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ARAŞTIRMA YAZISI / RESEARCH ARTICLE

AF64A TARAFINDAN ALZHEİMER BENZERİ DEMANS OLUŞTURULAN RATLARDA BETAİN ve PİPERİNİN ETKİSİ

THE EFFECT OF BETAINE AND PIPERINE ON RATS WITH CREATED ALZHEIMER- LIKE DEMENTIA BY AF64A

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ÖZ

AMAÇ: Demans hastalarının büyük çoğunluğu (yaklaşık %60-70) Alzheimer hastalığı (AH)'ndan muzdariptir. AH'nın ayırt edici patolojik belirtileri senil plaklar (SP'ler), nörofibriler yumaklar (NFTS), ve nörodejenerasyondur. Bu çalışmada; nörolojik anomalileri indükleyen Acetylcholine Mustard Aziridin İon (AF64A)'nın neden olduğu hasar ve bu zararın antioksidan piperin ve betain'nin tedavi edici etkisinin belirlenmesi amaçlanmıştır.

GEREÇ VE YÖNTEM: Bu çalışmada; 24 Sprague-Dawley erkek sıçan kullanılmış ve 4 grup oluşturulmuştur: Sağlıklı sıçanlardan oluşan grup 1 (kontrol, n=6); AF64A ile deneysel demans oluşturulan grup 2 (n=6), betain tedavisi yapılan grup 3 (AF64A betain, n=6) ve piperin tedavisi yapılan grup 4 (AF64A piperin, n=6). Hipokampus dokusunda mitojenle aktifleşmiş protein kinaz-1(MAPK-1) mRNA düzeyi, karaciğer ve kan serum örneklerinde Malondialdehit (MDA) düzeyleri ile karaciğer ve eritrosit örneklerinde redükte glutatyon (GSH) düzeyleri araştırılmıştır. İlaveten, morris su labirent testi kullanılarak davranışsal yönden kaynaklanan farklılıklar süre açısından belirlenmiştir.

BULGULAR: Karaciğer ve eritrositlerdeki en yüksek GSH düzeyleri piperin uygulanan grup 4'de belirlenmiştir ($p<0.01$). Karaciğer ve plazma MDA düzeyleri bakımından en yüksek sonuçlar grup 2'de ve en düşük sonuçlar grup 4'de kaydedilmiştir ($p<0.05$). Beyin doku patoloji bulgularında da en iyi sonuçlar piperin uygulanan grupta gözlenmiştir ($p<0.05$). Hipokampüs MAPK-1 mRNA düzeylerinde grup 2'de belirgin artış, grup 4'de ise baskılanma izlenmiştir.

SONUÇ: Belirlenen patolojik, biyokimyasal ve genetik analizler ile davranış testi sonucundaki reaksiyon süresinin uzaması göstermiştir ki, AF64A kullanılması beyin sinir hücresini önemli düzeyde tahrip etmektedir. Fakat özellikle piperin uygulamasının, betainin etkisine kıyasla, tüm negatif işaretlerin kontrol seviyesine getirilmesiyle AF64A'nın hasar verici etkisi üzerinde neredeyse geri dönüşümlü bir etki yarattığı gözlenmiştir. AF64A uygulaması, sıçanlarda önemli düzeyde beyin hasarına yol açarak Alzheimer'a benzer etki yaratmaktadır. Alternatif tedavi kapsamında betain ve piperin uygulamasının AF64A'ün tüm olumsuz sonuçlarını, ki özellikle piperin uygulaması, neredeyse tamamen normal seviyelere indirdiğini göstermektedir. Bu bulgular, demans ve özellikle AH'nda oksidan etkilerin azaltılmasında ve/veya geriletilmesinde antioksidan özellikteki piperin kullanımının faydalı olabileceği sonucunu işaret etmektedir**.**

ANAHTAR KELİMELER: AF64A, betaine, alzheimer, piperin, demans

ABSTRACT

OBJECTIVE: The great majority of dementia patients (about 60-70%) suffer from Alzheimer disease (AD). The distinctive pathological signs of AD are senile plaques (SPs), neurofibrillary tangles (NFTS), synaptic loss and neurodegeneration. In this study; it is aimed to determine the damage caused by Acetylcholine Mustard Aziridin Ion (AF64A), which induces neurological anomalies, and the therapeutic effect of antioxidant piperine and betaine.

