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Investigation of the Effects of Acetylsalicylic Acid Administration at Different Doses on Behavioral Disorders in Rats

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Apoptosis, Aspirin, Behavioral disorders, Inflammation, NSAIDs

Abstract: Aspirin is one of the most widely used non-steroidal anti-inflammatory drugs worldwide. Neurodegenerative diseases adversely affect the central nervous system, leading to cognitive decline. Aspirin has different pharmacological activities at different doses. Therefore, this study aimed to determine the effects of acetylsalicylic acid (ASA), the active ingredient of aspirin, administered at different doses on the parameters that play a role in cognitive function using molecular and histological methods and behavioral tests. For this purpose, 28 Wistar rats were divided into 4 groups. Control, ASA-low dose (1mg/kg), ASA-moderate dose (10mg/kg) and ASA-high dose (100mg/kg). ASA was intragastrically administered as a single dose, and an open field test was performed 3 hours later. Subsequently, hippocampus tissues were obtained, and the hippocampus tissue structure was analyzed by analyzing the parameters involved in antioxidant capacity, inflammation, apoptosis, and memory. ASA, especially at moderate doses, increased antioxidant capacity and partially reduced inflammation and apoptotic damage. At high doses, the opposite effect was observed, and the damage levels. Similar effects were detected by histological examination. Although there were no structural defects at low or moderate doses, structural defects were observed at high doses. Although there was no difference in the open field test findings between the groups, the time spent in the center, distance traveled, and speed were slightly higher in the ASA moderate-dose group. In conclusion, ASA may contribute to the improvement of cognitive function at low and moderate doses. However, high doses may cause cognitive impairment.

Farklı Dozlarda Asetilsalisilik Asit Uygulamasının Sıçanlarda Davranış Bozuklukları Üzerindeki Etkilerinin Araştırılması

Anahtar Kelimeler
Apoptozis, Aspirin, Davranışsal bozukluklar, İnflamasyon, NSAID

Öz: Aspirin, dünya genelinde en yaygın kullanılan non-steroid anti-inflamatuvar ilaçlardan biridir. Norodejeneratif hastalıklar merkezi sinir sistemini olumsuz etkileyerek bilişsel fonksiyonların gerilemesine neden olur. Aspirin farklı dozlarda farklı farmakolojik aktiviteye sahiptir. Bu nedenle bu çalışmadaki amaç, farklı dozlarda uygulanan aspirin etken maddesi asetilsalisilik asitin (ASA) bilişsel fonksiyonlarda rol oynayan parametreler üzerindeki etkilerinin moleküler ve histolojik yöntemlerle ve davranış testleri ile belirlenmesidir. Bu amaç doğrultusunda 28 Wistar rat 4 gruba ayrılmıştır. Kontrol, ASA-düşük doz (1mg/kg), ASA-orta doz (10mg/kg) ve ASA-yüksek doz (100mg/kg). ASA tek doz intragastrik olarak uygulandı ve 3 saat sonrasında açık alan testi uygulandı. Ardından hipokampüs dokuları alınarak antioksidan kapasite, inflamasyon, apoptozis ve hafızada rol oynayan parametrelerin analizi ile hipokampüs doku yapısı incelendi. ASA özellikle orta dozda antioksidan kapasiteyi arttırıp, inflamasyon ve apoptotik hasarı kısmı olarak azaltmıştır. Yüksek dozda ise tersi etki göstererek hasar düzeylerini arttırmıştır. Benzer etkiler histolojik incelemede de tespit edilmiş olup düşük ve orta dozda yapısal bozulmalar yok iken yüksek dozda yapısal bozukluklar görülmüştür. Açık alan testi bulgularında gruplar arası fark yok iken merkezde geçirilen süre, kat edilen mesafe ve hız ASA orta doz uygulanan grupta kısmen daha yüksektir. Sonuç olarak, ASA düşük ve orta düzeyde bilişsel fonksiyonların iyileşmesine katkı sağlayabilir ancak yüksek dozda bilişsel fonksiyonların bozulmasına neden olabilir.

1. INTRODUCTION

Neurodegenerative diseases affecting the central nervous system (CNS) cause damage to nerve cells and neurological problems, placing significant burdens on patients, their relatives, and the healthcare sector. Cognitive impairment caused by Alzheimer's disease (AD) has a significant negative impact on the quality of life of middle-aged and older individuals worldwide [1]. Neurodegenerative diseases, such as AD, begin with the gradual onset of memory loss. Over time, they manifest themselves with cognitive and behavioral problems, such as impairments in language, planning ability, and visuospatial skills [2].

Antibiotics and salicylates are the drugs of choice in both outpatients and inpatients; common side effects include diarrhea and gastrointestinal irritation, while toxic effects on the CNS are less well documented [3]. Acetylsalicylic acid (ASA, aspirin) is a non-steroidal anti-inflammatory drug (NSAIDs) commonly used to relieve pain, fever, and inflammation [4]. ASA is consumed globally at an average of 30 grams per person per year, with daily consumption reaching 35,000 kilograms in the United States alone; it is prescribed as secondary prophylaxis after heart attack in both Asian and Western societies for its antiplatelet effects; and low-dose long-term ASA is also used to reduce the risk of heart attack and stroke in individuals without a history of cardiovascular disease [5].

