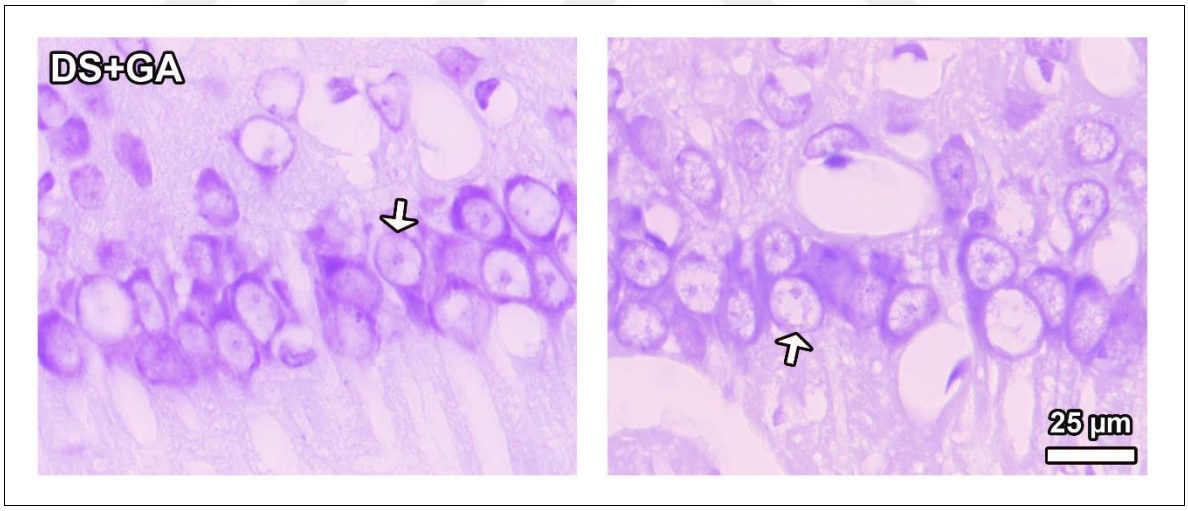


Figure 32. Histological images of the CA1 layer of the hippocampus stained with cresyl violet staining are seen. The arrows indicate healthy pyramidal cells. Original magnification: X100

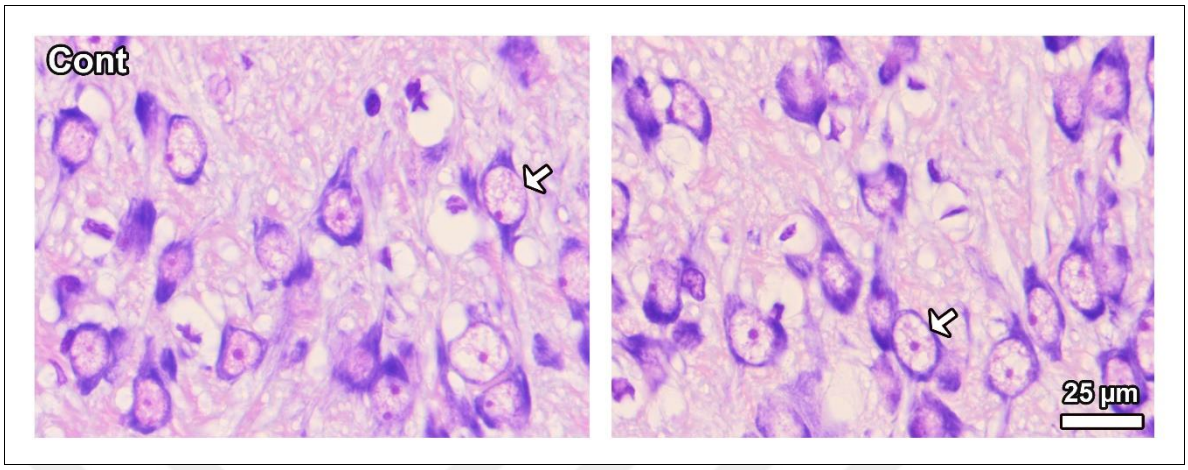


Figure 33. Histological images of the CA3 layer of the hippocampus stained with cresyl violet staining are seen. The general structure of the hippocampus is fine. The arrows indicate healthy pyramidal cells. Original magnification: X100

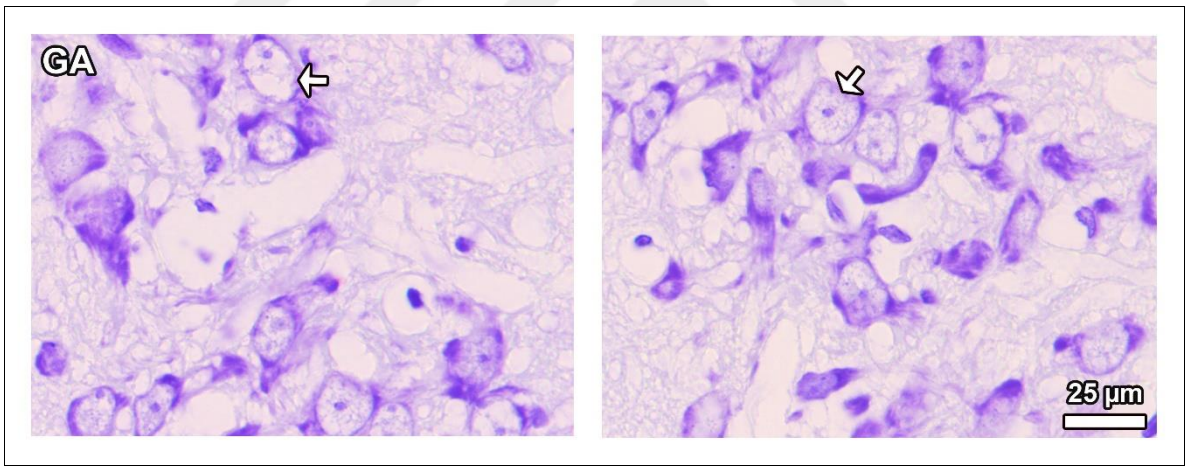


Figure 34. Histological images of the CA3 layer of the hippocampus stained with cresyl violet staining are seen. The arrows indicate healthy pyramidal cells. Original magnification: X100

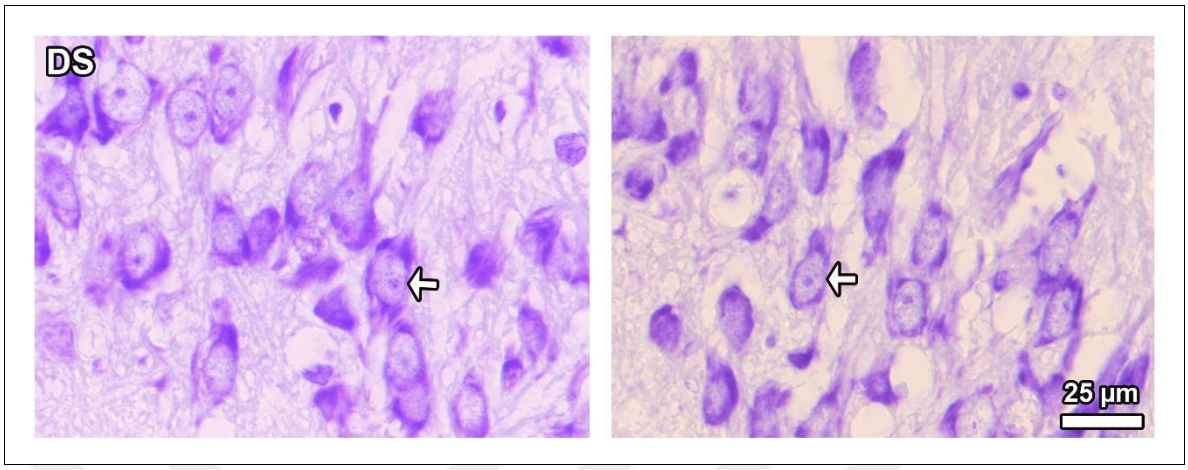


Figure 35. Histological images of the CA3 layer of the hippocampus stained with cresyl violet staining are seen. The arrows indicate healthy pyramidal cells. Original magnification: X100

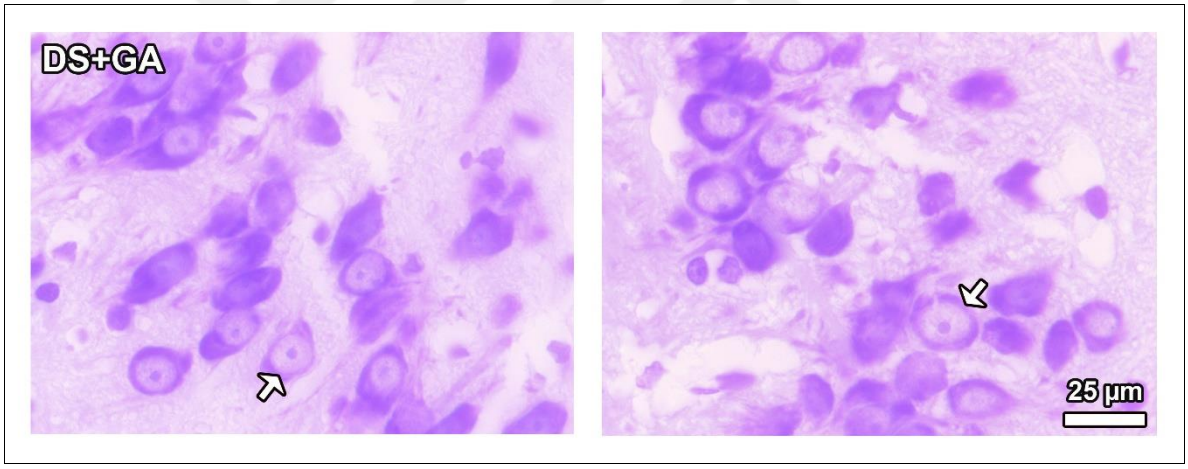


Figure 36. Histological images of the CA3 layer of the hippocampus stained with cresyl violet staining are seen. The arrows indicate healthy pyramidal cells. Original magnification: X100

4.4.2. Light Microscopic Findings by Toluidine Blue Stained Sections

Resin sections of the hippocampus from all groups were taken and stained by toluidine blue. Following the examination of the CA region, the findings are given below.

Light microscopic images obtained from the different regions of the hippocampus in the control group showed that pyramidal cells and granular cells have normal architectures; their nuclei are clearly observed. A few numbers of the dark cells and axons are found. Images taken from the (CA) layer of the hippocampus showed that the general structure of the hippocampus is intact. Each pyramidal cell has an oval to round shaped nucleus, nuclei of pyramidal cells, and the cell borders are clearly seen. Pyramidal cells in the CA3 region were of large size and less densely arranged in comparison to the CA1 region. Granular neurons (Gc) are observed; they were of medium size and densely compacted. Axons were seen in horizontal and longitudinal sections (**Figure 37, 38, 39, 40, 41 and 42**).

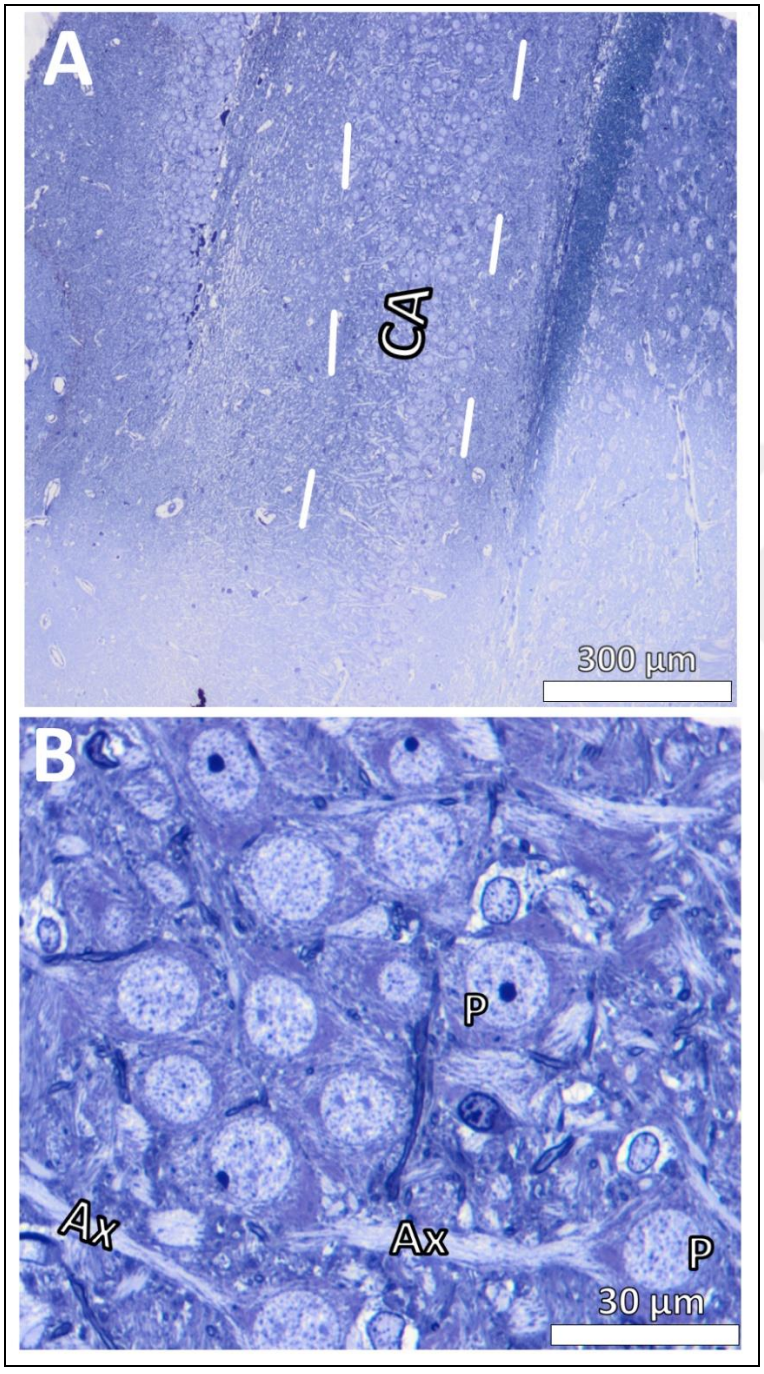


Figure 37. (A) Images taken from the hippocampal region of the control group are displayed. The general structure of the hippocampus is intact, the Cornu Ammonis (CA) layer of the hippocampus is presented, and (B) pyramidal cells (P) show the large size and less densely compacted indicates CA3 region. Ax: Axons are observed (Ax). Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification for (A) and (B): X10 and X100 respectively

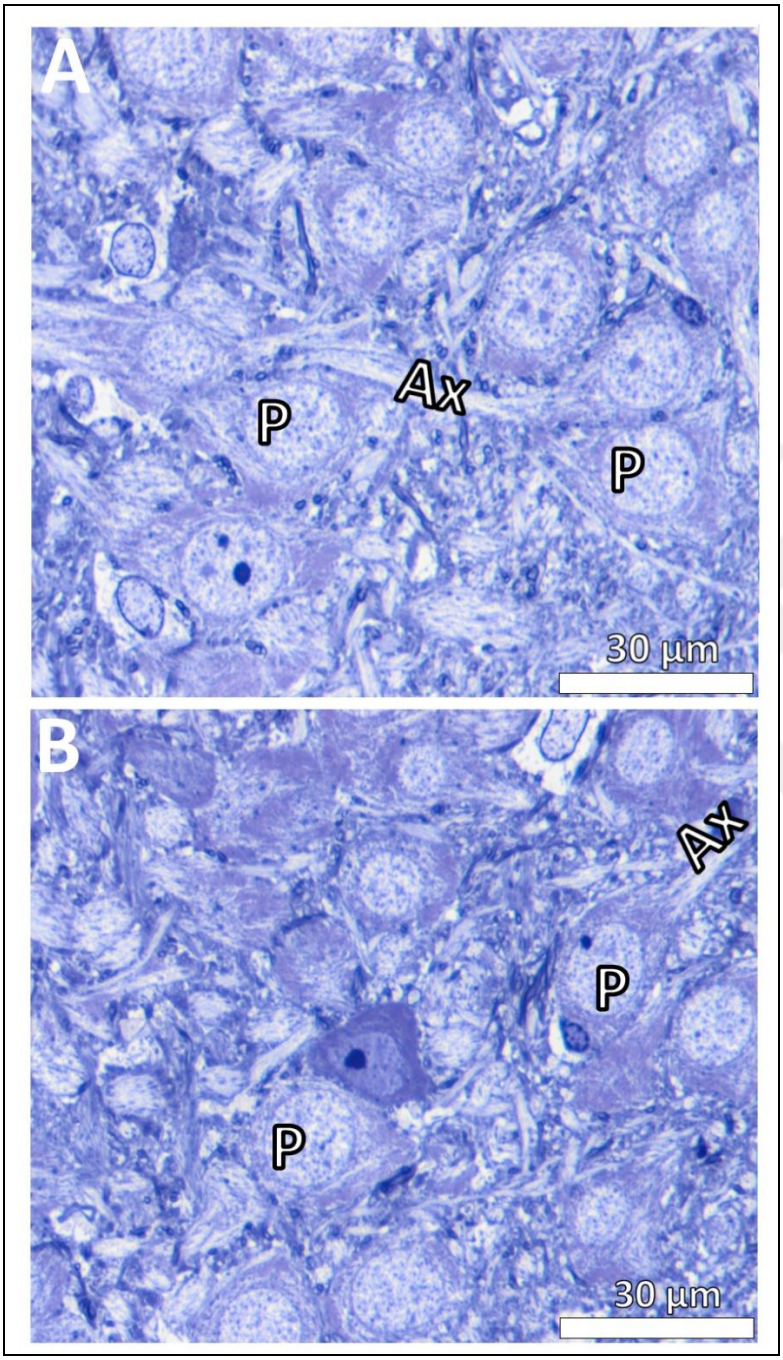


Figure 38. (A, B) images of the CA layer of the hippocampal region of the control group are seen. The general structure of the hippocampus is intact, and cells show the large size and less densely compacted. Nuclei of pyramidal cells (P) and the cell borders are apparent. Axon, Ax; toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

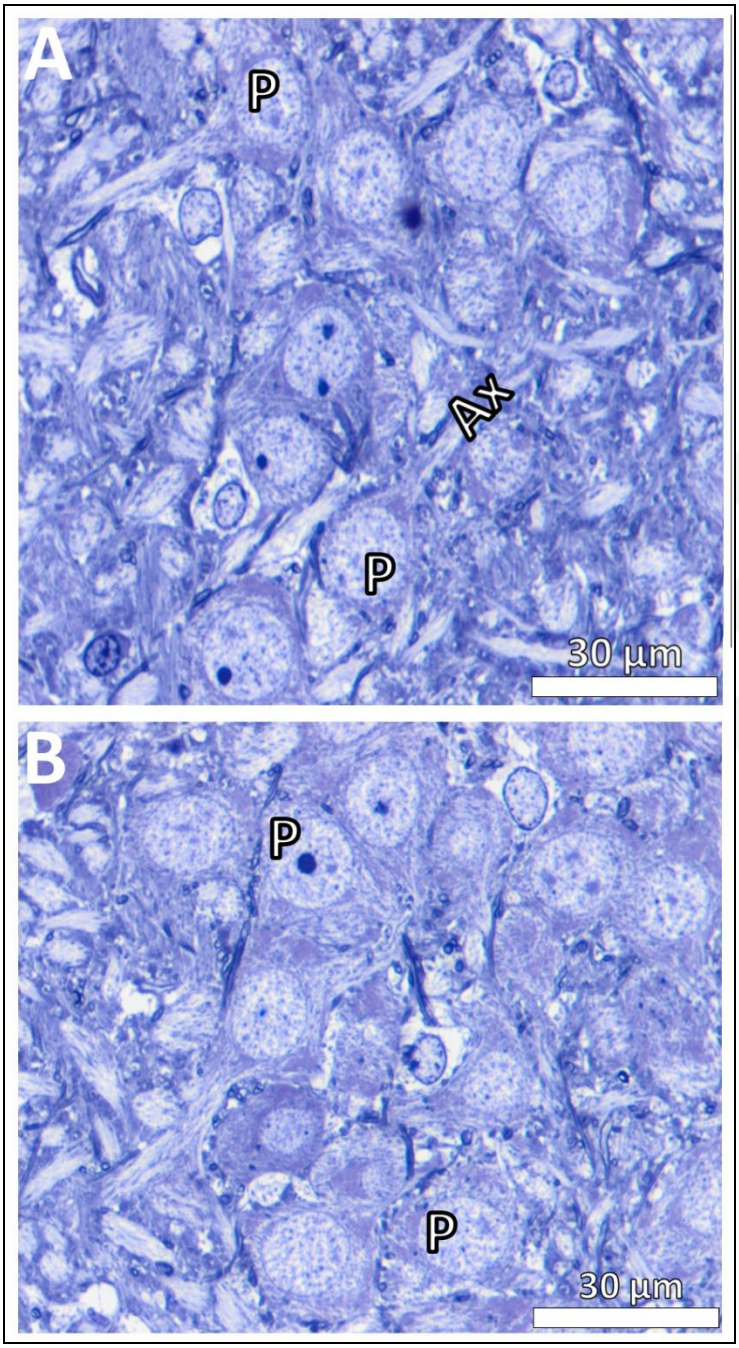


Figure 39. (A, B) images of the CA layer of the hippocampus of the control group are shown. And cells show the large size and less densely compacted indicating CA3 region. Nuclei of pyramidal cells (P) and the cell borders are evident. The arrows indicate healthy pyramidal cells. Axon, Ax; toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

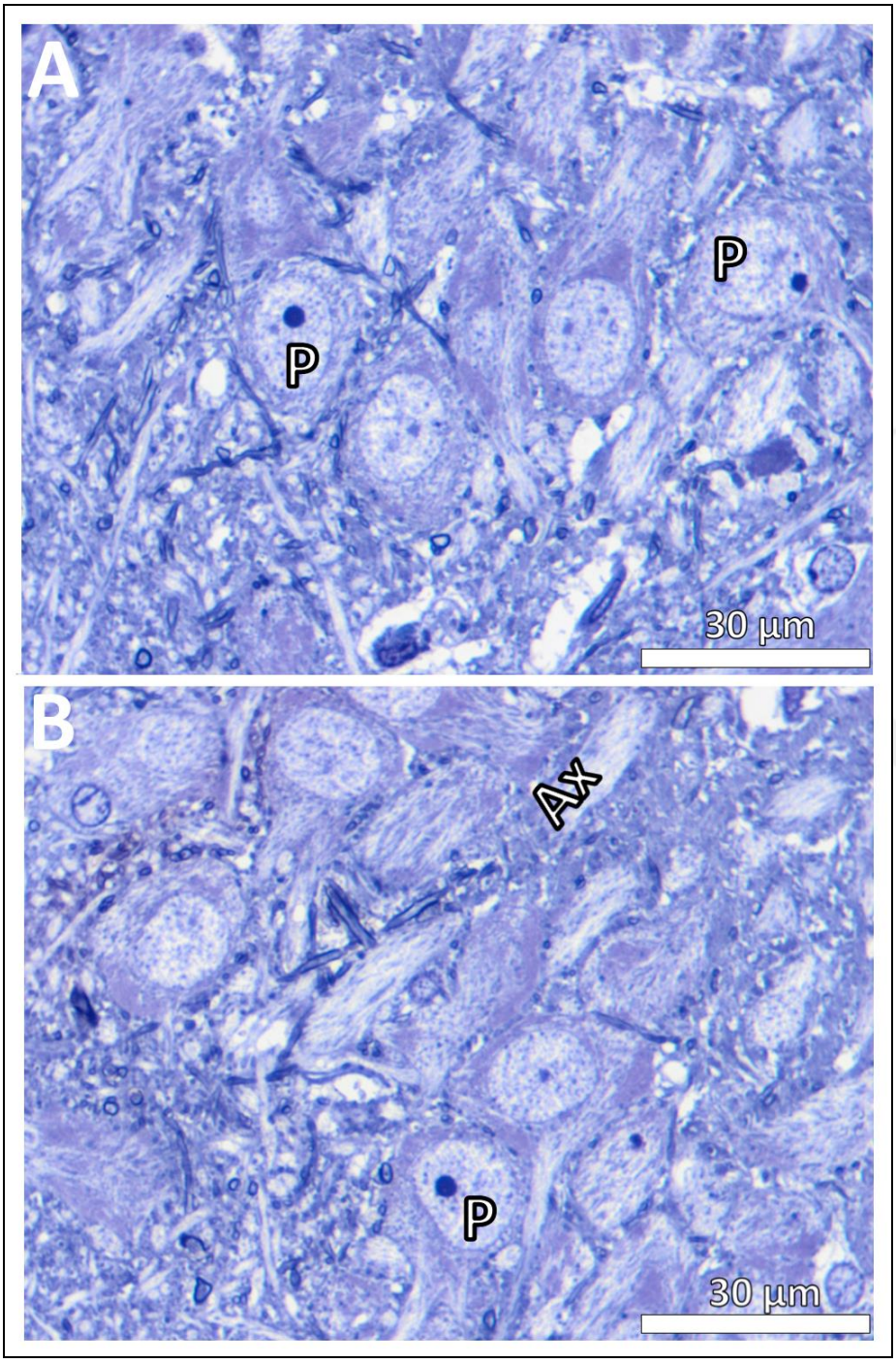


Figure 40. (A, B) Images were taken from the hippocampal region of the control group. Collections of the pyramidal neurons (P) are noticed. Each pyramidal cell has an oval to round shaped nucleus. Some axons belong to neurons are spotted. Axon, Ax; toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

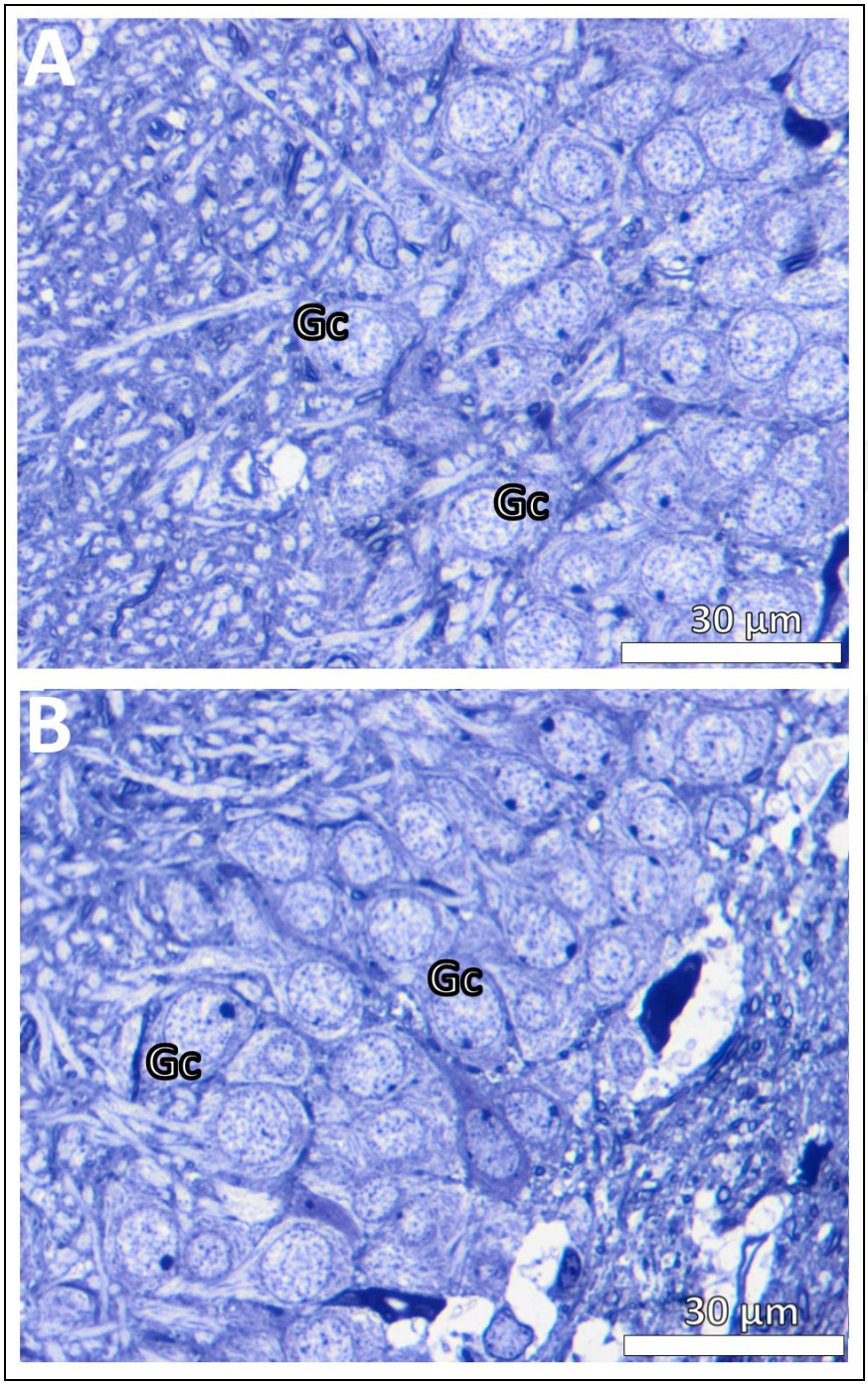


Figure 41. (A, B) Images taken from the dentate gyrus of the hippocampus belong to the control group. Granular neurons (Gc) are observed; they are medium-sized and densely compacted, indicating the dentate gyrus region. Some axons belong to neurons are identified. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

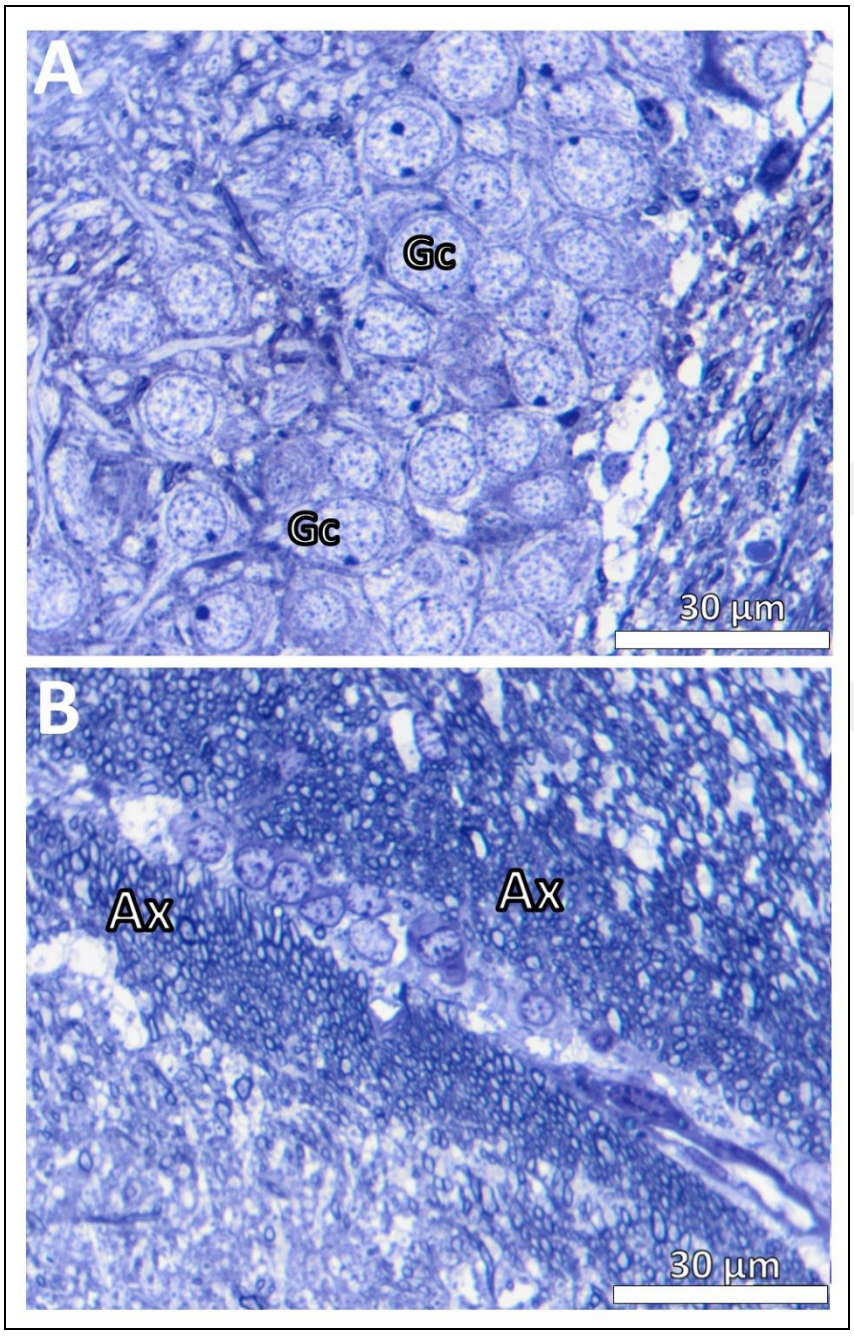


Figure 42. (A) Image was taken from the hippocampal region of the control group. Granular neurons (Gc) are seen; they are medium-sized and densely compacted, indicating the dentate gyrus region. (B) Some of the axons (Ax) belong to neurons are identified; they appeared in horizontal and longitudinal sections; this section was taken from the white matter. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

In the GA group, light microscopic images obtained from the different regions of the hippocampus, the general structure was intact, however. There were some degrees of disorganization as well as vacuolization and cell loss in some pictures (**Figure 43, 44, 45, 46, 47, 48, 49, 50, and 51**).

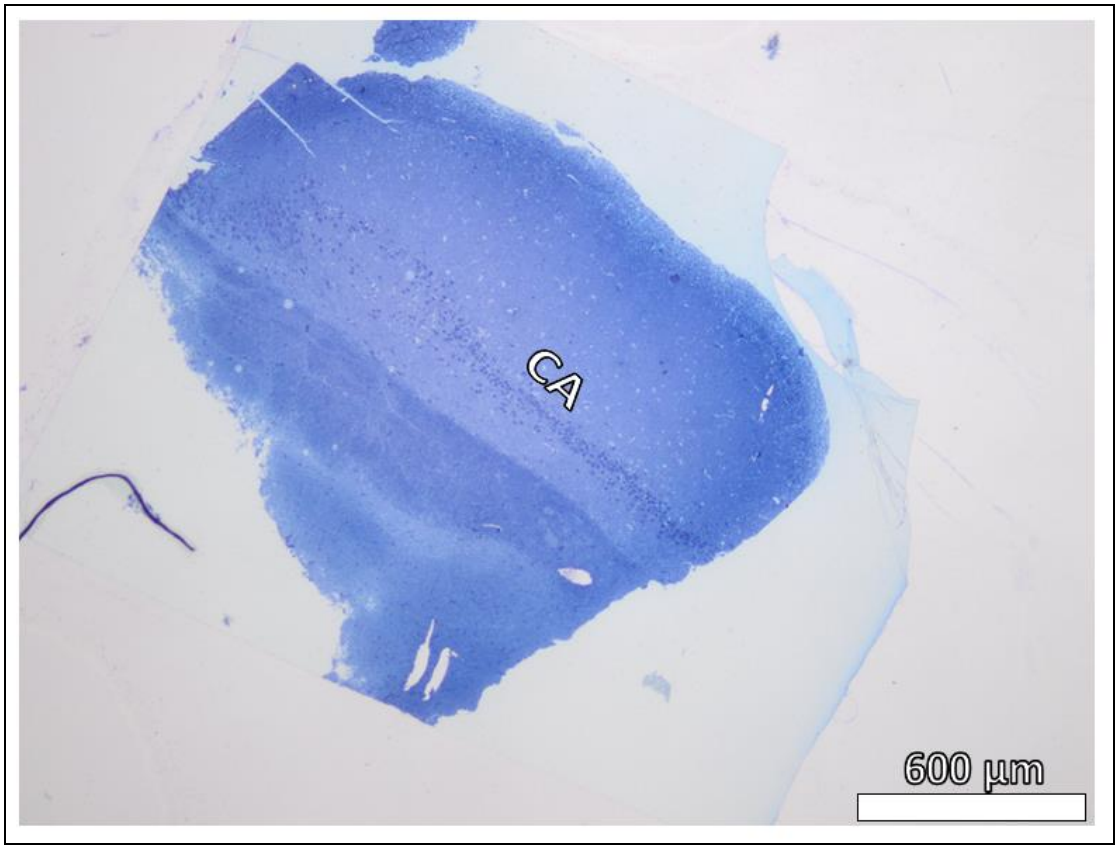


Figure 43. Image was taken from the CA region of hippocampal belong to the GA group is shown. The general structure of the hippocampus seems intact. Pyramidal cells are observed. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X4

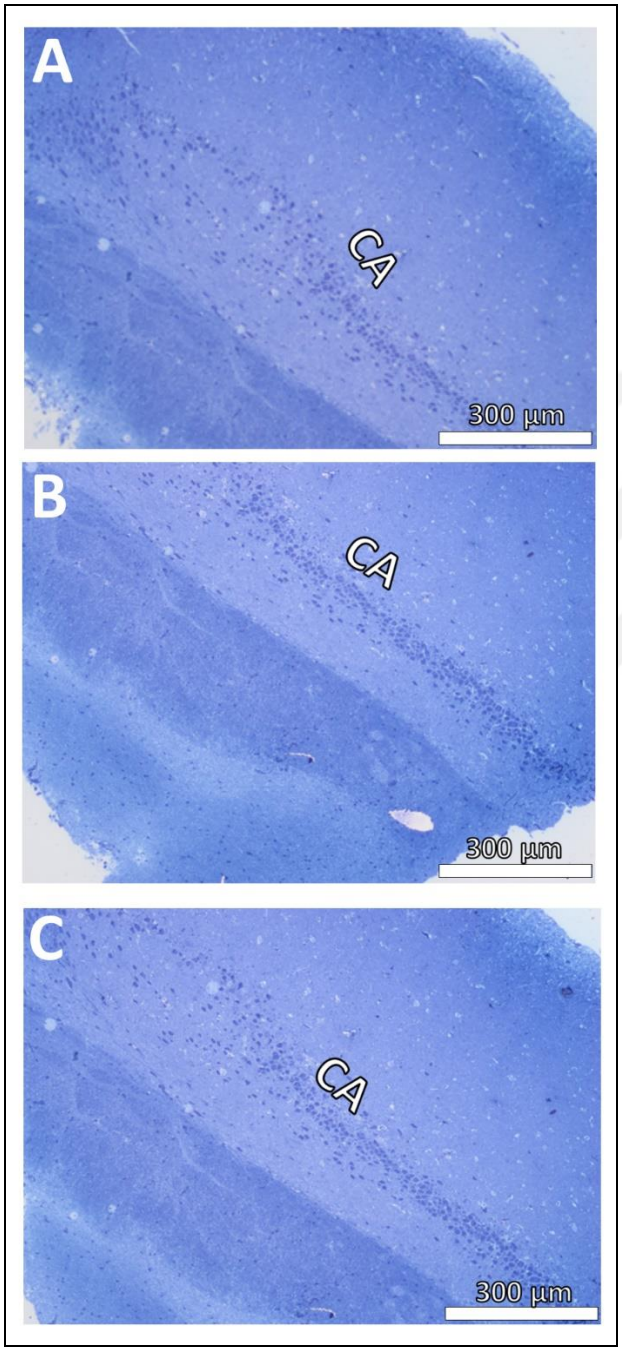


Figure 44. (A, B, and C) images of the CA layer of the hippocampus are shown in the GA group. The general structure of the hippocampus is intact. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X10

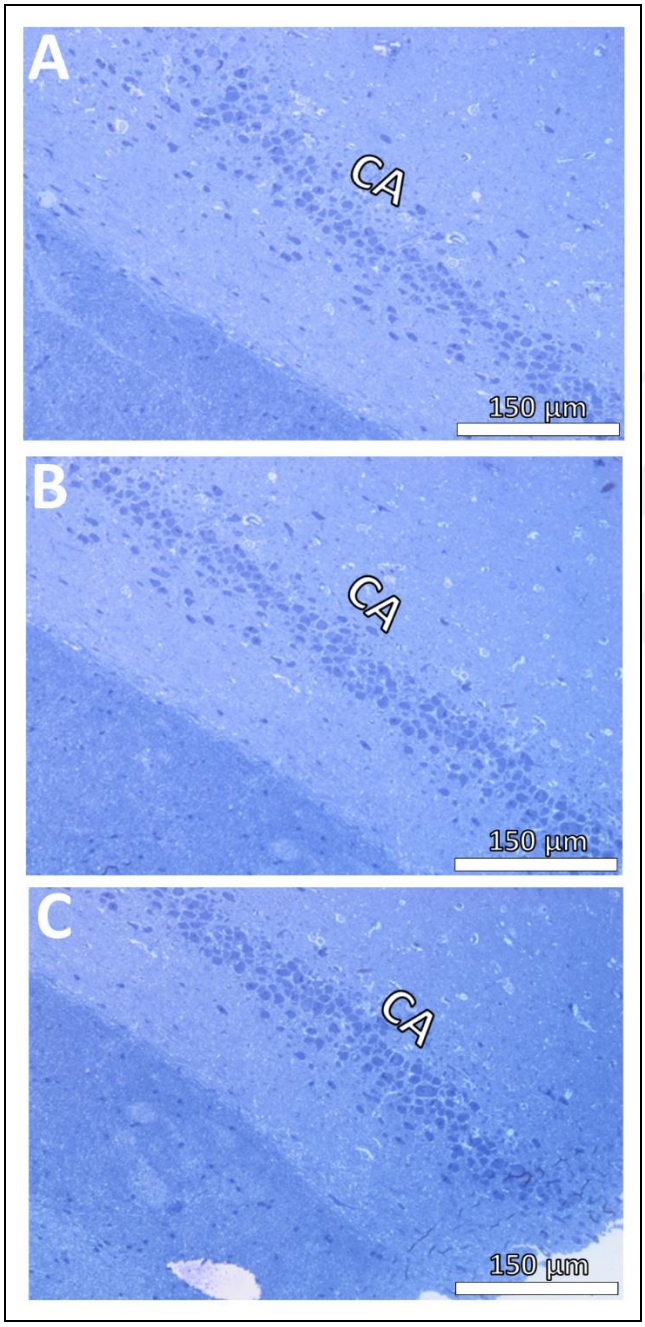


Figure 45. (A, B) images of the CA layer of the hippocampus are shown in the GA group. The general structure of the hippocampus is intact, there is some vacuolization, and cells are medium-sized and are densely compacted in a wide row, indicating CA1 region. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X10