MATERIAL AND METHODS: In this study; 24 Sprague-Dawley male rats were used and 4 groups were formed: Group 1 consisting of healthy rats (control, n = 6); Group 2 (n = 6) with experimental dementia induced by AF64A, group 3 (AF64A betaine, n = 6) treated with betaine and 4 (AF64A piperine, n = 6) treated with piperine. The mRNA levels of mitogen activated protein kinase-1 (MAPK-1) in hippocampus tissue, Malondialdehyde (MDA) levels in liver and blood serum samples and reduced glutathione (GSH) levels in liver and erythrocyte samples were investigated. In addition, behavioral differences were determined in terms of duration using the morris water maze test.

RESULTS: The highest GSH levels in liver and erythrocytes were determined in piperine-treated group 4 ($p <0.01$). The highest results were recorded in group 2 and the lowest results were recorded in group 4 ($p <0.05$) in terms of liver and plasma MDA levels. The best results in brain tissue pathology findings were also observed in the piperine applied group ($p <0.05$). There was a significant increase in hippocampus MAPK-1 mRNA levels in group 2 whereas a decrease in group 4.

CONCLUSIONS: Determined pathological, biochemical and genetic analyzes beside the longest reaction time in the behavior test result showed that the use of AF64A significantly destroys the brain nerve cell. But especially piperine treatment create almost reversible effect onto AF64A damaging act via bring down all negative signs into control level compare to the betaine effect. AF64A application causes a significant level of brain damage in rats, creating a similar effect to Alzheimer's. As an alternative treatment, it shows that the application of betaine and piperine reduces all the negative consequences of AF64A, especially the application of piperine, to almost completely normal levels. These findings indicate that the use of antioxidant piperine may be beneficial in reducing and/or regressing oxidant effects in dementia and especially in AD.

KEYWORDS: AF64A, betaine, alzheimer, piperine, dementia

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INTRODUCTION

Alzheimer's disease (AD) was defined as presenile dementia for the first time by German psychiatrist Alois Alzheimer in 1906. The main pathological signs of AD are synaptic loss, senile plaques (SPs), neurofibrillary tangles (NFTS) and neurodegeneration. The progressive cognitive deficits in AD is associated with the synaptic and neuronal loss (1-3).

Dementia occur via damage of neuron cells exist in the brain. This situation results in the loss of person's memory, behavior, abilities and mind. The large majority group of AD is over 65 years old (4,5).

Studies on twins and families showed that the genetic factors play an important role in at least 80% of AD patients. The dominant genes, like the amyloid precursor protein gene (APP gene); the presenilin-1 (PSEN1) gene and the presenilin-2 (PSEN2) gene have an trigger effect on the genetic mecanism of AD. It has been suggested that; when the PSEN1 is mutated, the pathological characteristics of AD such as the memory loss, proliferation of amyloid beta (Aβ) plaques, the formation of neurofibrillary tangles and the disruption of calcium homeostasis emerge. It is known that the activated mitogen activated protein kinases (MAPK) signaling pathways promote the pathogenesis of AD through various mechanisms such as equilibration and phosphorylation of APP. It has been shown that APP and beta-secretase 1 (BACE1) codes a transmembrane protease, which has catalyzes the first step in the formation of amyloid beta peptide from amyloid precursor protein. Amyloid beta peptides are the main constituent of amyloid beta plaques cause a devastating effect on memory loss in mice and indicating Aβ has an important role in learning and memory (6-9).