Some correlative studies indicate an unclear relationship between aspirin treatment and memory [6]. There is no evidence that low-dose aspirin improves cognitive performance in healthy women aged 65 years and older. Higher doses that inhibit COX enzymes may be more effective than the low doses of 75 mg used in the AD2000 study, but they also carry a higher risk of toxicity. In addition, these higher doses were found to have similar properties to NSAIDs [7]. Neuroinflammation is a major driver of many neurodegenerative diseases, such as multiple sclerosis, AD, and Parkinson's disease. While cytokines, growth factors, and reactive oxygen species (ROS) are released to aid neuronal repair, these substances can damage healthy tissues in chronic conditions, and oxidative stress is manifested in many of these diseases by an imbalance between ROS production and antioxidant defense [8] High doses of aspirin lead to increased levels of malondialdehyde (MDA), a product of lipid peroxidation (LPO), which can cause serious changes in the brain. LPO affects cell functions involving polyunsaturated fatty acids, impairing the activity of membrane-bound enzymes and can lead to atrophy or death of neurons. MDA level is used to assess ROS-induced brain damage in aging and neurodegenerative diseases and may affect learning and memory functions in the hippocampus [7]. Brain damage and neuronal loss are also associated with activation of microglia, astrocytes and invasive macrophages that overproduce toxic substances such as tumor necrosis factor (TNF)-α, and interleukin (IL)-1β [9]. The reasons for adverse effects of aspirin may be due to variations in experimental models or specifically to the concentrations of aspirin administered. It is well-established that low, moderate, and high doses of aspirin target different molecules [10]. This suggests that ASA may have different pharmacological and biological effects at different doses.

The aim of this study was to compare the effects of ASA, which is recommended for use in CNS-related diseases, on cognitive functions and brain memory markers at different doses. For this purpose, biochemical, molecular, and histological methods were used.

2. MATERIAL AND METHOD

2.1. Chemicals

ASA (≥99.0%, CAS Number: 50-78-2) and other chemicals were obtained from Sigma (St. Louis, USA).

2.2. Ethics Committee Aprproval

Ethical approval was obtained from Necmettin Erbakan University Local Animal Experiments Ethics Committee (25.09.2024, 2024-87).

2.3. Experimental Groups and Procedures

In the experiment, 28 *Wistar albino* rats weighing 220-250 g and aged 10-12 weeks were used. Animals were kept in cages in a controlled room with a constant temperature of 24-25ºC and a twelve (12 h) hour dark-light cycle (07:00-19:00 light; 19:00-07:00 dark). They were given unlimited access to water and standard chow. All animal experiments were performed at the KONÜDAM Experimental Medicine Application and Research Center (Konya / Türkiye). Rats were randomly divided into 4 groups with 7 rats in each group.

1: Control Group: 1 ml of physiologic saline was administered via intragastric gavage.
2: ASA - low dose (ASA-L): ASA 1 mg/kg was administered via intragastric gavage.
3: ASA - moderate dose (ASA-M): ASA 10 mg/kg was administered via intragastric gavage.
4: ASA - high dose (ASA-H): ASA 100 mg/kg was administered via intragastric gavage.

The protocol for ASA administration and tissue collection time was determined from the study by Senol et al. [7] and the doses were determined based on the study of Espiridiao et al. [11].

2.4. Open Field Test

The open field apparatus was made of water-resistant material and had a square shape with a base size of 80 × 80 cm and a height of 40 cm. The base was marked with 16 equal squares, and the 4 squares in the center were classified as the "center area" and the 12 squares on the edges as the "peripheral area". During the experiment, rats were placed in the center of the area and allowed to explore the area freely for 5 min; video recording was performed during this time. The time spent in the center, the number of transitions to the center, the distance moved

in the center, and the velocity were recorded in the four squares in the center area to assess anxiety levels. After the experiment, the open field apparatus was cleaned with ethanol in each rat to eliminate any remaining odors [12].

2.5. Tissue Collection

Three hours after ASA administration, the animals were decapitated under light sevoflurane anesthesia, and brain tissue samples were collected. Then, some brain tissue samples were stored at -80°C until genetic analyses were performed. The other part was left in 10% formaldehyde solution for use in histological analyses.

2.5. RT-PCR

At the end of the experiment, relative mRNA transcript levels of the gene regions listed in Table 1 were determined in hippocampus tissue samples using qRT–PCR. A QIAzol Lysis Reagent (79306; Qiagen) was used for total RNA isolation from hippocampus tissue samples. cDNA synthesis from the obtained total RNA was performed using the OneScript Plus cDNA Synthesis Kit (ABM, G236, Richmond, Canada). The prepared cDNAs were combined with primer sequences and BlasTaq™ 2X qPCR MasterMix (ABM, G891, Richmond, Canada) to form a reaction mixture. This mixture was run on a Rotor-Gene Q (Qiagen) device in the cycle and temperature program described according to the manufacturer's instructions. After the completion of the cycles, gene expression was normalized to β-Actin and evaluated by the $2^{-\Delta\Delta CT}$ method [13].

