ISSN: 2717-8161 RESEARCH ARTICLE

New Trend Med Sci 2023; 4(2): 58-65. https://dergipark.org.tr/tr/pub/ntms

Effect of a TNF-Alpha Inhibitor on Anxiety and Depression-Like Behaviors in a Mouse Chemobrain Model

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Article History
Received 19 Jan 2023
Accepted 07 Mar 2023
Published Online 26 May 2023

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Doi: 10.56766/ntms.1239435

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Abstract: Although chemotherapy increases the survival rate of cancer patients, it causes significant side effects such as deterioration in cognitive functions that generate a decline in their living standards. In our study, the effect of adalimumab (TNF-Alpha inhibitor) on anxiety and depression-like behaviors in mice with cognitive impairment with methotrexate was investigated. In our study, a total of 24 mice, 6 mice in each group, were used, and the first group was considered as the control. A single dose of methotrexate (40 mg kg^{-1}) was administered intraperitoneally to the other two groups, and a chemobrain model was created. Adalimumab (10 mg kg^{-1}) was administered twice, 1 hour and 5 days before methotrexate and/or vehicle administration. Anxiety-like behaviors were measured with elevated plus maze (EPM) test and open field test (OF), depression-like behaviors were measured with tail suspension test (TST), and hippocampal tissue was examined histopathologically. Methotrexate decreased the time spent in the central zone in the open-field arena, the time spent in the open arms in the elevated plus maze test, and increased the duration of immobility in the tail suspension test in rats. Methotrexate caused a decrease in the number of neuronal cells in the CA3 region of the hippocampus, as well as neurodegenerative and atrophic changes. Adalimumab improved the time spent in open arms in the OF test and the number of open arm entries in the EPM, immobility time on TST, and histopathological changes. In this study, it was shown that methotrexate-related anxiety and depression-like behavioral disorders were prevented by adalimumab treatment, but further studies are recommended to investigate the mechanisms mediating the therapeutic effect of adalimumab. ©2023 NTMS.

Keywords: Methotrexate; Adalimumab; Anxiety; Depression; Neuroinflammation.

1. Introduction

Associated with the increase in the prevalence of malignant diseases, the use of different types of drugs at more aggressive doses for therapeutic purposes, as well as the development of a large number of new chemotherapeutic agents, has led to reduced recurrence and increased survival rate for many types of cancer. However, it is inevitable to observe different complications that lead to a decrease in living standards. One of these is cognitive dysfunction associated with the use of systemic chemotherapy, which includes mild cognitive impairments, also known as 'chemophog' or 'chemobrain'. Such

Cite this article as: Oz M and Akaras N. Effect of a TNF-Alpha Inhibitor on Anxiety and Depression-Like Behaviors in a Mouse Chemobrain Model. *New Trend Med Sci*. 2023; 4(2):58-65. Doi:10.56766/ntms.1239435

conditions are non-fatal but persistent, so long-term survivors' quality of life is significantly impaired and clinically important as they prevent a return to their pre-cancerous state [1]. Methotrexate (Mtx) is a chemotherapeutic that reduces the growth and proliferation of tumor cells and is widely used in the treatment of types of cancer, and significantly increases the survival rates of patients with acute lymphoblastic leukemia and lymphoma [2]. Mtx is a folic acid antagonist that negatively affects important cellular bioprocesses such as DNA, RNA and protein synthesis by inhibiting the dihydrofolate reductase enzyme, which acts by changing the intracellular folate distribution [3]. One of the important complications of Mtx is the pathophysiological changes that lead to neurodegenerative processes in the brain, especially in areas where cognitive functions are carried out, such as the hippocampus. Thus, in addition to improving survival, Mtx therapy is associated with persistent impairments in survivors' cognitive functions such as attention, reasoning, memory, learning, and executive function [4]. The pathophysiological changes associated with this neurotoxicity are multifaceted and are of increasing interest by researchers. Numerous mechanisms have been suggested to explain the underlying mechanism of Mtx therapy-induced cognitive impairment. Mtx causes increased hippocampal endoplasmic reticulum stress[5], decreased hippocampal antioxidant enzyme activity, such as SOD, CAT, GPx and GSH, and decreased levels of some neurotrophic factor, such as BDNF, increased lipid peroxidation [6], induced apoptosis and associated disruption of hydrogen sulfide production in the hippocampal CA1 region [5, 7], altered dendritic branching and spine morphology [8], reduced cell proliferation, survival and cell differentiation in the hippocampal DG region [9–11], increased amount of proinflammatory cytokines that cause neuroinflammation [12, 13].