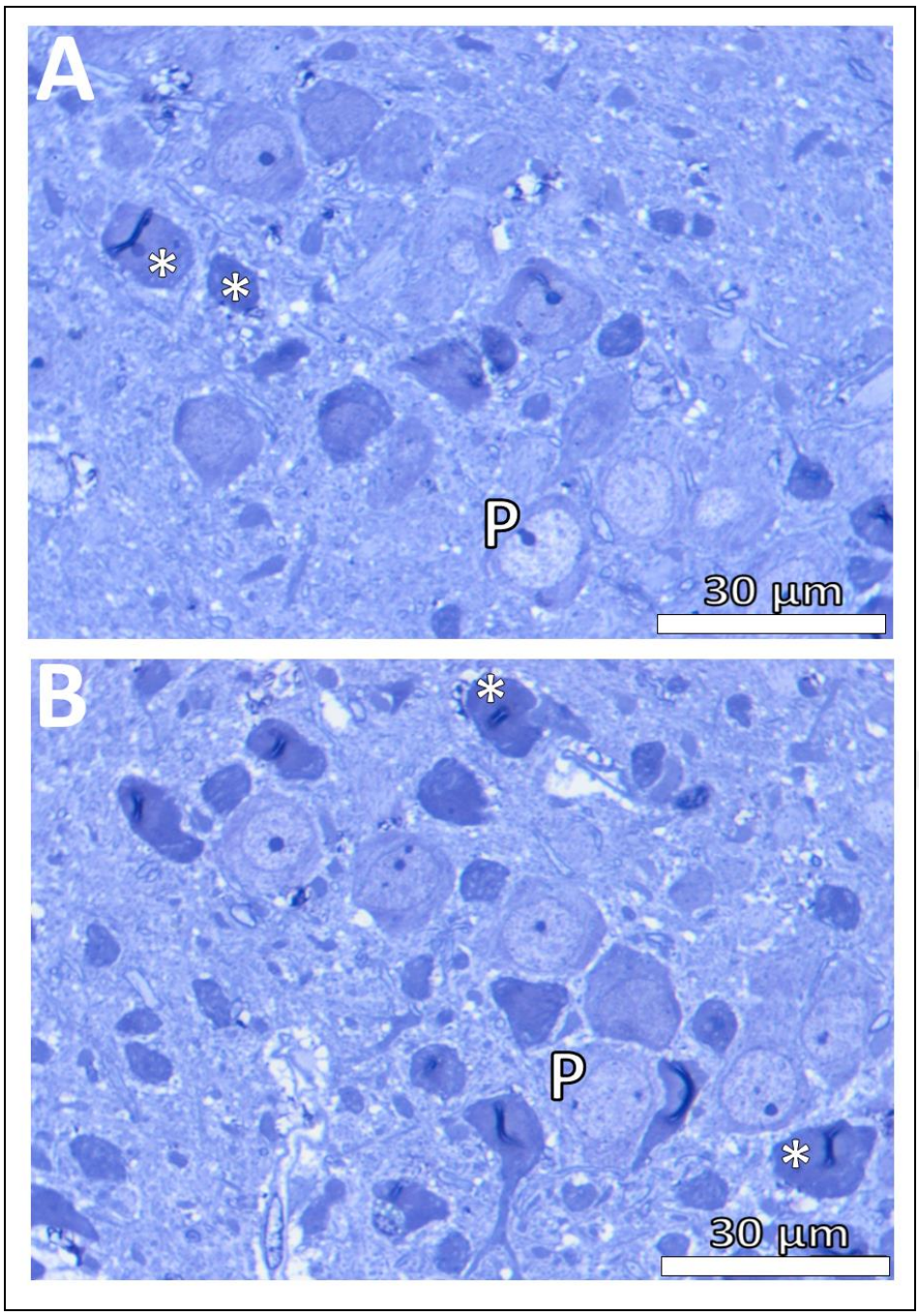


Figure 46. (A, B) images of the CA layer of the hippocampus are seen in the GA group. Nuclei of pyramidal cells (P) are vesicular and rounded; cells show variable degrees of staining, some axons are shown as well as a capillary. Darkly stained cells (*). Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

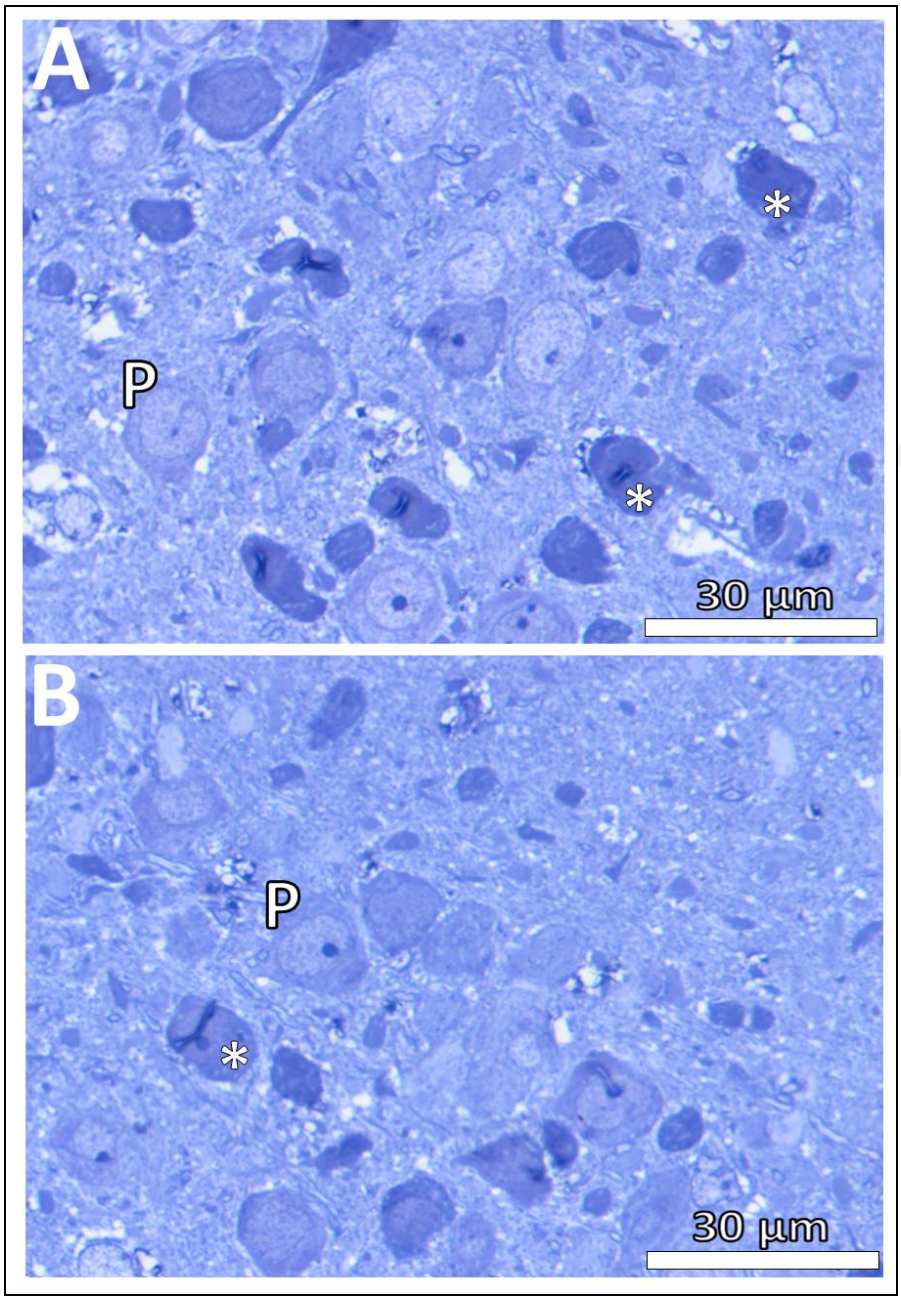


Figure 47. (A, B) images of the CA layer of the hippocampus are observed in the GA group. Nuclei of pyramidal cells (P) are vesicular and rounded; cells show variable degrees of staining, cells are densely packed, suggesting CA1 region, there are some vacuolizations. Also, some axons are shown as well as a capillary. Darkly stained cells (*), toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

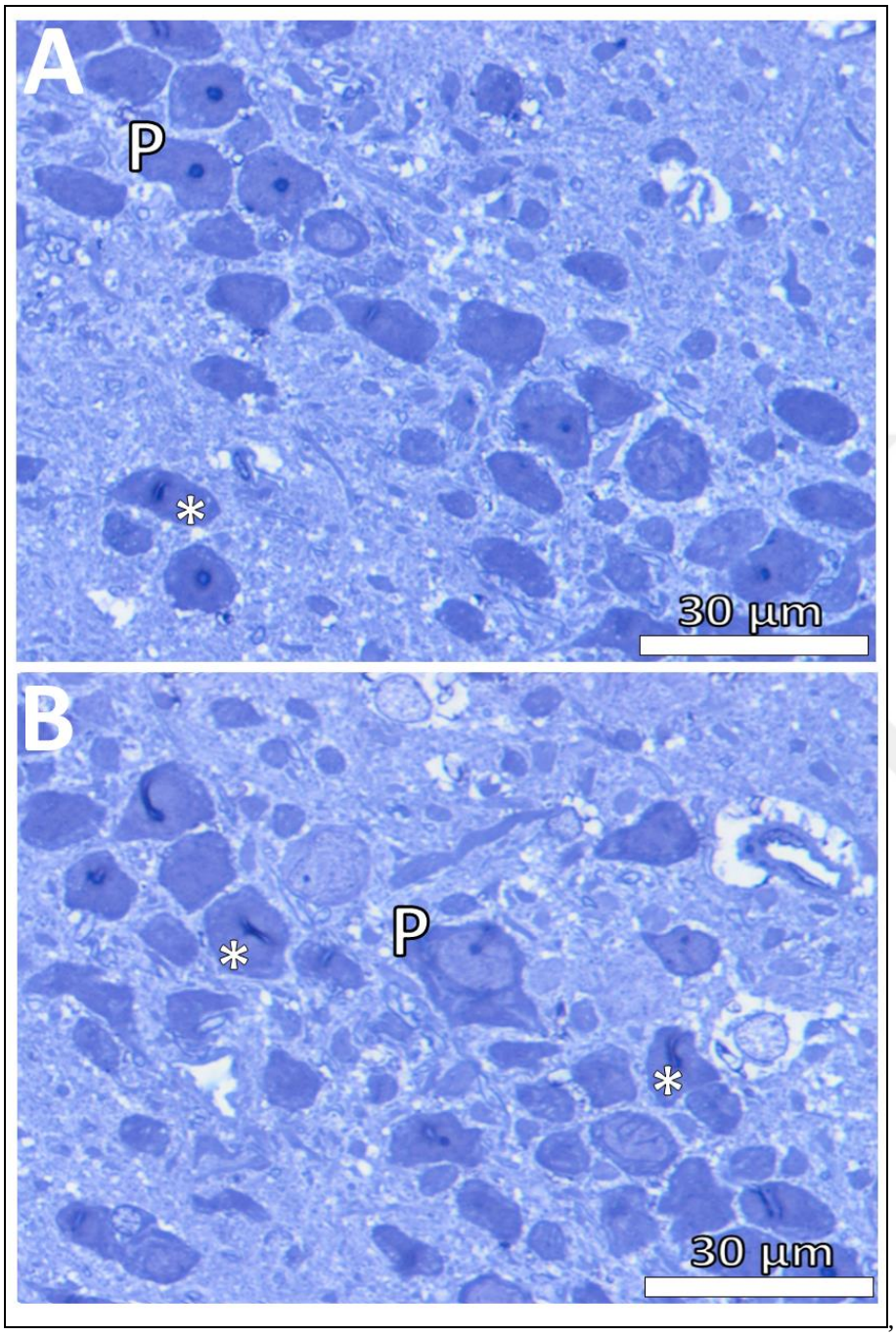


Figure 48. (A, B) images of the CA layer of the hippocampus are seen in the GA group. Nuclei of pyramidal cells (P) are vesicular. They show oval or rounded shapes; cells are with varying degrees of staining, they are densely packed suggesting CA1 region, there are some vacuolizations. Also, some capillaries are observed. Darkly stained cells (*), toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

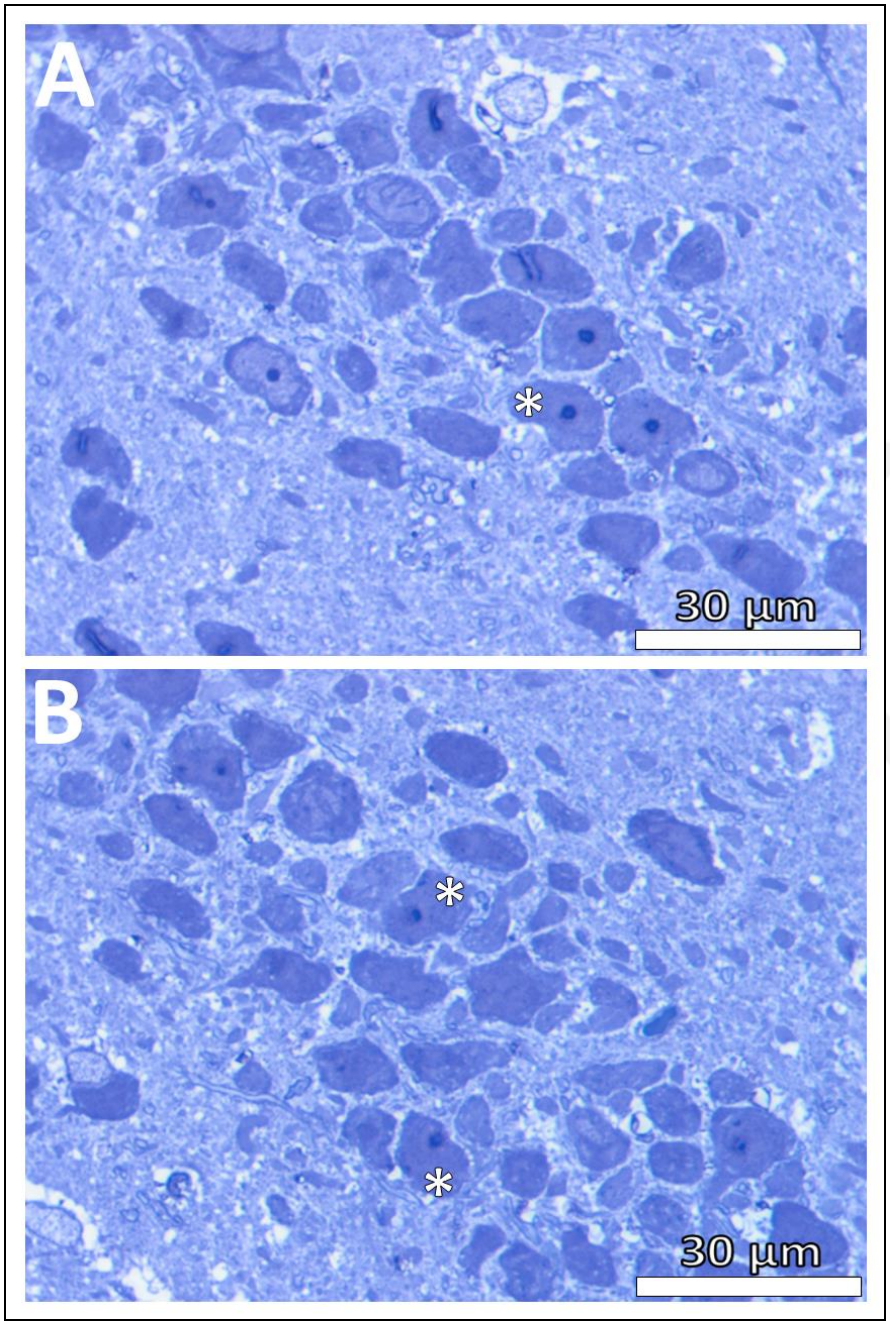


Figure 49. (A, B) images of the CA layer of the hippocampus are seen in the GA group. There is a degree of disorganization as well as vacuolization. Nuclei of pyramidal cells are vesicular. They show oval or rounded shapes; cells are with varying degrees of staining, they are densely packed suggesting CA1 region. Also, some capillaries are observed. Darkly stained cells (*), toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

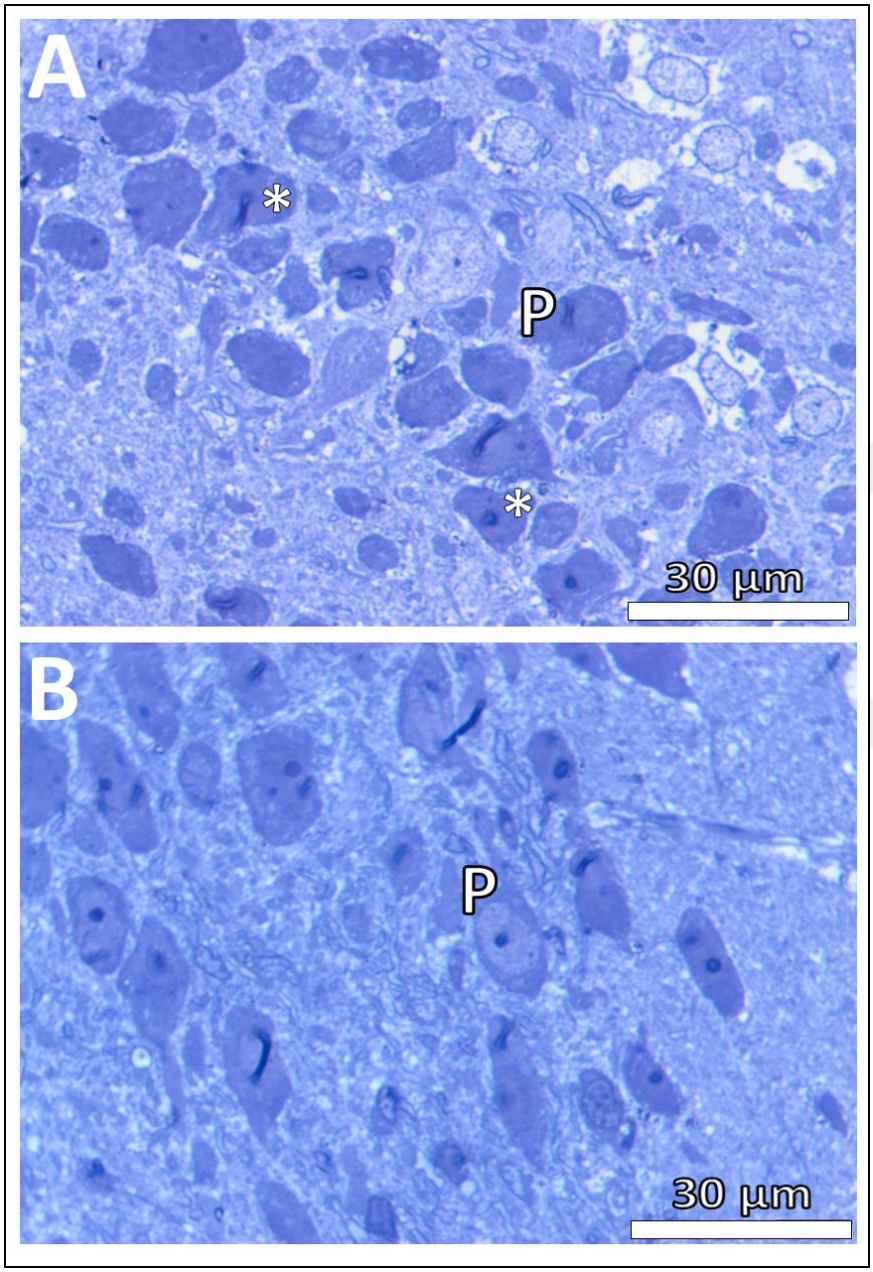


Figure 50. (A, B) images of the CA layer of the hippocampus are seen in the GA group. There is a degree of disorganization as well as vacuolization and cell loss. Nuclei of pyramidal cells (P) are vesicular. They show oval or rounded shapes; cells are with varying degrees of staining, they are densely packed suggesting CA1 region. Also, some capillaries are observed. Darkly stained cells (*), toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

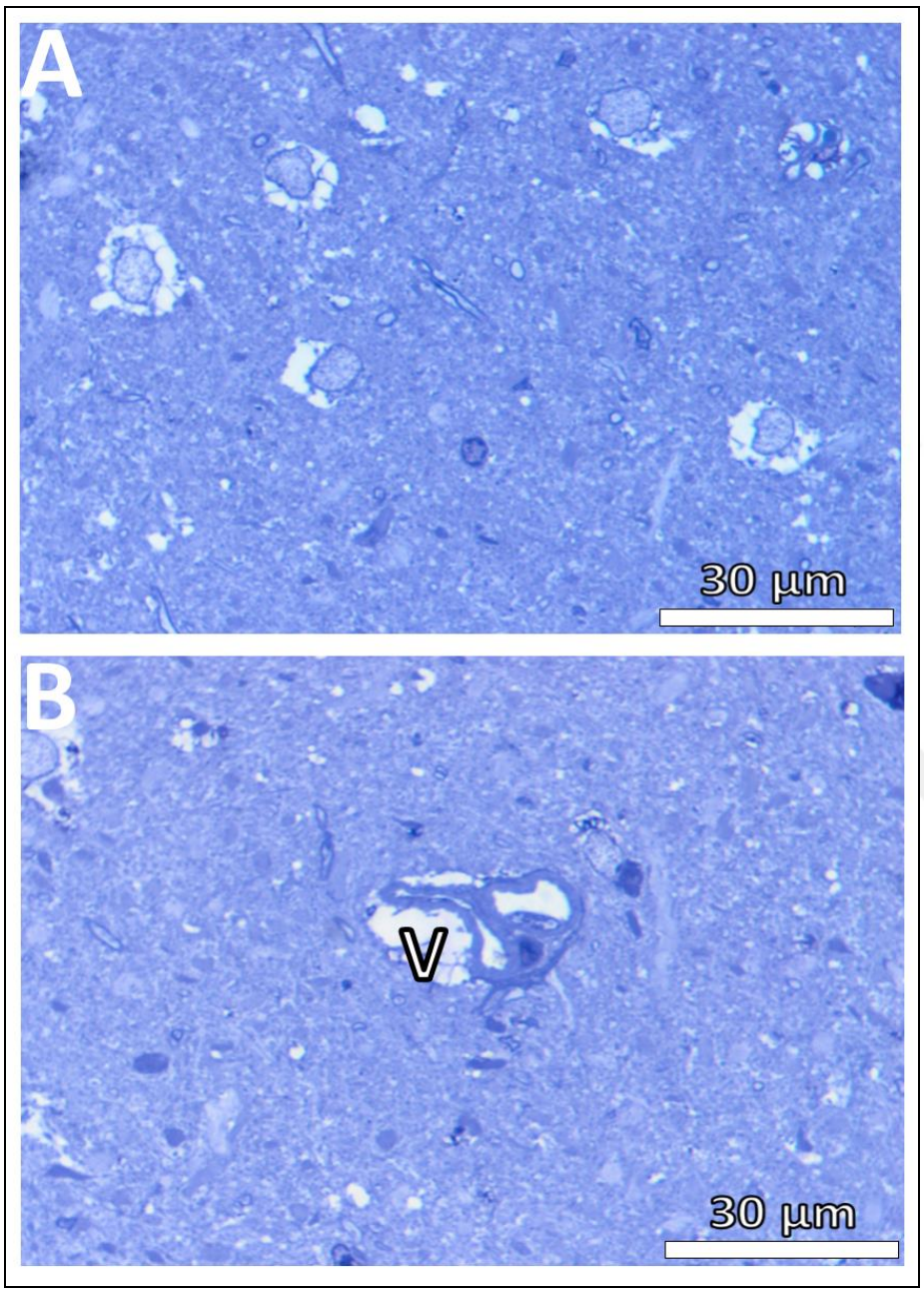


Figure 51. (A, B) images of the brain from the GA group are seen. There is a degree of disorganization as well as vacuolization and cell loss. Many axons are seen as well as capillaries. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

In the DS group, light microscopic images obtained from the different regions of the hippocampus, the general structure seemed damaged (**Figure 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, and 62**).

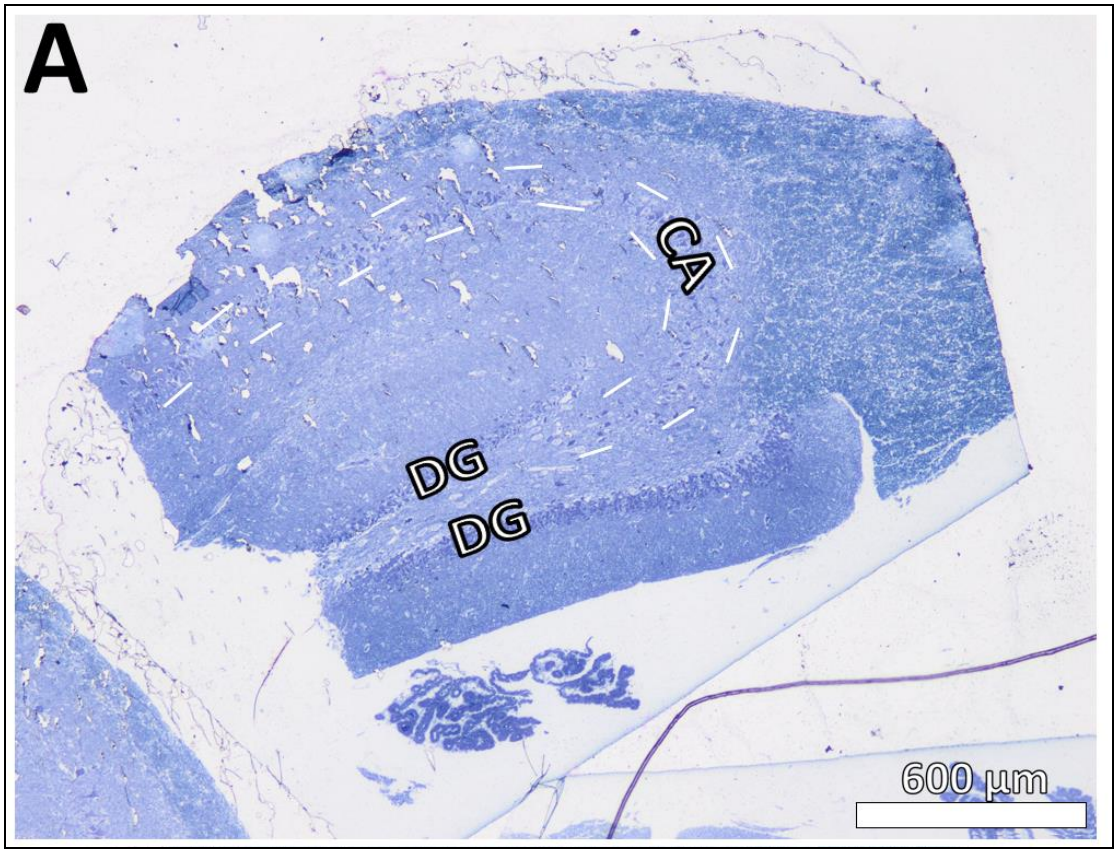


Figure 52. Image taken from the hippocampal region of the DS group is seen. The general structure of the hippocampus seems damaged. CA, cornu ammonis; DG, dentate gyrus, toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X4

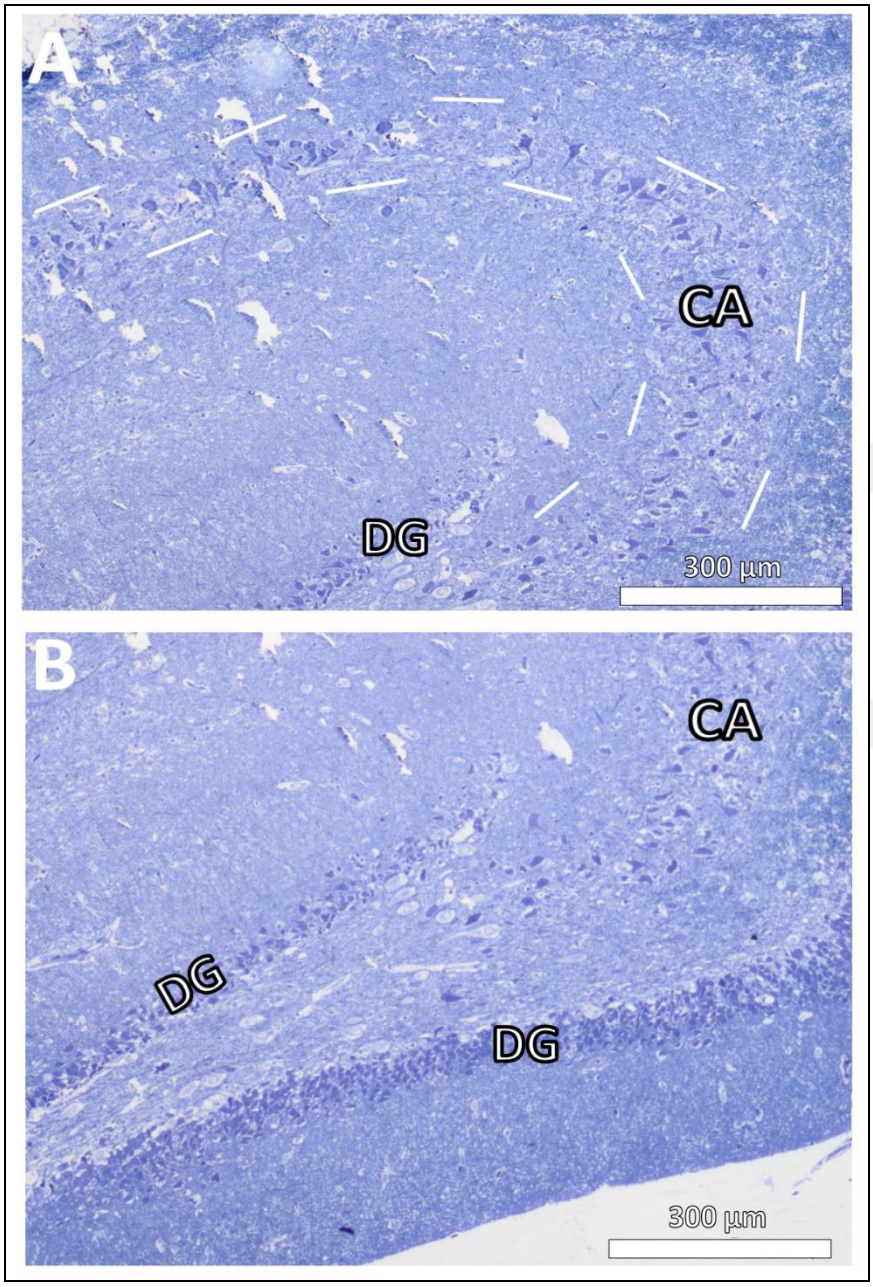


Figure 53. (A, B) images of the CA1 and CA3 layer of the hippocampus are displayed in the DS group. The general structure of the hippocampus is impaired, and cells show the large size and less densely compacted. CA, cornu ammonis; DG, dentate gyrus, toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X10

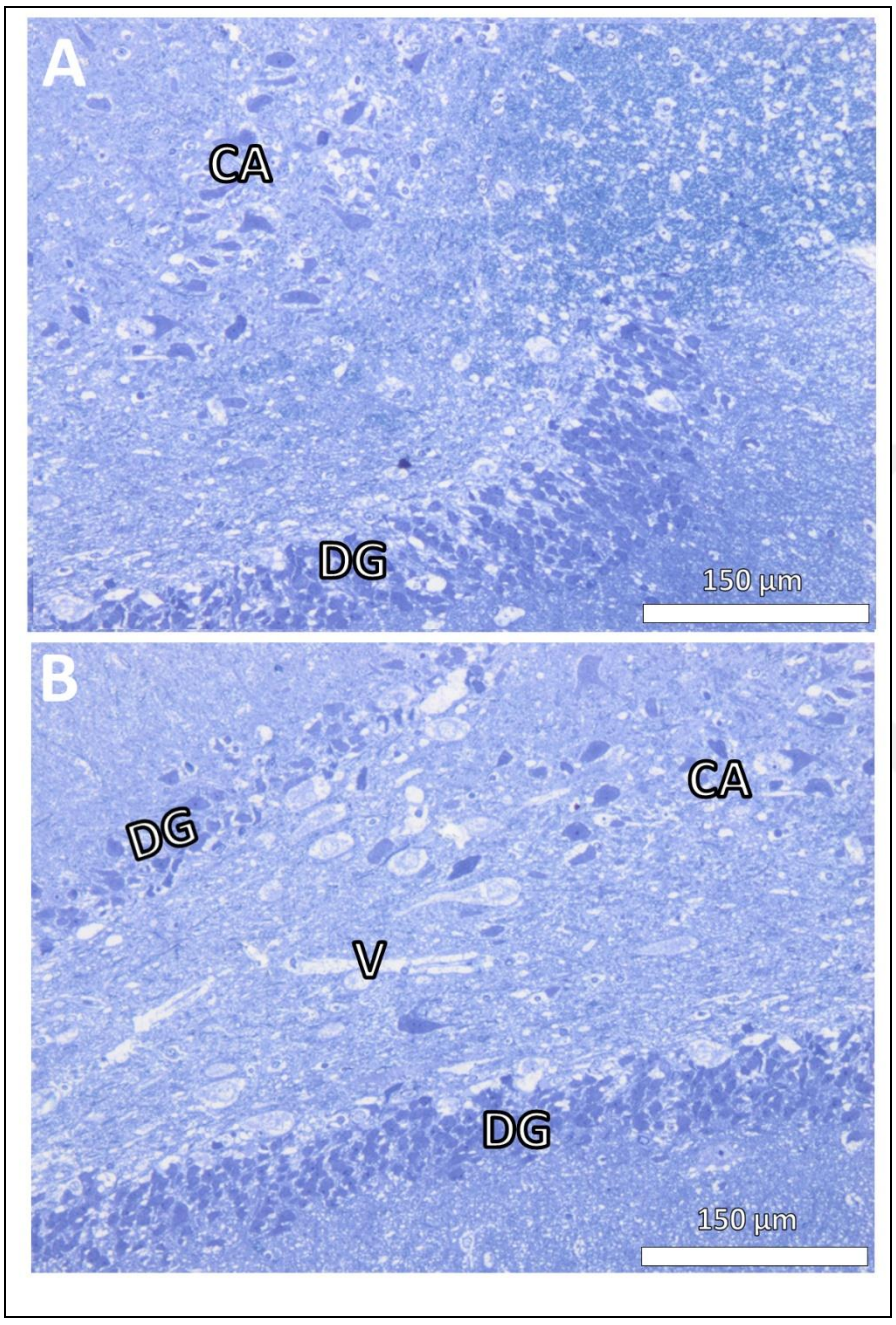


Figure 54. (A, B) Images of the granular cell layer of dentate of the DS group are shown with pyramidal cells from the CA3 region of the hippocampus. CA, cornu ammonis; DG, dentate gyrus; V, vessel; toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X20

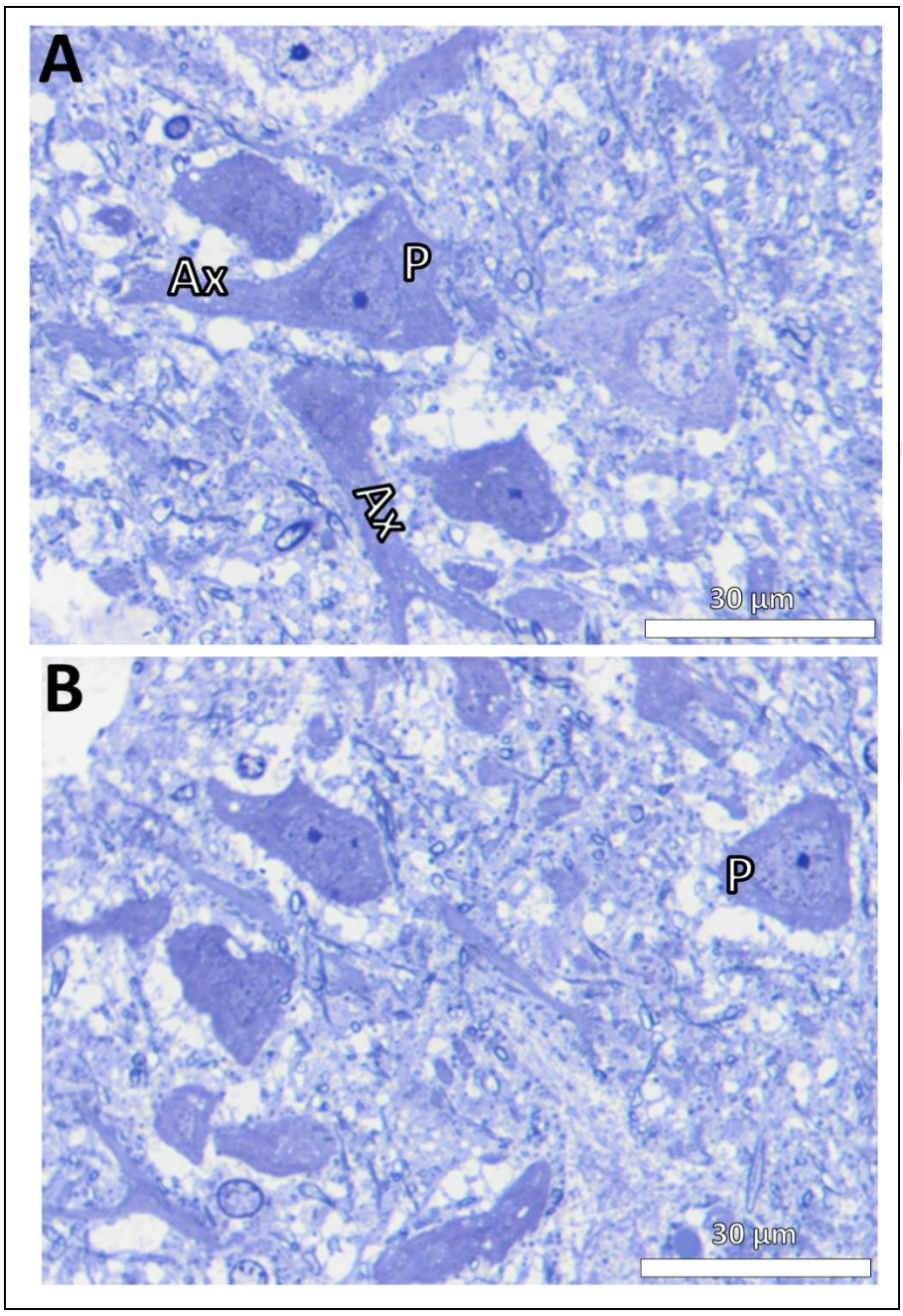


Figure 55. (A, B) Images were taken from the CA region of the DS group hippocampus. Collections of the pyramidal neurons (P) are noticed. Each pyramidal cell has an oval to round shaped nucleus. Most of the pyramidal neuron has an impaired shaped. Some of the axons (Ax) belong to neurons are spotted. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