Table 1. Primer sequences

Gene	Sequences (5'-3')	Product length
Cu-Zn SOD	F: AGTCCCGCCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC	387
CAT	F: ATGGCAACTGTCCCTGAACT R: AGTGACACTGCCTTCCTGAA	670
GPx	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCAGTT	290
NF-κB	F: AGTCCCGCCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC	106
IL-1β	F: ATGGCAACTGTCCCTGAACT R: AGTGACACTGCCTTCCTGAA	197
TNF-α	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCAGTT	139
Caspase-3	F: ACTGGAATGTCAGCTCGCAA R: GCAGTAGTCGCCTCTGAAGA	270
Bax	F: TTTCATCCAGGATCGAGCAG R: AATCATCCTCTGCAGCTCCA	154
Bcl-2	F: GACTTTGCAGAGATGTCCAG R: TCAGGTACTCAGTCATCCAC	214
Glutamat	F: ACAGCAGAGTTCCAGGACAG R: GTCTATGTGAAGGTCACGCC	212
NMDA-R	F: TAGTTAGCCACCCGCCTACT R: GTCCGCGCTTGTTGTCATAG	357
CREB	F: TGCACAGACCACTGATGGAC R: CTGTGTAGGAAGTGCTGGGG	160
HSP27	F: GTCAACCACTTCGCTCCTGA R: CGACTCTGGGCCTCCAATTT	288
β-Actin	F: CAGCCTTCCTTCCTGGGTATG R: AGCTCAGTAACAGTCCGCCT	360

2.6. Histopathological Examination

Hippocampus tissue samples were fixed in 10% neutral formalin buffer for 24 h. After tissue tracking was completed, 5 μm thick sections were prepared from the paraffin blocks using a microtome. These sections were placed on slides, stained with hematoxylin and eosin (H&E), examined, and photographed using an Olympus Cx 43 microscope (Japan).

2.6. Statistical Analysis

One-way ANOVA followed by Tukey's post hoc test (SPSS 20.0; Chicago, USA) was applied to analyze the differences and significance levels between the groups. All results are presented as mean ± SE. $p<0.05$ was taken as the significance level.

3. RESULTS

3.1. Open Field Test Findings

In the open-field test (Figure 1), the distance moved in the center (A), velocity (B), time spent in the center (C), and the number of transitions from the edge to the center (D) were determined. For all these parameters, the highest level was observed in the ASA-M group, and the lowest level was observed in the ASA-H group. The differences between the groups were not significant ($p>0.05$).

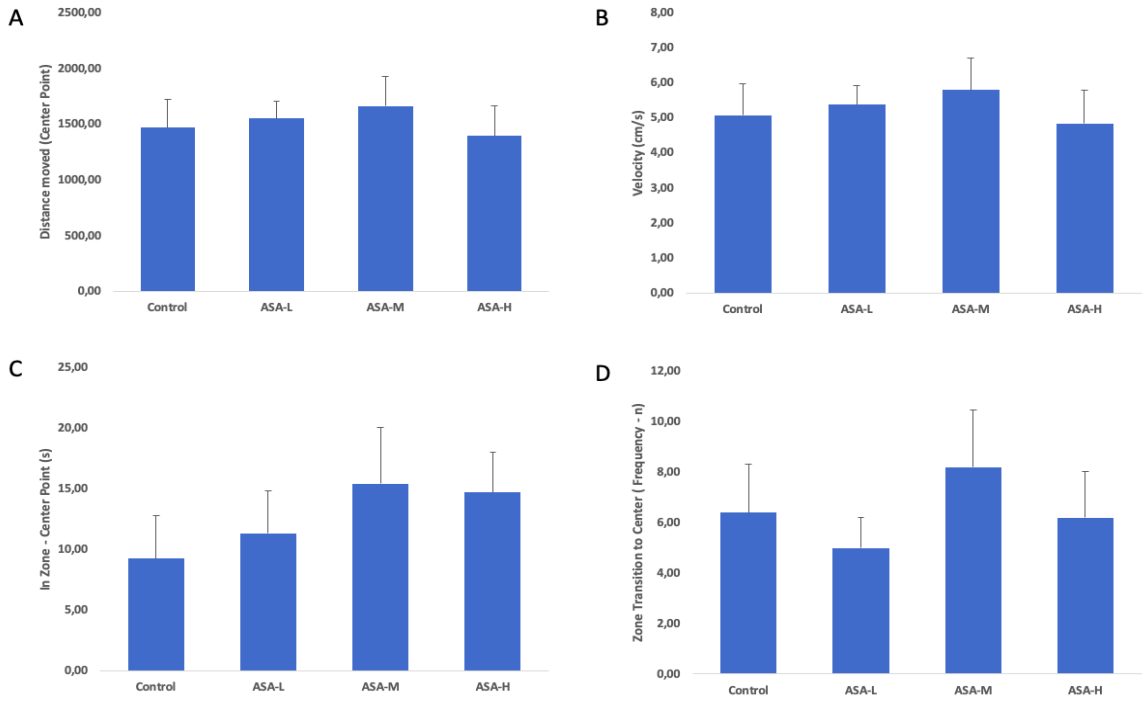

Figure 1. Open field test results

3.2. Antioxidant Capacity Findings

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were determined to determine the level of antioxidant capacity in hippocampus tissues (Figure 2). The highest activities of SOD, CAT, and GPx were detected in the ASA-M group, whereas the lowest activities were detected in the ASA-H group ($p<0.05$).