It has been reported that the cholinergic neuron systems have an important role in occurrence of cognitive impairment, associated with aging and neurodegenerative diseases. There was a strong correlation between AD and learning disability, memory and cholinergic deficit. In this study; we used Acetylcholine Mustard Aziridin Ion (AF64A) as an selective colinotoxin **(Figure1 1)**.

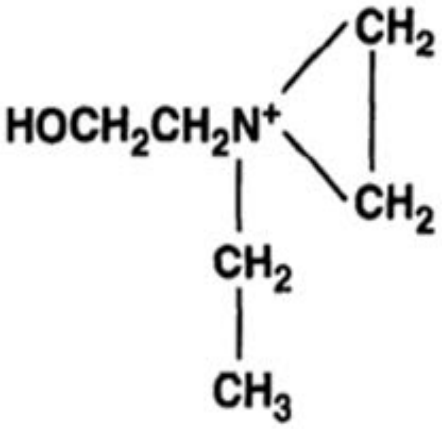

Figure 1: The molecular structure of AF64A (10)

Also AF64A cause the striking decrease into reduced glutathione (GSH) levels. GSH, produced endogenously, serves to limit of cellular toxicity in response to in vivo oxidative stress. AF64A can generate free radicals and can trigger oxidative stress via lipid peroxidation, causing to cellular damage (10-12).

In our study; the betaine and the piperine were used as an antioxidant treatment agents aiming to repair of AD mechanism' destroy effect on neurons.

The betaine (N, N, N, are -trimetilglisin) play a role as a methyl donor in a pathway of Betaine Homocysteine Methyl Transferase (BHMT) mechanism (**Figure 2)**.

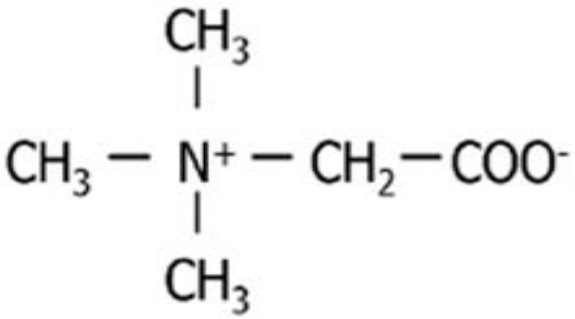

Figure 2: Betaine structure (15)

The betaine can be obtaine from the wheat, spinach and beet at higher levels [13-15]. The protective efficiency of herbal products, especially the spices on overall health, is good as well as their anti inflammation, antimicrobial, chemoprotective and neuroprotective effects have an greater importance onto digestive system. Among the various spices, the black pepper (Piper nigrum L.) and its active ingredients can be used as a supporting agent for treatment va-

rious diseases associated with central nervous system such as depression, AD, epilepsy etc. Piperine is present in 5-7% in black pepper (16-18) **(Figure 3)**.

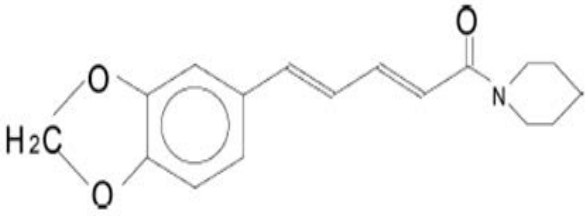

Figure 3: The chemical structure of Pipperine (16)

This study were performed to determine the therepeutic effects of piperine and betaine on Alzheimer's disease created by AF64A in rats.