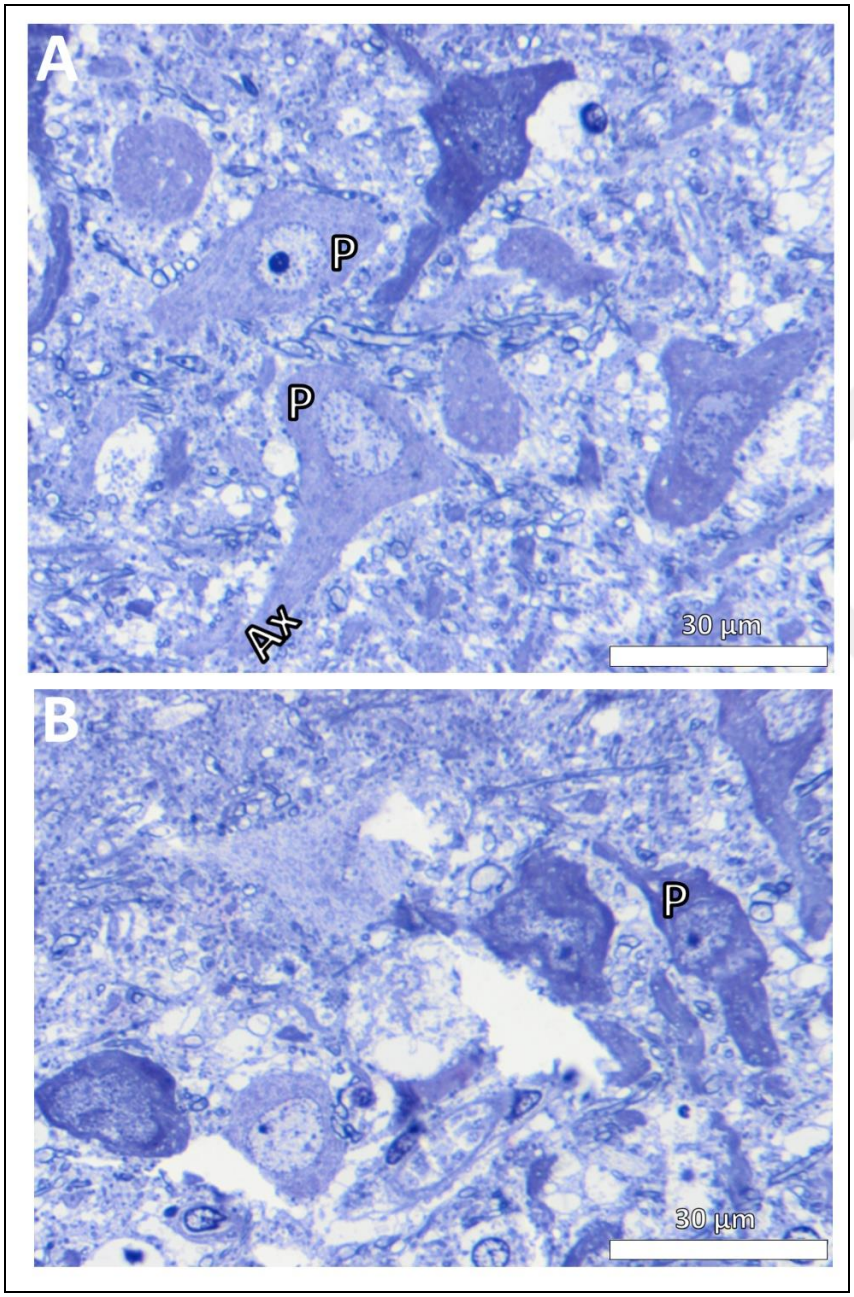


Figure 56. (A, B) Images were taken from the CA layer of the hippocampus of the DS group. Pyramidal neurons (P) are observed; they are medium-sized and loosely compacted, indicating the CA3 region. Most of the neurons does lose their normal shape. Some of the axons (Ax) belongs to neurons are identified. Nuclei are round to oval. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

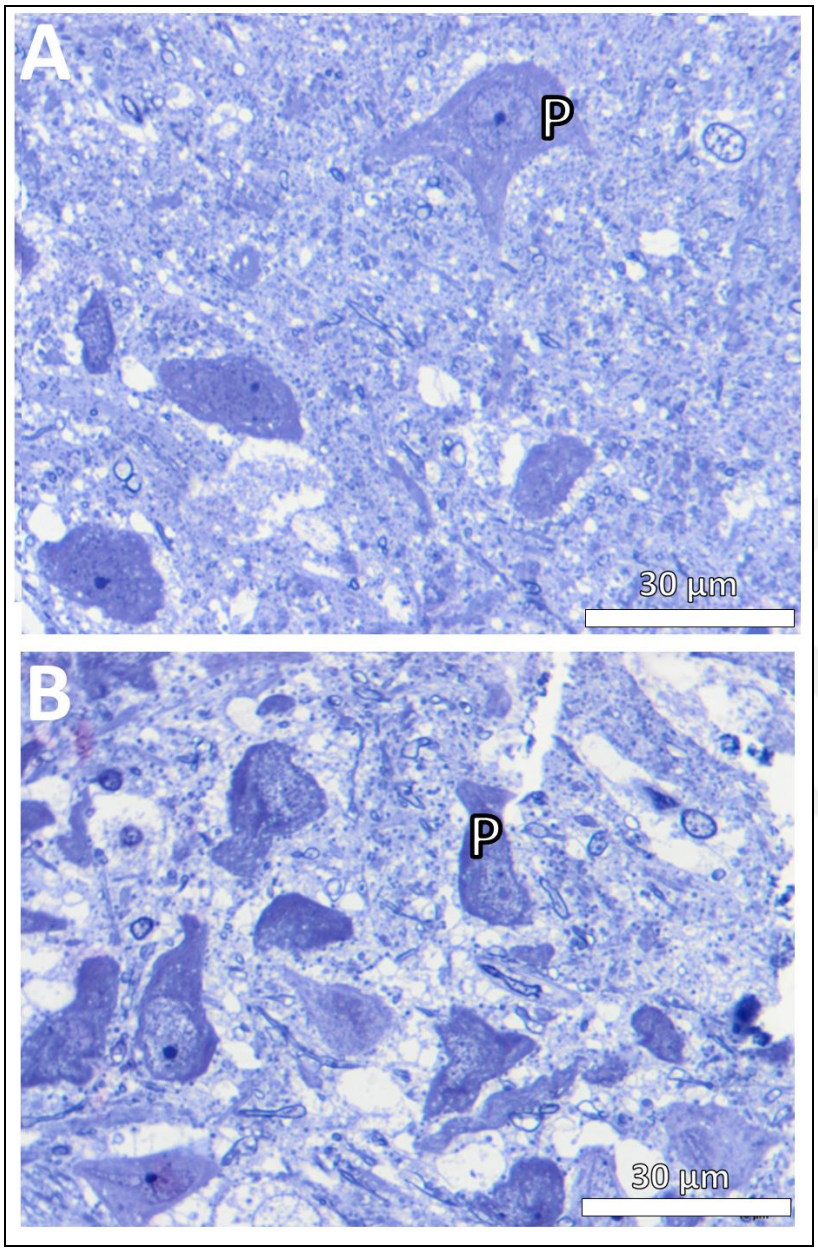


Figure 57. (A, B) Images were taken from the CA region of the hippocampus of the DS group. Pyramidal neurons (P) are seen; they are medium-sized and loosely compacted, indicating the CA3 region. Most of the pyramidal neurons lost their normal shape and their cytoplasm are densely stained. Nuclei are round to oval and some of the axons belong to neurons are identified. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

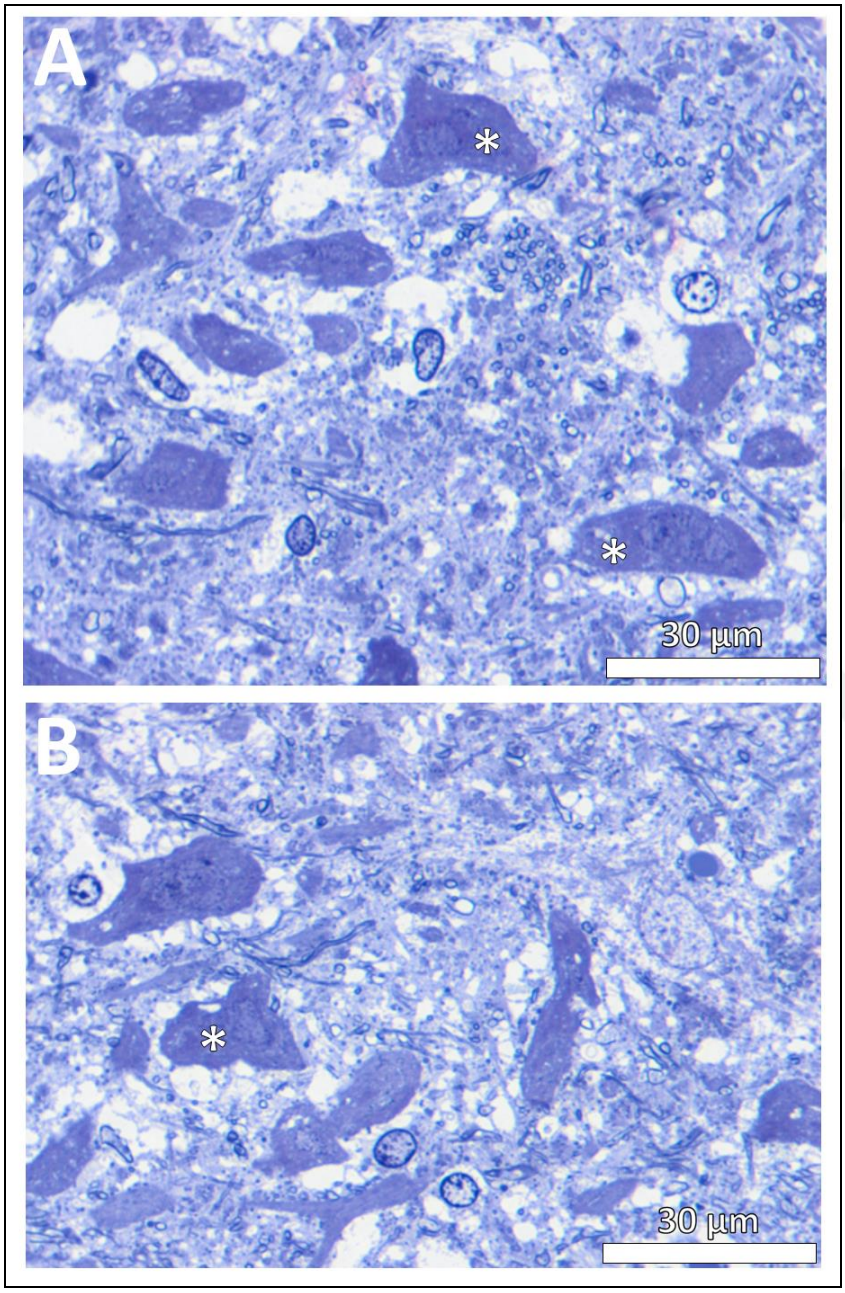


Figure 58. (A, B) Images were taken from the CA region of the hippocampus belong to the DS group. Pyramidal neurons (*) are seen, they are medium-sized and form, and there are spaces in-between cells. Nuclei are round to oval, and some of the axons belong to neurons are identified. Most of the pyramidal neurons lost their normal shape and their cytoplasm are densely stained. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

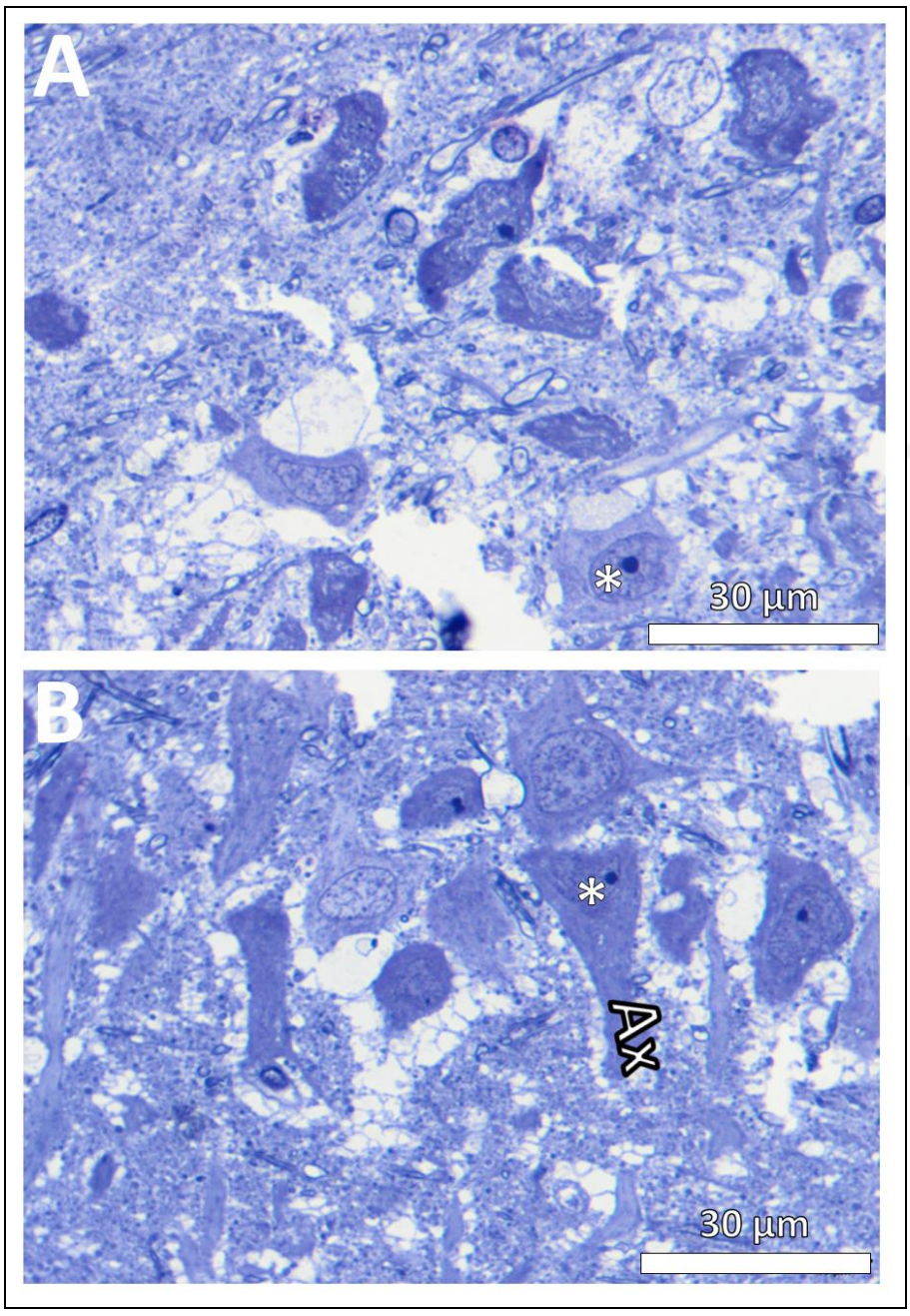


Figure 59. (A, B) Images were taken from the CA layer of the hippocampus of the DS group. Pyramidal neurons (*) are observed, they are medium-sized, some cells show no clear boundaries, the spaces in-between cells are more numerous and larger. Nuclei are round to oval, and some of the axons belong to neurons are identified. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

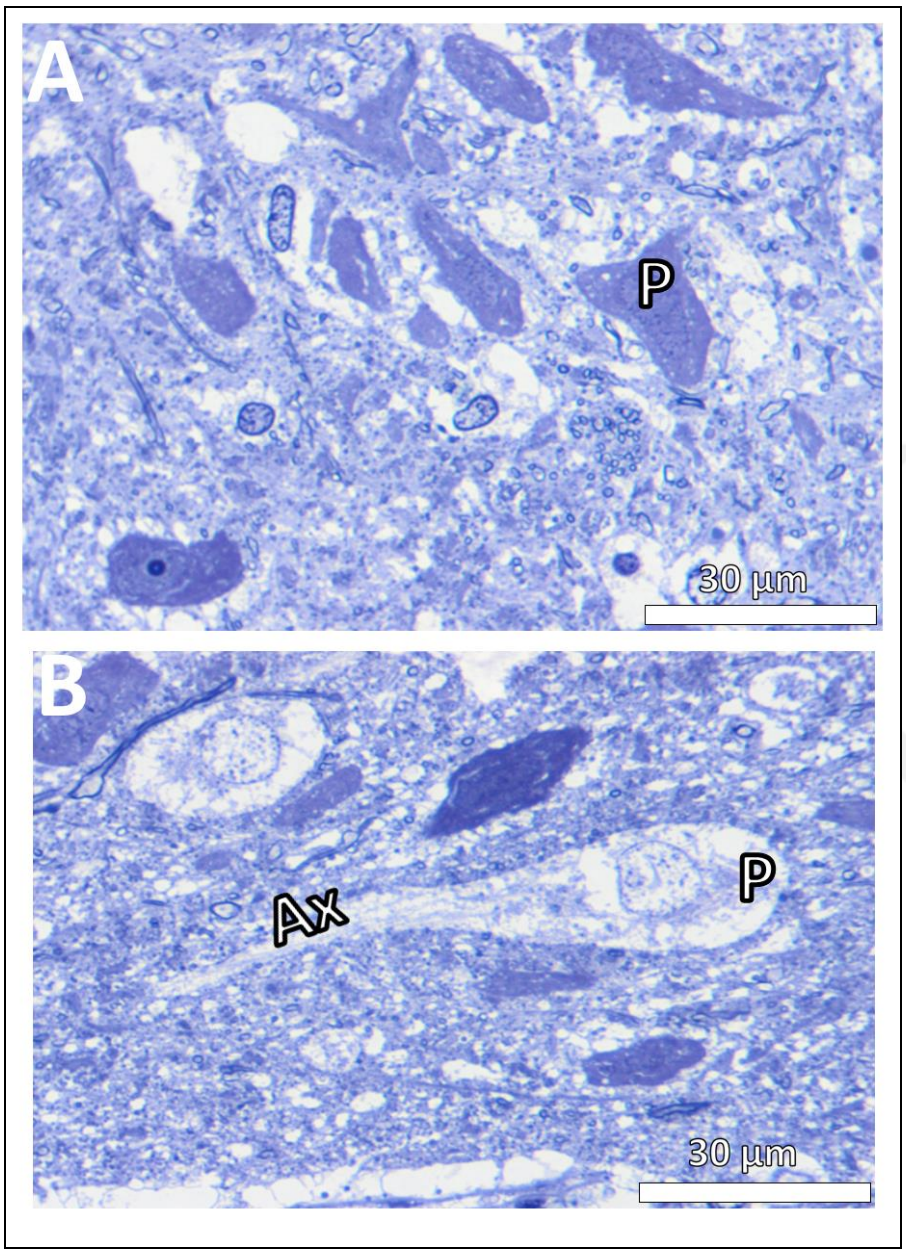


Figure 60. (A, B) Images were taken from the CA layer of the hippocampus of the DS group. Pyramidal neurons (P) are observed; they look distorted; the spaces in-between cells are many and large-sized. Most of the pyramidal neurons lost their normal shape and their cytoplasm are densely stained. Nuclei are round to oval and nucleoli are not clear. Ax, axon; toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

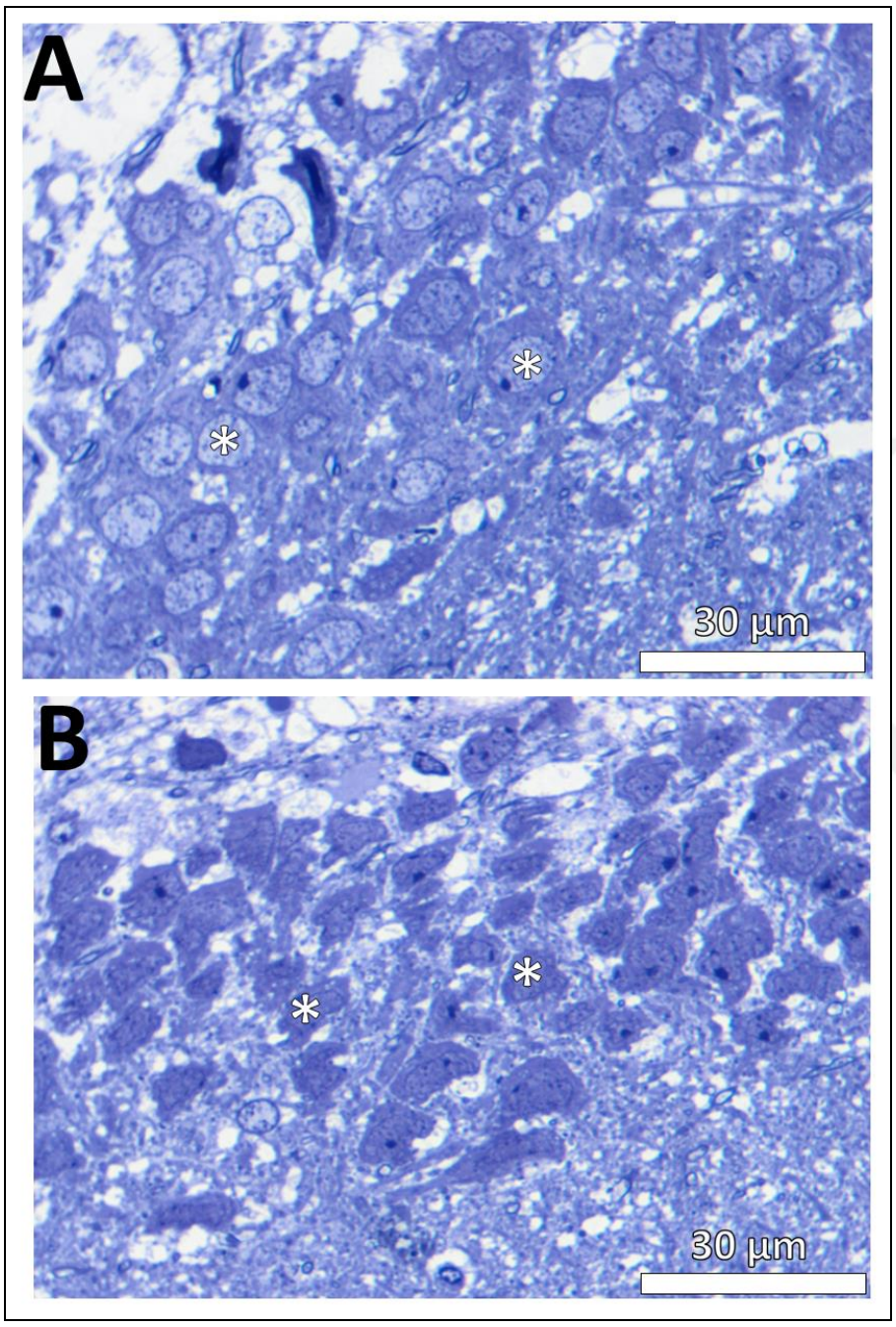


Figure 61. (A, B) Images taken from the dentate gyrus of the hippocampus of the DS group. Granular neurons (*) are observed, the spaces in-between cells are many and large-sized, and the cells are crowded. Nucleoli are not clear in many cells. Most of the granular neurons lost their normal shape, spherical, and their cytoplasms are densely stained. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

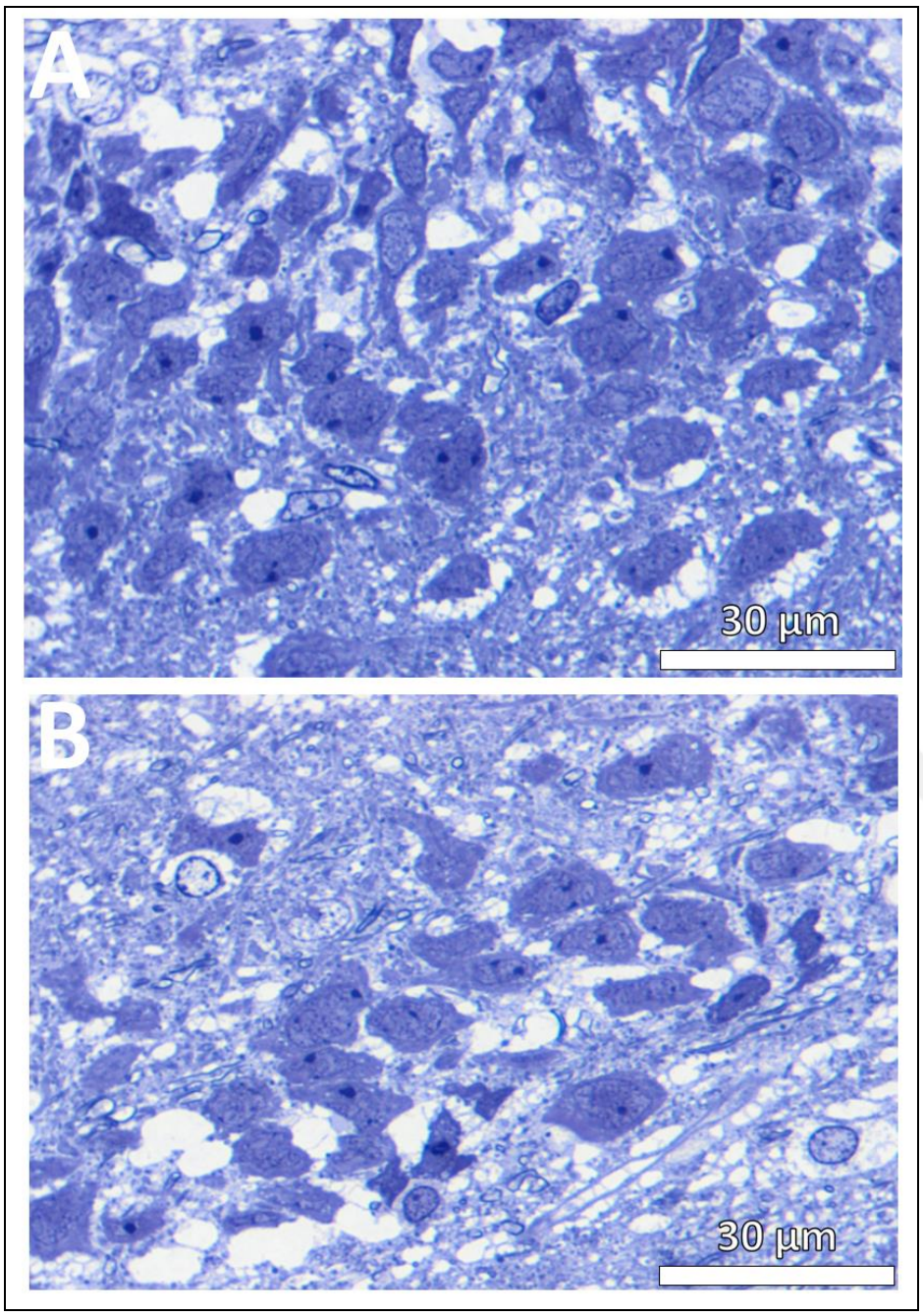


Figure 62. (A, B) Images taken from the dentate gyrus of the hippocampus of the DS group. Granular neurons are seen; the spaces in-between cells are many. Nucleoli are not clear in many cells. Most of the granular neurons lost their normal shape, spherical, and their cytoplasms are densely stained. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

In the DS+GA group, light microscopic images obtained from the different regions of the hippocampus, the general structure seemed intact (**Figure 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, and 75**).

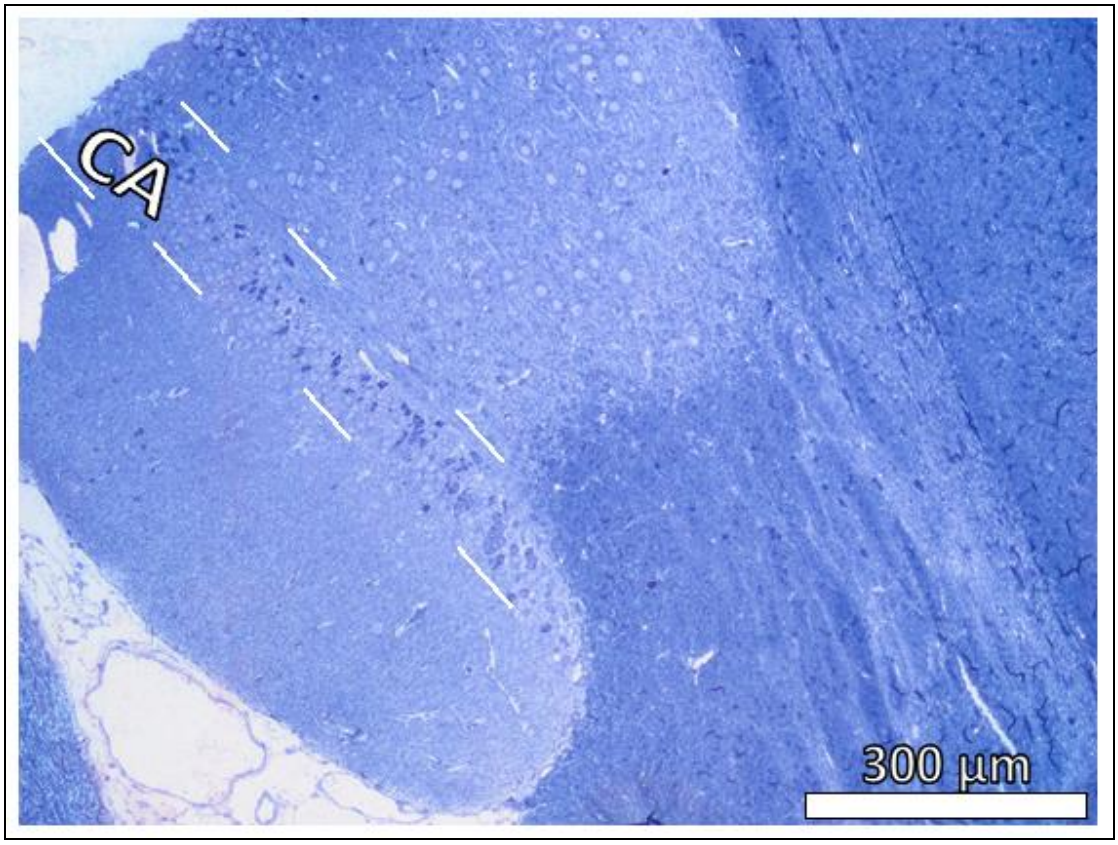


Figure 63. Image taken from the hippocampal region of the DS+GA group is shown. The general structure of the hippocampus seems intact. There is also a large capillary along with other small capillaries (left lower). Many of pyramidal neurons lost their normal shape, and their cytoplasms are densely stained. CA, cornu ammonis, Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X10

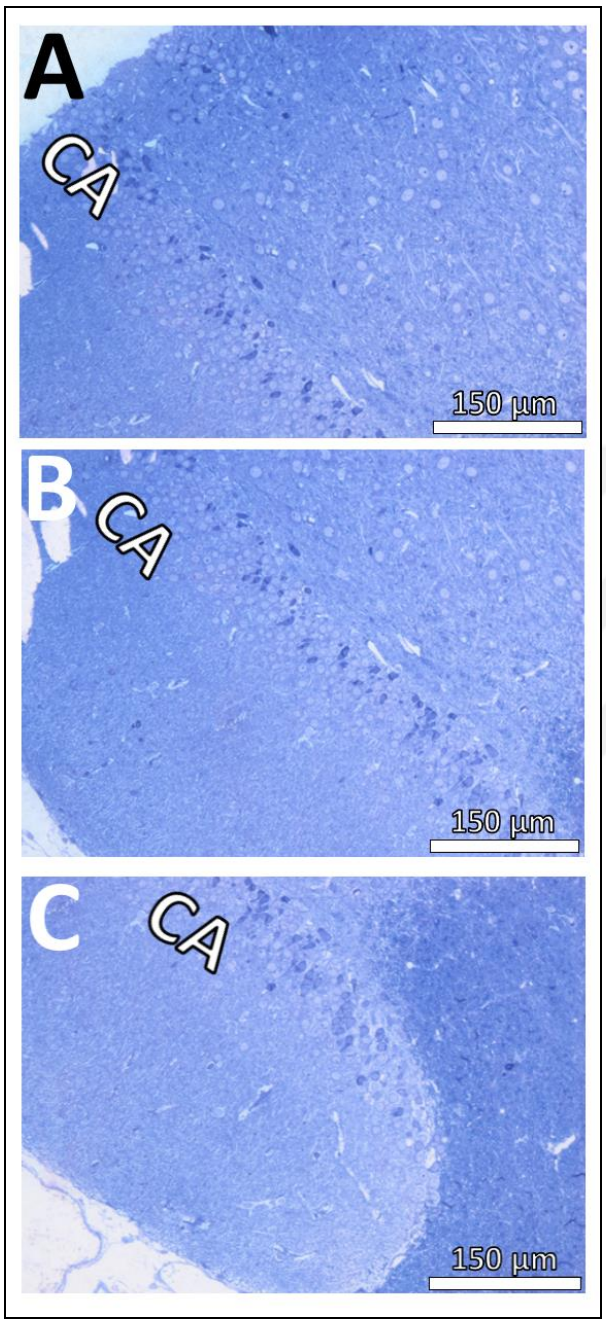


Figure 64. (A, B, and C) Images of the CA layer of the hippocampus are displayed in the DS+GA group. The general structure of the hippocampus is intact, and cells show medium to small size and are densely compacted, indicating the CA1 region. Many of pyramidal neurons lost their pyramidal shape, and their cytoplasms are densely stained. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X20

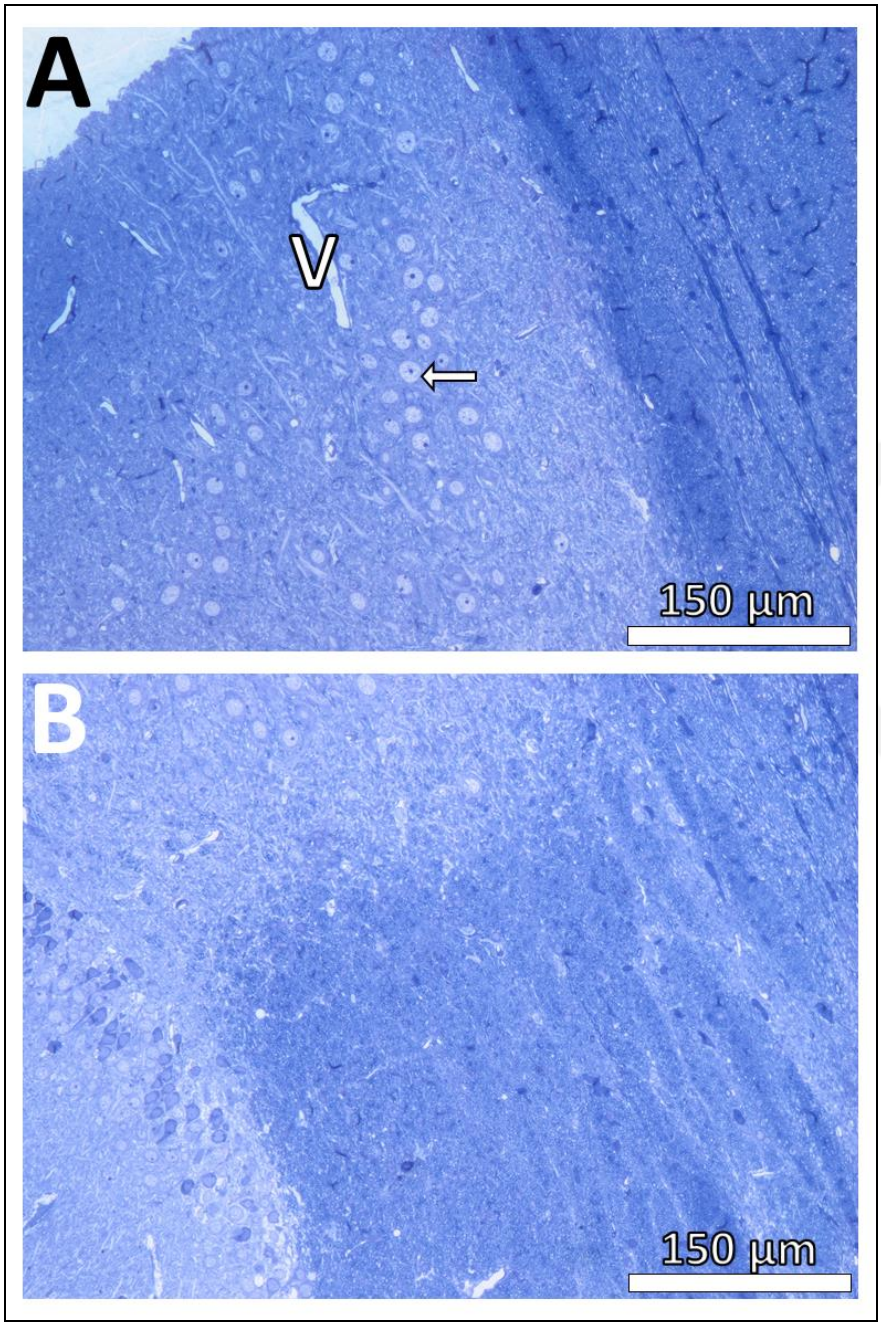


Figure 65. (A, B) Images of the CA region of the hippocampus are shown in the DS+GA group. The general structure of the hippocampus is intact, pyramidal cells (P) are medium-sized and are densely compacted in a wide row, indicating CA region. V, vessel. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X40

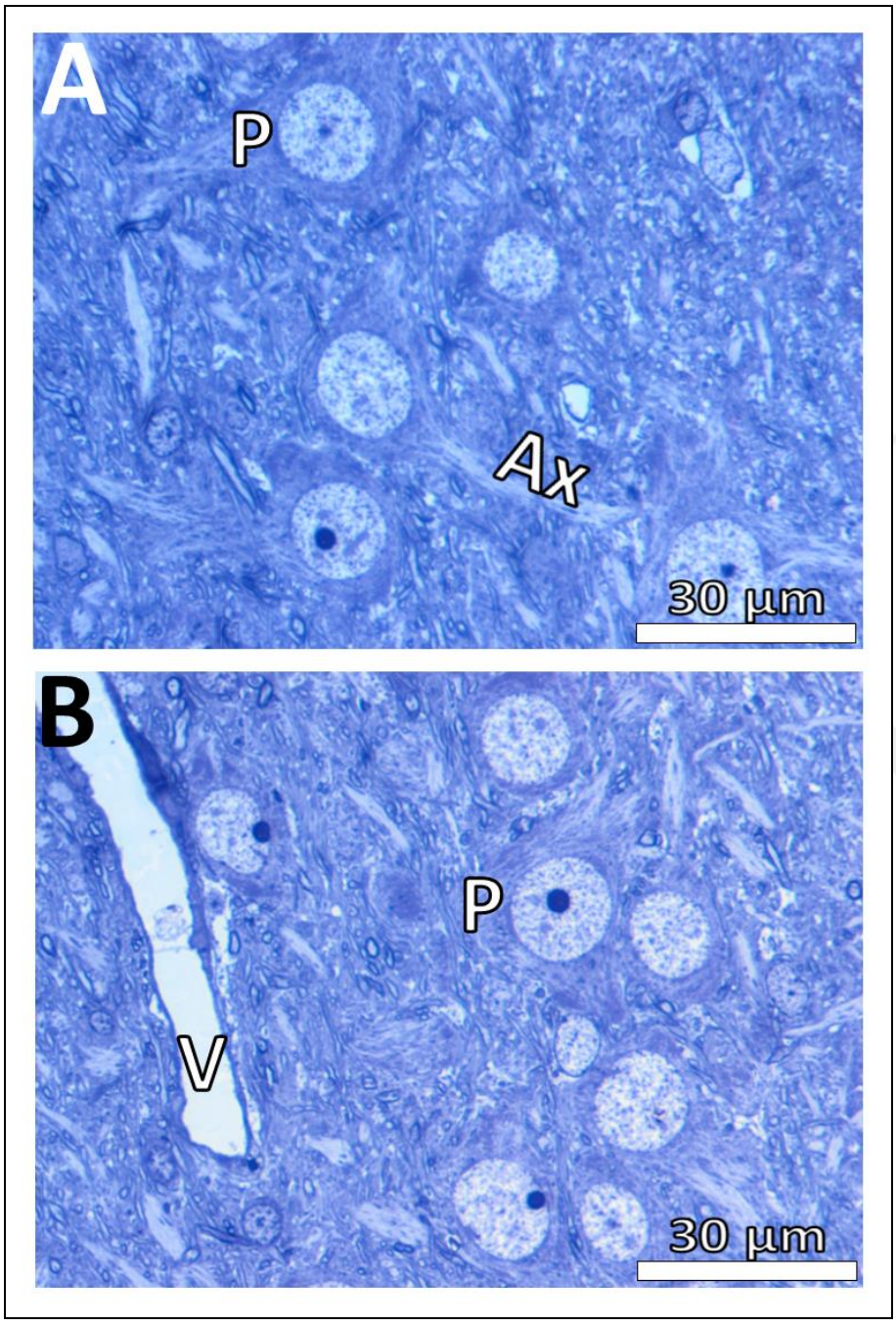


Figure 66. (A, B) Images of the CA region of the hippocampus are seen in the DS+GA group. Pyramidal cells are seen in health (P), and their nuclei are vesicular and rounded; some axons are shown (Ax) as well as a capillary (V). Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