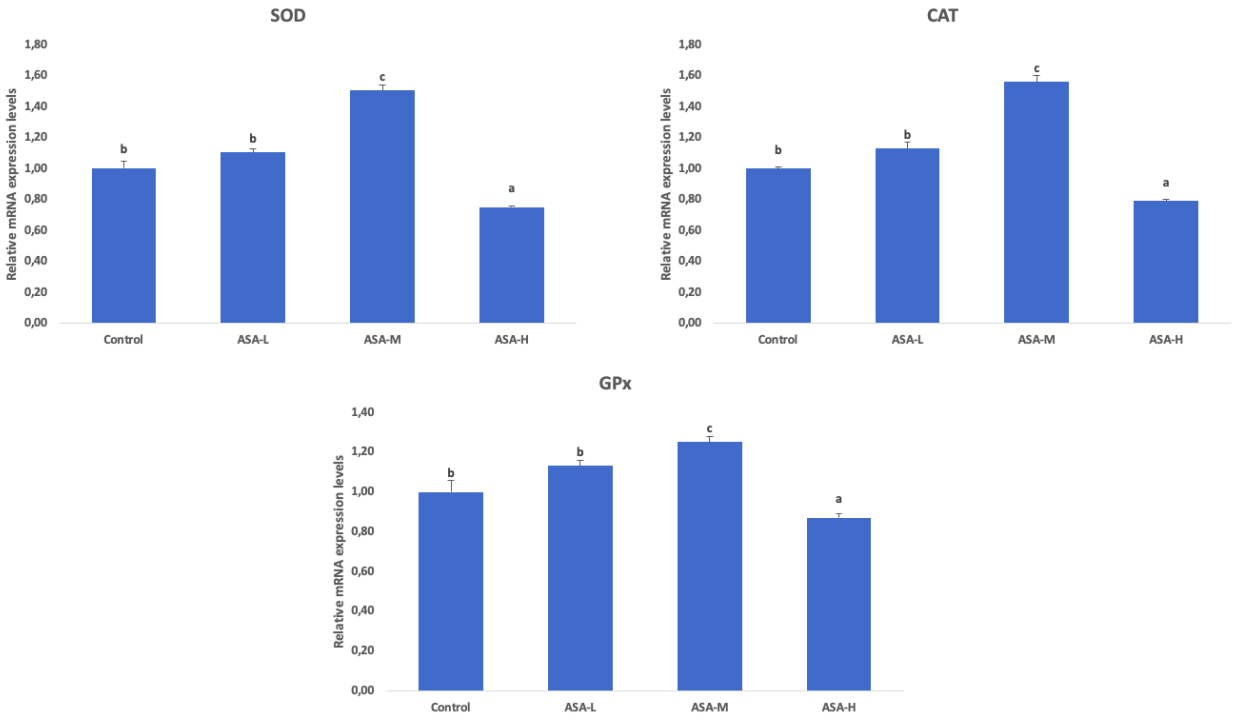

Figure 2. Effects of different doses of ASA on antioxidant enzymes in rat hippocampus tissues. Values are given as mean ± SE. Different letters indicate statistical difference: *$p<0.05$

3.3. Apoptosis Findings

To determine the extent of apoptotic damage in hippocampus tissues, apoptotic cysteine-aspartic acid protease-3 (Casp-3) and BCL2-associated X (Bax) and antiapoptotic B-cell lymphoma 2 (Bcl-2) levels were determined (Figure 3). The highest levels of Casp-3 and Bax and the lowest levels of Bcl-2 were detected in the

ASA-M group, whereas the lowest levels of Casp-3, Bax, and Bcl-2 were detected in the ASA-H group ($p<0.05$).