The genes involved in the generation of the inflammatory response are normally suppressed, but become active when cells sense information about infection or injury, and this response must be strengthened to mount an effective immune response and initiate antimicrobial or antiviral activity. Some of the important enhancers of this immune response are proinflammatory cytokines, such as TNF-alpha, and IL-1beta [14]. TNF-alpha, a 25 kDA type II transmembrane protein, is produced by active macrophages, as well as by different types of cells such as kupffer cells of the liver, ovarian cells, beta and T cells in the immune system, and astrocytes in the brain [15]. Neuroinflammation, which plays an important role in the etiology of neurodegenerative disorders that cause deterioration in cognitive activities such as anxiety, depression and learning-memory, is closely related to the excessive increase in the expression of these proinflammatory cytokines [12, 13]. In the literature, there are studies reporting improvement in cognitive functions as a result of suppressing or reversing neurodegenerative processes by inhibiting proinflammatory cytokines, especially TNF-alpha, with plant-derived [16] or different chemicals [17]. Adalimumab (Ada), a recombinant monoclonal human antibody, is a tnf-alpha inhibitor, it can exert its effect in two ways, binds directly to tnf-alpha, preventing it from binding to its receptor or it dissolves to avert TNF-alpha from binding with cell surface receptors and inhibits the functions of TNF-alpha by binding to its receptors [18, 19]. Ada cannot cross the blood-brain barrier, but a recent study has shown that the TNF-alpha inhibitor, which cannot cross the blood-brain barrier, is as effective as its analogue that can cross the blood brain barrier [20]. The number of studies showing the positive effects of Ada use in critical pathologies where inflammation plays an important role is increasing, and Ada has also been proven to be effective in cognitive disorders due to neuroinflammation [17, 19]. Therefore, we hypothesized that Ada would be effective in the "chemobrain" model, which develops due to chemotherapeutic use and in which neuroinflammation participates. In addition, we could not find any study in the medical literature showing the effect of Ada on methotrexate-induced anxiety and depression-like behaviors in mice.

With this background, this study was planned to evaluate the effect of Ada, a TNF-alpha inhibitor, on anxiety-like and depression-like behaviors in a chemotherapeutic drug-induced "chemobrain" mouse model, as well as histopathological changes in the brain hippocampal region.

2. Material and Methods

2.1. Experimental Animals and Design

In this study, 10-week-old male Swiss Albino mice obtained from Aksaray University Experimental Animals Unit were used. Mice weighed an average of 32.29±2.03 g, groups were formed with 6 mice in each cage, and the mice were taken to the cages 10 days before the study and adapted to each other. Throughout the study, mice were kept in a quiet and noise-free environment and standard laboratory conditions (light-dark period, 55% humidity, 22-24 °C temperature), with unlimited access to water and food. The experimental procedure was carried out with care and attention, in accordance with the care and use guidelines of experimental animals, after the approval of Aksaray University Experimental Animals Local Ethics Committee (Ethics Committee Date and Number: 19.09.2022-44). The study consisted of a total of 4 groups, with 6 mice in each group; the first group is considered as the control group, no drug was administered, the second group is the Mtx group; A single intraperitoneal dose of 40 mg/kg methotrexate was administered 24 hours before the behavioral tests. The third group is Mtx plus Ada; It consisted of mice administered both methotrexate and Ada. The fourth group is Ada; Two doses of 10 mg/kg Ada were administered intraperitoneally as described above.