MATERIALS AND METHODS

In this study; 24 Sprague-Dawley male rats with each 3-3,5 months old and 280-320 g weight were used. Rats were randomly divided into four groups;

Group 1 (n = 6): Control
Group 2 (n = 6): AF64A
Group 3 (n = 6): AF64A + Betaine
Group 4 (n = 6): AF64A + Piperine

AF64A was applied at a dose of 2 nmol/2µl/day for 5 weeks (from 1 µmol/L stock solution 2 µl AF64A for each rats were given via adding into drinking water). In addition to AF64A administration of 2 nmol/2ul/day to rats, betaine was added to drinking water at 12,5/kg/day for 5 weeks. In addition to 2 nmol/2ul/day AF64A administration, 10 mg/kg/day piperine was added to drinking water for 5 weeks.

ISTATISTICAL ANALYSIS

The obtained plasma samples and erythrocyte packets were stored at -20 degrees until the working day. MDA levels (as a marker for lipid peroxidation) were studied in plasma and in liver samples. GSH levels were studied in erythrocyte tissue and liver samples. Both of them were analysed with HPLC method using HPLC fluorescence detector kit supplied from the CHROMSYSTEMS Diagnostics (Munich/Germany) (Ex: 385 Em: 515 nm) and the results were given as µmol/L.

PATHOLOGIC EXAMINATION

The all left brain hemisphere were excised. The hippocampal region of tissue was blocked in paraffin and stained with Cresyl Violet. The results were evaluated on light microscopy, using 5 ICC Zeiss camera and ZEN software imaging (Zeiss axiolab.a1) programme.

GENETIC ANALYSIS

The RNA isolation and Real Time QPCR Analysis: The total RNA was isolated (using Thermo Scientific High Pure RNA Isolation Kit, Roche, Germany) in paraffined left brain hemispheres samples from each group. 30 mg of tissue were weighed into RNAase free ependorfs. 300 µl of Lysis Buffer Solution and 600 µl of Proteinase K solution was used with duplication of react with wash buffer 1 and 2. The isolated RNAs were kept at -70 ° C and the process was completed.

cDNA Sythesis: The cDNA synthesis was done with reverse transcription method using a kit (Transcriptor High Fidelity cDNA Synthesis Kit, Germany). After 2 µl cDNA samples were distributed to the wells on the real-time microplate, real-time PCR was performed on the Light cycler Roche 480 instrument following the reaction steps.

Primer Design: The primers of ACTB gene were used with 05532957001 catalog number and assay ID: 500153 from Roche The primers of MAPK1 gene were used with 05532957001 catalog number and assay ID: 500184 from Roche **(Table1)**.

Table 1: Primer sequences

	Primer	Length	Position	Tm	%GC	Sequence
MAPK-1	Left Primer	20	156-175	60	50	gcgcttcagacatgagaaca
	Right Primer	20	205-224	59	45	ttcatctgctcaatggttgg
	UPL 63	8		72		5'-Fam-G+G+CA+T+CAA-Tamra-3'
		Amplication (69nt)				
		gcgcttcagacatgagaacatcatcggcatcaatgacatcatccgggcaccaaccattgagcagatgaa				
ACTB	Left Primer	19	46-64	59	58	ctggctcctagcaccatga
	Right Primer	20	102-121	60	50	tagagccaccaatccacaca
	UPL 63	8		72		5'-FAM-ct+c+ct+c+ct-Tamra-3'
		Amplication (76nt)				
		ctggctcctagcaccatgaagatcaagatcattgctcctcctgagcgcaagtactctgtgtggattggtggctct				

MAPK-1: Mitogen-activated protein kinase-1; ACTB: Actin Beta gene

Amplification was performed according to the manufacturer's instructions **(Table 2)**.

Table 2: PCR protocol

Parameter	Denaturation	Amplication			Cooling
Analysis mode	No	Quantification Mode			No
Cycle	1	45			1
Target [°C]	95	95	60	72	40
Time [hh: mm: ss]	00:10:00	00:00:10	00:00:30	00:01:00	00:00:30
Ramp. Rate [°C/s]	20	20	20	20	20
Acquisition Mode	No	No	No	Single	No

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 method and relative quantitation (Target gene/reference gene) results were obtained (19). 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 test (Learning test): Morris water maze test, developed by Richard Morris was used to analyse the learning (20,21) . This system has a wide circular tank, filled with water, containing a hidden platform.