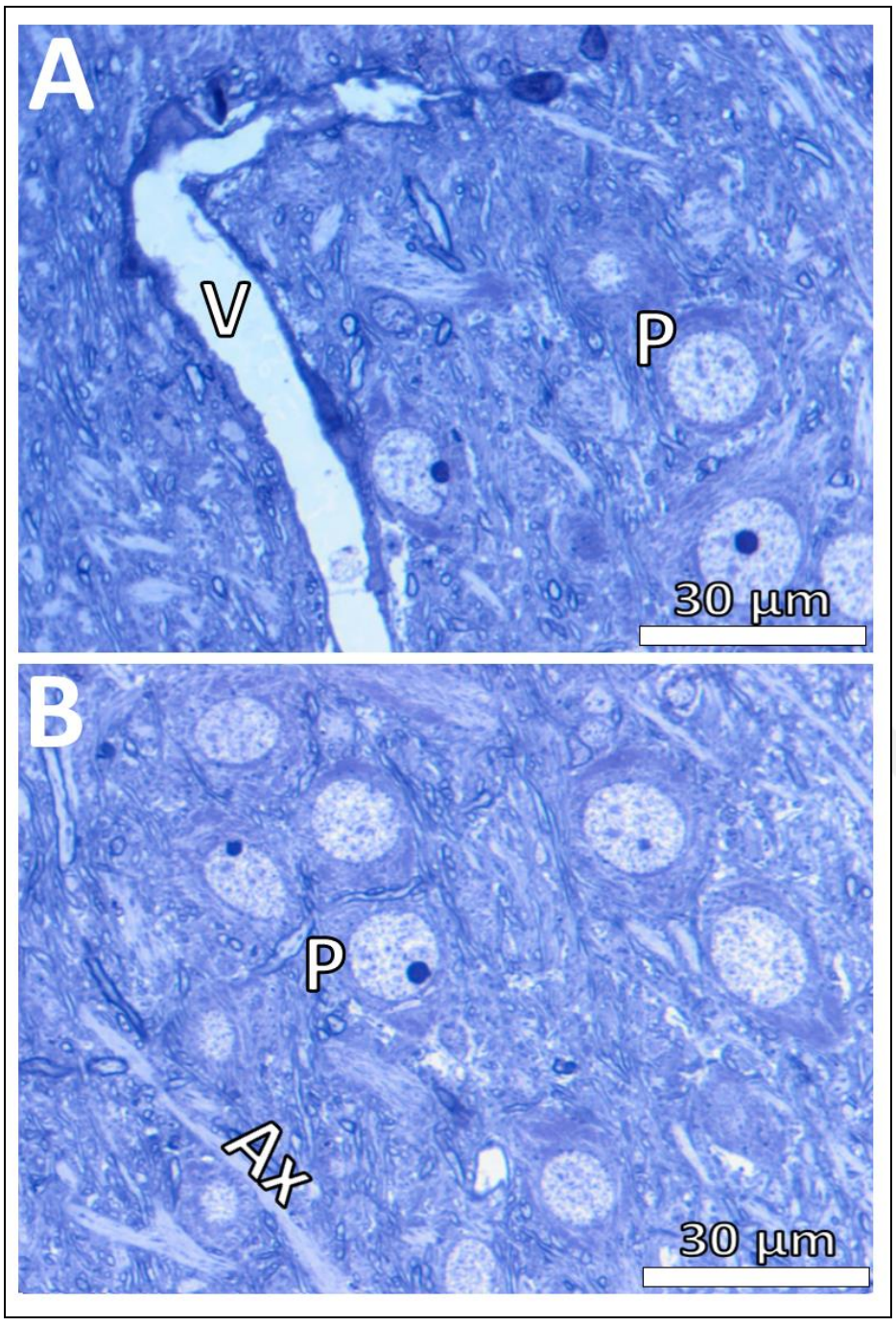


Figure 67. (A, B) Images of the CA region of the hippocampus are seen in the DS+GA group. The pyramidal cells (P) are less densely compacted, and their nuclei are vesicular with a rounded or oval shape, some axons (Ax) are shown as well as a capillary (V). Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

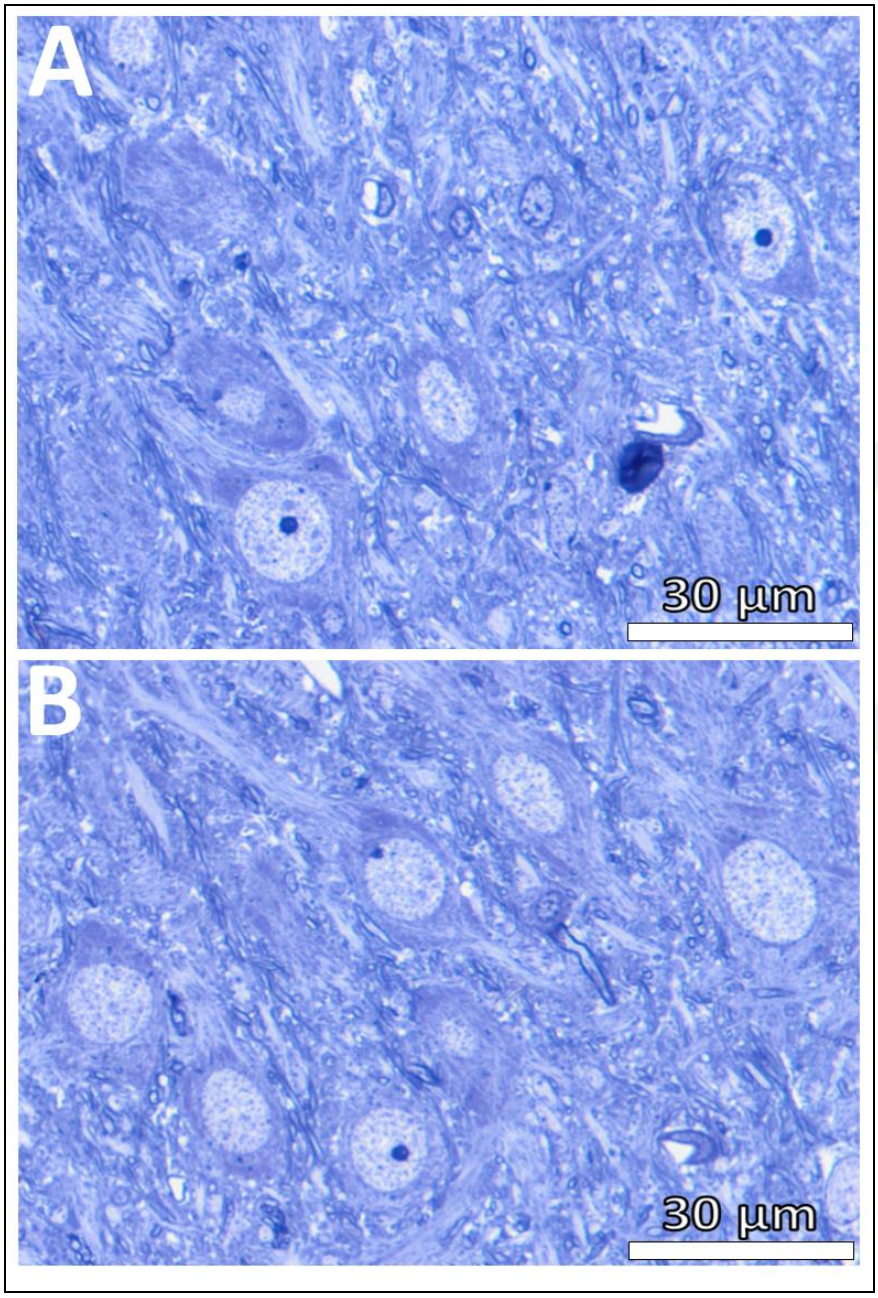


Figure 68. (A, B) Images of the CA region of the hippocampus are seen in the DS+GA group. The pyramidal cells are less densely compacted, and their nuclei are rounded or oval, some axons are shown as well as a capillary. The cytoplasm of pyramidal neurons is stained slightly dark. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

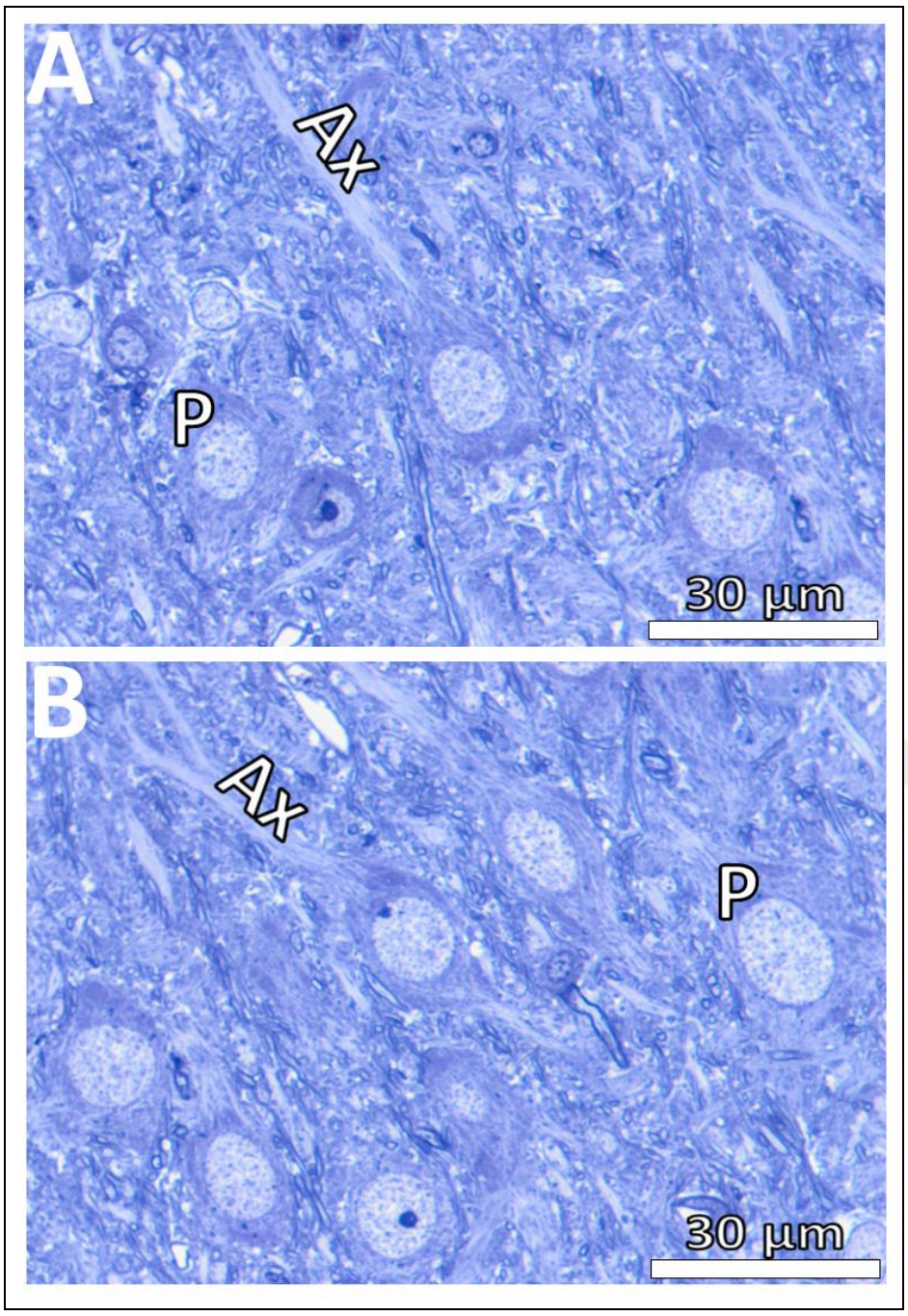


Figure 69. (A, B) Images of the CA layer of the hippocampus are seen in the DS+GA group. The pyramidal cells (P) are less densely compacted, and their nuclei are rounded or oval, some axons (Ax) are shown as well as a capillary. This region might belong to the CA3 region. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

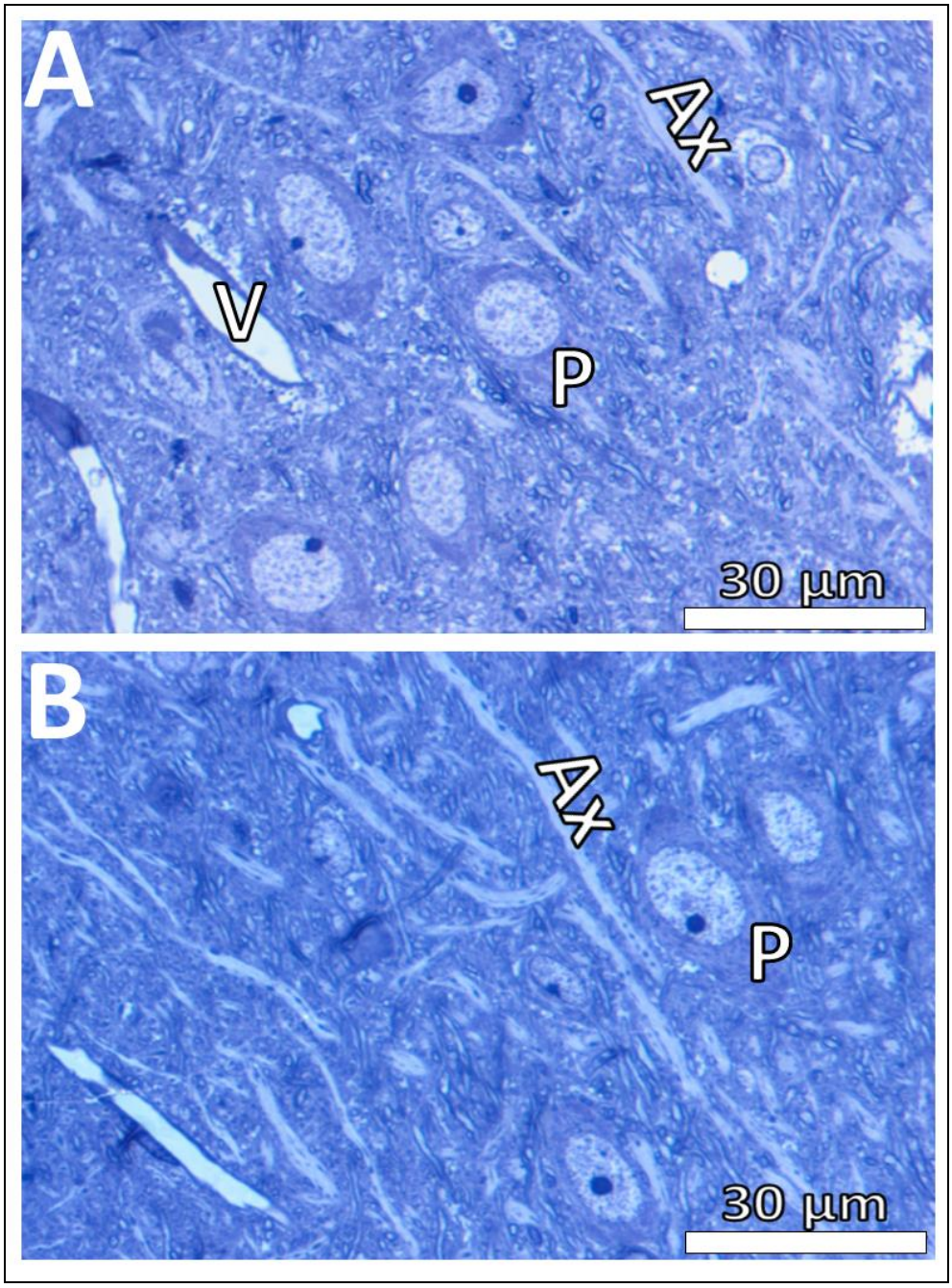


Figure 70. (A, B) Images of the CA layer of the hippocampus are seen in the DS+GA group. There are vacuoles making pyramidal cells (P) less densely compacted, and their nuclei are rounded or oval, some axons (Ax) are seen as well as a capillary (V). This region might belong to the CA3 region since the distance between neurons is large. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

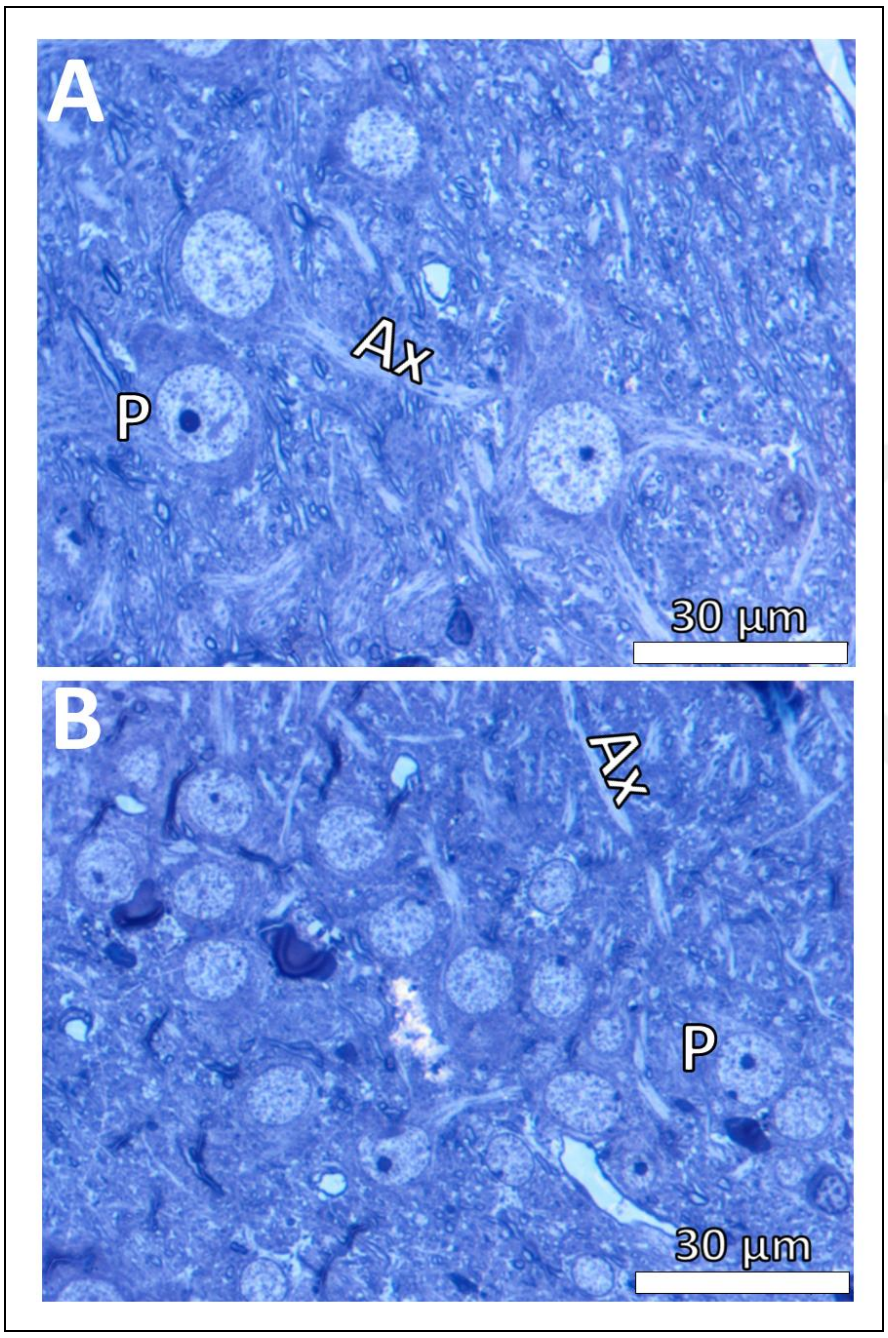


Figure 71. (A, B) Images of the CA layer of the hippocampus are seen in the DS+GA group. There are many distances between neurons that making pyramidal cells (P) less densely packed, and their nuclei are vesicular with rounded or oval shape, some axons (Ax) are shown as well as a capillary (V). Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

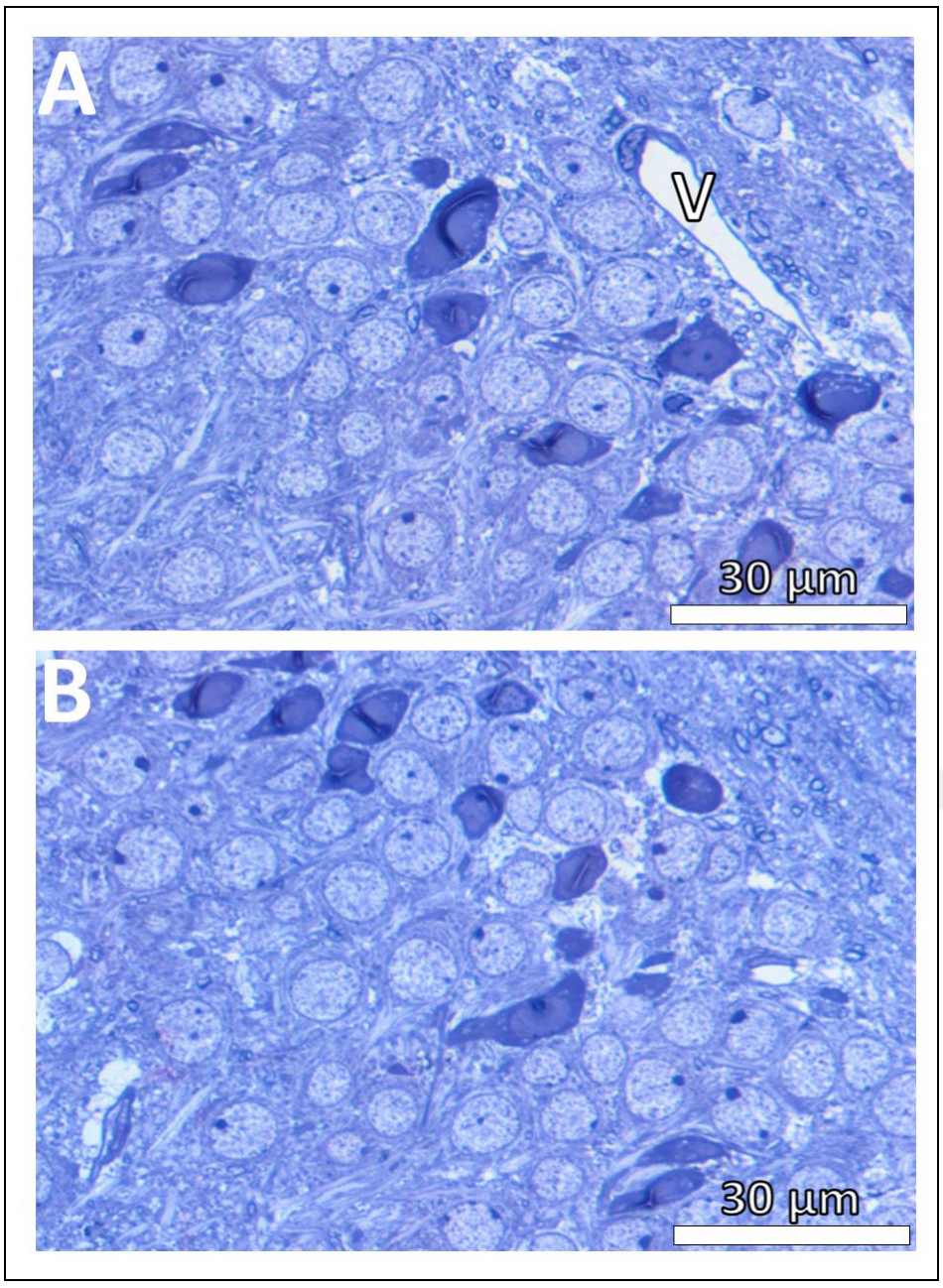


Figure 72. (A, B) Images of the DG layer of the hippocampus are seen in the DS+GA group. Nuclei of granular cells are vesicular with a rounded shape. Some of the cells are dark-stained and they loosely compacted. They show medium to large size. Some axons are seen as well as capillaries (V). Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

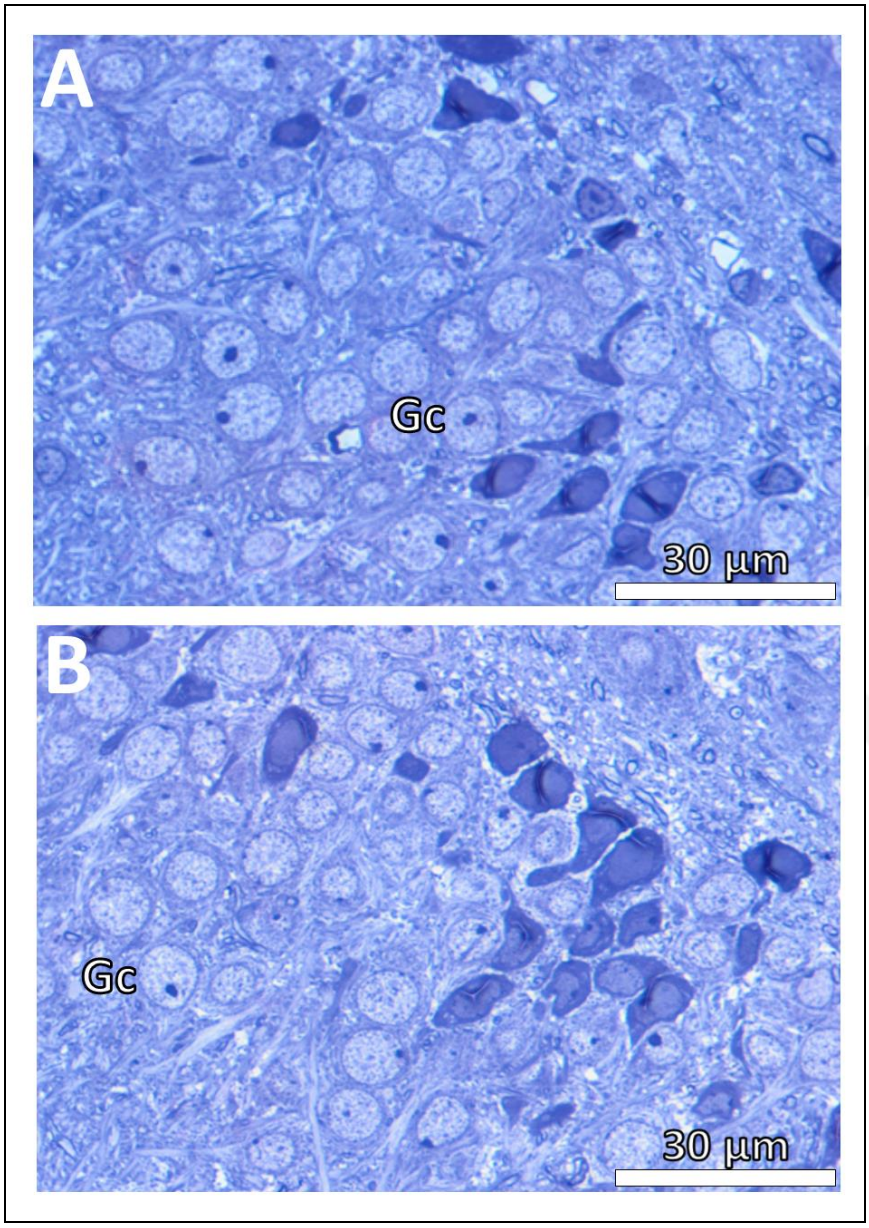


Figure 73. (A, B) Images of the DG layer of the hippocampus are seen in the DS+GA group. Nuclei of granular cells (Gc) are vesicular with a rounded shape, some cells are dark-stained, the cells are loosely compacted, and they show medium to large size, some axons are seen. Darkly stained neurons might be going to die since before removing non-functional cells, they are condensing. After this stage, they will be removed by microglia. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

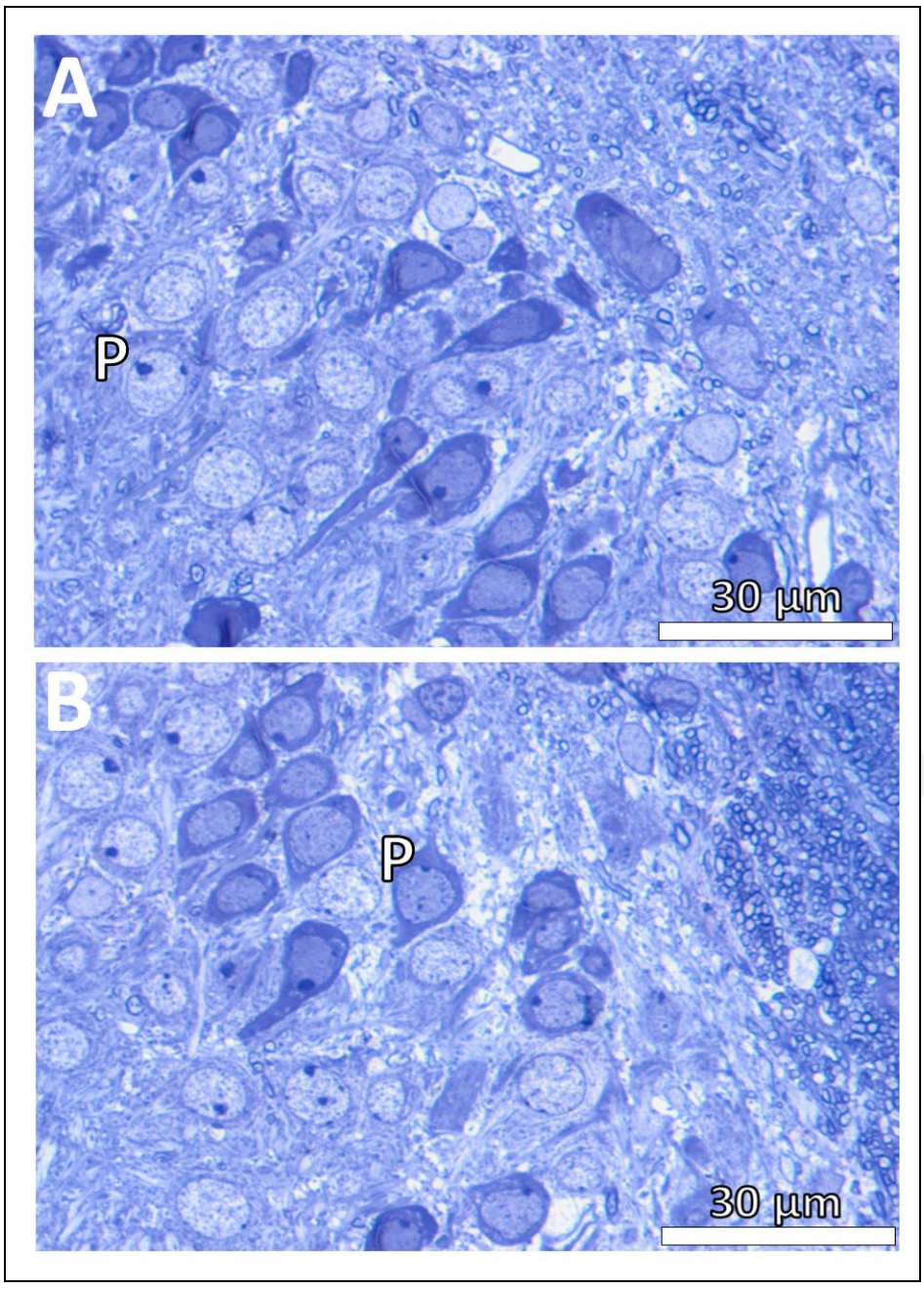


Figure 74. (A, B) Images of the CA layer of the hippocampus are seen in the DS+GA group. Nuclei of pyramidal cells (P) are vesicular with a rounded shape. Some cells are dark-stained, they densely compacted and show medium to small size indicating CA1 region. Some axons are shown as well as a capillary. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

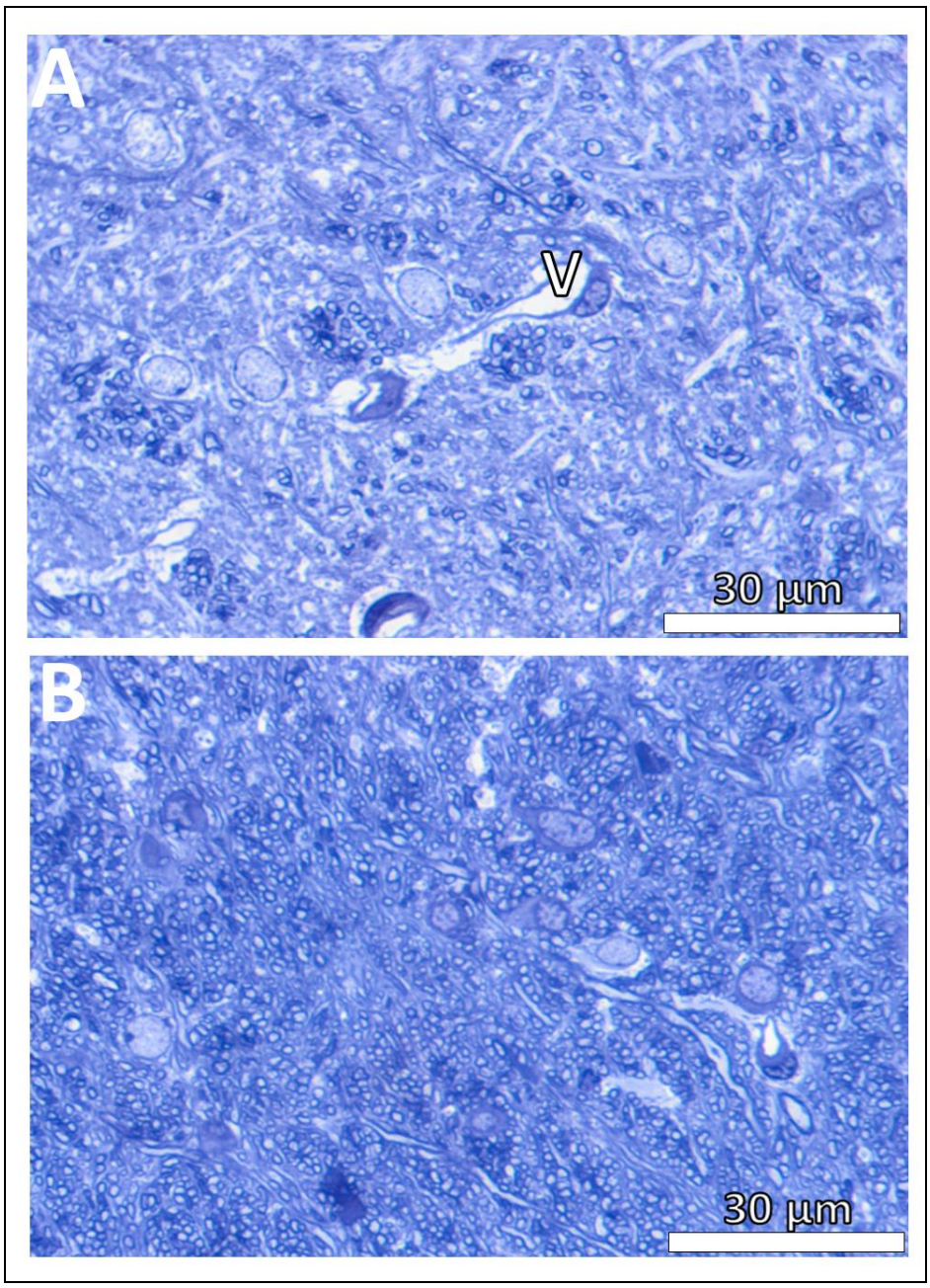


Figure 75. (A, B) Images from the brain are seen in the DS+GA group. Many axons, cross or longitudinal sectioned are seen in healthy as well as capillary. Toluidine blue stained semi-thin resin section (0.5 µm thickness). Original magnification: X100

4.5. Electron Microscopic Findings

Images taken by EM from the control group are seen below. It was found that the pyramidal cells of the hippocampus have oval shape nucleus and a health cytoplasm (**Figure 76, 77, 78, 79, 80, 81, 82, 83, 84, and 85**).

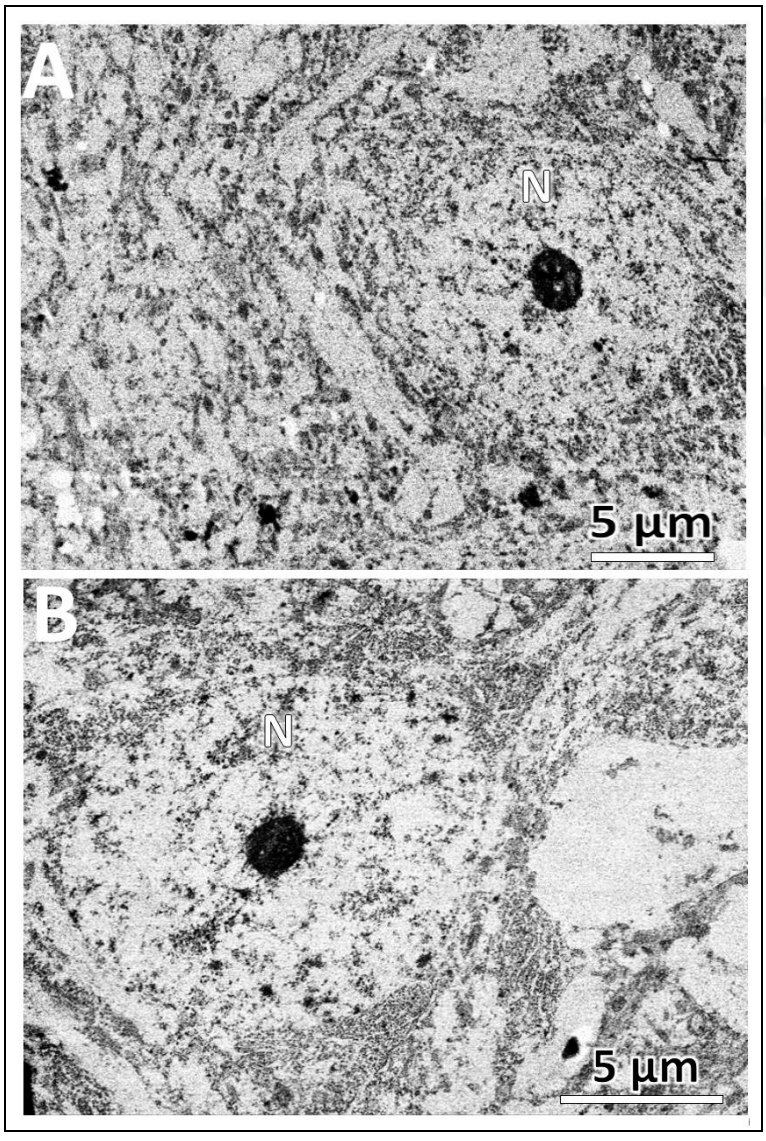


Figure 76. (A, B) Images taken from the CA region of the hippocampus belong to the control group are seen. A well-preserved nucleus and cytoplasm of a pyramidal cell (N) are observed. The nucleolus of the pyramidal cell is large-sized and darkly stained

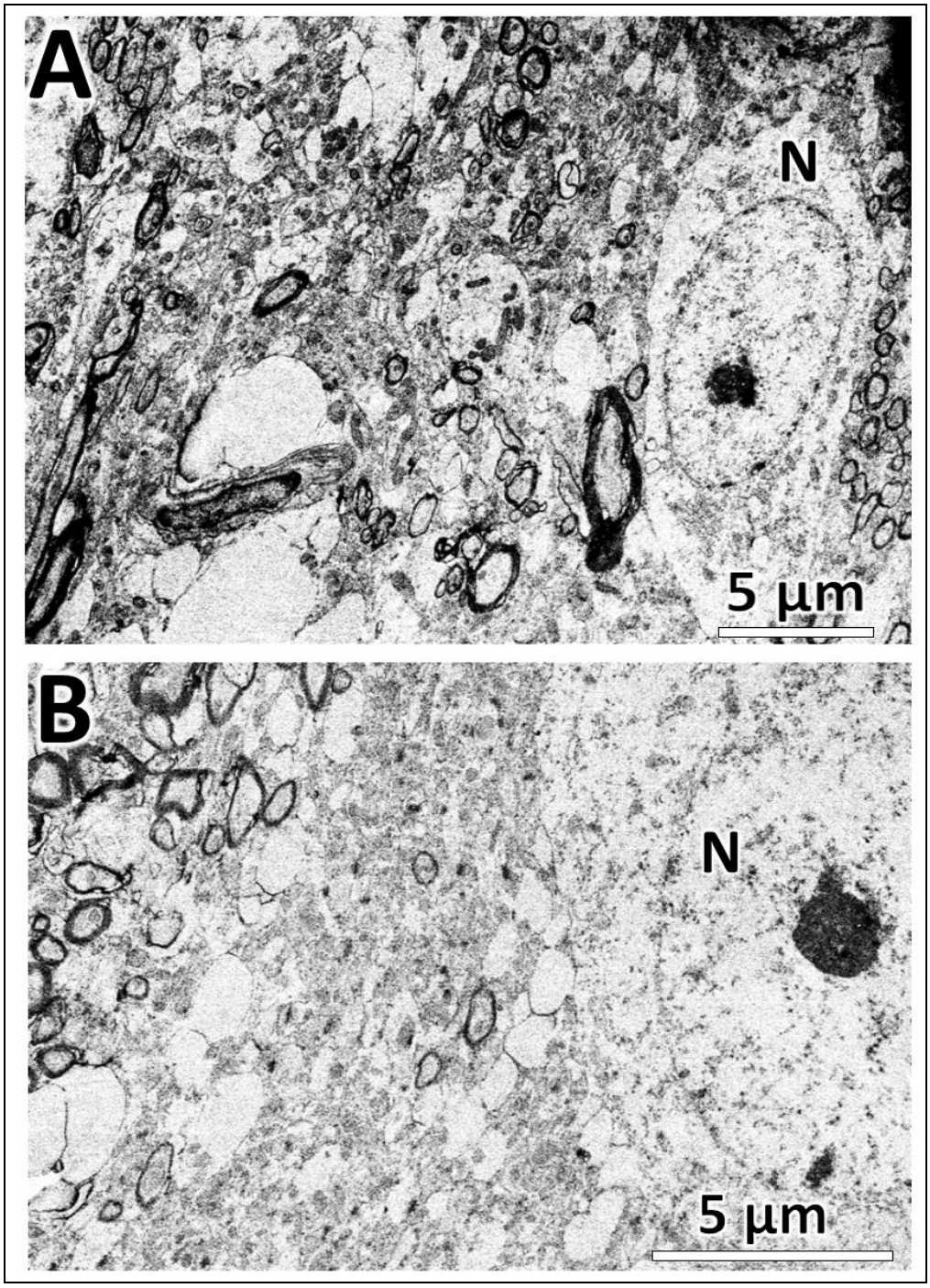


Figure 77. (A, B) Images taken from the CA region of the hippocampus belong to the control group are seen. A well-preserved nucleus and cytoplasm of pyramidal cells (N) are observed. Their nucleoli are a large-sized and darkly stained. Many axons are observed in cross-sections