2.2. Drug administration

All the drugs used in our study were prepared fresh just before use and the remaining drugs were evaluated as medical waste. Mtx (Metoart Con, Kocak Farma Drug and Chemical Industry A.Ş. Istanbul, Turkey) was administered intraperitoneally as a single dose of 40 mg/kg, since it is the lowest dose of Mtx that causes depression and anxiety-like behaviors in mice [12]. While the half-life of Ada (Humira, AbbVie Tıbbi İlaçlar San. Tic. Ltd. Şti, Turkey) is about 2 weeks in humans, this period is shorter in rodents [21]. For this reason, Ada was administered at a dose of 10 mg/kg[22] in two doses, 5 days apart (1 hour and 5 days before Mtx injection). The drugs were diluted with physiological saline, mice not treated with Mtx and/or Ada were injected with the same volume of saline to avoid the placebo effect. Behavioral tests were started 24 hours after MTX administration, with a 2-hour break between each test. 2 hours after the end of behavioral tests, brain tissues were removed from all mice under end-stage anesthesia (ketamine & xylazine) and immediately placed in 10% formaldehyde solution.

2.3. Open Field Test (OF)

The OF test was achieved to evaluate the anxiety-like behaviors and locomotor activities of the mice. The OF arena consisted of an empty 40 x 40 x 40 cm box made of water and chemical resistant wood, and the albino mice were contrastingly dark. Before the study, the floor of the open field arena was marked as 16 equal squares, 4 squares in the center and 12 squares on the side were coded as perimeters. Each mouse was placed in the middle of the arena and allowed 5 minutes to explore the area. Time spent in the central zone, vertical movements (Rearing count; mice standing up on their hind legs) and horizontal movements (crossing number; at least three paws in the same square) were evaluated [23]. After each experiment, the OF arena was cleaned so that residual odor did not affect the next mouse.

2.4. Elevated Plus Maze (EPM)

In our study, the EPM paradigm was used to measure anxiety-like behaviors. The EPM arena consisted of 4 arms, 35x5 cm in length, two of which were open arms and the other two closed arms. These 4 arms were combined with a 5x5 cm square in the center, giving the arena a cross shape. The closed arms were open at the top and had 36 cm high walls. The arena was positioned 45 cm above the floor. Each mouse was placed in the square in the middle of the arena, facing the open arm, and their behavior on the EPM arena was recorded for 5 minutes. During this time, the total time spent in open arms and the number of entries in each arm were measured [24].

2.5. Tail Suspension Test (TST)

The TST was achieved to assess the depression-like behavior of the mice. For this purpose, in the compartment made of black material resistant to water and chemicals, there was a hook for hanging each mouse by its tail with the help of an adhesive tape. Four mice were able to do this test at the same time, but the mice were prevented from seeing each other with a wall placed between them. The mice suspended by their tails were video-recorded for 6 minutes, during which time they remained motionless was recorded. Moments when the mice remained passive, made no effort to escape, and remained completely motionless were considered as immobility.

2.6. Histopathological examination of brain tissues

As part of the histopathological analysis, mouse brains were stored in 10% formalin for fixation for 48 hours. After the fixed brain tissues were washed in water overnight, blocks were prepared by performing routine histological procedures. Sections of 4 μm thickness were taken from the pre-prepared blocks using microtone and Hematoxylin-Eosin (H&E) staining was applied. Histopathological analyzes were photographed with a computer-assisted microscope (Olympus Cx 43; Japan) and reviewed by the histologist.

2.7. Statistical analysis

All data from the study were presented as mean ± SD. One-way analysis of variance and post hoc test were used to determine statistical differences between groups (ANOVA and TUKEY)

The differences observed when the p was less than 0.05 were considered significant.

3. Results

3.1. Effect of Ada on locomotor activity in a mouse chemobrain model

In our study, OF was used to evaluate locomotor activity. In the statistical analysis between the groups, no significant discrepancy was found in locomotor activity evaluated by crossing number (Figure 2A, $p>0.05$). Thus, it can be said that the cognitive data obtained from all mice are independent of locomotor activity.