The statistical Analysis: The Statistical Package for the Social Sciences (SPSS) 17.0 were used for the statistical analysis. The results are expressed as mean ± standard deviation. ANOVA analysis was used for comparing the difference between the groups. The Kolmogorov-Smirnov test are applied to test whether normally distributed continuous variables, and p <0.05 value was set for significance level. The continuous and multivariate comparisons between groups in the pathological examination was performed using Kruskal-Wallis test according to distribution.

Mann-Whitney U-test was used for statistical differences show differences observed between the group according to the Kruskal-Wallis test, and smaller values then p <0.05 were considered statistically significant.

ETHICS COMMITTEE

This study was carried out with etics rapor no: AKUHADYEK-195-13; obtained from Afyon Kocatepe University, Animal experiments local ethics committee (AKUHADYEK).

RESULTS

The Liver and Plasma MDA Levels

The statistically significant differences (p<0,05) were obtained from AF64A and AF64 A+Betaine groups, compared to the control group on the basis of MDA results of liver (Figure 4). AF64A levels were obtained as significantly high.

The results have emerged as a result of AF64A and AF64A+Betaine groups exposed to high oxidative damage compared to the control groups. Also it means that betaine has no significant treatment effect agains to AF64A' oxidative damage. The result of AF64 A + Piperine group was statistically significant (p<0.05) in compare to AF64A group **(Figure 4)**.

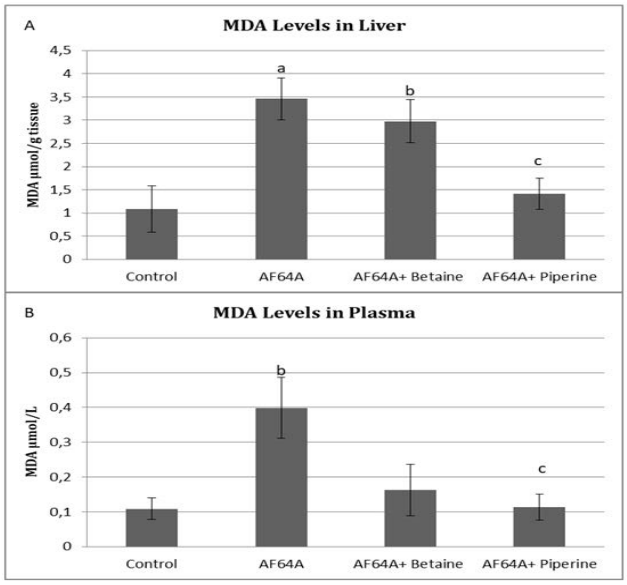

Figure 4: The MDA levels of liver (A) and plasma (B). (a: p< 0,01 ve b: p< 0,05 comparison with control, and c: p< 0,05 comparison with AF64A. It was not seen statistically importance in the other groups).

Which means that the piperine has an significant reducing effect on the AF64A-induced oxidative damage, compare to betaine antioxidant effect; the piperine comes into prominence.

Reduced Glutathione (GSH) Levels in Liver and Erytrocyte

Low results were observed betweeen AF64A and AF64A+Betaine groups compare to control and AF64A+Piperine groups on the basis of the reduced glutathione (GSH) levels (p<0.01) **(Figure 5)**.