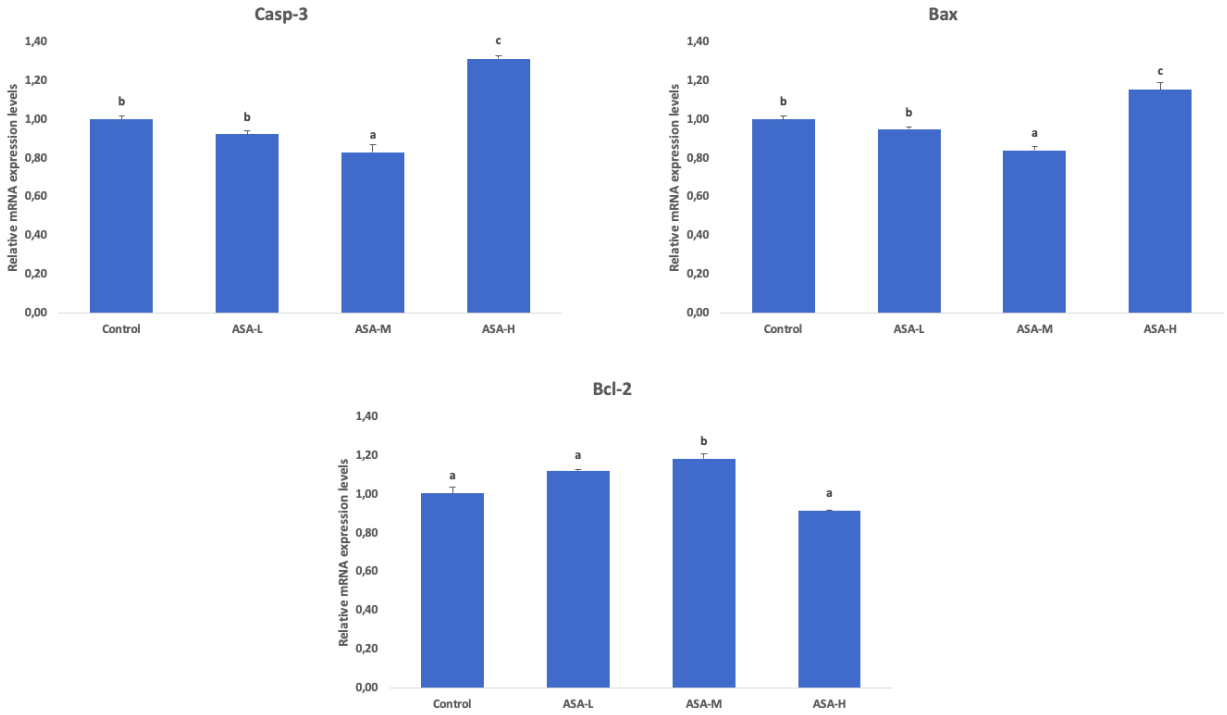

Figure 3. Effects of different doses of ASA on apoptotic and antiapoptotic markers in rat hippocampus tissues. Values are given as mean ± SE. Different letters indicate statistical difference: *$p<0.05$

3.4. Inflammation Findings

Nuclear factor kappa B (NF-κB), TNF-α, and IL-1β mRNA transcription levels were determined to determine inflammation damage in hippocampus tissues (Figure 4). The lowest NF-κB, TNF-α, and IL-1β levels were detected in the ASA-M group ($p>0.05$), whereas the highest NF-κB, TNF-α, and IL-1β mRNA transcription levels were detected in the ASA-H group ($p<0.05$).

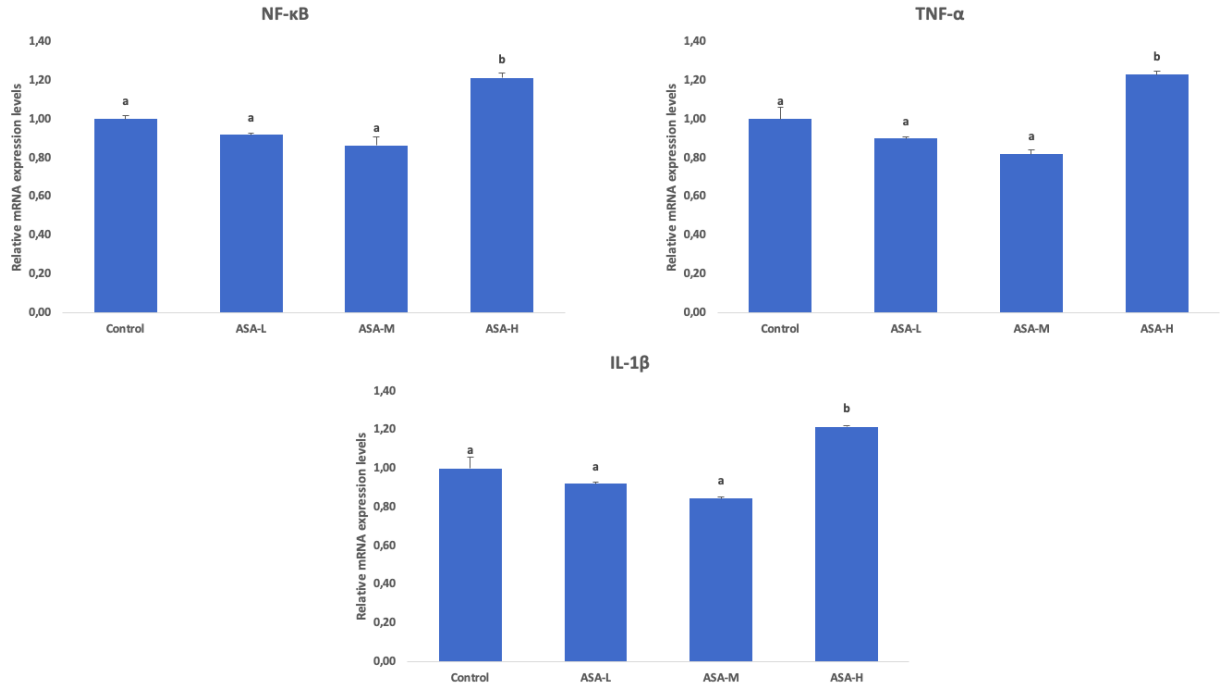

Figure 4. Effects of different doses of ASA on inflammatory markers in rat hippocampus tissues. Values are given as mean ± SE. Different letters indicate statistical difference: *$p<0.05$

3.5. Memory markers expressions

Glutamate, cAMP-response element binding protein (CREB), and N-methyl-D-aspartate (NMDA) mRNA transcription levels associated with memory were analyzed (Figure 5). The highest glutamate and CREB levels were observed in the ASA-M group, whereas the lowest levels were observed in the ASA-H group ($p<0.05$). The highest level of NMDA was observed in the ASA-H group, whereas the lowest level was observed in the ASA-M group ($p<0.05$).