3.2. Effect of Ada on anxiety-like behaviors in a mouse chemobrain model

Anxiety-like behaviors were assessed using the OF and the EPM test. The time spent in the central area of the mice that received only Mtx injection was found to be lower than the control mice (Figure 2B, $p<0.001$) in the OF test. This indicates that Mtx administered at a dose of 40 mg/kg 24 hours before the behavioral test causes anxiety-like behaviors. Ada treatment improved mtx-induced reduced time spent in the central area of the OF test (Figure 2B, $p<0.001$). The findings in mice receiving only Ada treatment were similar to the control group. Mice standing on their hind legs and looking around in the open field arena is considered exploratory behavior (Rearing number) 23. In our study, no significant difference was found in terms of the number of rearing (Figure 2C, $p >0.05$).

In the EPM test, mice that received an injection of Mtx had a shorter open-arm stay compared to the control

(Figure 3A, p<0.001). The decrease in the open arm time in this test is associated with anxiety-like behaviors 24. In mice receiving Mtx, Ada treatment improved their time in the open arm (Figure 3A, p<0.001). This time was higher than for mice in the Mtx group, but not enough to reach the level of mice in the control and Ada group (Figure 3A). In the EPM test, the number of open arm entries was similar in the control and Ada groups, and it was found to be significantly higher than the Mtx and Mtx plus Ada groups. (Figure 3B, p>0.05).

3.3. Effect of Ada on depression-like behaviors in a mouse chemobrain model

The TST was used to evaluate depression-like behaviors in the mouse chemobrain model induced by Mtx. In this test, all mice are hung from their tails to a special apparatus and video recording is made for 6 minutes. Immobility time is used as a measure of depression-like behavior. In our study, it was found that mice in the Mtx group showed higher immobility time than the control group (Figure 4, p<0.001). This result suggests that Mtx injection induced depression-like behavior in mice. On the other hand, Ada treatment administered in two doses 1 hour and 5 days before the Mtx injection resulted in the improvement of depression-like behaviors (Figure 4, p<0.001). In the Ada-only group, the immobility time was the same as in the control and Mtx plus Ada groups (p>0.05), but lower than the mice in the Mtx group (Figure 4, p<0.001).

3.4. Effect of Ada on histopathological changes in brain tissue

When the H&E staining results were evaluated, it was seen that the arrangement and density of neuron cells in the hippocampus region of the mice in the control group were normal. As a result of histological examination in the dentate gyrus (DG) and corpus ammonis (CA) regions, the morphology of the neurons was pyramidal, healthy and easily distinguishable (Figure 5A). When the hippocampus images of the mice in the Mtx-treated group were examined, the pyramidal cells were sparse, irregular, and the images were unclear compared to the control. In addition, degenerative and atrophic changes were observed in neurons in this group (Figure 5B). In the groups given Mtx and Ada together, it was observed that the pathological changes related to Mtx decreased and the number of neuron cells increased. Improvements were detected in neuron and glial cells in mouse hippocampus in this group (Figure 5C). There was no significant change in any of the neurons belonging to the hippocampus parts of the groups that were given only Ada, and it was determined that they showed a characteristic arrangement close to the control.