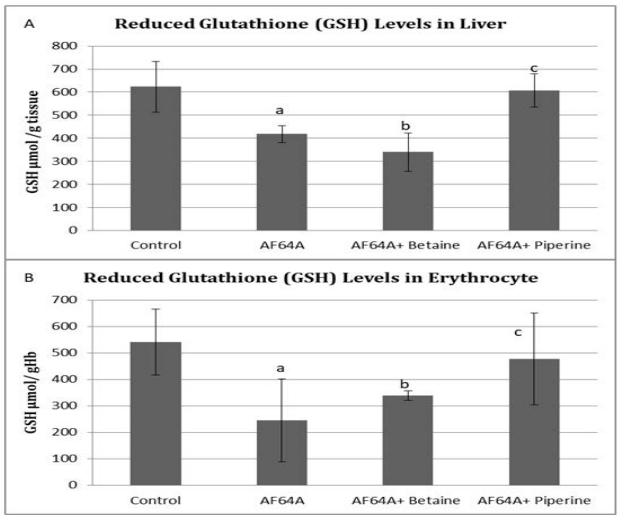

Figure 5: The GSH levels of liver (A) (a: p< 0.01, b: p< 0.001 comparison with control group, and c: p< 0.01 comparison with AF64A groups. It was not seen statistically importance in the other groups) and Erythrocyte (B) (a: p< 0.001, b: p< 0.05 comparison with control group, and c: p< 0.01 comparison with AF64A groups. It was not seen statistically importance in the other groups) levels

Especially the lowest level was obtained in the AF64A group on the basis of GSH erythrocyte criterias. This can be explained as AF64A causes oxidant stress. The significantly higher result was recorded in the AF64A + Piperine group compare to the AF64A and AF64A+Betaine groups. It is clear that the piperine demonstrated its antioxidant features and reducing effect of oxidants influence caused by AF64A.

Moris Water Maze Test

The platform was found easily by the rats in 3rd and 4th days of the test exercises. At the end of trial week; the rats reached to the exit platform much easier and faster rate (about 1 minute).

No significant changes were observed in the first week. But coming weeks the behavioral changes were observed in terms of arriving time to the escape platform in AF64A applications compare to the control. Applying AF64A shows destruction to the brain cell causing to disremember of escape platform. Because of this; reaching time to this platform enlarged in AF64A group compare to control. On the comparison of using betaine and piperine as an antioxidant; piperine groups rats reaching time much shorter compare to the betaine effect **(Figure 6)**.

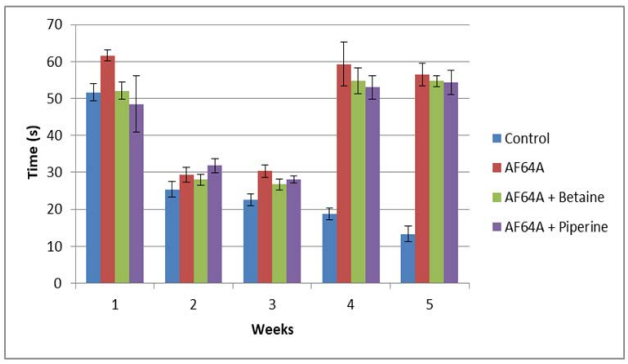

Figure 6: The Moris water maze test results in 5 weeks periods

Using piperine; in the recession, reduction or in treating of oxidative damage caused by AF64A, significantly is taking more attention and its success comes forward compare to betaine. These changes are described in biochemical parameters and the hippocampus region of rat brain tissue pathological findings also supports these results as well.

Pathological examination result

On the basis of histopathological examination, several important neural pathological changes were seen at the CA1 region of hippocampus. A lot of different necrobiotic changes were seen in many neurons cell have cytoplasm in a dark blue color with a angular shrinks. The results were scored in the form of semi-quantitatively according to percentage of these cells including degenerative-necrotic changes in that area, as if the percent is more than 50% (3), is between 25-50% of (2), is between 5-25% (1) and is less than 5% (0) respectively **(Figure 7)**.