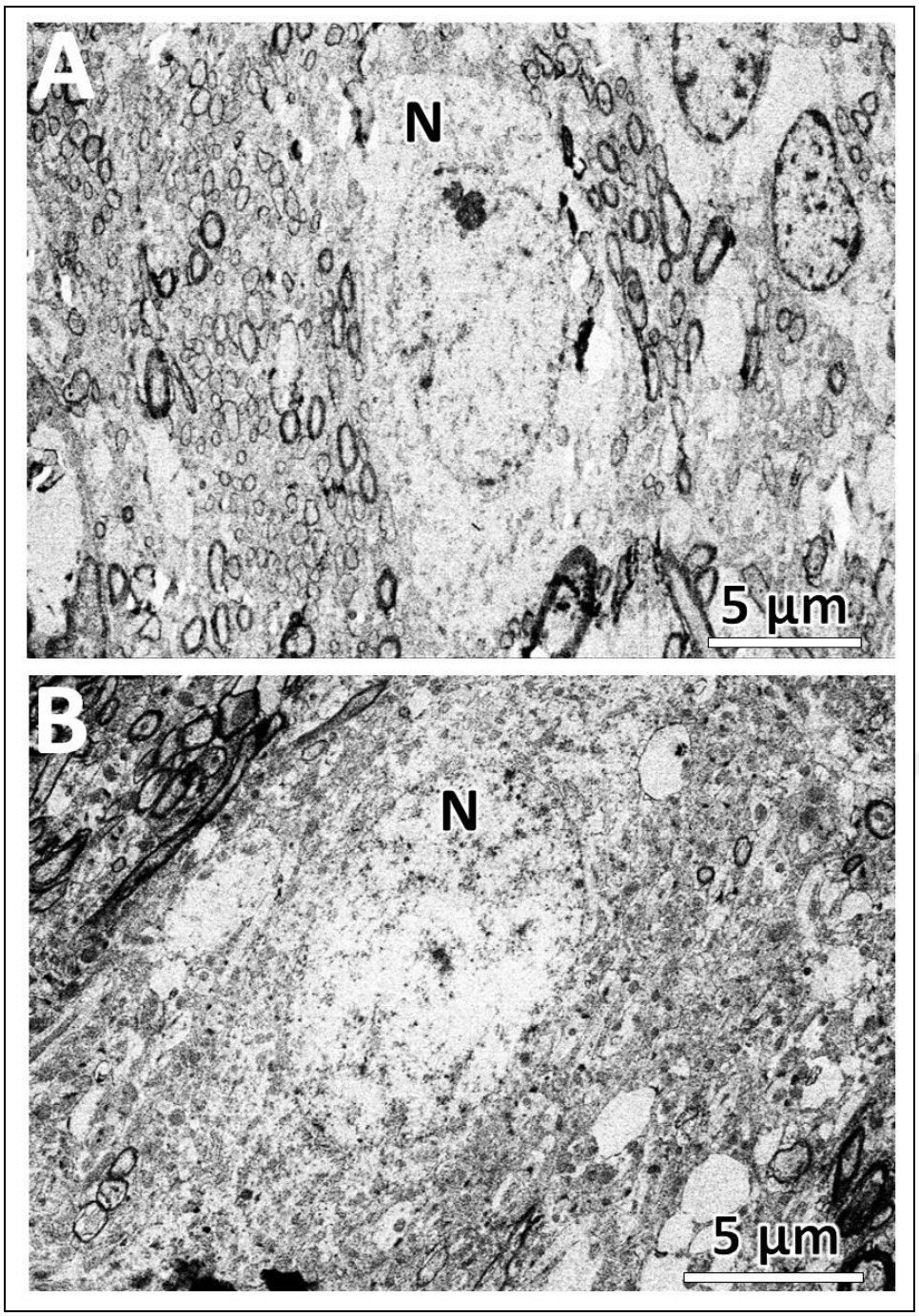


Figure 78. (A, B) Images taken from the CA region of the hippocampus belong to the control group are observed. A well-preserved nucleus and cytoplasm of pyramidal cells (N) are observed. Their nucleoli are a large-sized and darkly stained. Also, here many axons are seen in cross-sections

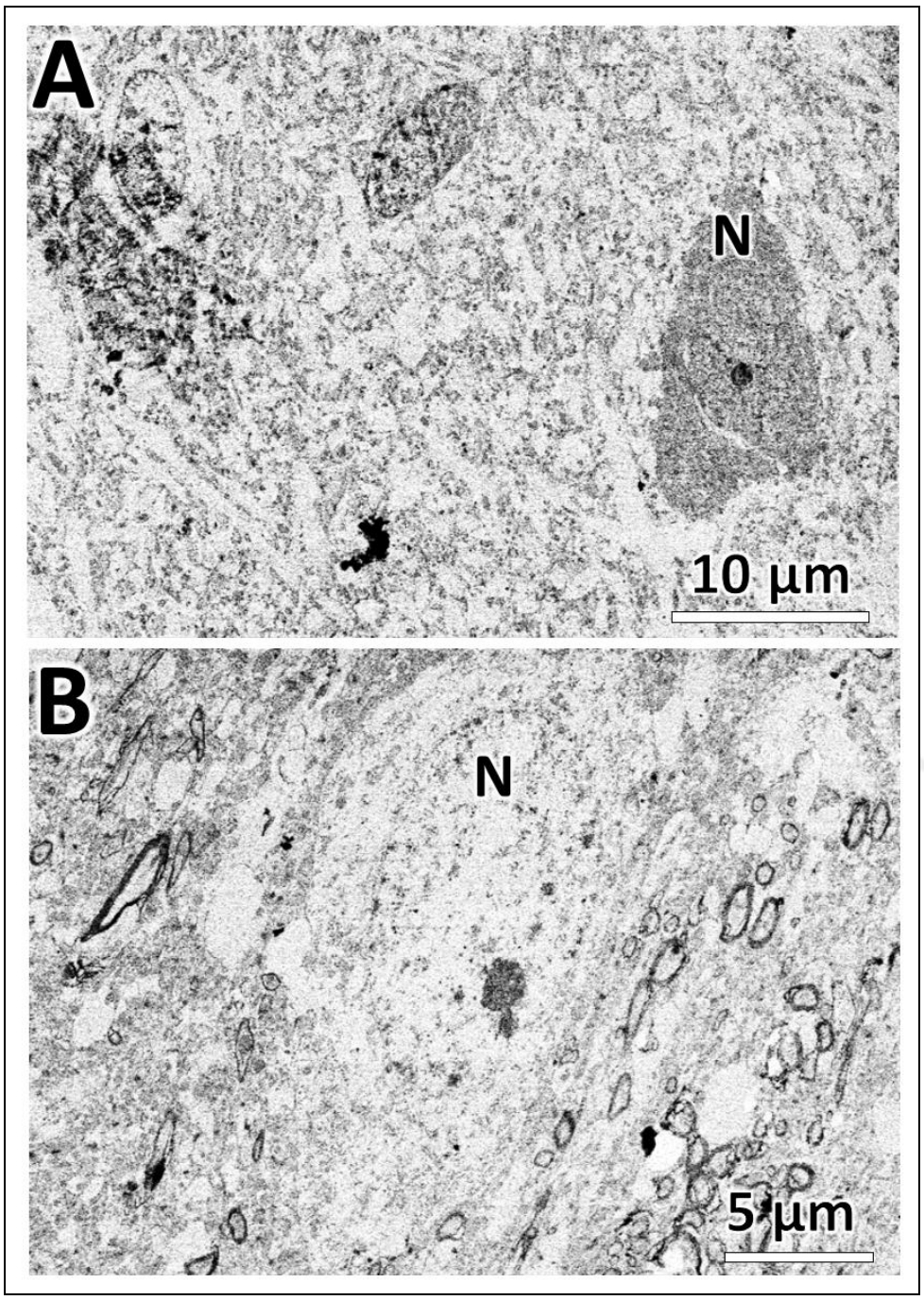


Figure 79. (A, B) Images taken from the CA region of the hippocampus belong to the control group are observed. Pyramidal cells (N) have an oval-shaped nucleus and a notable nucleolus. Axons of pyramidal cells are seen, and some of the axons are sectioned in longitudinal and horizontal sections. The borders of cells are clearly seen, and cells have a healthy appearance

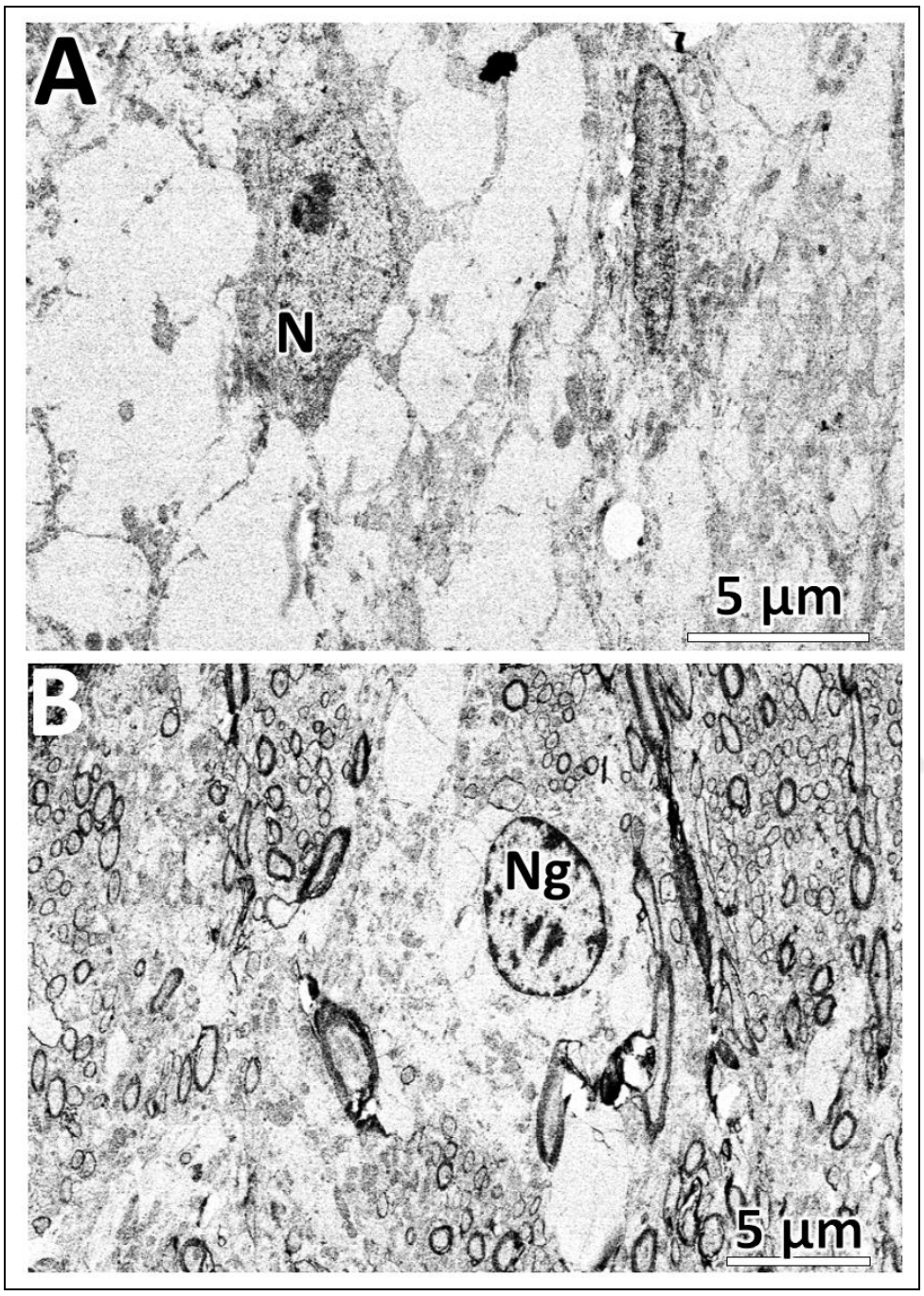


Figure 80. (A, B) Images taken from the CA region of the hippocampus belong to the control group are seen. The shape of cells and nucleus is typically pyramidal-shaped (N); they have condensed cytoplasm. In (B) a neuroglia (Ng) is seen

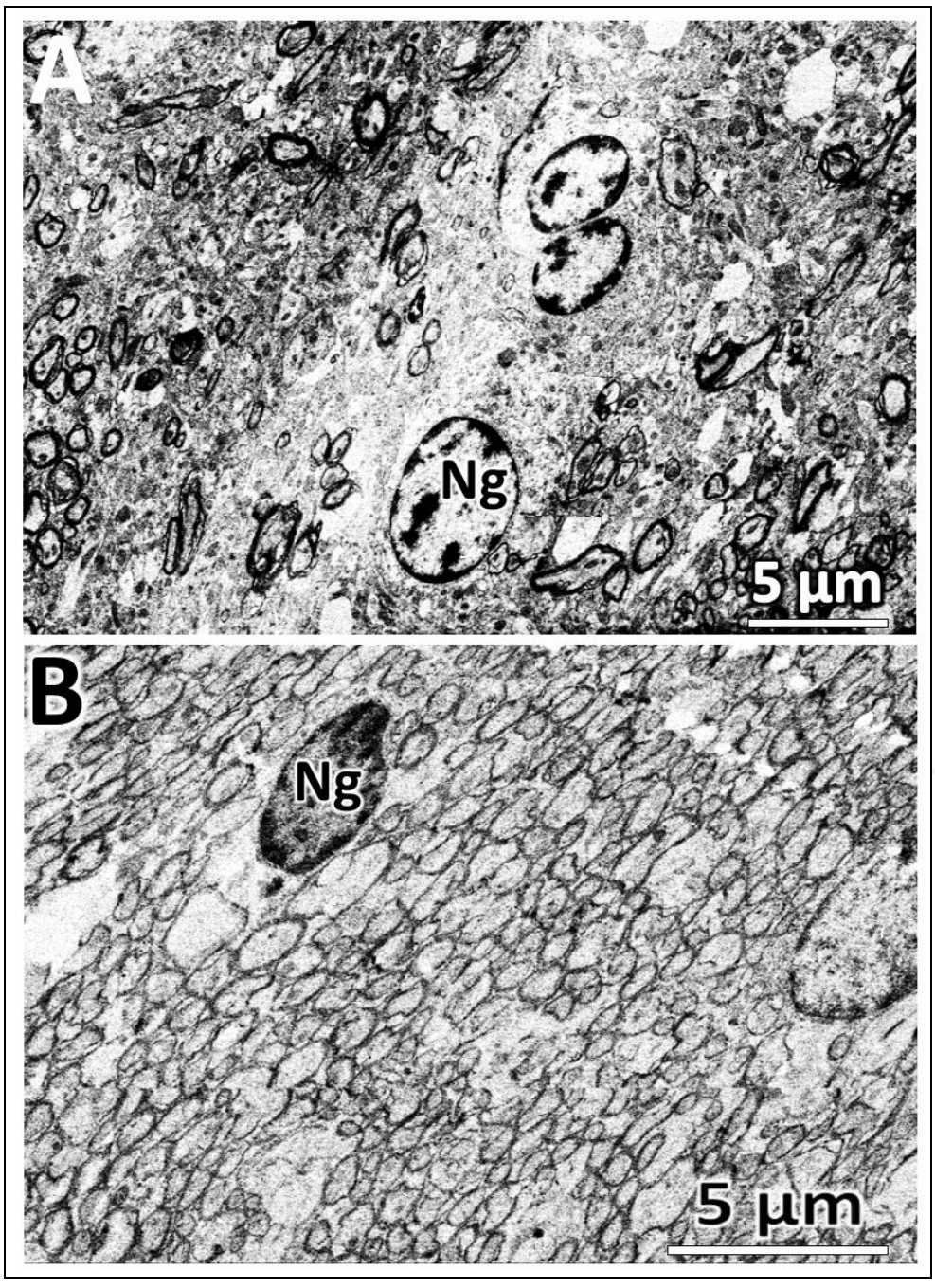


Figure 81. (A, B) Images taken from the CA region of the hippocampus belong to the control group are observed. A well-preserved nucleus and cytoplasm of neuroglia (Ng) are observed. Between these cells, some longitudinal and cross-sections of myelinated axons are observed, and also double unit membranes around the nucleus are found in a normal structure. Their nucleoli are a normal sized

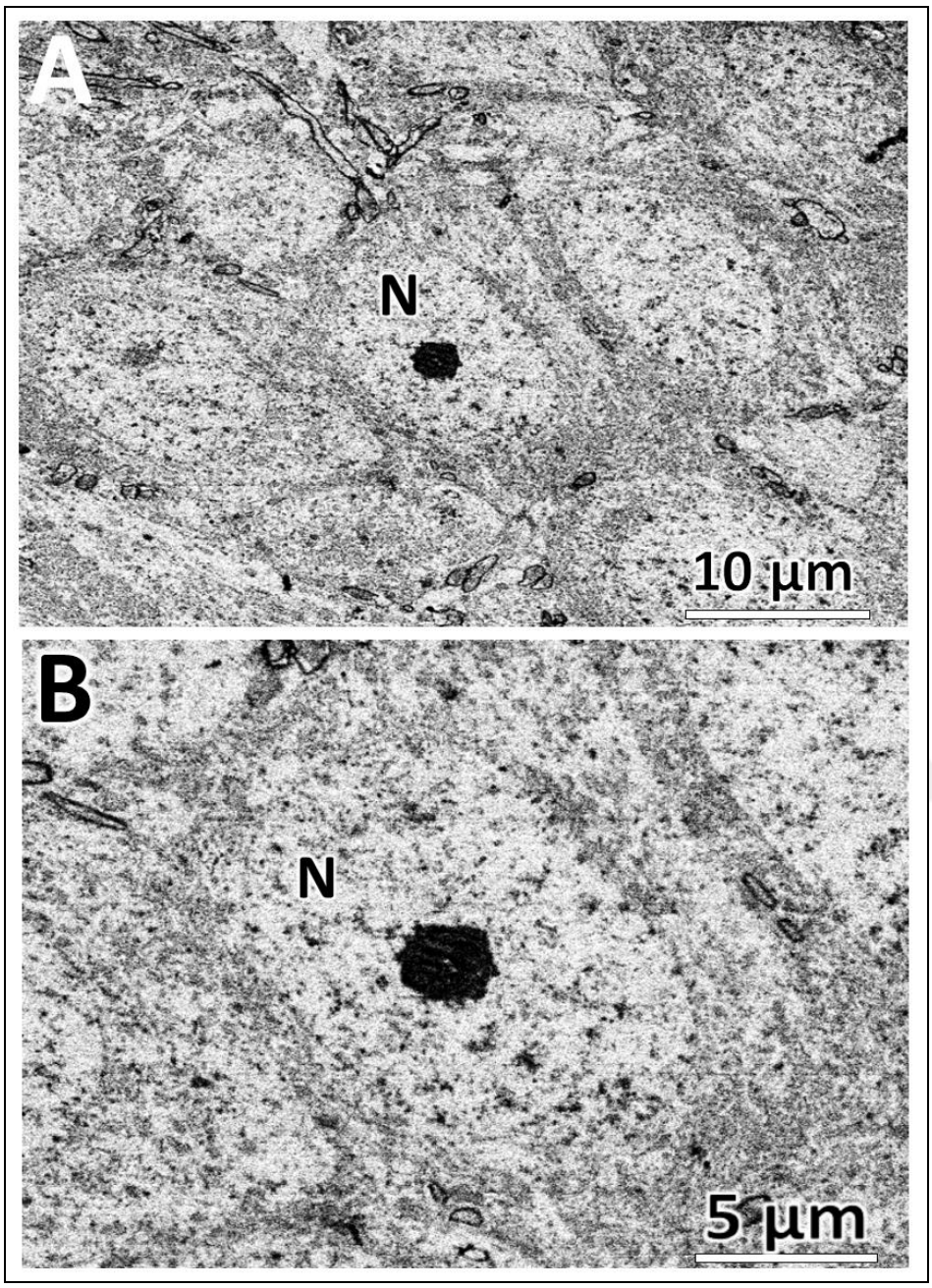


Figure 82. (A, B) Images taken from the CA region of the hippocampus belong to the control group are seen. A well-preserved nucleus and cytoplasm of pyramidal cells (N) are observed. In this area, the cytoplasm density of pyramidal cells is lightly stained

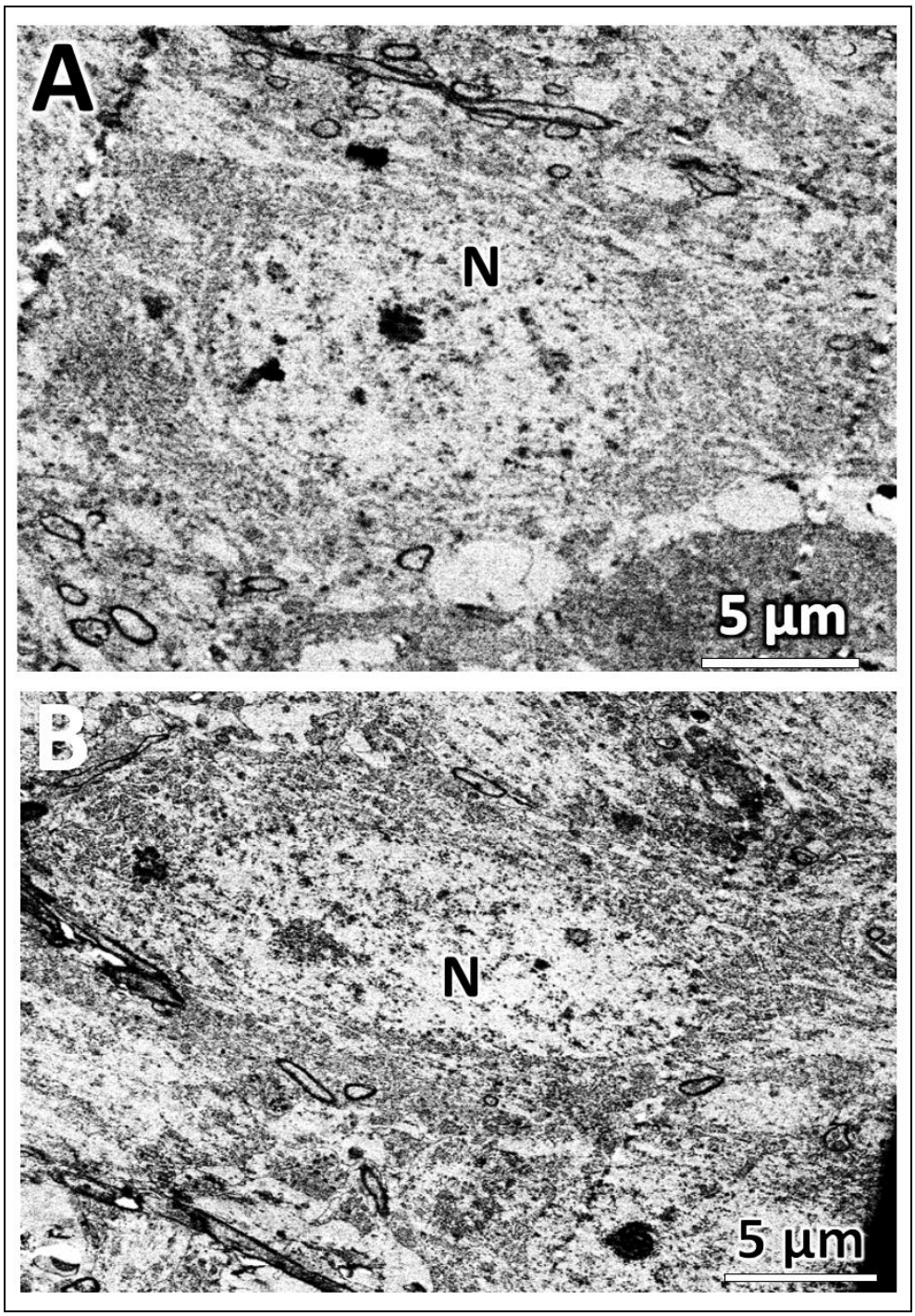


Figure 83. (A, B) Images taken from the CA region of the hippocampus from the control group are seen. The pyramidal neurons (N) have a condensed cytoplasm. Cross and longitudinally sectioned axons are observed

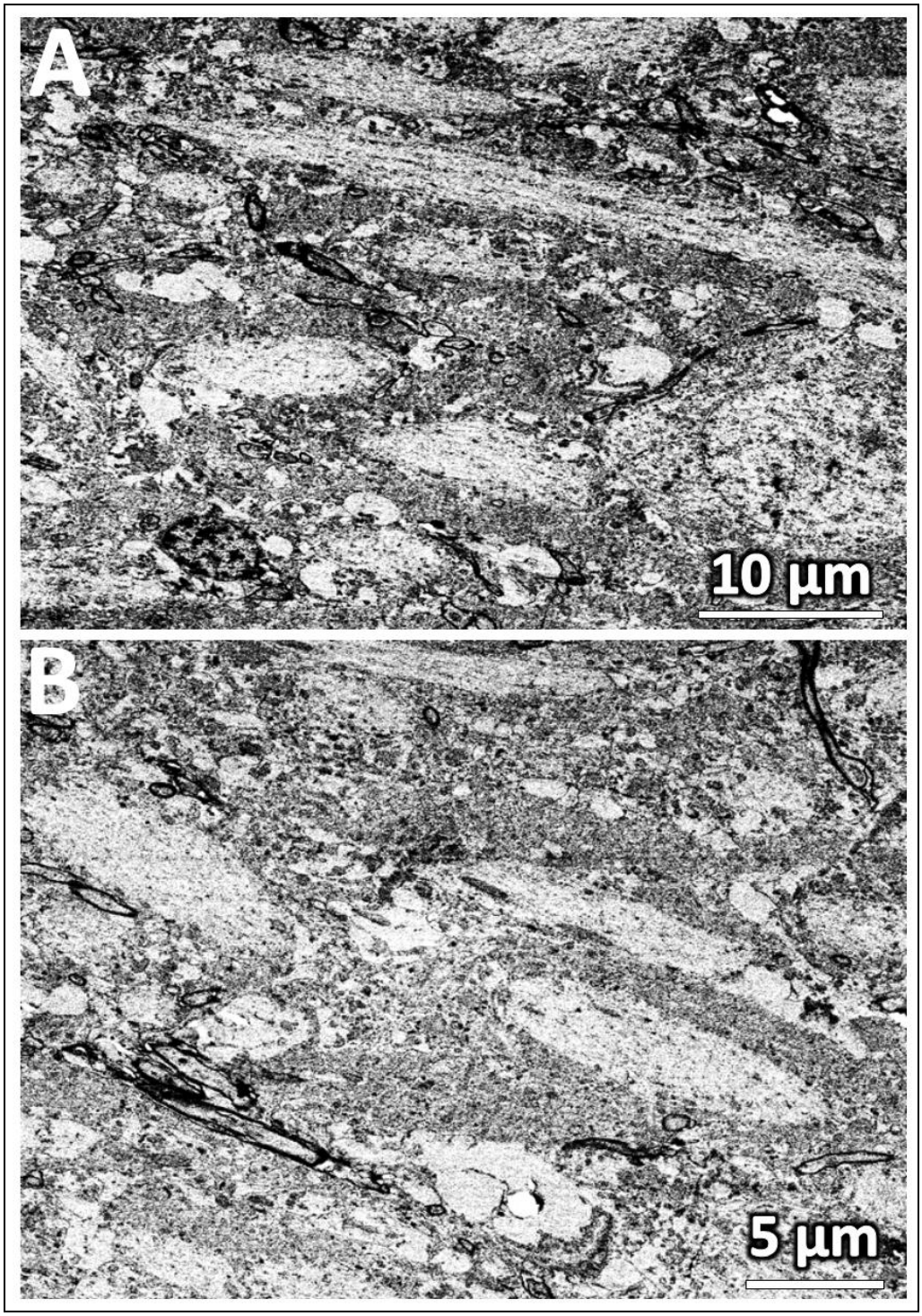


Figure 84. (A, B) Images taken from the CA region of the hippocampus belong to the control group are observed. A well-preserved nucleus and cytoplasm of pyramidal cells are seen. Cross and longitudinal section of axons are also observed

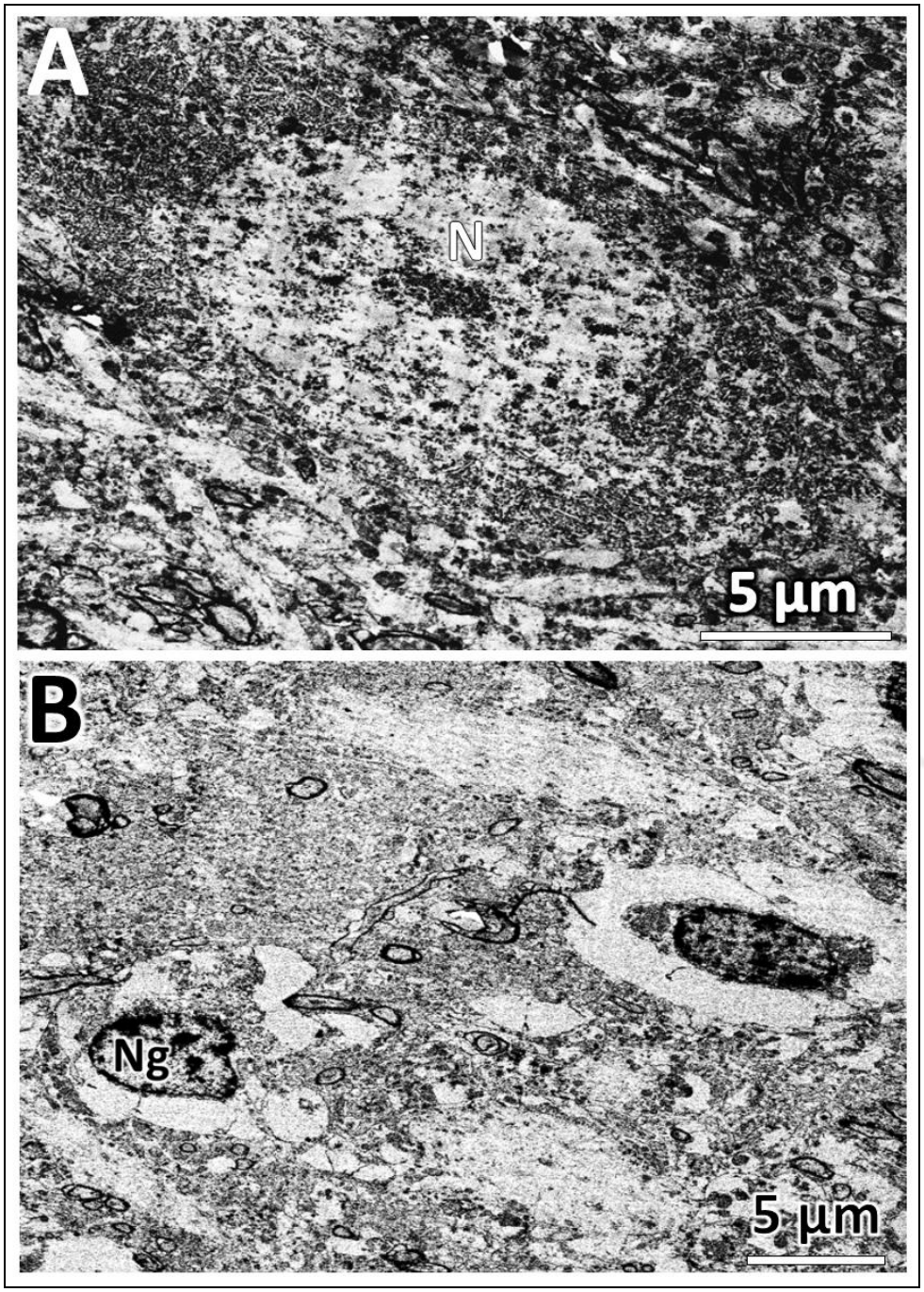


Figure 85. (A, B) Images taken from the CA region of the hippocampus belong to the control group are seen. Pyramidal cells (N) look healthy, and the distance between them is normal. Their axons and nucleus can be clearly observed. At the border of pyramidal cells, some of the cross-sectioned of axons are found. (B) Neuroglia cells (Ng) are seen

Images taken by EM from the GA group demonstrated the oval shape of pyramidal cells and their condensed cytoplasm (**Figure 86, 87, 88, and 89**).

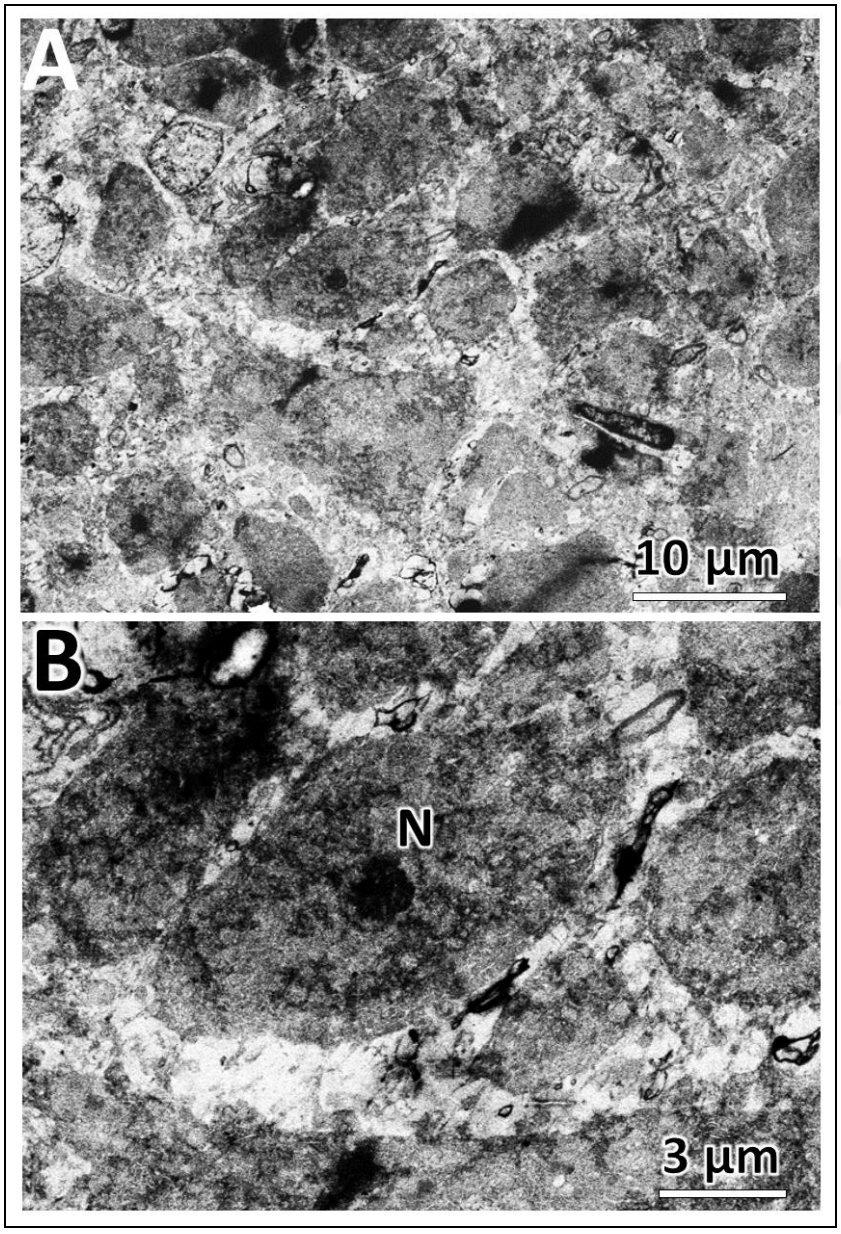


Figure 86. (A, B) Images taken from the CA region of the hippocampus belong to the GA group are seen. Highly damaged pyramidal cells (N) are observed. The bodies of pyramidal cells were seriously shrinkage; the border of them does not have a smooth line. Few numbers of axon cross-sectioned are seen. The distances between neurons are substantially increased

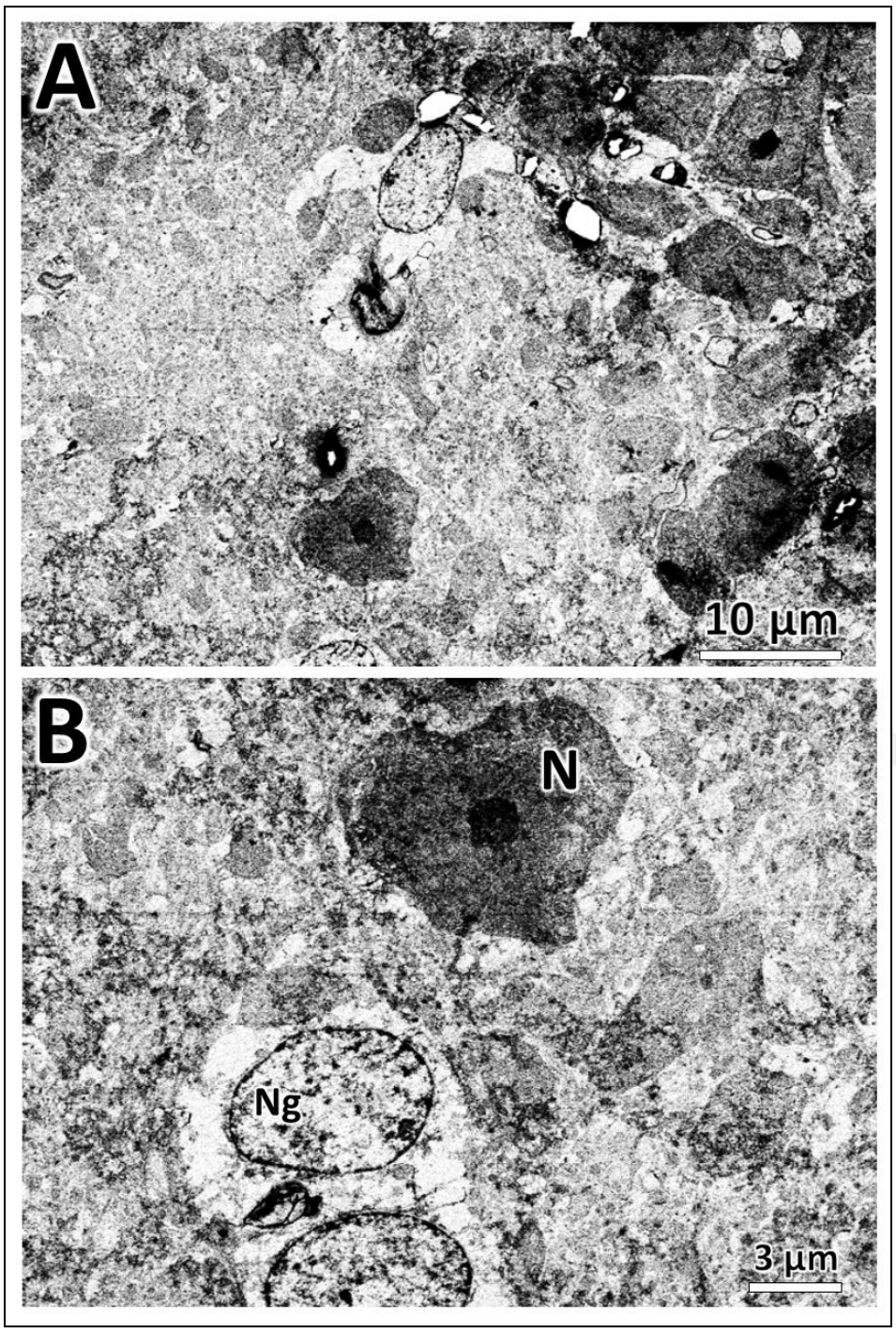


Figure 87. (A, B) Images taken from the CA region of the hippocampus belong to the GA group are seen. Many of darkly stained pyramidal cells (N) that are going to die are observed. Many of empty spaces (vacuoles), few numbers of axons and partly a healthy neuroglia (Ng) are seen