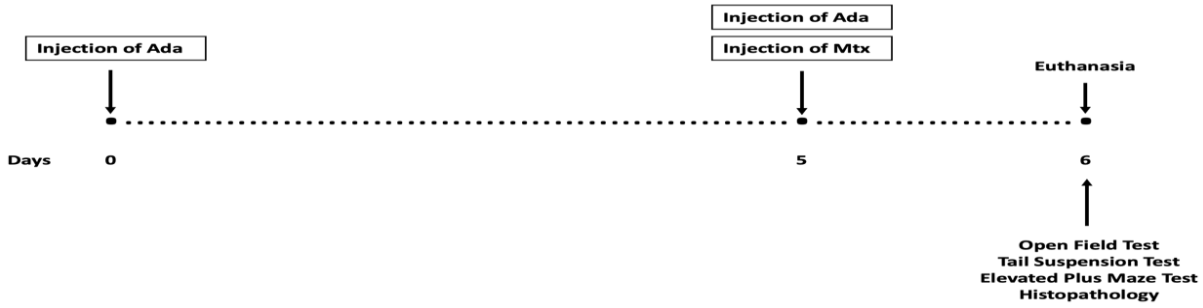
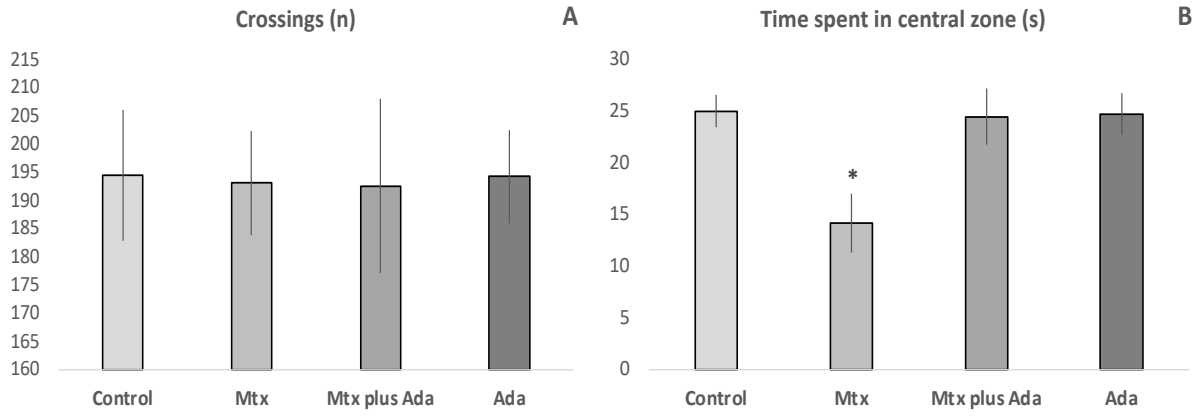


Figure 1: Schematic presentation of the protocol used for the study.



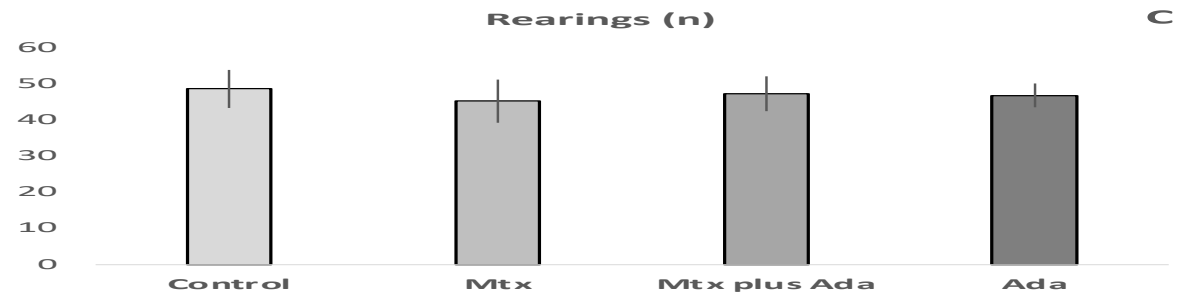


Figure 2: Effect of Ada on (A) time spent in central zone (B) number of rearing (C) number of crossing in the open field test in mice. The data are expressed as the Means±SD. Asterisk (*) indicates significance compared with other groups. p<0.001; one-way ANOVA.

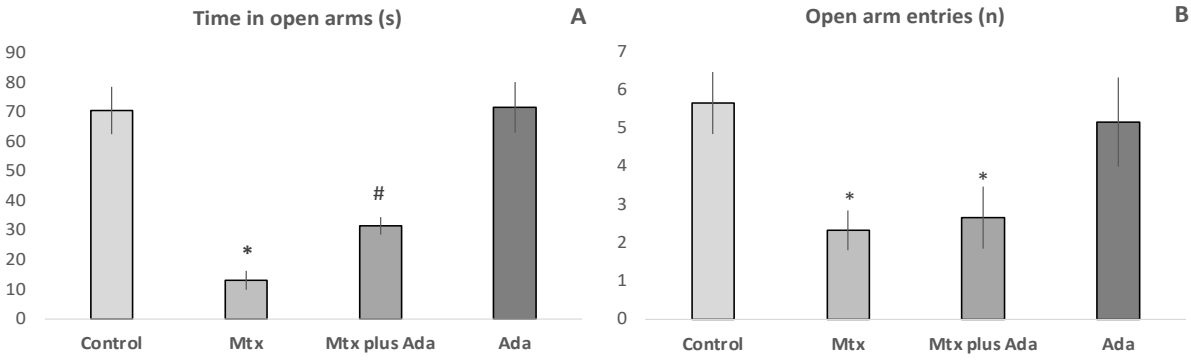


Figure 3: The effects of Ada on open arm time (A) and open arm entries (B) in the elevated plus maze test. Data presented as means ± SD. Asterisk (*) indicates significance compared with other groups. Hash (#) indicates significance compared with other groups. p<0.001; one-way ANOVA.

