Determination of experimentally given homocysteine causes to alzheimer-like dementia in rats on the basis of different parameters

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Abstract
Alzheimer’s disease (AD) is the most seen form of neurodegenerative disease with specific pathological findings like; senile plaques (CPs), synaptic loss, neurofibrillary tangles (NFTs) and neurodegeneration. Homocysteine is a naturally occurring amino acid and have a role in the body’s methylation process. In this study, total 12 rats, were used and divided into 2 groups. Group 1 Control group (n = 6): No rats were treated. Group 2 Application of High-Dose Homocysteine (n = 6): For 5 weeks, 4 mg / kg / day; to create an experimental Alzheimer model. Morris Water Maze test, the MDA, homocysteine (Hcy), reduced glutathione (GSH) and oxidised glutathione (GSSG) levels in rat liver, serum and erythrocyte samples were studied. Mitogen activated protein kinase (MAPK) and beta-secretase (BACE1) mRNA levels and pathological examinations were performed in rat hippocampus samples. The reaching time to exit platform was longer in the Hcy group according to Morris Water Maze test. Neurodegenerative areas were observed in the hippocampus CA1 region of Hcy group. Liver and serum Hcy and MDA levels and liver and erythrocyte GSSG levels were significantly higher (p <0.01) while GSH levels were lower in the liver and erythrocyte samples compared to control (p <0.01). MAPK and BACE1 mRNA levels of the hippocampus were significantly higher in the Hcy group (p <0.01). Different studies results indicate that; increased plasma homocysteine level was found to be a strongly independent risk factor for the progress of dementia and Alzheimer’s disease. However, there is also contradictory evidence, and it is still controversial whether HHcy is a risk factor for AD or just a bio producer. This study was carried out to determine homocystein role in Alzheimer process. According to findings It is very important to keep homocysteine level in lower grade to reduce or eliminate AD possibility.

Keywords: Homocystein, alzheimer, dementia and markers

Introduction
Dementia is a general term and nomenclature used for progressive organic diseases that lead to inability to perform one’s daily activities, loss of intellectual abilities and serious memory loss. Dementia is the damage to the cells in the brain called neurons. Depending on the severity of the damage, the neuron dies or loses its ability to perform normal functions. This can change the person’s memory, behavior, abilities and thoughts. Doctors usually diagnose according to the Diagnostic and Statistical Manual of Mental Disorders (DSM). In 2013, the American Psychiatric Association (APS) categorized dementia as major and mild cognitive disease in the 5th edition of the DSM [1].

Globally, more than 26 million people have been diagnosed with AD. The majority of the group is over 65. The incidence of the disease in people above 65 years of age increases logarithmically. The estimated frequency in 2050 for this period getting shorter and the occurrence and progression of this disease will be lower to every 33 seconds. Considering the fact that the AD mostly affects individuals over the age of 65, a small change such as 1 year from the onset of the disease means that there will be less than 9.2 million fewer cases by 2050 and less cost expenses for this disease. There is tremendous effort to identify risk factors for AD and to improve treatment pathways [2].

The specific pathological findings of Alzheimer’s disease which has been defined for more than 100 years are as follows; senile plaques (SPs), neurofibrillary tangles (NFTs), synaptic loss and neurodegeneration. Senile plaques are composed of amyloid-β (Aβ) and are surrounded by microglia, the primary immune effector cell in the central nervous system.

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Brain images of healthy person and person with AD [3].

**Homocystein (Hcy)**

In 1932, Burtz and du Vigneaud discovered a new amino acid with the treatment of methionine amino acid with sulfuric acid. Du Vingneaud, who has investigated the coline and homocysteine instead of methionine, which is essential for the growth of animals, had little information about the role of homocysteine in vascular diseases in the 1950s. It has been suggested that homocysteine is associated with atherosclerosis in monkeys in later years [4].

Homocysteine (HSCH2CH2CH [NH2] CO2H) is a form of methionine, an essential amino acid containing sulfur.

![Structures of the sulfur-containing amino acids](image)

Structures of the sulfur-containing amino acids [5].

Hcy is metabolised by two important catabolic pathways: remethylation and transsulfuration. When methionine-rich protein is taken up, the transsulfuration pathway is activated, which develops in the direction of reducing the Hcy level. In the protein-free diet, the remethylation pathway of Hcy is activated.