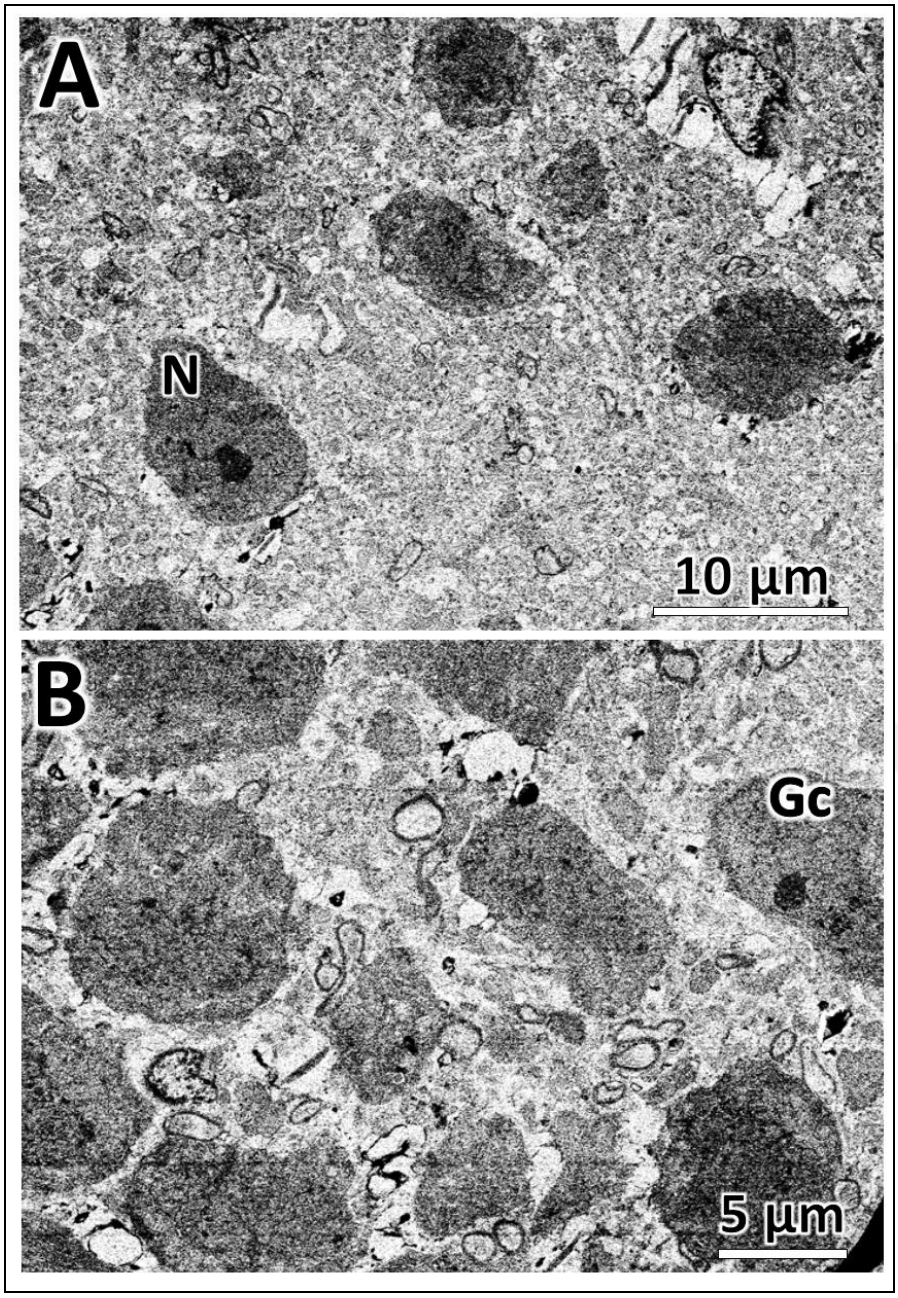


Figure 88. (A) An image taken from the CA region of the hippocampus belong to the GA group is observed. (B) Image taken from the dentate gyrus of the hippocampus belong to the GA group is seen. Severely damaged granular neurons (Gc) are found. The border of neurons (N) and their nucleus were not clearly seen. Increased vacuoles around them are also obvious. A few numbers of axons cross-sectioned in this region are seen. The distance between granular neurons are highly increased

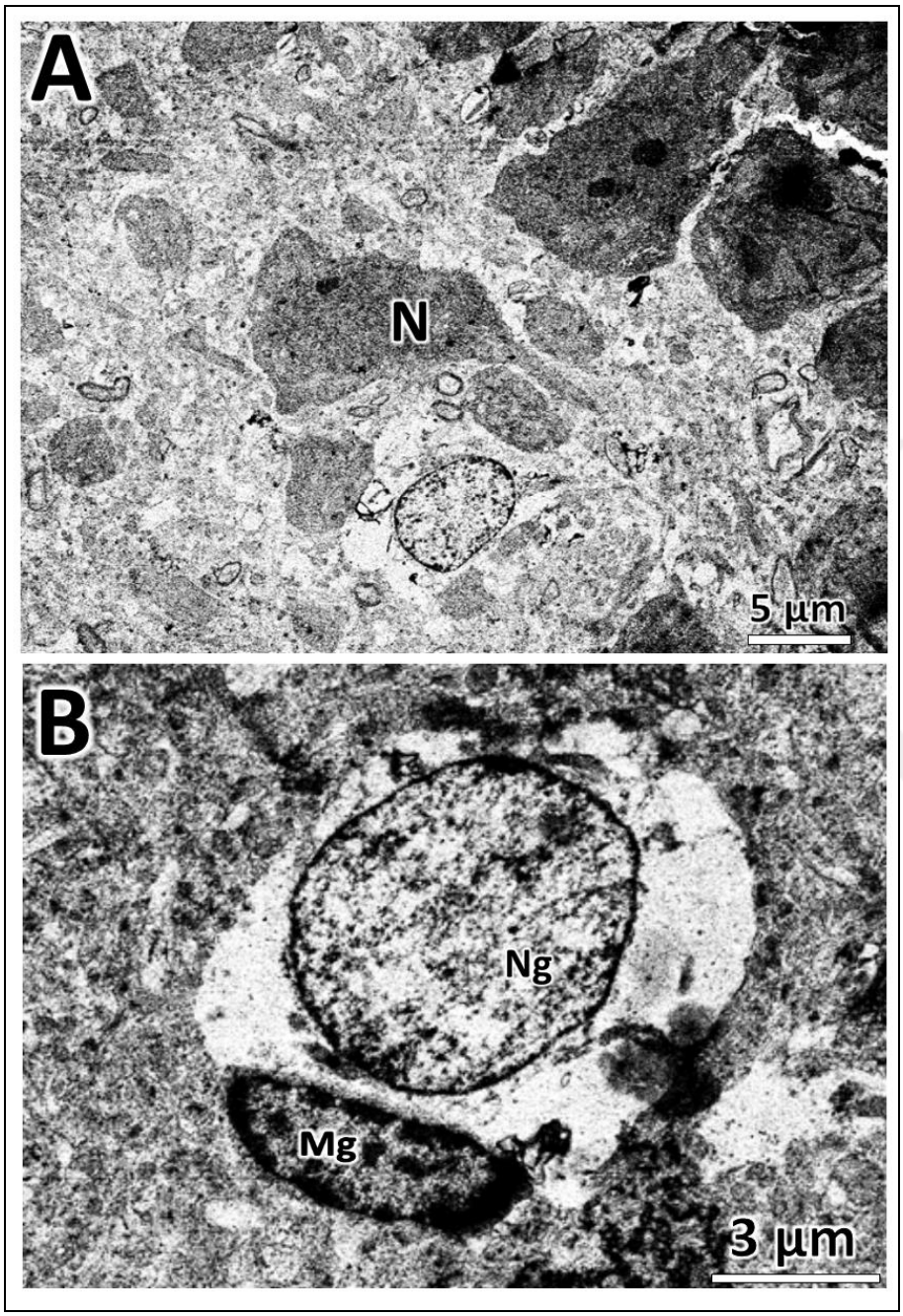


Figure 89. (A, B) Images taken from the CA region of the hippocampus belong to the GA group are seen. (A) Severely damaged of pyramidal neurons (N) that have a condense body are observed. The borders of the cell and its nucleus were not seen clearly, and increased vacuoles around them are also observed. A few numbers of axons in this region are seen. (B) Microglia (Mg) and neuroglia (Ng) are observed that might be an astrocyte

Images taken by EM from the DS group demonstrated that neurons in this group have oval-shaped pyramidal cells and a condensed cytoplasm. The bodies of cells were shrinkage, and some nuclei showed varying degrees of damage (**Figure 90,91, 92, 93, 94, 95, and 96**).

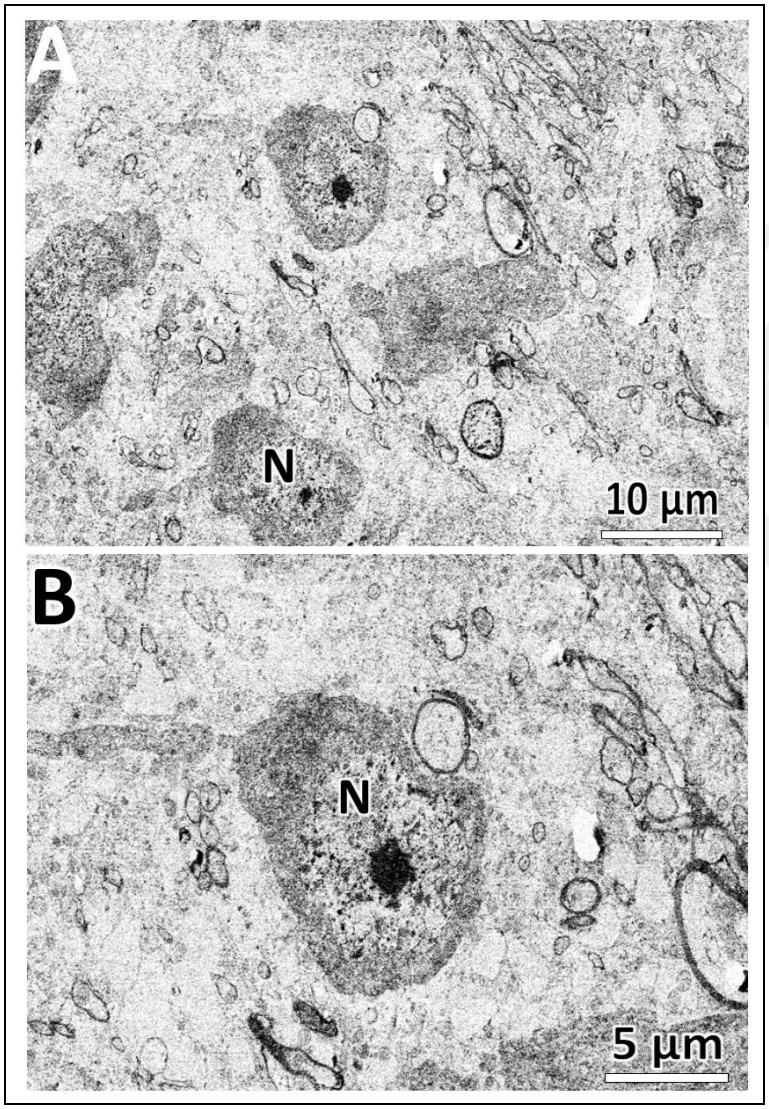


Figure 90. (A, B) Images taken from the CA region of the hippocampus belong to the DS group are seen. A highly damaged nucleus and cytoplasm of a pyramidal cell (N) are observed. The bodies of pyramidal cells were seriously shrinkage; the border of them does not have a smooth line. The distance between pyramidal neurons was increased because of substantial cell loss. Few numbers of axons section are seen

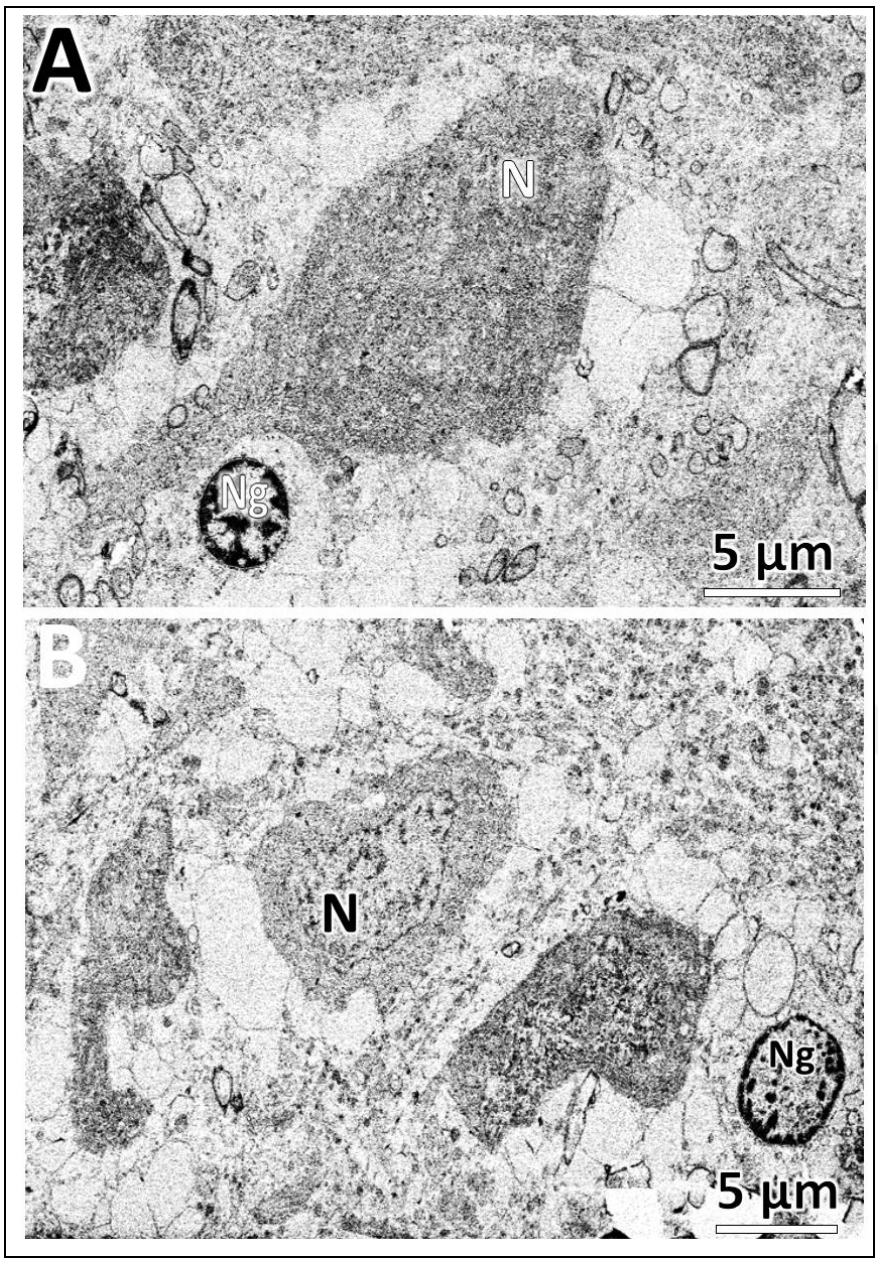


Figure 91. (A, B) Images taken from the CA region of the hippocampus belong to the DS group are seen. (A) A darkly stained pyramidal cell (N) that might be near death is observed. Many empty spaces (vacuoles), few numbers of axons, and partly a healthy neuroglia (Ng) are seen around it. (B) An increased number of darkly stained pyramidal neurons is found. The distance between them was increased, and the shapes of them were severely impaired. Vacuolization around the neurons is a typical character of these group tissues. Many of cross-sections of axons are seen but their myelin sheaths are very thin

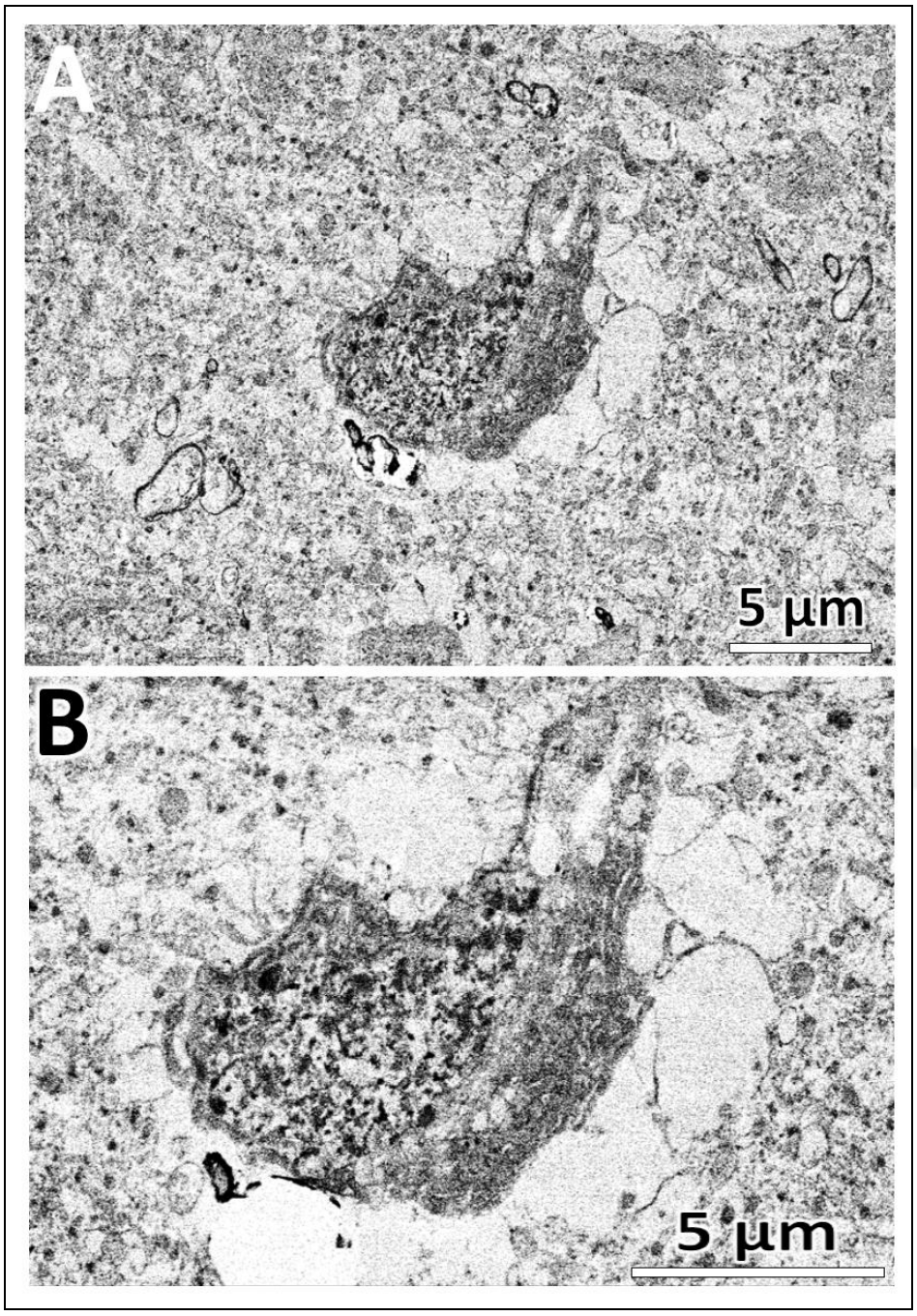


Figure 92. (A, B) Images taken from the CA region of the hippocampus belong to the DS group are observed. A severely damaged pyramidal neuron (N) is observed. The border of the cell and its nucleus was not clearly seen. Increased numbers of vacuoles around neuron are also observed

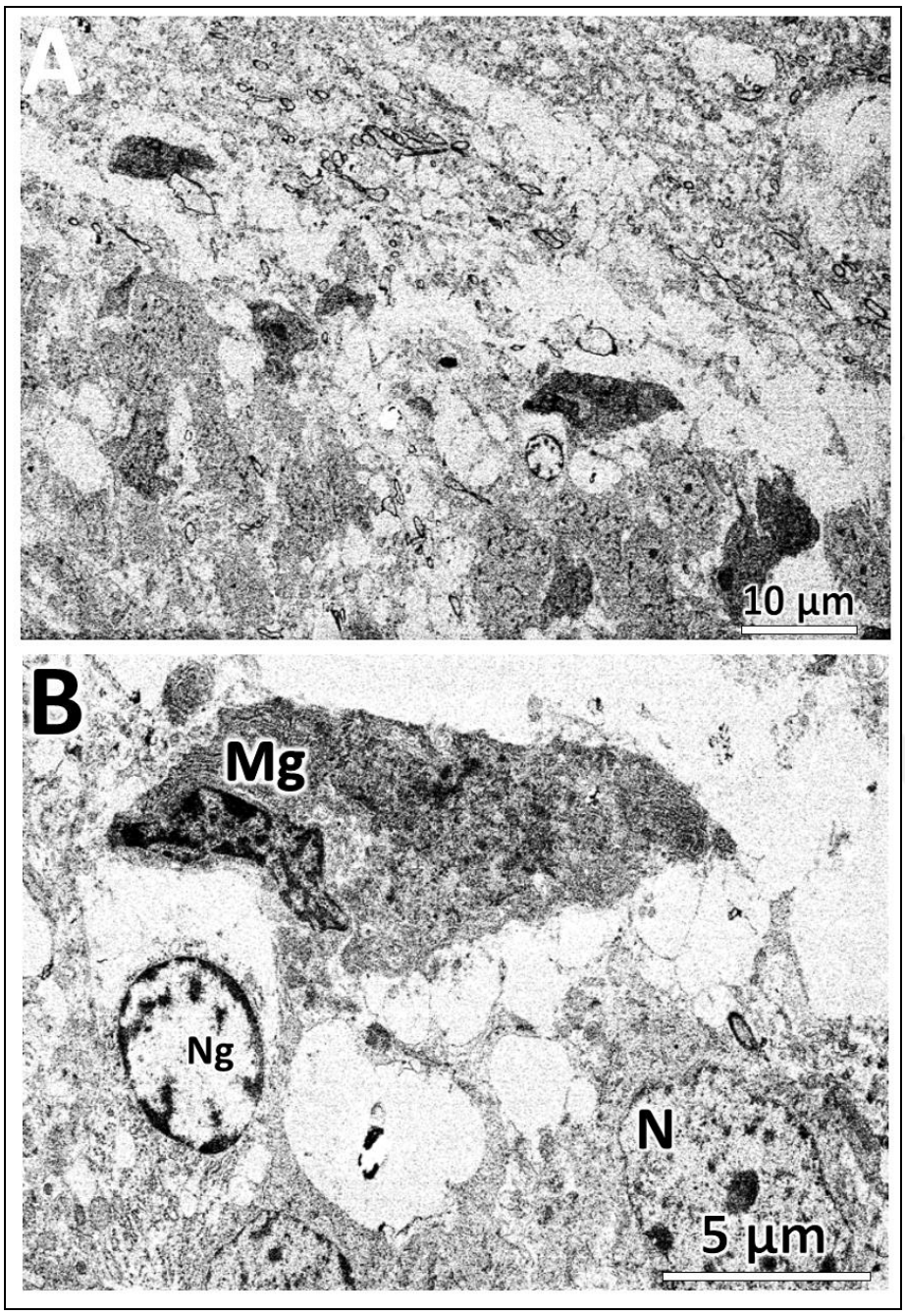


Figure 93. (A, B) Images taken from the CA region of the hippocampus belong to the DS group are observed. Pyramidal cells in this region were severely damaged since they lost their nucleus as well as their shape. Most of the cells between darkly stained cells were removed; remaining cells in this region might lose their function. (B) A magnified of (A) is seen, darkly stained, and nearly died, phagocytes by microglia (Mg). Different sizes of vacuoles are seen around the cells. Neuroglial (Ng) cells are seen

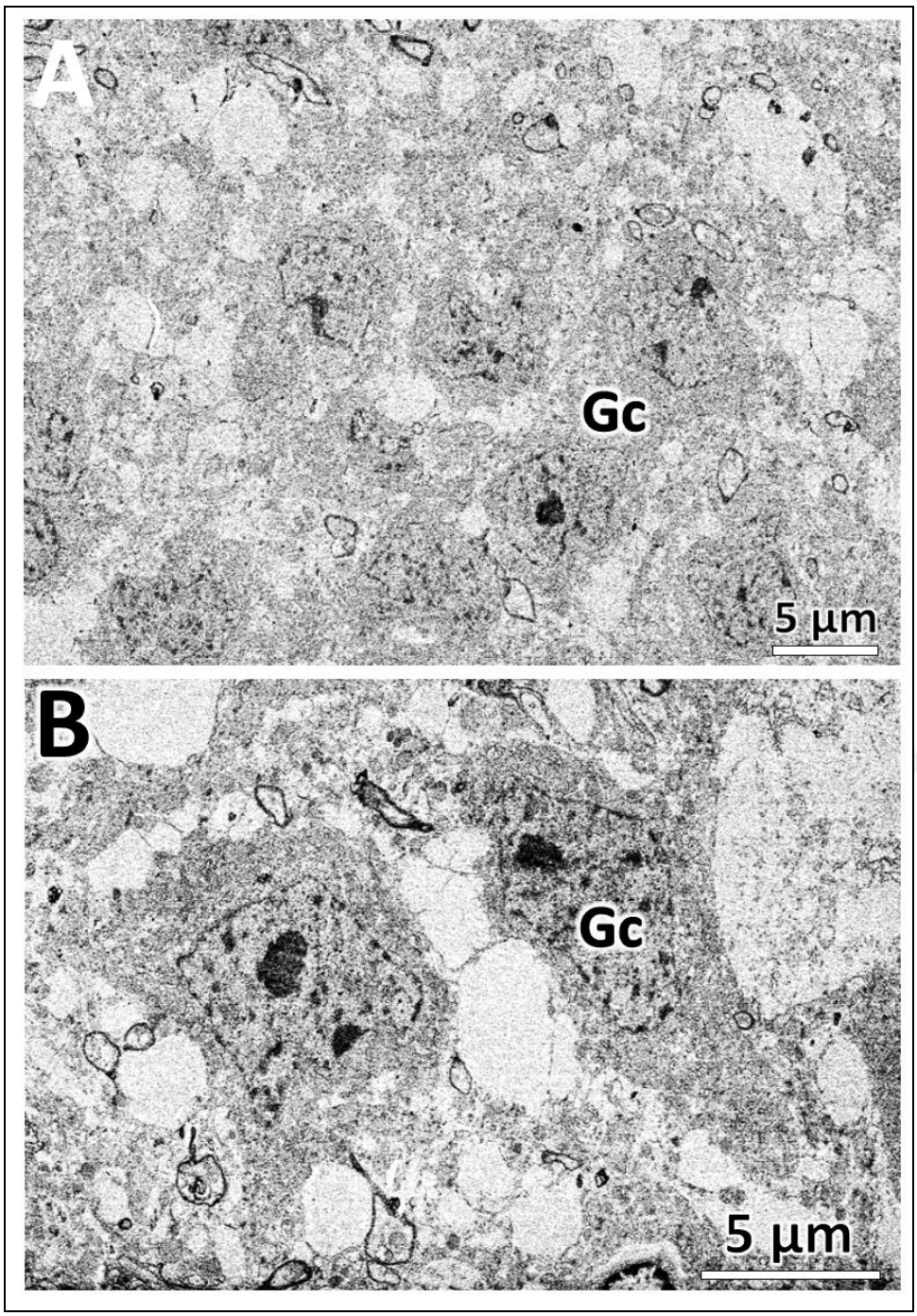


Figure 94. (A, B) Images taken from the dentate gyrus of the hippocampus belong to the DS group are seen. Most of the granular cells (Gc) lost their spherical shape, and highly increased spaces around them are observed. Increased spaces around/between dark granular cells may be a result of removing dead neurons in this region. Few numbers of axons sections are found

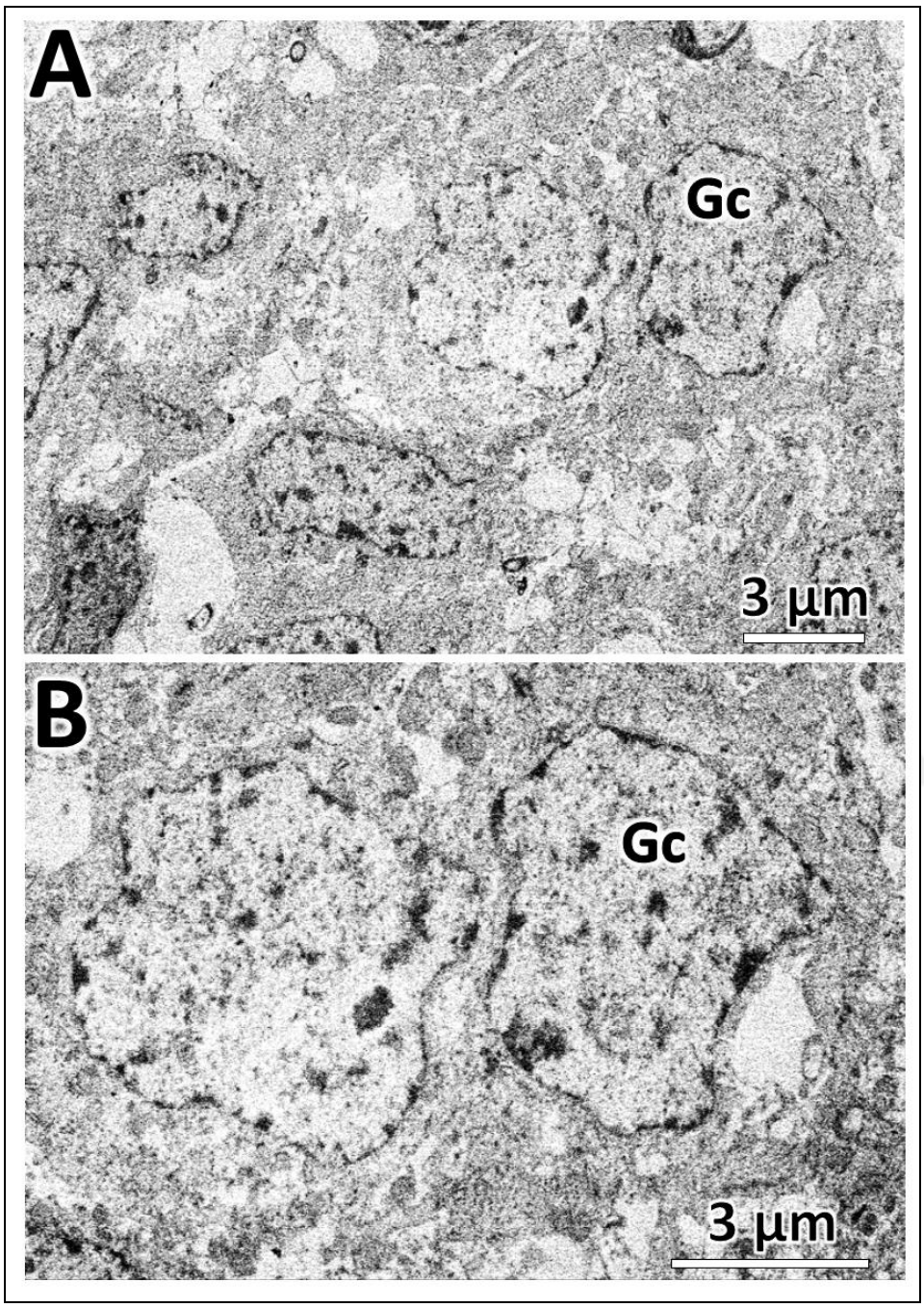


Figure 95. (A, B) Images taken from the dentate gyrus of the hippocampus belong to the DS group are seen. Most of the granular cells (Gc) in this region lost not only their spherical shape but also their integrity. There are many spaces around them observed. Increased spaces around darkly stained granular cells may also belong to removing dead neurons in this region. Few numbers of axons sections are found

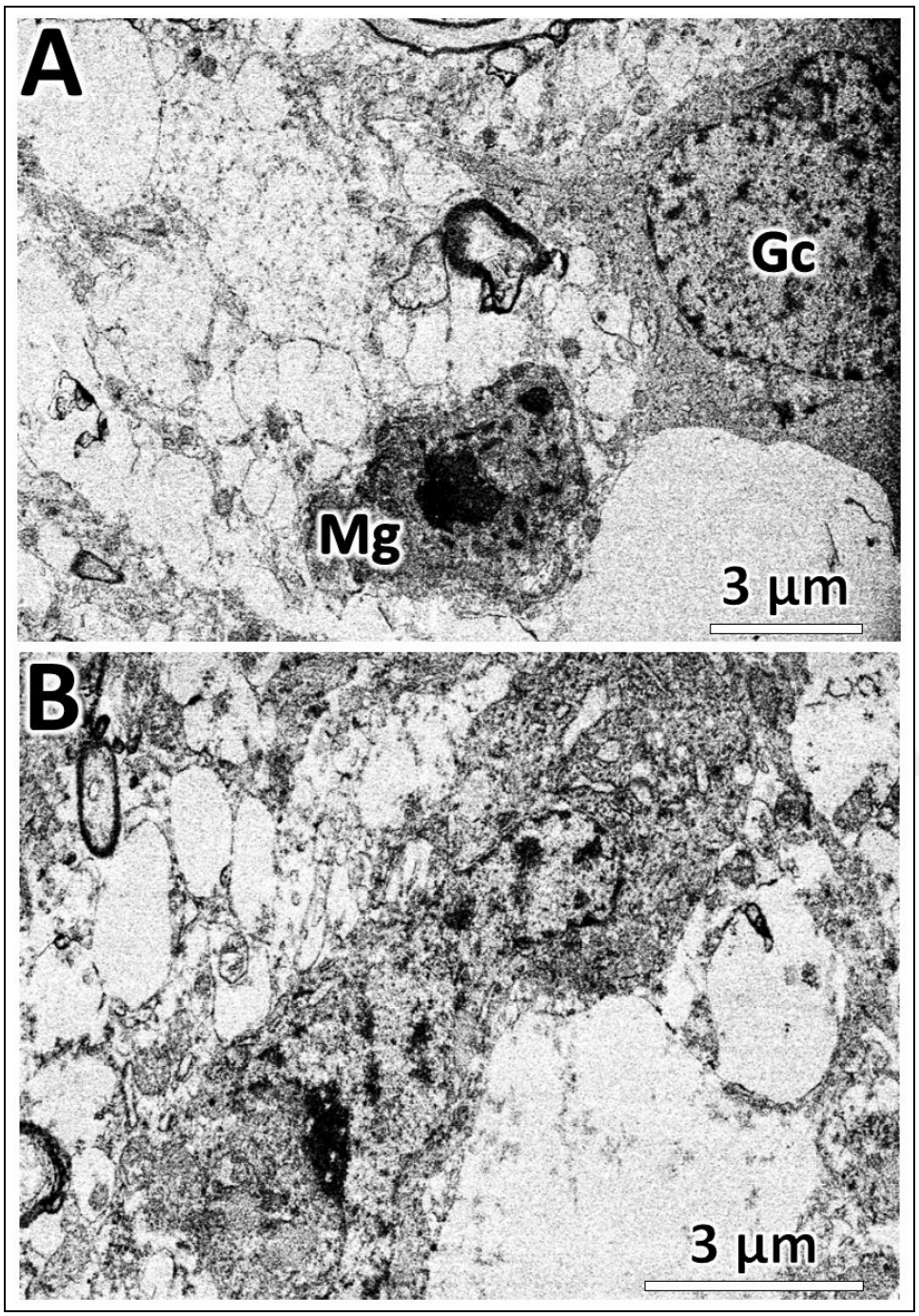


Figure 96. (A, B) Images taken from the dentate gyrus of the hippocampus belong to the DS group are observed. (A) A dark granular cell (Gc) and microglia (Mg) are seen; notice the heterogeneity of its cytoplasm. (B) These cells might be microglia since they have a heterogeneous cytoplasm and its nucleus has a fusiform shape

Images taken by EM from the DS+GA group demonstrated the oval shape of pyramidal cells and their condensed cytoplasm. The bodies of cells were shrinkage, and some nuclei showed varying degrees of damage (**Figure 97, 98, 99, 100, 101, 102, 103, and 104**).

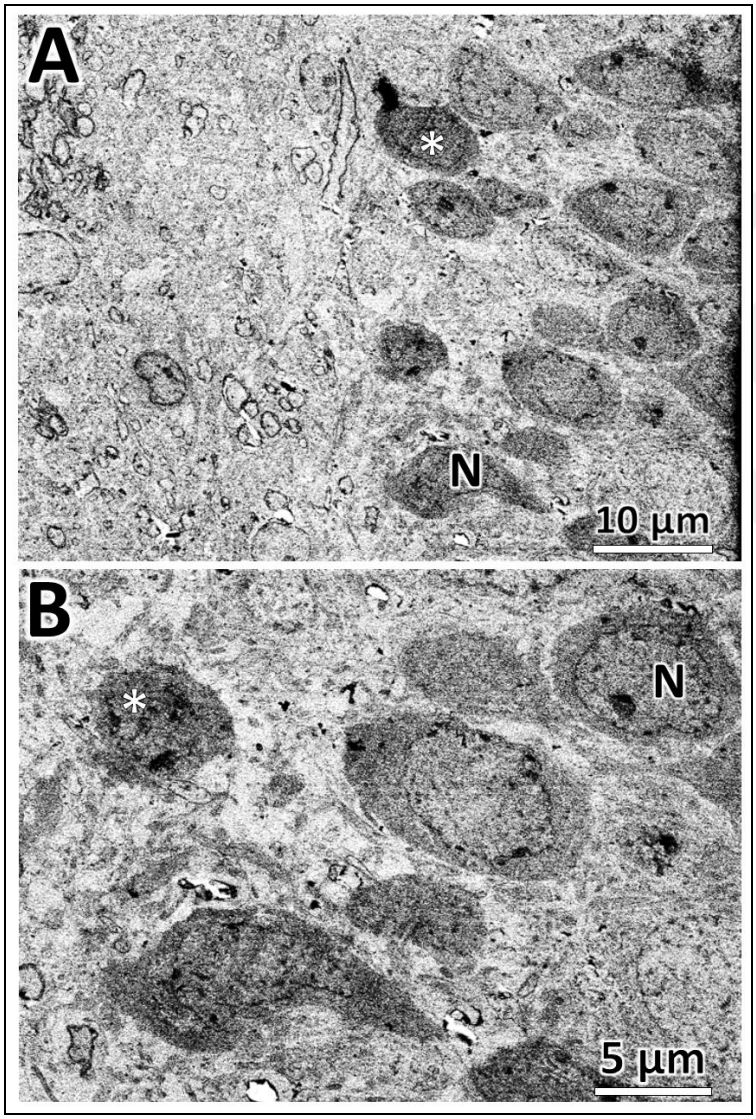


Figure 97. (A, B) Images taken from the CA region of the hippocampus belong to the GA+DS group are seen. Although the distance between pyramidal neurons (N) was increased because of some cells lost, most of the pyramidal cells (N) look functional. Few of them were darkly stained; they might be going to death (*). Few numbers of axons section are seen

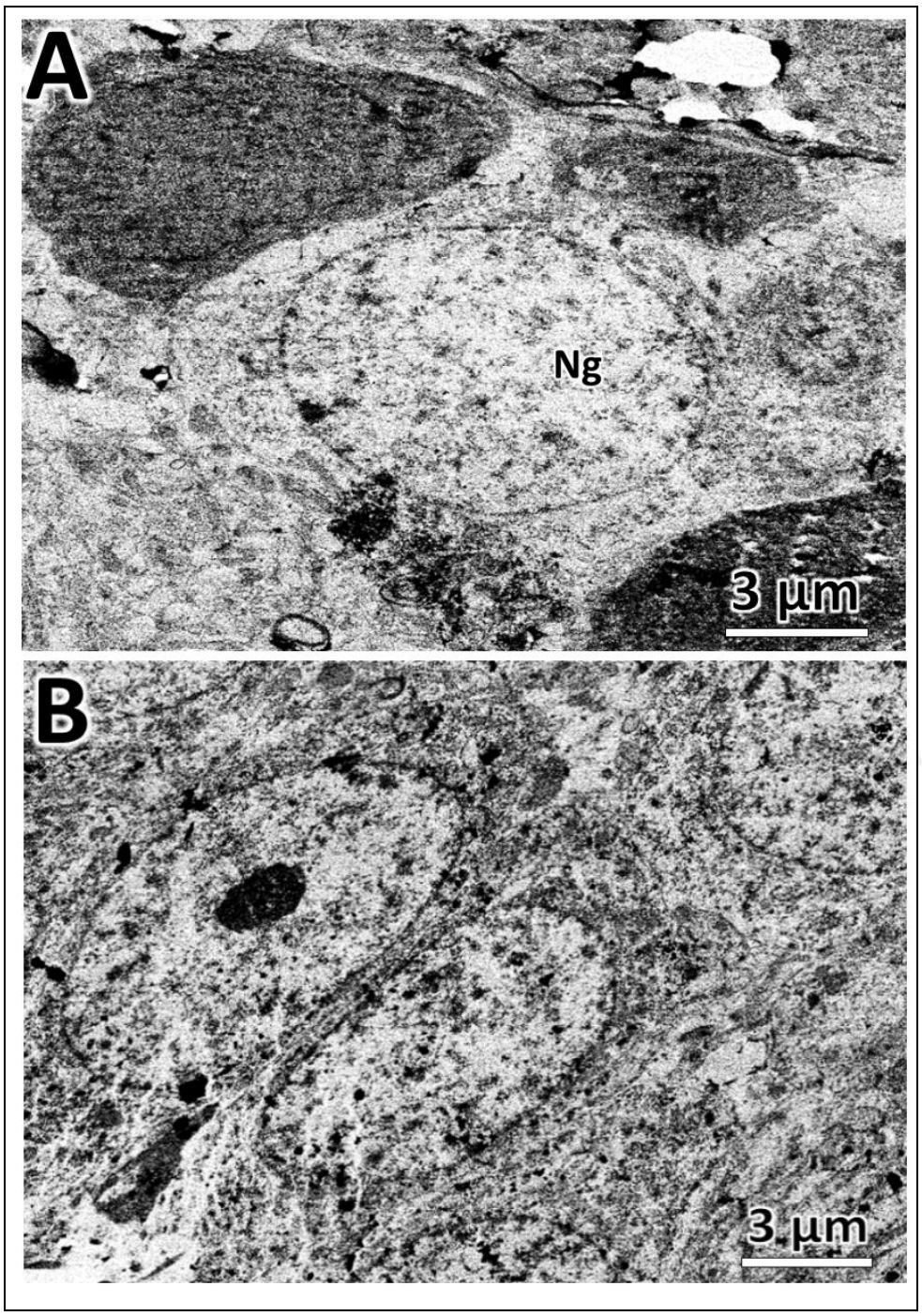


Figure 98. (A, B) Images taken from the CA region of the hippocampus belong to the GA+DS group are seen. (A) A neuroglia (Ng) and a darkly stained neuron are observed. (B) Healthy pyramidal neurons are found; their nuclei and nucleolus look very intact