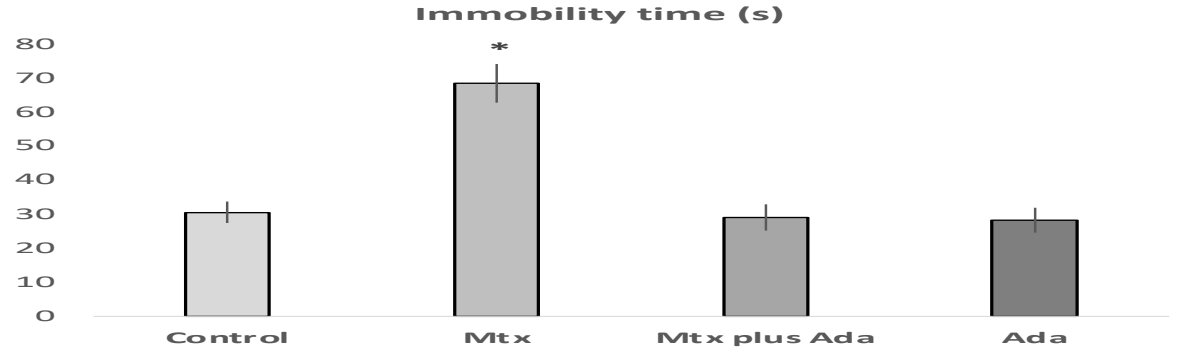


Figure 4: Changes in the immobility time in tail suspension test among each group. The data are expressed as the Means±SD. Asterisk (*) indicates significance compared with other groups. p<0.001; one-way ANOVA.