![Hcy metabolism](image)

Figure 1. Hcy metabolism. Converting to methionine via remetilation and into cysteine via transsulfuration [6]

Hcy metabolism depends on folic acid, vitamin B6 and B12 levels. Deficiencies of folic acid, vitamin B6 and B12 cause to disfunction of Methylene tetrahydrofolate reductase (MTHFR), GIS and MS enzymes and as a result Hcy metabolism disorders occur. Hcy causes the accumulation of Hcy in tissues known as hyperhomocysteinemia (HHcy) after disruption of metabolism. HHcy; can be classified as light, moderate and severe form. Hyperhomocystein (HHcy) levels induce neurological abnormalities such as cerebral atrophy and seizures. Deficiency of vitamins leads to increased levels of homocysteine in which AD is involved in vascular mechanisms [7].

According to different studies, elevated levels of homocysteine are found related to Alzheimer’s, dementia, poor concentration, diminished memory, reduced judgment and mood. Also these studies results indicate that; increased plasma homocysteine level was found to be a strongly independent risk factor for the progress of dementia and Alzheimer’s disease. When the Hcy level is higher than 14 μM, the risk of AD almost reach to twice in people over 60 years of age. However, there is also contradictory evidence, and it is still controversial whether HHcy is a risk factor for AD or just a bio producer [8].

The aim of treatment of classic homocysteinuria is to decrease the accumulation of homocysteine. This study was carried out to determine homocystein role in Alzheimer process. For this aim; homocysteine (Hcy) used as an oxidant and was given in a high-dose to perform experimental Alzheimer model and was carried out to see and put forth Hyperhomocysteinemia (HHcy) effect and its possible result similarities with AD.

**Material and Methods**

**Animals**

In this study, total 12 rats, with 3-3.5 months and 280-320 g Sprague-Dawley male rats were used. The animals were obtained from the Animal Experiments Units of Afyon Kocatepe University. The rats were fed a standard diet and there was no restriction on drinking water. Environmental conditions were kept under control in all experimental groups (12 hours / 12 hours light / dark cycle, 25-28 ° C ambient temperature).

According to the study procedure, rats were divided into 2 main groups as described below.

**Group 1 Control group (n = 6):** No rats were treated in the control group and their normal daily lives were preserved.

**Group 2 Application of High-Dose Homocysteine (n = 6):** For 5 weeks, 4 mg / kg / day homocysteine was applied by mixing the drinking water of rats.

In order to create an experimental Alzheimer model, homocysteine (Hcy) as an oxidant was used daily for 5 weeks in drinking water and replaced with new one every day.

Alfamin were administered to all rats intraperitoneally (i.p) 24 hours after the last loading. Rats were fasted for approximately 12 hours before the operation. The rats were placed on their back and the operation table was located on the four extremities. After opening the abdomen, approximately 5 ml blood were taken with heparinized syringe by intracardiac method and placed in heparinized tubes after that rats were decapitated.
Preparation of erythrocyte package: Heparinized blood is centrifuged at 3000 rpm for 5 minutes at 4 degrees. The plasma and the white blood cell layer are aspirated. It is then treated three times with isotonic NaCl and centrifuged. After each centrifugation, the supernatant is aspirated to obtain the erythrocyte package. The obtained plasma samples and erythrocyte samples were taken in separate tubes and stored at -20 °C until the working day.

Liver tissue samples were weighed 0.2 g of tissue samples after tissue collection and homogenized for 1 minute at 24,000 rpm with Ultra Turrax (IKA Works, USA) brand homogenizer by adding 2 ml of 0.1 M pH: 7.4 phosphate buffer. The homogenates were then subjected to a 1 minute ultrasonication of 20,000 cycles / sec with a Dr. Hielscher (Germany) sonicator. Subsequently, the homogenates were separated by supernatant at 10,000 g by 15 centifugation and stored at -20 degrees until work. GSH and MDA levels were studied from liver tissue samples. The results are given per gram of protein.

Pathological Examination
The removed brains were cut into longitudinally between the right and left hemispheres and divided into two equal parts. All left brain hemispheres were detected in buffered neutral 15% formaldehyde solution for 72 hours. The hippocampal regions were transferred to the cassettes by tissue cutting. The tissues were washed in running water for 8 hours. Tissue was passed through a series of alcohol and xylenes and blocked in paraffin via tissue tracer (Leica TP 1020). 6-8 microns thick sections were obtained by Rotary microtome (Leica RM 2155). Sections stained with Cresyl Violet. The values was evaluated by ZEN imaging software in light microscope (Zeiss Axiolab.A1) with Zeiss ICC 5 camera and microscopically images were taken with digital camera when necessary. The other half sphere, reserved for the study of genetic tests, was embedded in paraffin embedded. 50 µm

Biochemical Analysis
MDA levels (lipid peroxidation) were studied from the plasma and liver samples. GSH (Reduce glutathione) and GSGG (oxide glutathione) levels were studied from erythrocyte tissue and liver samples. All of them were defined with HPLC method using HPLC fluorescence detector kit supplied from the CHROMSYSTEMS Diagnostics (Munich / Germany) (Ex: 385 Em: 515 nm) and the results is evaluated as µmol/L.