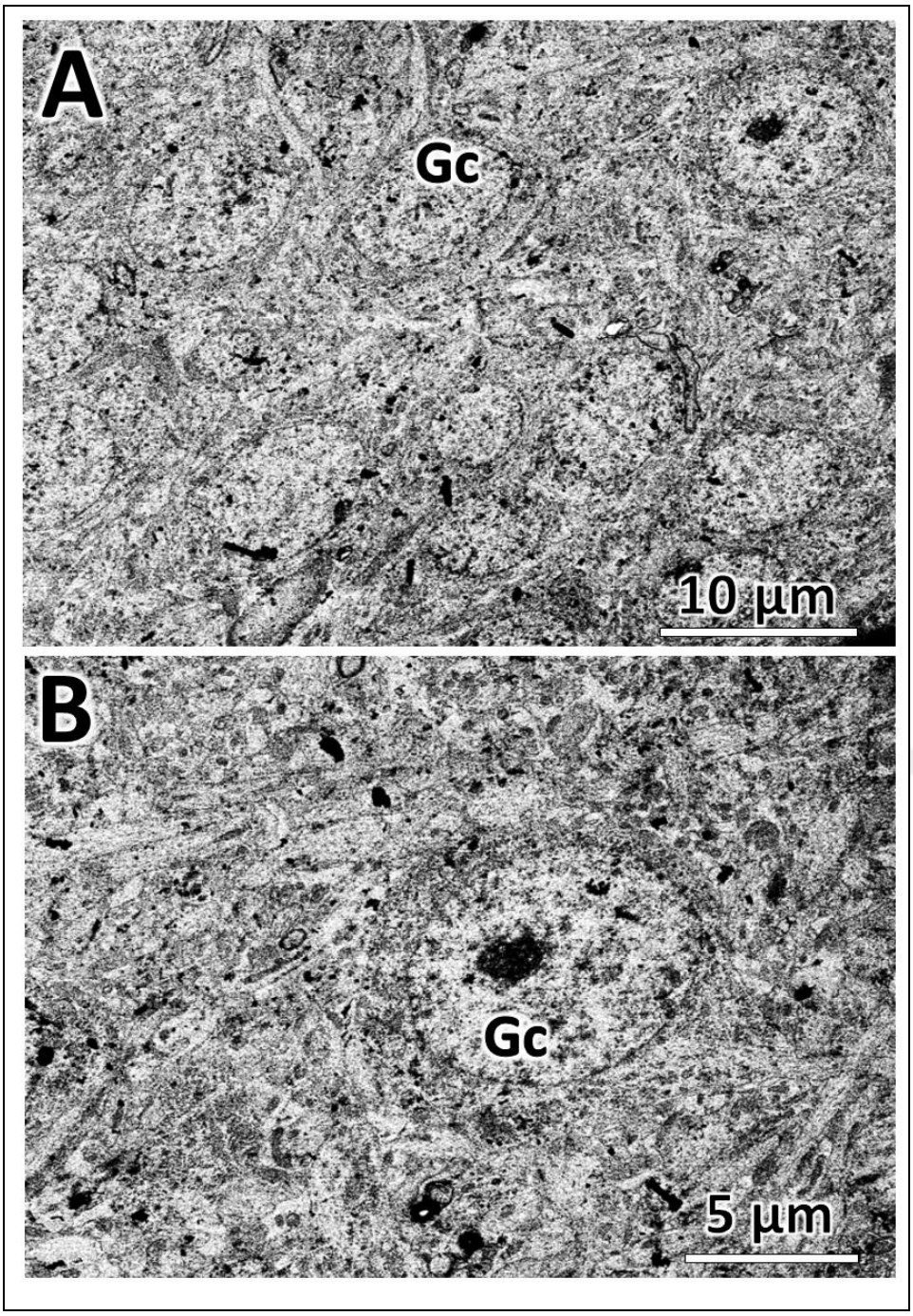


Figure 99. (A, B) An image was taken from the dentate gyrus of the hippocampus belong to the GA+DS group is observed. Most of the granular cells (Gc) look healthy; although their nuclei borders are seen clearly, cell borders are not clear. No dark cells in this region were found

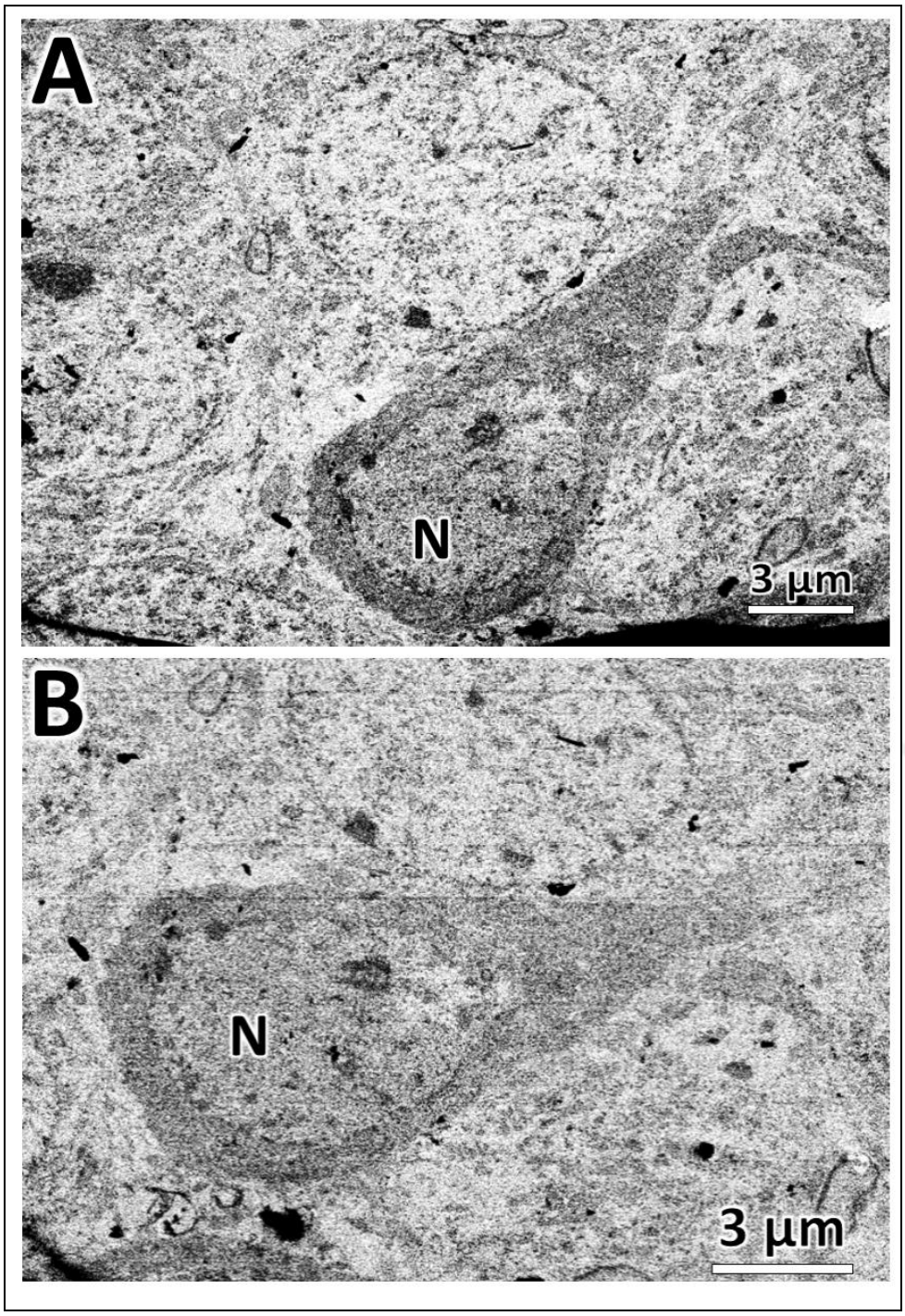


Figure 100. (A, B) Images taken from the CA region of the hippocampus belong to the GA+DS group are seen. (A) Although slightly dark stained pyramidal neuron (N) is observed, but most of the neurons around it look healthy since well-preserved nuclei, and cytoplasm of the cells are found

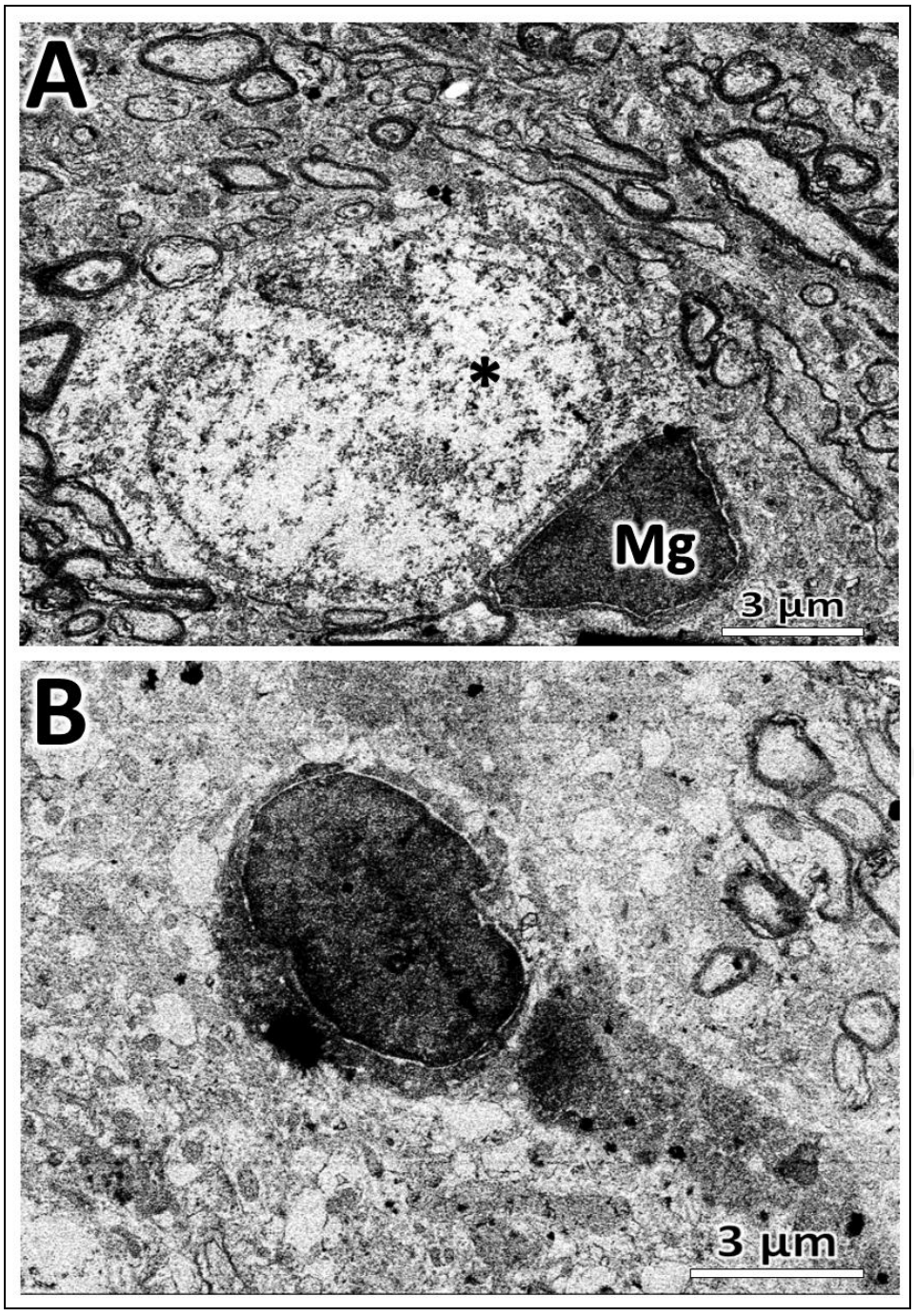


Figure 101. (A, B) Images taken from the CA region of the hippocampus belong to the GA+DS group are seen. (A) A healthy astrocyte (*) and microglia (Mg) are observed. There are many cross-and longitudinal sections of axons around them. (B) Based on its appearance, this cell might be microglia; many cross-sections of axons are also observed

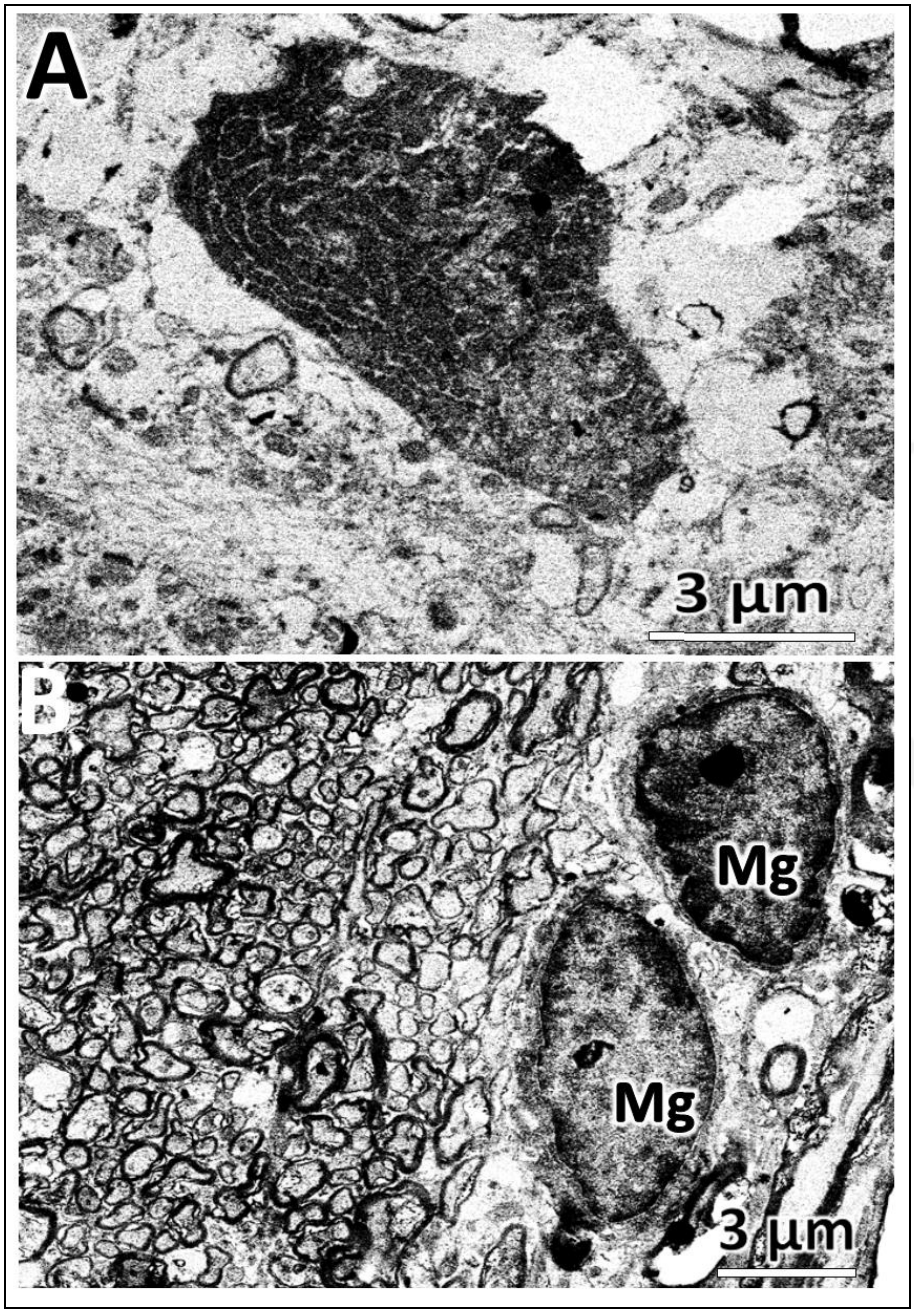


Figure 102. (A, B) Images taken from the brain of the GA+DS group are seen. (A) A darkly stained cell might be a remnant of a neuron that lost its function and will be removed by microglia that is a macrophage of the central nervous system. Many of the different sizes of vacuoles around this cell are also seen. (B) Two microglia (Mg) at the right side of the image and many of cross-sectioned myelinated axons are observed at the right side

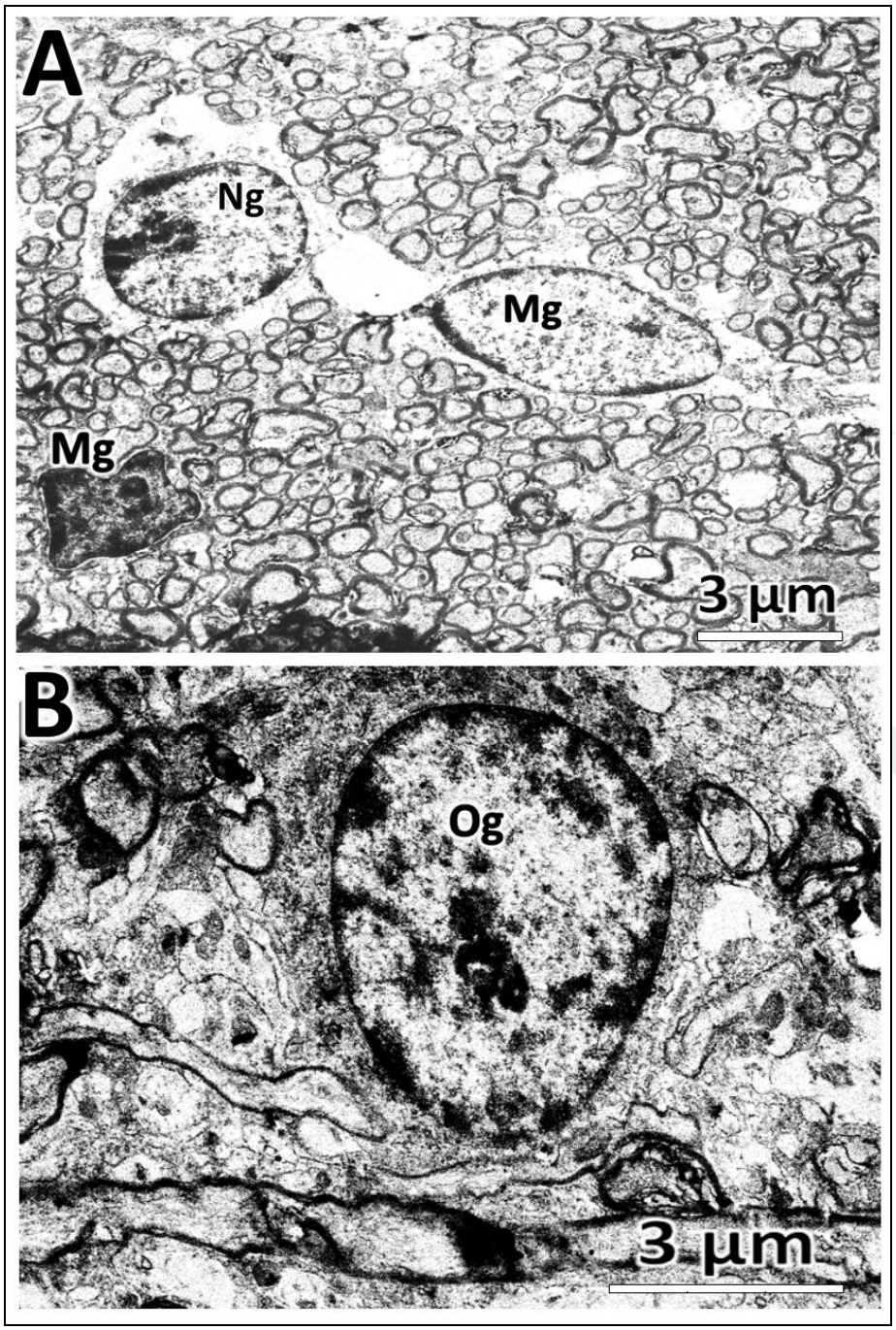


Figure 103. (A, B) Images taken from the brain belong to the GA+DS group are observed. (A) Two different types of neuroglia are seen in this image, astrocyte (Ng), and microglia (Mg). (B) This cell might be oligodendroglia (Og) since it has a heterogeneous cytoplasm, and its nucleus has a spherical shape. Many cross and longitudinal sections of axons around it are seen

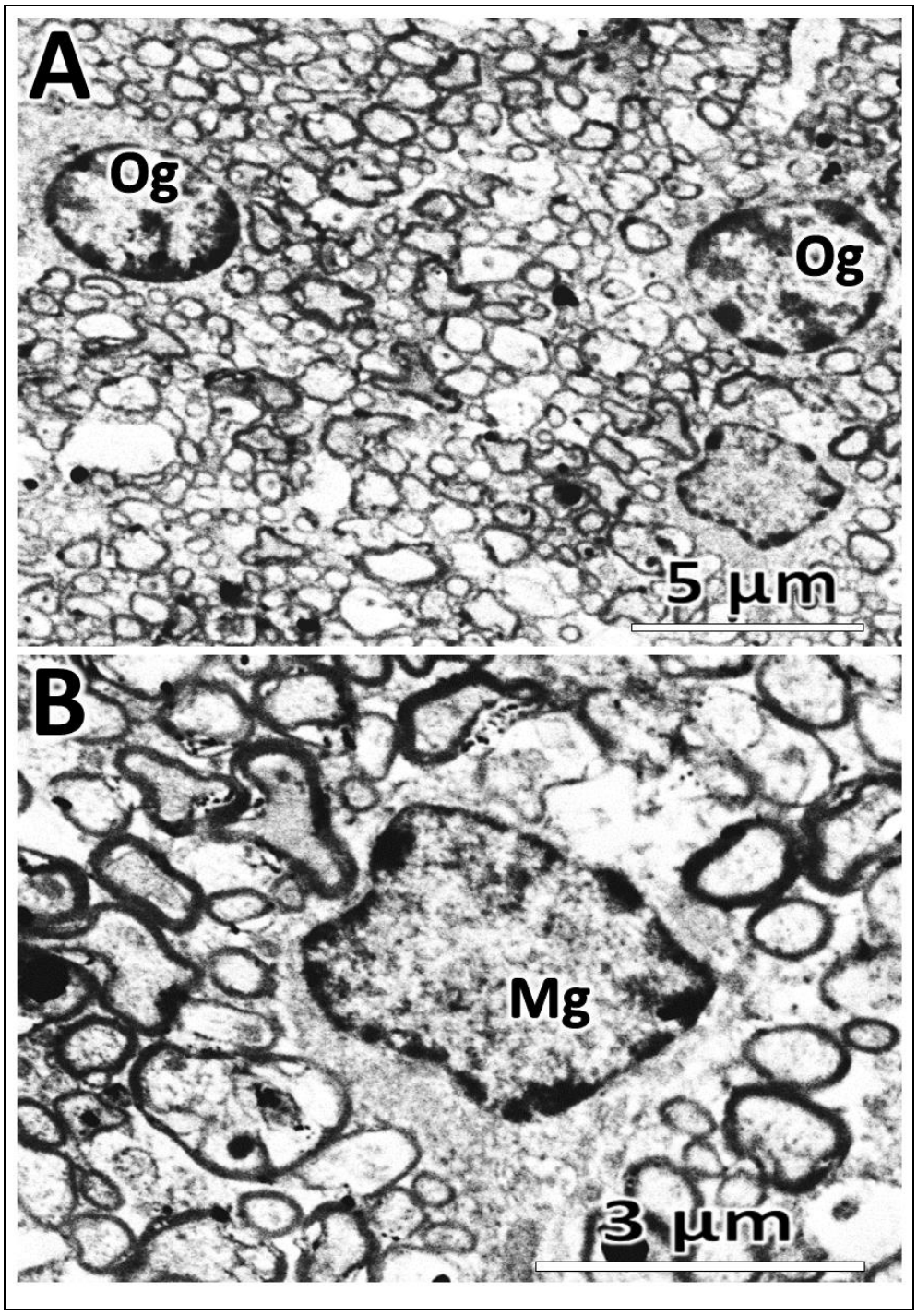


Figure 104. (A, B) Images taken from the brain of the GA+DS group are observed. (A) Two oligodendroglia (Og) and microglia (Mg) are seen; Og has a spherical shape nucleus. (B) Microglia (Mg) and many axons sections are observed around it

5. DISCUSSION

The present study was conducted to investigate the possible protective effects of GA on minimising the neurodegenerative effects of DS on rat hippocampus. Behavioural, biochemical, stereological, light and electron microscopic analyses were conducted.

The hippocampal formation (HF) is essential for memory, spatial navigation, emotions and pain processing (Sadeghi et al., 2019). The morphological characteristics of the hippocampus in terms of volume, numerical density and the total number of cells reflect its functional status. Thus, obtaining quantitative data with statistical reliability is crucial to gain accurate and unbiased results. Stereology lies at the heart of the finest tools in this manner. It is a science of estimating three-dimensional information from two-dimensional images based on mathematical and statistical approaches. Histological sections usually deliver qualitative analysis that can tell if the tissue is under pathological changes or not. However, the impact of this change in terms of statistically significant numbers can only be obtained through stereology. In which statistically significant changes can tell about the degree of the pathological changes or the effect of the experimental drug under study.

Behavioural tests were conducted to investigate the effect of both DS and GA. The MWM test is one of the useful tests for testing spatial learning and memory; it was developed more than 30 years ago (Morris et al., 1982). It is a sensitive test to see the hippocampal lesions and information on the size of the damage and the hippocampal activity (D'Hooge and De Deyn, 2001; Zhao et al., 2016). As shown in (**Figure 20**), the performance of the control group in this experiment looked better than that of the DS group. However, this difference is not reaching statistical significance, probably because of the short duration of the drug administration (15 days). When looking at the number of pyramidal cells in CA1 region (**Figure 24**), one can find that the total number of pyramidal cells in the control group is a little higher than that of DS group. Although this difference is not also reaching a statistically significant level. The pyramidal cells in CA1 region are more involved in cognitive functions as memory and learning (Dupret et al., 2010) and can affect MWM test results when it is impaired (Akbari et al., 2006). It would be speculated

that the presented low number of cells in the CA1 region and low performance at MWM test of DS group implies that if one applies a longer duration, statistically significant results between the control and diclofenac group would have been seen. The other behavioural test conducted was the passive avoidance test; it is informative regarding memory retention (Sarkaki and Rezaiei, 2013). However, the results show no significant difference between groups in both initial latency and step-through latency in passive avoidance tests as shown in (**Figures 21 and 22**). A prenatal study of the effect of diclofenac on the hippocampus was conducted. No side effect on the spatial memory was reported (1 mg/kg subcutaneously for 15 days from day 5 to day 20 during intrauterine life). However, other effects were noted regarding learning which was reported to be slower than the control group and emotional effects as higher anxiety (Elibol et al., 2019). Postnatal studies on the effect of DS showed no adverse effect on the spatial memory of adult male Sprague Dawley rats (in MWM test). The administered dose was 5 mg/kg injected intraperitoneally for 5 days (Emad et al., 2017). However, in this latter study, both indomethacin and DS were investigated. It was found that indomethacin enhanced memory and spatial learning better than DS exposed group. In a study involved Wistar albino rats, 5 mg/kg was administered for two weeks. Administration was through oral gavage, the study observed an enhancement in the performance of the rats in the MWM test (Ali and Arafa, 2011). In the previous studies, it was found that long-term memory was not affected by DS exposure. Previous studies administered DS prenatally or postnatally. The doses ranged between 1 mg/kg subcutaneously (prenatal), 5 mg/kg intraperitoneally (postanal) and 5 mg/kg through oral gavage (postanal). The duration ranged from 5 to 15 days the results (Elibol et al., 2019; Emad et al., 2017; Ali and Arafa, 2011). The presented study results are in agreement with those studies.

Regarding stereological analysis, the total number of pyramidal cells in the control group was 775468±188890 (CE 0.036). the total number in the literature is ranged between 632,000 for one-month-old Wistar rats, 930,000 for two-months, and 687,000 for 14-months old rats (Schmitz and Hof, 2005). Thus, the total number of pyramidal cells in this study falls in the normal range for control groups. However, there were no significant

differences between groups concerning the number of pyramidal cells and the hippocampal volume.

Stereological techniques were proven to be effective in detecting changes in the total number of pyramidal cells in the hippocampus under OS. The physical disector was used to estimate the total number of pyramidal cells in the hippocampus of rats exposed to monosodium glutamate. This leads to OS and black garlic extract, which have antioxidant properties (Hermawati et al., 2015). Stereological studies revealed that the CA1 region is more affected by the combined administration of monosodium glutamate and black garlic extract. The results were supported by the MWM test (Hermawati et al., 2015). In another study, the optical fractionator counting technique was used on the hippocampus. The purpose was to investigate the effect of melatonin against OS in the hippocampus of Wistar rats exposed to the electromagnetic field. The results show a protective effect of melatonin (Erdem Koc et al., 2016). Optical fractionator technique was used in another study regarding the hippocampus. Grape seed polyphenols were proven as an antioxidant of benefit against age-related OS and neuronal loss in the CA1 region of the hippocampus of male Wistar rats. This was concluded using the optical fractionator technique, which revealed the effectiveness of this antioxidant against neuronal loss (Abhijit et al., 2018). However, the study concluded that no significant difference in the total number of pyramidal cells or the volume of the hippocampus was observed.

Biochemical analysis was performed. Serum SOD and CAT levels were investigated, SOD is the major antioxidant, while GSH is the main antioxidant in the CNS (Gandhi and Abramov, 2012). Moreover, the hippocampus relays mainly on CAT and GSH as scavengers more than SOD (Santos et al., 2009). However, biochemical analysis of enzymes activities shows no significant difference between groups. It contradicts the results obtained by (Al-Yahya et al., 2009) in which the GA treated group showed restoration of GSH bladder contents. The dose of GA used in the mentioned study was (7.5 gm/kg/day) in drinking water, which is similar to our dose. We even used a longer duration, and our dose was administered by oral gavage. However, the biochemical analysis done by Al-Yahya and co-workers was performed in tissue samples, while our biochemical analysis was performed in the blood sample, which may explain the contradiction between two studies.

The lack of significance on the biochemical results may be attributed to the toxicity of diclofenac requires a higher dose and/or different route of administration. However, studies show that the toxicity of diclofenac may not be confined to OS; this drug may affect the transport systems in the cell membrane. This lead to an accumulation of neurotoxins as 1-methyl-4-phenylpyridinium. OS does not seem a sole reason of cell death, as indicated by the inefficiency of administration of an antioxidant and a radical scavenger in minimizing the effect of diclofenac (Morioka et al., 2004).

Microscopic findings using light microscopy in paraffin sections stained with cresyl violet were obtained. Also, toluidine blue stained resin sections and electron microscopes were used for evaluation. Images taken in toluidine blue staining showed normal appearance in the control group. Each pyramidal cell, have an oval to round shaped nucleus, nuclei of pyramidal cells, and the cell borders are clearly seen. Pyramidal cells in CA3 were large-sized and less densely compacted in comparison to the CA1 region. Granular neurons (Gc) are observed; they were medium-sized and densely compacted. Axons were seen in horizontal and longitudinal sections; the same findings were noted in the GA group. However, in DS and GA+DS groups, there were some degrees of disorganization as well as vacuolations and cell loss in some pictures. The general structure seemed damaged, but the severity of damage was less in the GA+DS. In electron microscopy, the same trend of varying degrees of damage in DS groups was observed. These results may imply that a longer duration of administration of DS will potentially show statistically significant damage when using stereological techniques.

To our knowledge, the role of GA on the hippocampus as an antioxidant hasn’t been investigated before. This role among other tissues was investigated and promising results were reported in the kidneys (Ali et al., 2013; Ali et al., 2014; Gado and Aldahmash, 2013; Al-Majed et al., 2002; Al-Majed et al., 2003), the liver (Gamal el-din et al., 2003), the heart (Abd-Allah et al., 2002) and the gonads (Fedail et al., 2016; Ahmed et al., 2016). However, some contradicted results emerged, while many reports supported the effectiveness of GA as an antioxidant (Kaddam et al., 2018). Some report suggested that its role is less significant than proposed (Cindoruk et al., 2007). It is reported that oral administration of GA in the drinking water for rats at a three graded concentration of 2.5, 5.0 or 10.0% w/v

for a duration of 8 days didn't lead to any significant change in the concentrations of antioxidants like GSH and superoxide dismutase in the kidney or liver. The report concluded that the beneficial effects of GA are unlikely to due to its antioxidant properties (Ali, 2004). It is also suggested that GA can pass through the gut and get absorbed to reach the adequate concentrations in the circulation to apply its antioxidant effect into other organs rather than the intestine (Ali, 2004). However, we administered GA orally to mimic its use in folk medicine and traditions in the local community. Our study adds to the literature by confirming that GA has no effect on the hippocampus as an antioxidant. To our knowledge, stereological investigation of the effect of GA on the histology of the nervous tissues was not done before. This is the first study on the subject.

Considering the vulnerability of the hippocampus to OS, many antioxidants have proven to be neuroprotective. As mentioned above, studies have explored the role of dehydroepiandrosterone, ascorbic acid, vitamin E, dendropanax morbifera Léveille leaf extract, black garlic extract, a combination of Boswellia sacra and folic acid, green tea mega EGCG^{TM} and grape seed polyphenols. All against OS induced by/associated with H_2O_2 and sodium nitroprusside, pilocarpine-induced seizers, OS associated with diabetes mellitus, OS produced by mercury, OS of monosodium glutamate, electromagnetic radiation (900-MHz) and age, respectively. Also, it was shown that melatonin has an antioxidant effect of minimising the toxic effects of diclofenac on developing hippocampus (Yurt et al., 2018a).

Yet, randomised controlled trials have concluded that antioxidants have no significant effects on PD and AD, both neurogenic diseases associated with OS. The probable explanation could be elaborated in the following factors: there are limitations regarding the applications of the driven results obtained from animals on humans. Animal models cannot mimic the same long duration of disease development or ageing processes (Gandhi and Abramov, 2012). Also, it looks crucial to administer antioxidants in early stages or prior to disease development. Other factors include issues related to the dosage differences between animal models and humans, using an antioxidant that scavenges ROS rather than eliminating the source of their production is also a contributing factor. Lastly,

there are different mechanisms of OS with different sources of ROS. Thus, the use of multiple antioxidants would be more beneficial (Gandhi and Abramov, 2012).

The dose of DS applied in our study was 3.6 mg/kg. It was kept as the same dose used by Yurt and co-workers (Yurt et al., 2018a). In which their study is the most similar one to ours in terms of using an antioxidant substance to investigate its effect on minimising damage caused by DS on the hippocampus. Examples of doses from other studies investigating the use effect of DS on the hippocampal tissue include 1 mg/kg, intraperitoneal administration for 15 days (Gokcimen et al., 2007). The use of different doses, i.e. 3.9, 9 and 18 mg/kg, intraperitoneal administration for seven days in which the effect was dose-dependent (Elibol et al., 2019). Other histological investigations on the hippocampus revealed a decrease in the number of neurons after prenatal administration of DS. Offspring of Wistar albino rats were arranged into two groups 4-weeks-old and 20-weeks-old after prenatal administration of DS (1 mg/kg for intraperitoneal administration 15 days) to their mothers. The 20-weeks-old group showed a decrease in the number of pyramidal cells. Also, there was found a decrease in the number of granule cells in the DG in the 4-weeks-old group while they were increased in the 20-weeks-old group. The latter results are because granular cells in the DG proliferate mostly postnatally, which indicates the possible role of diclofenac in increasing their rate of proliferation (Gokcimen et al., 2007). The same results were obtained when administering diclofenac postnatally. The dose of 15 mg/kg through intramuscular for seven days was applied postoperatively to rats with acute subdural hematoma (ASDH) (Turkmen et al., 2016). Also, diclofenac was administered intraperitoneally to Wistar rats at a dose of 12.5 mg/kg for three days. The results on the nervous system were manifested in the cerebral cortex as well as the cerebellum as the presence of ballooned red neurons. This phenomenon was also (but less observed) detected in the hippocampus (Ilic et al., 2011). However, some of these studies were prenatal while others were postnatal. Many factors are reported to contribute to the effect of diclofenac on the nervous system, including the dose, route of administration and the duration (Elibol et al., 2019). Those studies used intraperitoneal and intramuscular injections while we used oral route for administration of DS. On the other hand, our study

was concerned about adult hippocampus rather than the developing one. All these factors may explain the lack of significant results in our study.

This is the first report on the effect of GA as an antioxidant to minimising the harmful effect of DS on the hippocampus. Behavioural, stereological, and biochemical analyses indicate no significant protective effect of GA since the used DS did not create a side effect on the number of pyramidal cells. But our qualitative results of LM and EM sections showed the opposite. The DS impaired most of the pyramidal neurons in the hippocampus. And a protective result was seen after GA exposure. This was indicated by the fact that most of the neurons in this region have a normal structure.

The use of multiple antioxidants would get significantly positive results. The administration of diclofenac through oral route also contributed to the negative results of the study. Most of the studies with positive results used intraperitoneal, intramuscular or intrathecal injections. Also, most of those results were done on developing hippocampus, i.e. prenatal exposure of DS. However, postnatal studies found no significant results at least at the level of behavioural tests, which indicates that the oral administration of diclofenac in such dose used in this study has no significant effect on the adult rat hippocampus.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

1. DS, although it can affect the developing hippocampus, it has no harmful effects on the structure of adult hippocampus and function when administered by oral gavage for 15 days at a dose of 3.6 mg/kg.

2. Two behavioural tests were used. MWM test for spatial memory and learning, and passive avoidance test for memory retention. The results show no significant difference between groups.

3. Administration of DS postnatally via other routes with higher doses and for a longer duration would give different results.

4. Biochemical investigations showed no change in antioxidants level following administration of GA by oral gavage for 15 days at a dose of 7.5 g/kg. On the other hand, the lack of significant biochemical results is possibly because the toxicity of diclofenac requires higher dose and/or different route of administration. However, studies show that toxicity of diclofenac (if any) may not be necessarily confined to OS at least used dose of DS.

5. However, our qualitative analysis showed a toxic effect of DS and GA on the pyramidal cells of the hippocampus; if GA is simultaneously used with DS, it shows a protective effect on the pyramidal cells, since the number of dark cells in hippocampus was decreased after GA exposure.

6. To our knowledge, stereological analysis of the effect of GA on the histology of the nervous tissues was not done before. This is the first study in this manner; many reports praise the antioxidant role of GA in the kidneys, liver, gonads, and heart, with other contradicting reports suggesting that there is no role for GA as antioxidant among oral administration.

6.2. Recommendations

Further investigations are required, considering the following points:

- Administration of DS via other routes with a higher dose and for a longer duration.
- Administration of GA through the same route and dose, but the starting date of administration should be before the DS exposure.
- Using multiple antioxidants rather than one.
- Biochemical analyses are to be done in tissue samples rather than a blood sample.
- For the sake of time and effort, an optical fractionator is recommended for stereological analysis.

REFERENCES

Abd-Allah AR, Al-Majed AA, Mostafa AM, Al-Shabanah OA, Din AG, Nagi MN. Protective effect of arabic gum against cardiotoxicity induced by doxorubicin in mice: A possible mechanism of protection. J Biochem Mol Toxicol 2002;16(5):254-259.

Abhijit S, Tripathi SJ, Bhagya V, Shankaranarayana Rao BS, Subramanyam MV, Asha Devi S. Antioxidant action of grape seed polyphenols and aerobic exercise in improving neuronal number in the hippocampus is associated with decrease in lipid peroxidation and hydrogen peroxide in adult and middle-aged rats. Exp Gerontol 2018;101:101-112.

Ahmed AA, Fedail JS, Musa HH, Kamboh AA, Sifaldin AZ, Musa TH. Gum arabic extracts protect against hepatic oxidative stress in alloxan induced diabetes in rats. Pathophysiology 2015;22(4):189-194.

Ahmed AA, Fedail JS, Musa HH, Musa TH, Sifaldin AZ. Gum arabic supplementation improved antioxidant status and alters expression of oxidative stress gene in ovary of mice fed high fat diet. Middle East Fertil S 2016;21(2):101-108.

Ahmed NA, Radwan NM, Aboul Ezz HS, Salama NA. The antioxidant effect of green tea mega egcg against electromagnetic radiation-induced oxidative stress in the hippocampus and striatum of rats. Electromagn Biol Med 2017;36(1):63-73.

Akbari E, Naghdi N, Motamedi F. Functional inactivation of orexin 1 receptors in ca1 region impairs acquisition, consolidation and retrieval in morris water maze task. Behav Brain Res 2006;173(1):47-52.

Al-Majed AA, Abd-Allah AR, Al-Rikabi AC, Al-Shabanah OA, Mostafa AM. Effect of oral administration of arabic gum on cisplatin-induced nephrotoxicity in rats. J Biochem Mol Toxicol 2003;17(3):146-153.

Al-Majed AA, Mostafa AM, Al-Rikabi AC, Al-Shabanah OA. Protective effects of oral arabic gum administration on gentamicin-induced nephrotoxicity in rats. Pharmacol Res 2002;46(5):445-451.

Al-Yahya AA, Al-Majed AA, Gado AM, Daba MH, Al-Shabanah OA, Abd-Allah AR. Acacia senegal gum exudate offers protection against cyclophosphamide-induced urinary bladder cytotoxicity. Oxid Med Cell Longev 2009;2(4):207-213.

Ali AA, Ali KE, Fadlalla AE, Khalid KE. The effects of gum arabic oral treatment on the metabolic profile of chronic renal failure patients under regular haemodialysis in central sudan. Nat Prod Res 2008;22(1):12-21.

Ali BH. Does gum arabic have an antioxidant action in rat kidney? Ren Fail 2004;26(1):1-3.

Ali BH, Al-Husseni I, Beegam S, Al-Shukaili A, Nemmar A, Schierling S, Queisser N, Schupp N. Effect of gum arabic on oxidative stress and inflammation in adenine–induced chronic renal failure in rats. PLoS One 2013;8(2):e55242.