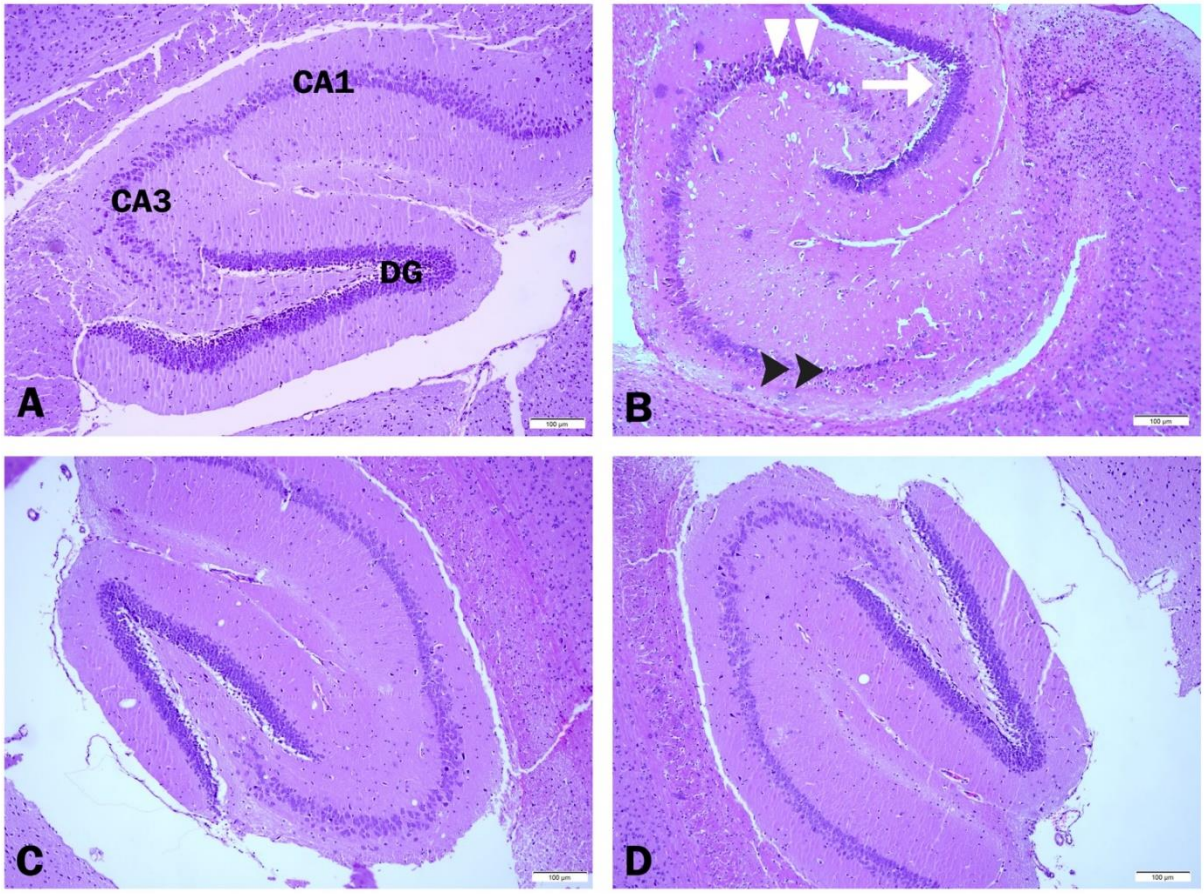


Figure 5: Light micrographs of brain hippocampus regions in mice. A) Control group; Corpus Ammonis 1 (CA1), Corpus Ammonis 3 (CA3) and Dentate Gyrus (DG) regions in normal morphology, (B) Mtx group; Neuronal degeneration in hippocampal region (white arrowhead), decrease in neuronal cells in CA3 region (black arrowhead), dentate gyrus structural disorder (arrow), (C) Mtx plus Ada group, (D) Ada group; H&E staining; scale bar=100 microns.

4. Discussion

In our study, the protective effects of Ada, a TNF-alpha inhibitor, on methotrexate-induced anxiety and depression-like behaviors were investigated. Administration of methotrexate to mice caused anxiety-like behaviors such as decreased time spent in the central zone in the OF, decreased time spent in the open arm and the number of open arm entries in the EPM, and depression-like behaviors such as increased immobility time in the TST. Ada administered in two doses before methotrexate injection increased the time spent in the central zone in the open field test, in the open arm in the EPM, and decreased immobility time in the TST. These results suggest that Ada may be a useful instrument to ameliorate mtx-dependent anxiety and depression-like pathologies.

One of the side effects of chemotherapeutic drugs that we cannot avoid in the treatment of malignant diseases is the deterioration of cognitive functions, which is called chemobrain [1]. Because cognitive impairment due to chemotherapeutic use negatively impacts post-survival life, researchers often use experimental animal models to better define the precise mechanism of this physiopathological phenomenon. Mtx, a folate inhibitor, is one of the chemotherapeutics commonly used for this purpose, causing cognitive dysfunction in both rats and mice [5, 12]. Mtx treatment causes mice to underperform in new object location and novel object recognition tests assessing spatial and recognition memory [6, 9, 11], and in the Morris water maze test, a well-established learning and memory paradigm in experimental animals [5, 7, 11], as well as the passive avoidance test, a hippocampal learning paradigm [12]. Mtx is not only acutely effective as in the well-organized studies mentioned above, but it has also been reported that cognitive deterioration still continues in the chronic period [25] even after 6-16 months [26] after the end of mtx administration. In addition to negatively affecting learning and memory performance, Mtx also affects depression-like behaviors. It has been shown that Mtx administered as a single dose of 40 mg/kg intraperitoneally leads to more immobility time in the forced swimming test, which is a test evaluating depression-like behaviors [12]. In our study, behavioral tests were performed 24 hours after injecting a single dose of 40 mg/kg Mtx into mice. Mtx decreased the time spent in the central zone in the OF test (Figure 2B), the time spent in the open arm in the EPM test (Figure 3A), while it increased the immobility time in the TST test (Figure 4). These results suggest that MTX induces anxiety and depression-like behaviors, respectively. There was no statistically significant difference