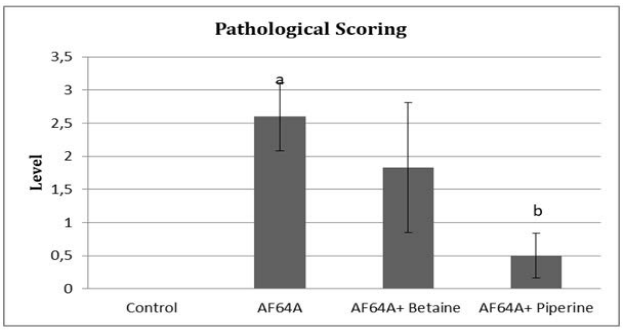

Figure 7: Pathological scoring results (a: p< 0.01 compare to control, and b: p< 0.05 compare to AF64A group)

According to the pathological scoring result; there were significant differences in AF64 group in comparison with the control group.

Using AF64A destroyed the brain nerve cell very badly. But especially piperine treatment create almost reversible effect onto AF64A damaging act via bring down pathological sign into control level compare to the betaine effect **(Figure 8)**.

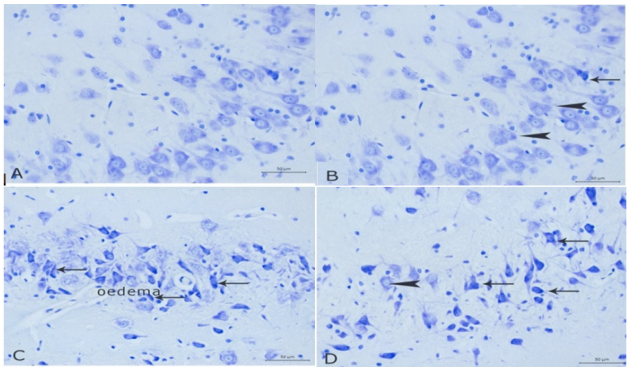

Figure 8: A-D The differences between groups, shown in CA1 part of hippocampus; degenerative necrotic primidal nöurons (arrow), normal nöurons (arrow head) **A:** The normal healty profile belongs to control group **B:** The much less degenerative effects in AF64A + Pipperine group rats. **C:** The necrotic regions in AF64A group rats **D:** The less degenerative effect were seen in AF64A + Betaine group rats compare to AF64A group.

Hippocampus MAPK mRNA Levels

Gene expression levels of MAPK in hippocampus of rats were found to be statistically different based on the groups. A significant increase was observed AF64A group. Down regulations were detected betaine and piperine applying groups. The best result was obtained in the AF64 A + Piperine group **(Figure 9)**.

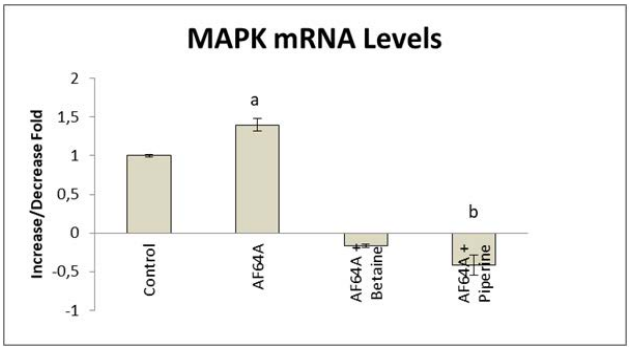

Figure 9: The comparison results in mRNA levels of MAPK in hippokampus of all groups (a: p< 0.05 and b: p< 0.05 comparison with control)

According to these findings; AF64A increased the mRNA expression levels MAPK. In contrast to this; piperine have an importantly higher antioxidant effect compare to the less regression effect of betaine. According to the results of this study; the MAPK pathway triggers to the cell death activated by AF64A. It was observed that piperine has an significantly important contribution into regression or decreasing of these effects.

DISCUSSION

The mechanism of AD has been tried to be explained and understand for along time, affects more than 26 million people globally, and the risk of becoming AD is increasing rapidly based on the life style (4,5). In our experimental rat model; we tried to perform experimental AD model affected by AF64A and piperine and betaine were used as an antioxidant for treating effects. Yu et al (22) reported that Aβ' induce ROS formation. It was showed that the AD-like behavior formed in the result of ROS, induced by Aβ' causing to extension of reaching time to exit platform in rats. This study results are in harmony with biochemical, behavioral and pathology results of our experimental model (22).