Homocysteine (Hcy) levels from rat plasma samples were determined by HPLC using a kit HPLC fluorescent detector (Ex: 385 Em: 515 nm) from Chromsystems Diagnostics (Munich / Germany). The results were evaluated as µmol/L.

Genetic Analysis
The RNA isolation from tissue: The RNA purification (using Thermo Scientific High Pure RNA Isolation Kit FFPET Roche Germany) was obtained from paraffinized left brain hemispheres samples from each group. Primer Design: These primers were used with 05532957001 catalog numbers and Assay ID: 500184 from Roche in the analysis of MAPK1 (R. NORVEGICUS) genes. In the analysis of BACE1 (R. NORVEGICUS) gene, primers numbered 05532957001 and Assay ID: 502827 were used.

Data analysis is performed using the LightCycler 480 instrument channel 465-510. The graph is formed by calculating the changing rates of the target gene mRNA expression levels using 2-ΔΔCt methods and using the values, obtained by relative quantitation analysis (Target gene / reference gene) [9]. The ΔΔCt= (Ct target gene-Ct actb) subject group - (Ct target gene-Ct actb) control group formula was used in the calculations.

Morris Water Maze (Learning Test)
The Morris water maze, developed by Richard Morris, was used to test learning. The Morris water labyrinth developed by Richard Morris is a behavioral experiment designed to test the spatial memory. The Morris water maze is a large, circular tank full of water, with a hidden platform inside. Tank diameter was 120 cm, height 60 cm and water height was 40 cm. The Morris water tank was filled with 24-26 degrees of water. The platform is placed 5 cm deep from the water surface and has a diameter of 10 cm and a distance of 17 cm from the edge of the pool. The top of the platform is covered with a fibrous fabric in such a way that the rats will feel secure and feel safe.

The rats underwent acclimatization before 1 week before the morris water maze test. In the first days they were expected to find the exit platform by themselves. At the end of one minute, the rats who could not find the exit platform were assisted and guided. After they found the platform, the rats were set up, first taken to the transition cage and then put back into their cages. Exercises of the rats in the morris water tank were carried out at the same time and in the same order every day. It was observed that Morris platform could easily find the platform themselves on the 3rd and 4th days of the experiment. In the last days of the trial week, it was observed that the rats could find the exit platform much easier and faster. After 7 days of training week, the rats started the experimental stage. The reason for taking the rats during the training week is to learn the pool of rats and to start the experiment with the same learning degree under the same conditions.

All animals at the same time every day was leaved one to the water and the platform output times are determined at the same time during the 5 weeks work.

Morris water test tank [10].

The statistical analysis
Statistical Package for the Social Sciences (SPSS) 17.0 was used for statistical analysis. Results are expressed as mean ± standard deviation. ANOVA variance analysis was performed to compare the differences between the groups. Kolmogorov Smirnov test was used to test whether the continuous variables were normally distributed and p <0.05 was determined as significance level.

In the pathological examination, continuous and multivariate comparisons between groups were performed using the Kruskal-Wallis test. Mann-Whitney U-test was used to show the difference
between groups according to Kruskal-Wallis test, and values less than p < 0.05 were considered statistically significant.

Result

Homocystein Results
Oxidative effects of Homocysteine (Hcy) level as expected were observed in the rat model of experimental Alzheimer. There was a statistically significant difference in Hcy levels between the control group and the high-dose homocysteine groups in liver tissue samples and serum Hcy levels and the differences were statistically significant (p < 0.01) (figure 2).

Liver and Plasma Homocystein (Hcy) levels

Chai and colleagues in the 14-day study showed that the Hcy application causes to Alzheimer-like effect and these effect formed by Hcy-induced ROS products. It has been reported that betaine decreases this oxidant effect in homocysteine-induced Alzheimer model. Epidemiological studies have shown that in more than 40% of patients, plasma hcy levels are high and cause a rapid neural atrophy. Hhcy levels are therefore an independent risk factor for AD. Lowering high Hcy levels can be a good strategy for AD [4].