between all groups in locomotor activity evaluated with the OF test (p> 0.001, Figure 2A), so the results obtained from our study are independent of locomotor activity. The first limitation of our study is that we did not evaluate well-defined learning and memory impairment in Mtx-related chemobrain models. However, we thought that targeting the physiopathology of anxiety and depression, which may adversely affect the pathogenesis of the disease due to its comorbid relationship with the disease, may contribute to better management of complications.

Possible mechanisms of cognitive impairment due to Mtx are various, such as decreased hippocampal tissue antioxidant enzyme activity and increased lipid peroxidation [6], decreased hippocampal neurogenesis [5, 10, 12, 25], and impaired cell proliferation in the hippocampal dentate gyrus [9–11]. In addition, a growing body of evidence indicates that neuroinflammation plays an important role in cognitive impairment due to Mtx [9–11]. The increase in proinflammatory cytokines responsible for neuroinflammation due to chemotherapeutic drugs has led to an increased interest in studies that can improve cognitive dysfunctions by suppressing these cytokines. We evaluated the effect of Ada, a tnf alpha inhibitor shown to ameliorate neuroinflammation in an experimental model of chronic cerebral hypoperfusion [17], on chemotherapy-induced anxiety and depression-like behaviors associated with increased proinflammatory cytokines. According to the findings of our study, Ada administered twice intraperitoneally (1 hour and 5 days before Mtx injection) at a dose of 10 mg/kg improved Mtx-related anxiety and depression-like behaviors. Ada increased the time spent in the central zone in the OF test (Figure 2B, p<0.001), the time spent in the open arm in the EPM test (Figure 3A, p<0.001), and decreased the immobility time in the FST test (Figure 4, p <0.001). Our results are consistent with similar studies, Ada improves cognitive functions by increasing hippocampal tissue BDNF level, decreasing TNF alpha and IL-6 levels in Alzheimer mouse model [19], reducing oxidative stress and inhibiting NF-κB signaling in chronic cerebral hypoperfusion model [17]. Although we have shown that Ada inhibits impaired anxiety and depression-like behaviors in the chemotherapy-induced chemobrain model, the second limitation of our study is that a physiopathological parameter related to the mechanism of this effect was not evaluated. However, Ada ameliorated the reduction in neuronal cells, neuronal degeneration, and atrophic changes in the CA3 region of the hippocampus due to methotrexate (Figure 5). In addition, this report is the first study in the literature to show the effect of Ada on anxiety and depression-like behaviors in an Mtx-induced chemobrain model.

5. Conclusion

Based on the results of this study, adalimumab supplementation prevented impaired anxiety and depression-like behaviors in a mouse model of Mtx-induced chemobrain. It also improved hippocampal tissue degenerative and atrophic changes. This suggests that Ada may be a therapeutic agent, especially in neurodegenerative disorders accompanied by neuroinflammation causing anxiety and depression.

Limitations of the Study

The main limitations of our study are that the learning and memory activities of the mice were not evaluated after the chemobrain model was created, and that tnf-alpha levels were not detected both in the brain tissue and in the blood. In addition, the anxiety and depression-producing mechanism of MTX and the inability to study the mechanism of the therapeutic role of Ada in this effect are other limitations.

Acknowledgement

None.

Conflict of Interests

There is no conflict of interest.

Financial Support

This study received no financial support.

Author Contributions

Conseption-Oz M; Design-Oz M; Supervision-Oz M; Materials-Oz M, AKARAS N; Data Collection and/or Processing-Oz M, AKARAS N; Analysis and/or Interpretation-Oz M, AKARAS N; Literature Review-Oz M, AKARAS N; Writing-Oz M; Critical Review-Oz M, AKARAS N.

Ethical Approval

The study was approved by Aksaray University Experimental Animal Ethics Committee (Approval Date: 19.09.2022 and Approval number: 44).

Data sharing statement

None.

Consent to participate

None.

Informed Consent

None.

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