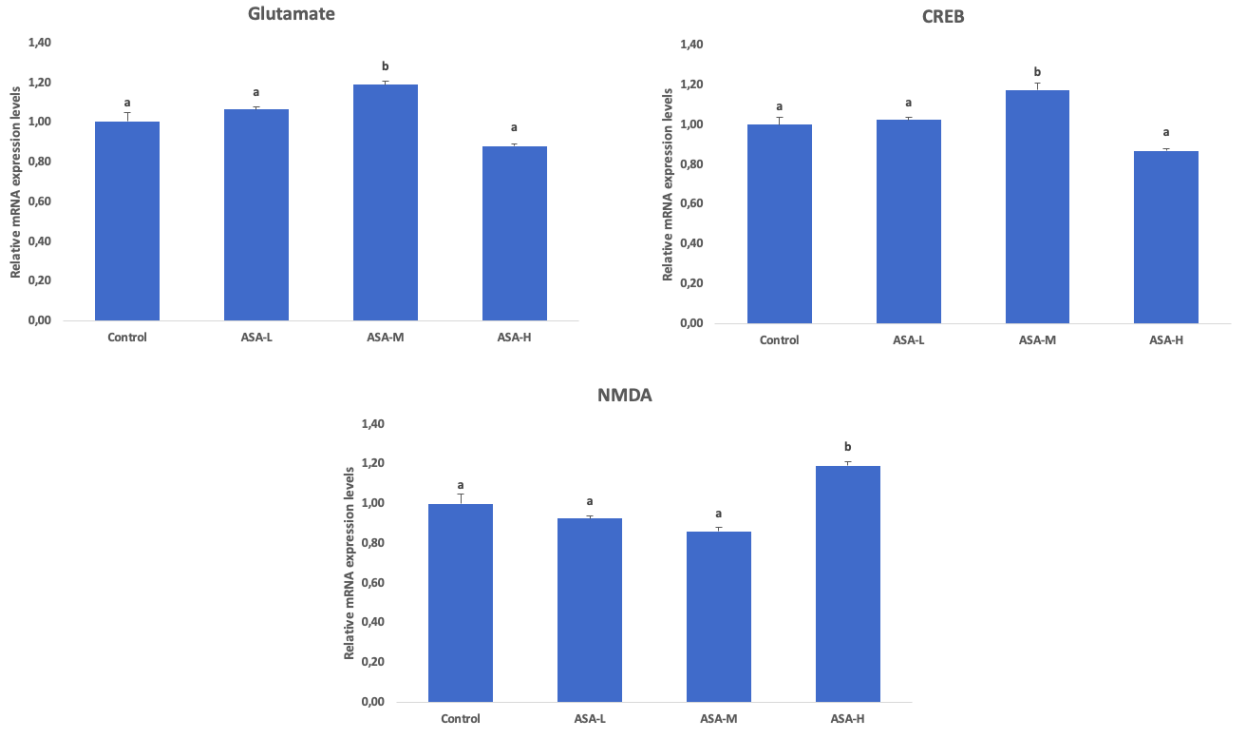

Figure 5. Effects of different doses of ASA on glutamate, CREB and NMDA mRNA transcription levels in rat hippocampus tissues. Values are given as mean ± SE. Different letters indicate statistical difference: *p<0.05

3.6. HSP27 exprenssions

HSP27 mRNA transcription, which protects against apoptotic damage in hippocampus tissues, was also determined (Figure 6). HSP27 levels increased in the ASA-L and ASA-M groups, with the highest level in the ASA-M group and the lowest level in the ASA-H group ($p<0.05$).

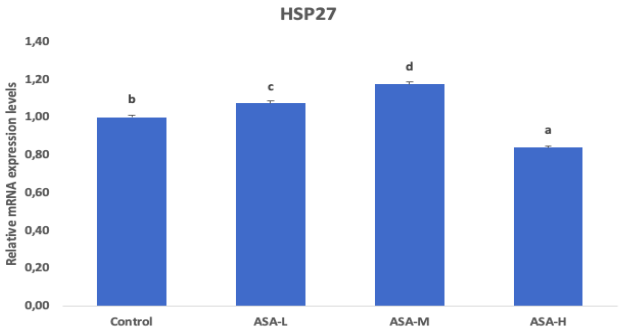

Figure 6. Effects of different doses of ASA on HSP27 mRNA transcription levels in rat hippocampus tissues. Values are given as mean ± SE. Different letters indicate statistical difference: *p<0.05