Ali BH, Al Za'abi M, Ramkumar A, Yasin J, Nemmar A. Anemia in adenine-induced chronic renal failure and the influence of treatment with gum acacia thereon. Physiol Res 2014;63(3):351-358.

Ali BH, Ziada A, Blunden G. Biological effects of gum arabic: A review of some recent research. Food and Chemical Toxicology 2009a;47(1):1-8.

Ali BH, Ziada A, Blunden G. Biological effects of gum arabic: A review of some recent research. Food Chem Toxicol 2009b;47(1):1-8.

Ali EH, Arafa NM. Comparative protective action of curcumin, memantine and diclofenac against scopolamine-induced memory dysfunction. Fitoterapia 2011;82(4):601-608.

Assady M, Farahnak A, Golestani A, Esharghian M. Superoxide dismutase (sod) enzyme activity assay in fasciola spp. Parasites and liver tissue extract. Iran J Parasitol 2011;6(4):17-22.

Ataizi ZS, Ertilav K, Naziroglu M. Mitochondrial oxidative stress-induced brain and hippocampus apoptosis decrease through modulation of caspase activity, ca(2+) influx and inflammatory cytokine molecular pathways in the docetaxel-treated mice by melatonin and selenium treatments. Metab Brain Dis 2019;34(4):1077-1089.

Atzeni F, Masala IF, Sarzi-Puttini P. A review of chronic musculoskeletal pain: Central and peripheral effects of diclofenac. Pain Ther 2018;7(2):163-177.

Aygun D, Kaplan S, Odaci E, Onger ME, Altunkaynak ME. Toxicity of non-steroidal anti-inflammatory drugs: A review of melatonin and diclofenac sodium association. Histol Histopathol 2012;27(4):417-436.

Ayranci E, Altunkaynak BZ, Aktas A, Ragbetli MC, Kaplan S. Prenatal exposure of diclofenac sodium affects morphology but not axon number of the median nerve of rats. Folia Neuropathol 2013;51(1):76-86.

Bannerman DM, Rawlins JN, Mchugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J. Regional dissociations within the hippocampus--memory and anxiety. Neurosci Biobehav Rev 2004;28(3):273-283.

Bas O, Odaci E, Kaplan S, Acer N, Ucok K, Colakoglu S. 900 mhz electromagnetic field exposure affects qualitative and quantitative features of hippocampal pyramidal cells in the adult female rat. Brain Res 2009;1265:178-185.

Bastianetto S, Ramassamy C, Poirier J, Quirion R. Dehydroepiandrosterone (dhea) protects hippocampal cells from oxidative stress-induced damage. Brain Res Mol Brain Res 1999;66(1-2):35-41.

Bayer SA. Hippocampal region. The rat nervous system,New York, Academic Press. 1985;335-352.

Bellissimo MI, Amado D, Abdalla DS, Ferreira EC, Cavalheiro EA, Naffah-Mazzacoratti MG. Superoxide dismutase, glutathione peroxidase activities and the hydroperoxide concentration are modified in the hippocampus of epileptic rats. Epilepsy Res 2001;46(2):121-128.

Biessels GJ, Kamal A, Urban IJ, Spruijt BM, Erkelens DW, Gispen WH. Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: Effects of insulin treatment. Brain Res 1998;800(1):125-135.

Bliss DZ, Stein TP, Schleifer CR, Settle RG. Supplementation with gum arabic fiber increases fecal nitrogen excretion and lowers serum urea nitrogen concentration in chronic renal failure patients consuming a low-protein diet. Am J Clin Nutr 1996;63(3):392-398.

Bort R, Ponsoda X, Jover R, Gomez-Lechon MJ, Castell JV. Diclofenac toxicity to hepatocytes: A role for drug metabolism in cell toxicity. J Pharmacol Exp Ther 1999;288(1):65-72.

Brandeis R, Brandys Y, Yehuda S. The use of the morris water maze in the study of memory and learning. Int J Neurosci 1989;48(1-2):29-69.

Bucci DJ, Chiba AA, Gallagher M. Spatial learning in male and female long-evans rats. Behav Neurosci 1995;109(1):180-183.

Canan S, Aktas A, Ulkay MB, Colakoglu S, Ragbetli MC, Ayyildiz M, Geuna S, Kaplan S. Prenatal exposure to a non-steroidal anti-inflammatory drug or saline solution impairs sciatic nerve morphology: A stereological and histological study. Int J Dev Neurosci 2008;26(7):733-738.

Cantoni L, Valaperta R, Ponsoda X, Castell JV, Barelli D, Rizzardini M, Mangolini A, Hauri L, Villa P. Induction of hepatic heme oxygenase-1 by diclofenac in rodents: Role of oxidative stress and cytochrome p-450 activity. J Hepatol 2003;38(6):776-783.

Cardoso A, Lukoyanova EA, Madeira MD, Lukoyanov NV. Seizure-induced structural and functional changes in the rat hippocampal formation: Comparison between brief seizures and status epilepticus. Behav Brain Res 2011;225(2):538-546.

Cindoruk M, Kerem M, Karakan T, Salman B, Akin O, Alper M, Erdem O, Unal S. Peroxisome proliferators-activated alpha agonist treatment ameliorates hepatic damage in rats with obstructive jaundice: An experimental study. BMC Gastroenterol 2007;7(1):44.

Colakoglu S, Aktas A, Raimondo S, Turkmen AP, Altunkaynak BZ, Odaci E, Geuna S, Kaplan S. Effects of prenatal exposure to diclofenac sodium and saline on the optic nerve of 4- and 20-week-old male rats: A stereological and histological study. Biotech Histochem 2014;89(2):136-144.

Conaghan PG. A turbulent decade for nsaids: Update on current concepts of classification, epidemiology, comparative efficacy, and toxicity. Rheumatol Int 2012;32(6):1491-1502.

Cullen DJ, Seager JM, Holmes S, Doherty M, Wilson JV, Garrud P, Garner S, Maynard A, Logan RF, Hawkey CJ. Pharmacoepidemiology of non-steroidal anti-inflammatory drug use in nottingham general practices. Aliment Pharmacol Ther 2000;14(2):177-185.

D'hooge R, De Deyn PP. Applications of the morris water maze in the study of learning and memory. Brain Res Rev 2001;36(1):60-90.

De La Garza Ii R, Asnis GM. The non-steroidal anti-inflammatory drug diclofenac sodium attenuates ifn-α induced alterations to monoamine turnover in prefrontal cortex and hippocampus. Brain Res 2003;977(1):70-79.

De La Garza R, 2nd, Asnis GM, Fabrizio KR, Pedrosa E. Acute diclofenac treatment attenuates lipopolysaccharide-induced alterations to basic reward behavior and hpa axis activation in rats. Psychopharmacology (Berl) 2005;179(2):356-365.

Dokter M, Von Bohlen Und Halbach O. Neurogenesis within the adult hippocampus under physiological conditions and in depression. Neural Regen Res 2012;7(7):552-559.

Dorofeeva NA, Barygin OI, Staruschenko A, Bolshakov KV, Magazanik LG. Mechanisms of non-steroid anti-inflammatory drugs action on asics expressed in hippocampal interneurons. J Neurochem 2008;106(1):429-441.

Dupret D, O'neill J, Pleydell-Bouverie B, Csicsvari J. The reorganization and reactivation of hippocampal maps predict spatial memory performance. Nat Neurosci 2010;13(8):995-1002.

Duvernoy HMFCP-YR. The human hippocampus: Functional anatomy, vascularization and serial sections with mri. 4 Ed, Springer Science & Business Media. 2013;

Eichenbaum H. Hippocampus: Mapping or memory? Curr Biol 2000;10(21):R785-787.

Ekİcİ F, Keskİn İ, Aslan H, Erİşgİn Z, Altunkaynak BZ, Gökçİmen A, Odaci E, Kaplan S. Does prenatal exposure to diclofenac sodium affect the total number of cerebellar granule cells in male juvenile and adult rats? J Exp Clin Med 2012;29(1):52-57.

El Falougy H, Kubikova E, Benuska J. The microscopical structure of the hippocampus in the rat. Bratisl Lek Listy 2008;109(3):106-110.

Elibol B, Aritan Ogur B, Dogru H. Prenatal exposure of diclofenac sodium alters the behavioral development of young wistar rats. Turk J Biol 2019;43(5):305-313.

Elibol E, Kaplan S, Türkmen AP, Aksoy A, Yurt K, Altun G, Önger ME, Altunkaynak BZ. Effect of different doses of diclofenac sodium on the hippocampus development during the first trimester of pregnancy. J Exp Clin Med 2014;31(2):136.

Emad S, Qadeer S, Sadaf S, Batool Z, Haider S, Perveen T. Attenuation of stress induced memory deficits by nonsteroidal anti-inflammatory drugs (nsaids) in rats: Role of antioxidant enzymes. Pharmacol Rep 2017;69(2):300-305.

Erdem Koc G, Kaplan S, Altun G, Gumus H, Gulsum Deniz O, Aydin I, Emin Onger M, Altunkaynak Z. Neuroprotective effects of melatonin and omega-3 on hippocampal cells prenatally exposed to 900 mhz electromagnetic fields. Int J Radiat Biol 2016;92(10):590-595.

Fattori V, Borghi SM, Guazelli CFS, Giroldo AC, Crespigio J, Bussmann AJC, Coelho-Silva L, Ludwig NG, Mazzuco TL, Casagrande R, Verri WA, Jr. Vinpocetine reduces diclofenac-induced acute kidney injury through inhibition of oxidative stress, apoptosis, cytokine production, and nf-kappab activation in mice. Pharmacol Res 2017;120:10-22.

Fedail JS, Ahmed AA, Musa HH, Ismail E, Sifaldin AZ, Musa TH. Gum arabic improves semen quality and oxidative stress capacity in alloxan induced diabetes rats. Asian Pac J Reprod 2016;5(5):434-441.

Fish R, Danneman PJ, Brown M, Karas A. Anesthesia and analgesia in laboratory animals.Academic press. 2011;

Fitting S, Booze RM, Hasselrot U, Mactutus CF. Differential long-term neurotoxicity of hiv-1 proteins in the rat hippocampal formation: A design-based stereological study. Hippocampus 2008;18(2):135-147.

Fitting S, Booze RM, Hasselrot U, Mactutus CF. Dose-dependent long-term effects of tat in the rat hippocampal formation: A design-based stereological study. Hippocampus 2010;20(4):469-480.

Freitas RM, Vasconcelos SM, Souza FC, Viana GS, Fonteles MM. Oxidative stress in the hippocampus after pilocarpine-induced status epilepticus in wistar rats. FEBS J 2005;272(6):1307-1312.

Fukuda M, Kitaichi K, Abe F, Fujimoto Y, Takagi K, Takagi K, Morishima T, Hasegawa T. Altered brain penetration of diclofenac and mefenamic acid, but not acetaminophen, in shiga-like toxin ii-treated mice. J Pharmacol Sci 2005;97(4):525-532.

Gado AM, Aldahmash BA. Antioxidant effect of arabic gum against mercuric chloride-induced nephrotoxicity. Drug Des Devel Ther 2013;7:1245-1252.

Gamal El-Din AM, Mostafa AM, Al-Shabanah OA, Al-Bekairi AM, Nagi MN. Protective effect of arabic gum against acetaminophen-induced hepatotoxicity in mice. Pharmacol Res 2003;48(6):631-635.

Gan TJ. Diclofenac: An update on its mechanism of action and safety profile. Curr Med Res Opin 2010;26(7):1715-1731.

Gandhi S, Abramov AY. Mechanism of oxidative stress in neurodegeneration. Oxid Med Cell Longev 2012;2012:428010.

Gao X, Björk L, Trajkovski V, Uggla M. Evaluation of antioxidant activities of rosehip ethanol extracts in different test systems. Journal of the Science of Food and Agriculture 2000;80(14):2021-2027.

Gokcimen A, Ragbetli MC, Bas O, Tunc AT, Aslan H, Yazici AC, Kaplan S. Effect of prenatal exposure to an anti-inflammatory drug on neuron number in cornu ammonis and dentate gyrus of the rat hippocampus: A stereological study. Brain Res 2007;1127(1):185-192.

Gómez-Lechón MJ, Ponsoda X, O'connor E, Donato T, Jover R, Castell JV. Diclofenac induces apoptosis in hepatocytes. Toxicol in Vitro 2003;17(5-6):675-680.

Grady MS, Charleston JS, Maris D, Witgen BM, Lifshitz J. Neuronal and glial cell number in the hippocampus after experimental traumatic brain injury: Analysis by stereological estimation. J Neurotrauma 2003;20(10):929-941.

Gundersen H, Jensen E. The efficiency of systematic sampling in stereology and its prediction. Journal of microscopy 1987;147(3):229-263.

Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Et Al. The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 1988;96(10):857-881.

Gundersen HJG. Stereology of arbitrary particles - a review of unbiased number and size estimators and the presentation of some new ones, in memory of thompson,william,r. J Microsc 1986;143(1):3-45.

Gundersen HJG, Jensen EBV, Kieu K, Nielsen J. The efficiency of systematic sampling in stereology—reconsidered. Journal of microscopy 1999;193(3):199-211.

Gurtskaia G, Tsiklauri N, Nozadze I, Nebieridze M, Tsagareli MG. Antinociceptive tolerance to nsaids microinjected into dorsal hippocampus. BMC Pharmacol Toxicol 2014;15(1):10.

Halliwell B. Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol 1995;49(10):1341-1348.

Hermawati E, Sari DC, Partadiredja G. The effects of black garlic ethanol extract on the spatial memory and estimated total number of pyramidal cells of the hippocampus of monosodium glutamate-exposed adolescent male wistar rats. Anat Sci Int 2015;90(4):275-286.

Hickey EJ, Raje RR, Redd VE, Gross SM, Ray SD. Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death. Free Radical Bio Med 2001;31(2):139-152.

Hirose K, Jyoyama H, Kojima Y, Eigyo M, Hatakeyama H, Asanuma F, Umehara H, Yamaguchi T. Pharmacological properties of 2-[4-(2-thiazolyloxy)-phenyl]-propionic acid (480156-s), a new non-steroidal antiinflammatory agent. Arzneimittelforschung 1984;34(3):280-286.

Hosseini-Sharifabad M, Nyengaard JR. Design-based estimation of neuronal number and individual neuronal volume in the rat hippocampus. J Neurosci Methods 2007;162(1-2):206-214.

Huang CW, Hung TY, Liao YK, Hsu MC, Wu SN. Underlying mechanism of regulatory actions of diclofenac, a nonsteroidal anti-inflammatory agent, on neuronal potassium channels and firing: An experimental and theoretical study. J Physiol Pharmacol 2013;64(3):269-280.

Huang T-T, Zou Y, Corniola R. Oxidative stress and adult neurogenesis—effects of radiation and superoxide dismutase deficiency Seminars in cell & developmental biology, Elsevier, 2012;738-744.

Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (sod), catalase (cat) and glutathione peroxidase (gpx): Their fundamental role in the entire antioxidant defence grid. Alex J Med 2018;54(4):287-293.

Ilic S, Drmic D, Franjic S, Kolenc D, Coric M, Brcic L, Klicek R, Radic B, Sever M, Djuzel V, Filipovic M, Djakovic Z, Stambolija V, Blagaic AB, Zoricic I, Gjurasin M, Stupnisek M, Romic Z, Zarkovic K, Dzidic S, Seiwerth S, Sikiric P. Pentadecapeptide bpc 157 and its effects on a nsaid toxicity model: Diclofenac-induced gastrointestinal, liver, and encephalopathy lesions. Life Sci 2011;88(11-12):535-542.

Inoue A, Muranaka S, Fujita H, Kanno T, Tamai H, Utsumi K. Molecular mechanism of diclofenac-induced apoptosis of promyelocytic leukemia: Dependency on reactive oxygen species, akt, bid, cytochrome and caspase pathway. Free Radic Biol Med 2004;37(8):1290-1299.

Islas-Flores H, Gomez-Olivan LM, Galar-Martinez M, Colin-Cruz A, Neri-Cruz N, Garcia-Medina S. Diclofenac-induced oxidative stress in brain, liver, gill and blood of common carp (cyprinus carpio). Ecotoxicol Environ Saf 2013;92:32-38.

Kaddam L, Fadl-Elmula I, Eisawi OA, Abdelrazig HA, Salih MA, Lang F, Saeed AM. Gum arabic as novel anti-oxidant agent in sickle cell anemia, phase ii trial. BMC Hematol 2017;17(1):4.

Kaddam L, Fdl-Elmula I, Saeed A. Gum arabic beneficial effects, clinical applications, and future prospective. Gum arabicElsevier. 2018;211-220.

Kaplan S, Esrefoglu M, Aktas A, Gul M, Onger ME, Altunkaynak ME, Ulkay MB, Ragbetli MC. The effect of prenatal exposure of a non-steroidal anti-inflammatory drug on the optic nerve of female rats: A stereological, histological, and electron microscopic study. J Matern Fetal Neonatal Med 2013;26(18):1860-1864.

Kaplan S, Odaci E, Canan S, Onger ME, Aslan H, Unal B. The disector counting technique. Neuroquantology 2012;10(1):44-53.

Kawase A, Hirata N, Tokunaga M, Matsuda H, Iwaki M. Gum arabic enhances intestinal calcium absorption in rats. Journal of Health Science 2007;53(5):622-624.

Keskin I, Kaplan S, Kalkan S, Sutcu M, Ulkay MB, Esener OB. Evaluation of neuroprotection by melatonin against adverse effects of prenatal exposure to a nonsteroidal anti-inflammatory drug during peripheral nerve development. Int J Dev Neurosci 2015;41:1-7.

Khoshvakhti H, Yurt KK, Altunkaynak BZ, Turkmen AP, Elibol E, Aydin I, Kivrak EG, Onger ME, Kaplan S. Effects of melatonin on diclofenac sodium treated rat kidney: A stereological and histopathological study. Ren Fail 2015;37(8):1379-1383.

Kim W, Kim DW, Yoo DY, Jung HY, Kim JW, Kim DW, Choi JH, Moon SM, Yoon YS, Hwang IK. Antioxidant effects of dendropanax morbifera leveille extract in the hippocampus of mercury-exposed rats. BMC Complement Altern Med 2015;15(1):247.

Kivrak EG, Altunkaynak BZ, Alkan I, Yurt KK, Kocaman A, Onger ME. Effects of 900-mhz radiation on the hippocampus and cerebellum of adult rats and attenuation of such effects by folic acid and boswellia sacra. J Microsc Ultrastruct 2017;5(4):216-224.

Kudo C, Kori M, Matsuzaki K, Yamai K, Nakajima A, Shibuya A, Niwa H, Kamisaki Y, Wada K. Diclofenac inhibits proliferation and differentiation of neural stem cells. Biochem Pharmacol 2003;66(2):289-295.

Kusuhara H, Komatsu H, Sumichika H, Sugahara K. Reactive oxygen species are involved in the apoptosis induced by nonsteroidal anti-inflammatory drugs in cultured gastric cells. Eur J Pharmacol 1999;383(3):331-337.

Limberaki E, Eleftheriou P, Vagdatli E, Kostoglou V, Petrou C. Serum antioxidant status among young, middle-aged and elderly people before and after antioxidant rich diet. Hippokratia 2012;16(2):118-123.

Lister JP, Blatt GJ, Debassio WA, Kemper TL, Tonkiss J, Galler JR, Rosene DL. Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. Hippocampus 2005;15(3):393-403.

Liu J, Wang A, Li L, Huang Y, Xue P, Hao A. Oxidative stress mediates hippocampal neuron death in rats after lithium-pilocarpine-induced status epilepticus. Seizure 2010;19(3):165-172.

Liu MG, Chen J. Roles of the hippocampal formation in pain information processing. Neurosci Bull 2009;25(5):237-266.

Logue SF, Paylor R, Wehner JM. Hippocampal lesions cause learning deficits in inbred mice in the morris water maze and conditioned-fear task. Behav Neurosci 1997;111(1):104-113.

Lojo N, Rasic Z, Zenko Sever A, Kolenc D, Vukusic D, Drmic D, Zoricic I, Sever M, Seiwerth S, Sikiric P. Effects of diclofenac, l-name, l-arginine, and pentadecapeptide bpc 157 on gastrointestinal, liver, and brain lesions, failed anastomosis, and intestinal adaptation deterioration in 24 hour-short-bowel rats. PLoS One 2016;11(9):e0162590.

Lorenzini CA, Baldi E, Bucherelli C, Sacchetti B, Tassoni G. Role of dorsal hippocampus in acquisition, consolidation and retrieval of rat's passive avoidance response: A tetrodotoxin functional inactivation study. Brain Res 1996;730(1-2):32-39.

Lorenzini CGA, Baldi E, Bucherelli C, Sacchetti B, Tassoni G. Role of ventral hippocampus in acquisition, consolidation and retrieval of rat's passive avoidance response memory trace. Brain Res 1997;768(1-2):242-248.

Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Fröhlich K-U. Oxygen stress: A regulator of apoptosis in yeast. The Journal of cell biology 1999;145(4):757-767.

Martins D, English AM. Catalase activity is stimulated by h2o2 in rich culture medium and is required for h2o2 resistance and adaptation in yeast. Redox biology 2014;2:308-313.

Marwah RG, Fatope MO, Al Mahrooqi R, Varma GB, Al Abadi H, Al-Burtamani SKS. Antioxidant capacity of some edible and wound healing plants in oman. Food Chemistry 2007;101(2):465-470.

Matsumoto Y, Yamaguchi T, Watanabe S, Yamamoto T. Involvement of arachidonic acid cascade in working memory impairment induced by interleukin-1 beta. Neuropharmacology 2004;46(8):1195-1200.

Mattson MP, Goodman Y, Luo H, Fu W, Furukawa K. Activation of nf- κb protects hippocampal neurons against oxidative stress- induced apoptosis: Evidence for induction of manganese superoxide dismutase and suppression of peroxynitrite production and protein tyrosine nitration. J Neurosci Res 1997;49(6):681-697.

Miki T, Harris SJ, Wilce PA, Takeuchi Y, Bedi KS. Effects of alcohol exposure during early life on neuron numbers in the rat hippocampus. I. Hilus neurons and granule cells. Hippocampus 2003;13(3):388-398.

Miki T, Harris SJ, Wilce PA, Takeuchi Y, Bedi KS. Effects of age and alcohol exposure during early life on pyramidal cell numbers in the ca1-ca3 region of the rat hippocampus. Hippocampus 2004;14(1):124-134.

Miki T, Satriotomo I, Li HP, Matsumoto Y, Gu H, Yokoyama T, Lee KY, Bedi KS, Takeuchi Y. Application of the physical disector to the central nervous system: Estimation of the total number of neurons in subdivisions of the rat hippocampus. Anat Sci Int 2005;80(3):153-162.

Minghetti L. Cyclooxygenase-2 (cox-2) in inflammatory and degenerative brain diseases. J Neuropathol Exp Neurol 2004;63(9):901-910.

Moore KL, Persaud T, Torchia MG. The developing human-e-book: Clinically oriented embryology.Elsevier Health Sciences. 2016;

Morioka N, Kumagai K, Morita K, Kitayama S, Dohi T. Nonsteroidal anti-inflammatory drugs potentiate 1-methyl-4-phenylpyridinium (mpp+)-induced cell death by promoting the intracellular accumulation of mpp+ in pc12 cells. J Pharmacol Exp Ther 2004;310(2):800-807.

Morishima T, Togashi T, Yokota S, Okuno Y, Miyazaki C, Tashiro M, Okabe N, Collaborative Study Group on Influenza-Associated Encephalopathy In J. Encephalitis and encephalopathy associated with an influenza epidemic in japan. Clin Infect Dis 2002;35(5):512-517.

Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11(1):47-60.

Morris RG, Garrud P, Rawlins JN, O'keefe J. Place navigation impaired in rats with hippocampal lesions. Nature 1982;297(5868):681-683.

Motawi TK, Ahmed SA, El-Boghdady NA, Metwally NS, Nasr NN. Protective effects of betanin against paracetamol and diclofenac induced neurotoxicity and endocrine disruption in rats. Biomarkers 2019;24(7):645-651.

Nishi KK, Antony M, Jayakrishnan A. Synthesis and evaluation of ampicillin-conjugated gum arabic microspheres for sustained release. J Pharm Pharmacol 2007;59(4):485-493.

Odaci E, Cihan OF, Aslan H, Ragbetli MC, Kaplan S. Prenatal diclofenac sodium administration increases the number of purkinje cells in female rats: A stereological study. Int J Dev Neurosci 2010;28(2):145-151.

Olesen MV, Wortwein G, Pakkenberg B. Electroconvulsive stimulation, but not chronic restraint stress, causes structural alterations in adult rat hippocampus--a stereological study. Hippocampus 2015;25(1):72-80.

Ong CK, Lirk P, Tan CH, Seymour RA. An evidence-based update on nonsteroidal anti-inflammatory drugs. Clin Med Res 2007;5(1):19-34.

Onishi T, Umemura S, Yanagawa M, Matsumura M, Sasaki Y, Ogasawara T, Ooshima T. Remineralization effects of gum arabic on caries-like enamel lesions. Arch Oral Biol 2008;53(3):257-260.

Ozdogan L, Ayerden T, Ornek D, Sastim H, Kilci O, Un C, Dikmen B. The neurotoxic effect of intrathecal diclofenac sodium in rats. Kafkas Univ Vet Fak 2011;17(2):309-314.

Ozyurt B, Kesici H, Alici SK, Yilmaz S, Odaci E, Aslan H, Ragbetli MC, Kaplan S. Prenatal exposure to diclofenac sodium changes the morphology of the male rat cervical spinal cord: A stereological and histopathological study. Neurotoxicol Teratol 2011;33(2):282-287.

Peltola V, Huhtaniemi I, Ahotupa M. Antioxidant enzyme activity in the maturing rat testis. J Androl 1992;13(5):450-455.

Phillips G, Williams P. Tree exudate gums: Natural and versatile food additives and ingredients. Food Ingredients and Analysis International 2001:26.

Popa-Wagner A, Mitran S, Sivanesan S, Chang E, Buga AM. Ros and brain diseases: The good, the bad, and the ugly. Oxid Med Cell Longev 2013;2013:963520.

Poulose SM, Miller MG, Scott T, Shukitt-Hale B. Nutritional factors affecting adult neurogenesis and cognitive function. Adv Nutr 2017;8(6):804-811.

Ragbetli MC, Ozyurt B, Aslan H, Odaci E, Gokcimen A, Sahin B, Kaplan S. Effect of prenatal exposure to diclofenac sodium on purkinje cell numbers in rat cerebellum: A stereological study. Brain Res 2007;1174:130-135.

Rajabalian S, Meimandi MS, Badinloo M. Diclofenac inhibits proliferation but not ngf-induced differentiation of pc12 cells. Pak J Pharm Sci 2009;22(3):259-262.

Rodrigues E, Mariutti LR, Faria AF, Mercadante AZ. Microcapsules containing antioxidant molecules as scavengers of reactive oxygen and nitrogen species. Food Chem 2012;134(2):704-711.

Ross MH, Pawlina W. Histology. 6 Ed, Lippincott Williams & Wilkins. 2011;

Sacerdote P, Monza G, Mantegazza P, Panerai AE. Diclofenac and pirprofen modify pituitary and hypothalamic beta-endorphin concentrations. Pharmacol Res Commun 1985;17(8):679-684.

Sadeghi A, Asghari H, Hami J, Mohasel Roodi M, Mostafaee H, Karimipour M, Namavar M, Idoon F. Volumetric investigation of the hippocampus in rat offspring due to diabetes in pregnancy-a stereological study. J Chem Neuroanat 2019;101:101669.

Sadler TW. Langman's medical embryology.Lippincott Williams & Wilkins. 2011;

Santos IM, Tome Ada R, Saldanha GB, Ferreira PM, Militao GC, Freitas RM. Oxidative stress in the hippocampus during experimental seizures can be ameliorated with the antioxidant ascorbic acid. Oxid Med Cell Longev 2009;2(4):214-221.

Sarkaki A, Rezaiei M. Improving active and passive avoidance memories deficits due to permanent cerebral ischemia by pomegranate seed extract in female rats. Malays J Med Sci 2013;20(2):25.

Scheff SW, Baldwin SA, Brown RW, Kraemer PJ. Morris water maze deficits in rats following traumatic brain injury: Lateral controlled cortical impact. J Neurotrauma 1997;14(9):615-627.

Schmitz C, Hof PR. Design-based stereology in neuroscience. Neuroscience 2005;130(4):813-831.

Shirasaki T, Harata N, Nakaye T, Akaike N. Interaction of various non-steroidal anti-inflammatories and quinolone antimicrobials on gaba response in rat dissociated hippocampal pyramidal neurons. Brain Res 1991;562(2):329-331.

Sies H. Oxidative stress: Oxidants and antioxidants. Exp Physiol 1997;82(2):291-295.

Silva RH, Abilio VC, Takatsu AL, Kameda SR, Grassl C, Chehin AB, Medrano WA, Calzavara MB, Registro S, Andersen ML, Machado RB, Carvalho RC, Ribeiro Rde A, Tufik S, Frussa-Filho R. Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. Neuropharmacology 2004;46(6):895-903.

Singh G. Gastrointestinal complications of prescription and over-the-counter nonsteroidal anti-inflammatory drugs: A view from the aramis database. Arthritis, rheumatism, and aging medical information system. Am J Ther 2000;7(2):115-121.

Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American journal of Enology and Viticulture 1965;16(3):144-158.

Snell RS. Clinical neuroanatomy. 7th Ed, Philadelphia, Lippincott Williams & Wilkins. 2010;

Standring S. Gray's anatomy e-book: The anatomical basis of clinical practice. 41 Ed, Elsevier Health Sciences. 2015;

Stella F, Cerasti E, Si B, Jezek K, Treves A. Self-organization of multiple spatial and context memories in the hippocampus. Neurosci Biobehav Rev 2012;36(7):1609-1625.

Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984;134(Pt 2):127-136.

Strauss KI. Antiinflammatory and neuroprotective actions of cox2 inhibitors in the injured brain. Brain Behav Immun 2008;22(3):285-298.

Teichberg S, Wingertzahn MA, Moyse J, Wapnir RA. Effect of gum arabic in an oral rehydration solution on recovery from diarrhea in rats. J Pediatr Gastroenterol Nutr 1999;29(4):411-417.

Terzi M, Altun G, Sen S, Kocaman A, Kaplan AA, Yurt KK, Kaplan S. The use of non-steroidal anti-inflammatory drugs in neurological diseases. J Chem Neuroanat 2018;87:12-24.

The European Agency for the Evaluation of Medicinal Products VMaI, Committee for Veterinary Medicinal Reports.Diclofenac summary report. The European Agency for the Evaluation of Medicinal Products 2003.

Trommer H, Neubert RH. The examination of polysaccharides as potential antioxidative compounds for topical administration using a lipid model system. Int J Pharm 2005;298(1):153-163.

Tsiklauri N, Pirkulashvili N, Nozadze I, Nebieridze M, Gurtskaia G, Abzianidze E, Tsagareli MG. Antinociceptive tolerance to nsaids in the anterior cingulate cortex is mediated via endogenous opioid mechanism. BMC Pharmacol Toxicol 2018;19(1):2.

Tural S, Koca I. Physico-chemical and antioxidant properties of cornelian cherry fruits (cornus mas l.) grown in turkey. Scientia Horticulturae 2008;116(4):362-366.

Turkmen AP, Kaplan S, Aksoy A, Altunkaynak B, Yurt K, Elibol E, Cokluk C, Onger M. Effects of diclofenac sodium on the hippocampus of rats with acute subdural hematoma: Histological, stereological, and molecular approach. Folia Neuropathol 2016;54(2):167-179.

Ukeda H, Kawana D, Maeda S, Sawamura M. Spectrophotometric assay for superoxide dismutase based on the reduction of highly water-soluble tetrazolium salts by xanthine-xanthine oxidase. Bioscience, biotechnology, and biochemistry 1999;63(3):485-488.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39(1):44-84.

Verbeken D, Dierckx S, Dewettinck K. Exudate gums: Occurrence, production, and applications. Appl Microbiol Biotechnol 2003;63(1):10-21.

Vieira V, Glassmann D, Marafon P, Pereira P, Gomez R, Coitinho AS. Effect of diclofenac sodium on seizures and inflammatory profile induced by kindling seizure model. Epilepsy Res 2016;127:107-113.

Viggiano A, Viggiano D, Viggiano A, Luca BD. Quantitative histochemical assay for superoxide dismutase in rat brain. Journal of Histochemistry & Cytochemistry 2003;51(7):865-871.

Vorhees CV, Williams MT. Morris water maze: Procedures for assessing spatial and related forms of learning and memory. Nat Protoc 2006;1(2):848-858.

Wang X, Michaelis EK. Selective neuronal vulnerability to oxidative stress in the brain. Front Aging Neurosci 2010;2:12.

Wapnir RA, Teichberg S, Go JT, Wingertzahn MA, Harper RG. Oral rehydration solutions: Enhanced sodium absorption with gum arabic. J Am Coll Nutr 1996;15(4):377-382.

Wapnir RA, Wingertzahn MA, Moyse J, Teichberg S. Gum arabic promotes rat jejunal sodium and water absorption from oral rehydration solutions in two models of diarrhea. Gastroenterology 1997;112(6):1979-1985.

Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA, Vane JR. Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: A full in vitro analysis. Proc Natl Acad Sci U S A 1999;96(13):7563-7568.

West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec 1991;231(4):482-497.

Williams PA, Phillips G. Gum arabic. Handbook of hydrocolloids (second edition)Woodhead Publishing. 2009;252-273.

Yamaguchi T, Kubota T, Watanabe S, Yamamoto T. Activation of brain prostanoid ep3 receptors via arachidonic acid cascade during behavioral suppression induced by delta8-tetrahydrocannabinol. J Neurochem 2004;88(1):148-154.

Yonguc GN, Dodurga Y, Adiguzel E, Gundogdu G, Kucukatay V, Ozbal S, Yilmaz I, Cankurt U, Yilmaz Y, Akdogan I. Grape seed extract has superior beneficial effects than vitamin e on oxidative stress and apoptosis in the hippocampus of streptozotocin induced diabetic rats. Gene 2015;555(2):119-126.

Yurt KK, Kaplan S. As a painkiller: A review of pre- and postnatal non-steroidal anti-inflammatory drug exposure effects on the nervous systems. Inflammopharmacology 2018;26(1):15-28.

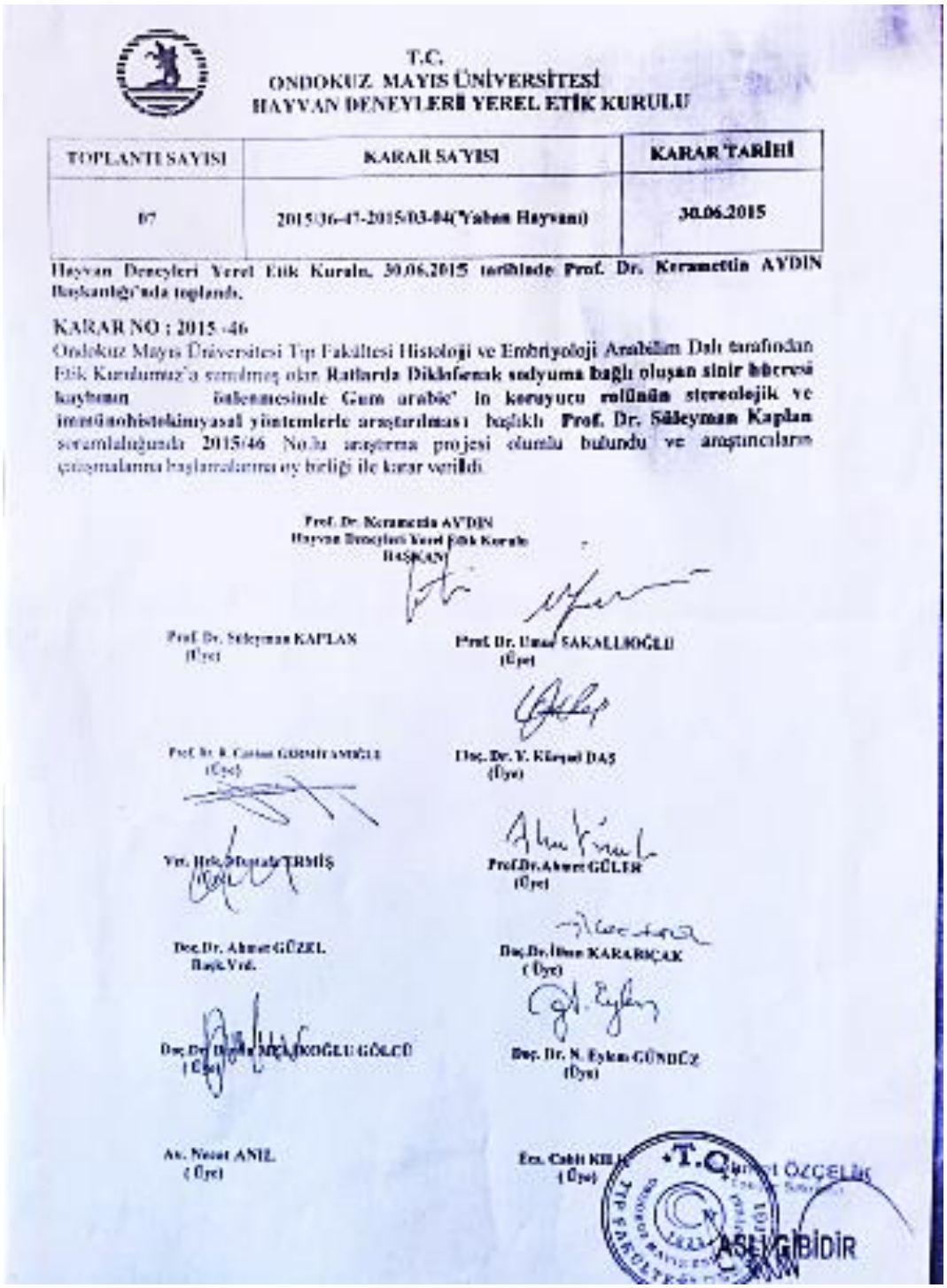
Yurt KK, Kaplan S, Kivrak EG. The neuroprotective effect of melatonin on the hippocampus exposed to diclofenac sodium during the prenatal period. J Chem Neuroanat 2018a;87:37-48.

Yurt KK, Kivrak EG, Altun G, Mohamed H, Ali F, Gasmalla HE, Kaplan S. A brief update on physical and optical disector applications and sectioning-staining methods in neuroscience. J Chem Neuroanat 2018b;93:16-29.

Zhao F, Li J, Mo L, Tan M, Zhang T, Tang Y, Zhao Y. Changes in neurons and synapses in hippocampus of streptozotocin-induced type 1 diabetes rats: A stereological investigation. Anat Rec (Hoboken) 2016;299(9):1174-1183.

APPENDICES

Ethical Committee Approval Certificate



T.C.
ONDOKUZ MAYIS ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU

TOPLANTI SAYISI	KARAR SAYISI	KARAR TARİHİ
07	2015/36-47-2015/03-04(Yaban Hayvanı)	30.06.2015

Hayvan Deneyleri Yerel Etik Kurulu, 30.06.2015 tarihinde Prof. Dr. Kerametin AYDIN Başkanlığında toplandı.

KARAR NO : 2015 -46

Ondokuz Mayıs Üniversitesi Tıp Fakültesi Histoloji ve Embriyoloji Anabilim Dalı tarafından Etik Kurulumuza sunulmuş olan Ratlarda Diklofenak sodyuma bağlı oluşan sinir hücresi kaybının önlenmesinde Gum arabic' in koruyucu rolünün stereolojik ve immünohistokimyasal yöntemlerle araştırılması başlıklı Prof. Dr. Süleyman Kaplan sorumluluğunda 2015/46 No.lu araştırma projesi olumlu bulundu ve araştırıcıların çalışmalarına başlamalarına oy birliği ile karar verildi.

Prof. Dr. Kerametin AYDIN
Hayvan Deneyleri Yerel Etik Kurulu
BAŞKAN

Prof. Dr. Süleyman KAPLAN
(Üye)

Prof. Dr. Ünsal SAKALLIOĞLU
(Üye)

Prof. Dr. A. Canan GÜNEŞ-ANADOL
(Üye)

Doç. Dr. V. Küçük DAŞ
(Üye)

Yrd. Doç. Dr. Ayşe ERMİŞ

Prof.Dr.Ahmet GÜLER
(Üye)

Doç.Dr. Ahmet GÜZEL
Başk.Vek.

Doç.Dr.İlhan KARAHÇAK
(Üye)

Doç. Dr. [illegible]
(Üye)

Doç. Dr. N. Eylem GÜNDÜZ
(Üye)

Ar. Nesat ANIL
(Üye)

Ecz. Cahit KILIÇ
(Üye)

T.C. [illegible] ÖZÇELİK

ASLI GİBİDİR

CURRICULUM VITAE

Full Name: Hosam Eldeen Elsadig Gasmalla MOHAMMED

Place of Birth: Khartoum - Sudan

Date of Birth: 17/06/1980

Marital Status: Single

Nationality: Sudanese

Educational Status (Institution and Year):

M.B.B.S: Al-Neelain University -Faculty of Medicine (1998-2004) Khartoum- Sudan.

M.Sc.: of Human Anatomy-University of Khartoum - Faculty of Medicine (2007-2010) Khartoum- Sudan.

MHPE: Master of health professions education of Human Anatomy-University of Gezira - Faculty of Medicine (2013-2017) Khartoum- Sudan.

Ph.D.: Ondokuz Mayıs University-Faculty of Medicine Department of Histology and Embryology 2015-2020- Samsun- Turkey

E-mail: hosamalwakeel@haotmail.com