In 2014, Li et al. reported that high Hcy levels cause to symptoms of Alzheimer’s disease, leading to memory loss, and high levels of Hcy may contribute to ER stress [11].

Biochemical Results
In our high-dose homocysteine group, tissue and serum MDA results were significantly higher obtained than control (Figure 5). Oxidized GSH (GSSG) levels in liver tissue samples and erythrocyte hemolysate samples were significantly higher in the YDHcy group than in the control group (Figure 4 p < 0.01 respectively). But reducte GSH levels in Erythrocyte and liver samples were significantly lower compare to control (Figure 3 p < 0.001). According to a study by Federico Cacciapuoti in 2013, he mentioned several factors that have an impact on Alzheimer’s disease and he showed HHcy levels as one of them. According to the study, HHCy levels contribute to the development of Alzheimer’s disease by acting on neurons in 2 different ways. One of these was caused by a decrease in the ratio of SAM / SAH, which led to a decrease in the antioxidant GSH levels [7].

Interpretation of biochemical results
In our study, malondialdehyde (MDA) levels (Figure 5) were obtained significantly higher in the high dose Hcy group compared to the control group. Oxidized GSH (GSSG) levels in liver tissue samples and erythrocyte hemolysate samples were significantly higher in the HDHcy group than in the control group (Figure 4 p < 0.01 respectively). On the other hand, liver and erythrocyte GSH levels were statistically lower (Figure 3 p < 0.001). According to a study by Federico Cacciapuoti in 2013, he mentioned several factors that have an impact on Alzheimer’s disease and he showed HDHcy levels as one of them. This indication supporting our finding that GSH antioxidant effect come to forward and lowered compare to control in HDHcy group.
the loss of alzheimer-like memory loss caused by the effect of Hcy’s oxidant effect on the rat hippocampus. The oxidant effect of homocysteine has shown itself from the second week onwards. In the fifth week HDHcy group reaching time to platform is getting decrease compare to earlier weeks because we think that as the weeks progress, the animals get used to the given substance, which leads to some degree of tolerance, although there is still a significant prolongation in time compared to the control, it is seen as an improvement in platform output compared to previous weeks.

**Interpretation of Moris water test result**

At the end of the second week, the first differences were observed between the groups (Figure 6-7 p <0.01). A significant difference was observed between the high Hcy group starting from the 2nd week compared to the control. This result shows that Hcy’s metabolism in the high Hcy group and its oxidant effects on the brain, especially in the hippocampus region, were statistically significant. At the end of the third, fourth and fifth weeks, the difference was more pronounced compare to the the control and it was interpreted that the high efficacy of high-dose Hcy alone was more dominant on the hippocampus and the adverse effects were more severe. This result shows that the effect of Hcy’s oxidant effect on the rat hippocampus caused alzheimer-like memory loss.

There was no difference between the groups in the first week.

![Figure 6. Moris water maze test second and third week results](image1)

![Figure 7. Moris water maze test fourth and fifth week result](image2)

**Results of Pathological Findings**

Histopathological examination revealed significant neuronal pathological changes in the CA1 region of the hippocampus. Nekrobiotic changes were observed in many neurons in varying amounts according to groups in the region. The cytoplasm of these cells was dark blue, shrunken and angular, and the nuclei were smaller and hyperchromatic in a view.

![Figure 8. changes in the CA1 region of the hippocampus according to groups](image3)

For this pathological examination; ZEN imaging software in light microscope (Zeiss Axiolab.A1) with Zeiss ICC 5 camera was used and microscopically images were taken with digital camera. The original magnification was x 50 and the scale bars represent 50 µm.

**Interpretation of pathological results**

As a result of a 22-year prospective study of 1368 female patients by Dimitri E.Zylberstein et al. in 2011, high Hcy levels were reported as an independent risk factor for dementia, especially in people with Alzheimer’s disease. The results of this study are consistent with our results and the results of rat hippocampus pathological examination support these findings as well (Figure 8) [12].

**Hippocampus MAPK and BACE mRNA Levels**

We found that BACE and MAPK activity results were significantly higher in the HDHcy group, indicating higher Aβ results accumulation which is one of the main reason to be AD (Figure 9 a: p <0.01) [13].