3.7. Histopathological examinations

In the histopathological study, the morphological findings of the hippocampus were examined at high magnification (200x) according to the hematoxylin and eosin (H&E) staining results and are presented in Figure 7. When hippocampus samples obtained from rats in the control group were examined, it was observed that they had a normal histological structure. In addition, the CA1, CA2, CA3, and CA4 regions and the dental gyrus region could be easily distinguished. To standardize our comparisons, the CA1 and dental gyrus regions were defined and examined at high magnification (Figure 7a, 7a1). In the low- and moderate-dose images of the ASA-treated groups, the regular, dense, and regular arrangement of neurons in the CA1 regions of the hippocampi was striking. In addition, neurons in the CA1 region had clear, dark nuclei and a light cytoplasm. In the dental gyrus region, the molecular, granular, and polymorphic cell

layers were regular; in particular, the cells in the granular layer were quite compactly arranged and had characteristic cell bodies (Figure 7b, 7b1, 7c, 7c1). High-dose ASA treatment had undesirable effects, such as irregular neuronal arrangement and decreased neuronal density, compared with the control. There were also vacuoles and degenerative changes in the pericellular areas of the cells. In addition, nuclei became unclear and smaller, and cytoplasmic borders lost their normal structure (Figure 7d, 7d1).

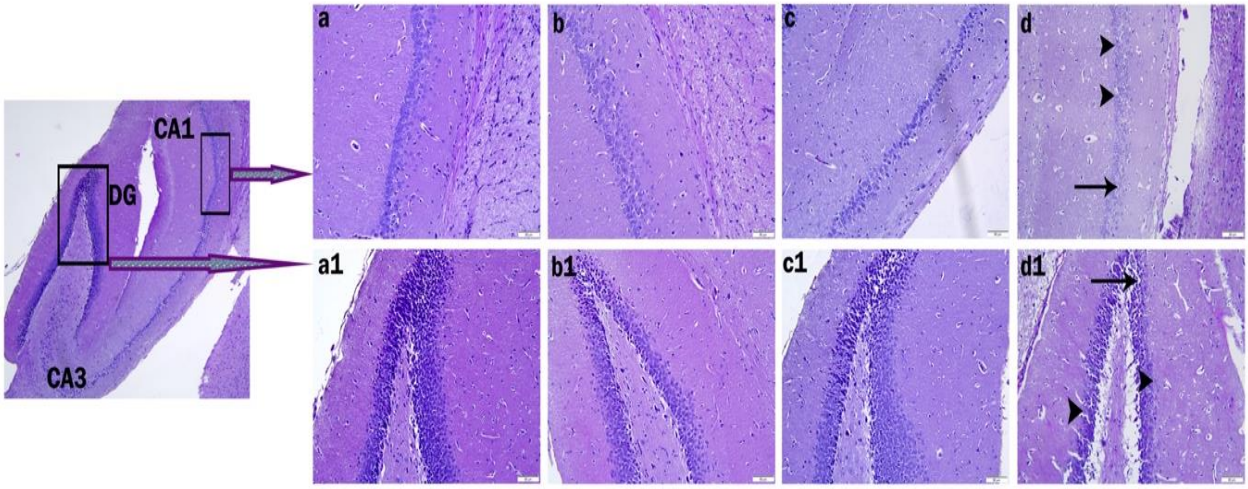

Figure 7. Representative photomicrographs of CA1 and dental gyrus regions of the hippocampus in all experimental groups. A,a1: Normal morphology of CA1 and dental gyrus regions in control group, b,b1: ASA-L group, c,c1: ASA-M group, d,d1: ASA-H group. Arrowheads: degenerating neurons, arrows: pericellular vacuolization. Hematoxylin and Eosin (H&E) staining, Original magnification: X200.

4. DISCUSSION AND CONCLUSION

NSAIDs are widely used worldwide to control pain and inflammation, with approximately 70 million prescriptions written each year in the US alone; these drugs usually have mild side effects at normal doses but can cause serious toxicity at excessive doses [14]. ASA is also an NSAID, and it can exert different pharmacological effects at different doses. Therefore, the effects of acute ASA administration at different doses on behavioral disorders were investigated using molecular, histological, and cognitive tests.

Inflammation and neutrophil migration are critical in the pathogenesis of NSAIDs [15]. Inflammation is the initial response of an organism to injury, which, when uncontrolled with the release of inflammatory mediators, can lead to inflammatory diseases and organ dysfunction [16]. NF-κB upregulation plays an important role in triggering inflammation [17]. NF-κB is involved in the stimulation of proinflammatory cytokines [18]. In the inflammatory process, NF-κB undergoes rapid phosphorylation by IkBα and IkB kinase-β (IKKβ), leading to the release of pro-inflammatory cytokines [19]. TNF-α and IL-1β, which are among these proinflammatory cytokines, are effective in the development of inflammation [20,21]. TNF-α leukocyte migration to the inflamed area in the tissues [22,23]. IL-1β triggers acute inflammatory responses by increasing the expression of adhesion molecules on endothelial cells, mobilizing inflammatory cells, and leading to the proliferation of circulating leukocytes [24]. In this study, the mRNA transcription levels of NF-κB, TNF-α, and IL-1β, which are effective against inflammation, were highest in the ASA-H group. In contrast, NF-κB, TNF-α, and IL-1β mRNA transcription levels were lower in the ASA-L and ASA-M groups than in the control group. These findings suggest that ASA can effectively alleviate inflammation at low and moderate doses and neuroinflammatory at high doses.