Pannangrong et al. (23) investigated the effect of AF64A into memory loss and impairment effect of rice berry. For this aim; the rice berry were given orally into young adult male Wistar rats, for total 3 weeks of AF64A, via bilateral intracerebroventricular administration. One week following AF64A administration the rats were evaluated for neuron density, spatial memory, hippocampal lipid peroxidation products and acetylcholinesterase activity. The results showed that the rice berry could significantly prevent memory impairment like as our material Piperin and betaine and hippocampal neurodegeneration in hippocampus. Also the decrease were seen at hippocampal acetylcholinesterase activity and lipid peroxidation product formation.

Gulyaev et al. (24) reported that AF64A application to brain cause to increase in lipid peroxidation levels. This study results, are in compliance with MDA levels in liver and plasma is our experimental model.

Bachurin et al. (25). declared the results that application of AF64A into brain, affect learning in a negative way, causing to weakens in learning and affect the Aβ levels in riser direction based on the morris water tank test results. In our study has been identified that AF64A negatively affect the learning and causing symptoms mimicking to Alzheimer disease as well (25). Chonpathompikunlert et al., (26) searched the effect of piperine, a main active alkaloid in fruit of Piper nigrum, on memory performance

and neurodegeneration in animal model of Alzheimer's disease. For this research the piperine were given orally at various doses ranging from 5, 10 and 20mg/kg BW at a period of 2 weeks before and 1 week after the intracerebroventricular administration of AF64A bilaterally to the adult male Wistar rats (180-220 g). The results showed that piperine has an important effect on memory impairment and neurodegeneration in hippocampus at all dosage range used in this study. The explanation for this mechanisms might be partly associated with the decrease of acetylcholinesterase enzyme and lipid peroxidation. Moreover, it was seen that piperine demonstrated the neurotrophic effect in hippocampus which is similar to our findings as well.

Yamada et al., (27) investigated whether nicotine (NCT) administration attenuated spatial learning deficits induced by intracerebroventricular AF64A treatment. AF64A produces various memory deficits in rats similar to some characteristics observed in Alzheimer's disease patients.

AF64A (6 nmol/6 µl)-or saline (SAL)-treated rats were trained in Morris water maze task. NCT (0.025-0.25mg/kg) was subcutaneously injected 5 min before the training every day. The results showed that moderate dose (0.10mg/kg) of NCT attenuated AF64A-induced prolongation of escape latency and also recovered the AF64A-induced decrease of time spent in the target quadrant in the probe test. According to these findings; NCT improves AF64A-induced spatial memory deficits, and can be used as a potential therapeutic agent for the treatment of memory deficits in dementia indicating similar impairment effect of piperine in our study.

János Varga et al. (28) have reported that APP and MAPK-1 levels increased with age and also the reaching time to Morris water tank test platform increased with age as well. As a reason is shown for that the reducing effect of MAPK, in inflammation risk that increased with age, which play a key role in the regulation of release of pro-inflammatory cytokines. In our experimental model, the reaching time to morris water tank platform were prolonged in the AF64A group, and the high levels of MAPK were observed in the same group. The high oxidative damage was observed in the group where high levels of MAPK obtained.

CONCLUSION

In our experimental alzheimer rat model, the oxidative and the destructive effects of oxidant AF64A at the molecular level were observed in the brain especially on the neuronal cells of the hippocampus region. Furthermore, the behavioral effects of the oxidant substance were also monitored by morris water tank test. Piperine and betaine, thought to have antioxidant properties, were used to reduce/regain oxidative effects. Although the betaine's efficiency is not fully monitored, piperine is reported to be more effective at reducing the oxidative effects of AF64A both at behavioral and molecular level.

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