**Interpretation of Genetic Results**

MAPK and BACE mRNA levels of hippocampus were significantly higher in high dose Hcy group compare to control in our study. This difference is seen especially for MAPK (Figure 9 p <0.01). High level of ROS induced by Hcy in the hippocampus cause to increased BACE expression and increased Aβ via γ -secretase activity. An increased Aβ and ROS levels leads to Alzheimer effect and these findings were supported by Morris water, pathological examination and genetic results also.

**Discussion**

On the basis of all these informations; it was understood that there is close relationship between elevated homocystein levels and Alzheimer and Dementia. So to support this indication and opinion several different studies were searched and summarized.

In 2012, Andrea Fusco et al. investigated the association between vitamin B deficiency and the mechanism of homocysteine and Alzheimer’s disease. B vitamin deficiency affects the Hcy increase side. This deficiency also results in increased DNA hypomethylation and BACE expression. As a result of this increase, Aβ accumulation, which is one of the leading factors in Alzheimer’s disease, occurs. The results of this study, meaning increased Hcy levels and the resulting increase in BACE expression and thus Alzheimer’s symptoms and effects are consistent with our study results [14].

![Figure 9.](image4)
According to a study by Federico Cacciapuoti in 2013, many factors that have an impact on Alzheimer’s have been mentioned, and HHcy levels have been shown as one of these. According to the study, HHcy levels contribute to the development of Alzheimer’s disease by acting on neurons in 2 different ways. One of them is the decrease in the ratio of SAM / SAH and consequently the decline in neuron functions; the other way is expressed as the destruction of the endothelial cells and the increase in platelet activity. It has been stated that decreasing SAM / SAH ratio affects the antioxidant GSH levels and increasing the harmful effects of ROS products and hence the progression of Alzheimer’s disease. It has also been reported that high Hcy levels contribute to hyperphosphorylation of tau protein by DNA hypomethylation and consequent oxidative damage, which is one of the causes of Alzheimer’s disease. The results of this study were consistent with our study, which is liver and erythrocyte GSSG levels were found to be statistically significant high at high Hcy levels [7].

Another review study indicate that; according to several different epidemiological studies there is a close relationship between hyperhomocysteinaemia and both histologically confirmed AD and disease progression and hyperhomocysteinaemia to be a strong, independent risk factor for dementia and AD. Thus, hyperhomocysteinaemia and AD could be linked by stroke or microvascular disease. Also there is known relations between B-group-vitamin deficiency and both hyperhomocysteinaemia and neurological dysfunction, give a possibility for prevention of AD with dietary modification or food fortification [15].

In the study of Khaled Nazef and colleagues in Algerian Alzheimer’s patients, it was reported that there was a statistically significant high difference between Hcy levels of patients and healthy individuals. These results are in line with our results [16].

Another study based on that Hyperhomocysteineimia (Hhcy) is an independent risk factor for Alzheimer’s disease (AD) and there is positive correlation between significant defect in AD patients and Hhcy. They indicated that Hhcy stimulate memory defect with AD-like amyloid-β (Aβ) and tau pathologies in the hippocampus, and supporting with folate and vitamin B12 prevents these AD-like pathologies. According to these reality and findings of Hhcy; our homocystein level and AD connection [20].

As a result of our study, high levels of HHcy levels in our experimental Alzheimer rats model are important in terms of revealing the destructive effects on cells in the brain tissue, and oxidative effect on cells of the hippocampus region of the brain tissue. On the other hand, further studies are needed to clarify the pathology of Alzheimer’s disease at the molecular level, and to broaden the treatment options and / or preventable pathways.

Conclusions

The complex metabolism of homocysteine within the body is highly dependent on vitamin derived cofactors, and deficiencies in vitamin B12, folic acid and vitamin B6 and raised levels of homocysteine are also linked to Alzheimer’s, dementia, poor concentration, declining memory, judgment and lowered mood. Results from human and animal studies suggest that moderate Hcy elevation especially in the elderly population is a potential risk factor for AD. When the Hcy level is higher than 14 µM, the risk of AD almost reach to twice in people over 60 years of age. According to our findings; an increased Aβ and ROS levels leads to Alzheimer effect and these findings were supported by Morris water maze test, pathological examination and genetic results as
well. Hyperhomocysteinemia (HHcy), the abnormal elevation of blood levels of homocysteine (Hcy), has been proposed to be a modifiable risk factor for AD. It is very important to keep homocysteine level in lower grade to reduce or eliminate AD possibility.

Conflict of interest
The authors declare that there are no conflicts of interest.

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Ethical approval
Consent of ethics was approved by the local ethics committee.

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