Oxidative stress is defined as the disruption of the balance between ROS and antioxidants in the presence of ROS [25,26]. In addition to neuroinflammation, oxidative stress has been suggested to contribute to the pathophysiological processes leading to neuronal loss in individuals with aging and neurological diseases, such as Alzheimer's disease and Parkinson's disease [27]. Oxidative stress activates different damage pathways [28–30]. The body has a defense system against the damage caused by free radicals [31]. Antioxidants are enzymatic and nonenzymatic agents [32]. Antioxidant enzymes such as SOD, CAT, and GPx are actively involved in scavenging oxygen-derived free radicals from oxidative stress to prevent further cell damage [33–35]. SOD, which converts superoxide to hydrogen peroxide (H_2O_2), is an important antioxidant enzyme involved in oxidative stress, whereas CAT, which converts H_2O_2 to oxygen and water, also plays an important role. Furthermore, GPx neutralizes the effects of cytotoxic lipid peroxides and H_2O_2 as a defense mechanism [36,37]. ROS, which are known as free radicals, cause oxidative damage by affecting macromolecules such as carbohydrates, proteins, nucleic acids, and lipids [38]. Increased ROS can cause cell death through proteolysis and lipid oxidation [39,40]. Owing to these properties, oxidative stress causes neuronal damage in brain tissue, which has at low defense against oxidative stress [41]. Potentially toxic drugs and their metabolites directly affect the biochemistry of the cell, firstly through increased oxidative stress and secondly through altered intracellular signaling pathways associated with apoptotic or necrotic

cell death [42]. According to the antioxidant capacity findings obtained in our study; ASA tried to resist neuronal damage by increasing antioxidant capacity at low and moderate doses, but at high doses it reduced antioxidant capacity and left the tissue vulnerable to neuronal damage.

Apoptosis, which protects the body by eliminating damaged or dangerous cells, can also cause stress or damage to healthy cells [43,44]. A relationship between intracellular ROS levels and apoptosis has also been reported [45]. Apoptosis pathways are activated by DNA or protein damage following excessive ROS production [46]. Apoptosis can also be induced by various stimuli such as cytokines, hormones, toxic insults, and viruses [47]. The mitochondrial pathway plays critical roles in apoptosis and is regulated by members of the Bcl-2 protein family, such as Bax and Bcl-2 [48,49]. Disruption of the balance between Bax and Bcl-2 in favor of Bax leads to the release of cytochrome C from mitochondria into the cytoplasm [50]. After cytochrome C passes into the cytoplasm, it combines with Apaf-1 to activate apoptotic caspase [51]. Caspases are inactive inside the cell; when a caspase becomes active, a chain reaction activates other pro-caspases [52]. Caspase-3, an important caspase, is also known as executioner caspase and is pro-apoptotic [53,54]. In this study, it was found that apoptotic activity in the ASA-H group was triggered by increased Casp-3 and Bax levels and a decrease in Bcl-2. On the other hand, in the ASA-L and ASA-M groups, apoptotic Casp-3 and Bax levels decreased while antiapoptotic Bcl-2 levels increased. When the apoptotic findings are evaluated together, ASA has ameliorative properties against apoptosis at low and moderate doses and increases at high doses.

Glutamate is an excitatory neurotransmitter in the brain that plays an important role in neuronal functions such as learning and memory. The activation of NMDA receptors leads to the formation of reactive oxygen and nitrogen species, which contribute to neuronal death [7]. Phosphorylation activation of CREB exhibits protective properties against apoptosis by accelerating Bcl-2 activation [55]. CREB is also one of the factors involved in memory and learning [56]. Glutamate and CREB mRNA transcription levels were increased in the ASA-M group, which may have contributed to the increase in neuronal and cognitive activity. The lowest NMDA level was detected in the ASA-M group. According to these findings, moderate-dose ASA may have a positive effect on cognitive function. High doses showed the opposite effect.

The open field test is commonly used to measure general locomotor activity and willingness to explore in rats [12]. Data on the effect of aspirin on cognitive functions are inconsistent; some studies suggest that ASA does not affect memory, while others argue that it positively affects neurocognitive behaviors. Furthermore, few placebo-controlled studies have failed to confirm the positive effects of aspirin on cognitive decline [57]. Increasing evidence suggests that aspirin may slow the progression of vascular pathology and cognitive loss in AD. However, even at low doses, there is a risk of serious side effects, especially in elderly patients [7]. Although there was no difference between the groups in the findings of the open field test, the time spent, distance moved, and velocity in the center area were observed in the ASA-M group.

A similar picture was observed in hippocampus tissue. In ASA-L and ASA-M groups, histologic features close to the control group were found. However, in the ASA-H group, the adverse effects caused irregular arrangement and decreased density of neurons. In this group, there were signs of vacuolization and degeneration in the areas around the cells. Cell nuclei became smaller and indistinct and the boundaries of the cytoplasm were disrupted. Decreased antioxidant capacity and increased inflammation and apoptotic damages detected especially in the ASA-H group may have played a role in the formation of these structural defects.

As a conclusion, ASA may be effective in the prevention of behavioral disorders at low and moderate doses, whereas, at high doses, it may have the opposite effect and contribute to the progression of behavioral disorders.

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