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Investigation of TheEffects of Trpc4/5 Inhibitor M084 in Experimental Alzheimer's-LikeDementia Model

Deneysel Alzheimer Benzeri Demans Modelinde Trpc4/5 İnhibitörü M084'ün Etkilerinin Araştırılması

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ÖZ

Amaç: Bu çalışmadaki amacımız skopolamin ile oluşturduğumuz deneysel Alzheimer benzeri demans modelinde N-butil-[lH]-benzimidazol-2-amin (M084)'in bilişsel fonksiyonlar üzerine etkilerini araştırmaktır.
Araçlar ve Yöntem: Çalışmamızda erkek BALB/c fareler beş gruba (n:8) ayrıldı. Kontrol grubu; 14 gün boyunca günde 1 ml 0.9% salin çözeltisi intraperitoneal (i.p.) olarak uygulandı. Skopolamin grubu: İlk 7 gün günde 1 ml 0.9% salin çözeltisi, ardından 8-14. günler arasında günde 3 mg/kg skopolamini.p. olarak uygulandı. Skopolamin + M084 grubu; İlk 7 gün günde 1 ml 0.9% salin çözeltisi uygulanan farelere 8-14. günler arasında günde 3 mg/kg skopolamini.p. + 10-14 günler arası günde 20 mg/kg M084 günde tek doz i.p. olarak uygulandı. M084 grubu; farelere 0-14. günler arası günde 20 mg/kg M084 günde tek doz i.p. uygulandı. Donepezil grubu: Pozitif kontrol grubu olarak farelere 0-14. günler arası günde 5 mg/kg donepezili.p + 8-14 günler arasında günde 3 mg/kg skopolamini.p. uygulandı. Çalışmanın 14 ve 15. günlerinde pasif sakınma, yeni obje tanıma ve modifiye yükseltilmiş artı labirenti testi uygulamaları yapıldı.
Bulgular: Pasif sakınma testinde karanlık odaya geçiş süreleri skopolamin grubunda kontrol grubuna kıyasla daha kısaydı. Modifiye Yükseltilmiş Artı Labirenti Testinde 2. gün kapalı kollardan birine geçiş süreleri skopolamin grubunda kontrol ve donezepil grubuna göre daha fazla bulundu. Yeni obje tanıma testinde diskriminasyon indeks değerleri kontrol, skopolamin + M084, donezepil ve diğer gruplardaki değerler skopolamin grubuna göre daha fazla bulundu.
Sonuç: Sonuç olarak Alzheimer benzeri demans modelinde M084 görsel bellek üzerine olumlu sonuçlar vermiş, fakat diğer bellek türleri üzerine etkisiz bulunmuştur.

AnahtarKelimeler: alzheimer; bellekfonksiyonu; M084; skopolamin

ABSTRACT

Purpose: This study aimed to research the effects of N-butyl-1H]-benzimidazole-2-amine (M084) on cognitive functions in an experimental Alzheimer-like dementia model.
Materials and Methods: In our study, male rats were divided into five groups (n:8). The control (C) had 1 ml 0.9% saline solution administered intraperitoneally (i.p.) for 14 days. Scopolamine (S) was administered intraperitoneally with 1 ml 0.9% saline solution for the first 7 days, followed by 3 mg/kg scopolamine on days 8-14.Scopolamine+M084 (SM) was administered i.p. dissolved in 1 ml 0.9% saline solution for the first 7 days, followed by 3 mg/kg scopolamine on days 8-14 and 20 mg/kg M084 on days 10-14. M084 (M) was administered i.p. 20 mg/kg M084 in a single daily dose from days 0-14. Positive control (D) had 5 mg/kg donepezil administrations on days 0-14 and 3 mg/kg scopolamine on days 8-14.
On days 14 and 15 of the study, passive avoidance, novel object recognition, and modified elevated plus maze tests were performed.
Results: In the passive avoidance test, transfer latencies were significantly lower in group S compared to group C. In the modified elevated plus maze test, the passing time to either closed arms on the 2nd-day test was significantly higher in group S compared to groups C and D. In the novel object recognition test, the values for groups C, SM, D, and other groups were significantly higher compared to group S.
Conclusion: In conclusion, in an Alzheimer-like dementia model, M084 provided positive results for visual memory; however, it was ineffective for other memory types.

Keywords: alzheimer's; memory functions; M084; scopolamine

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INTRODUCTION

Alzheimer's disease (AD) is a degenerative disorder of the central nervous system characterized by progressive disruption of cognitive functions. It is estimated that there are more than 24 million cases in the world in general.[1]Considering the aging of the global population, it is expected that the number of cases will double by 2040. There is an apparent increase in AD incidence after age 65 years.[1] Memory loss is the first symptom observed.[2]The most prominent feature in the histopathology of AD is the formation of senile amyloid plaques (SP) in the amygdala, hippocampus, and neocortex in the brain[3] Additionally, hypotheses like the cholinergic and calcium were proposed for AD occurrence, and a variety of studies were performed about these topics.[4-6]

Ach is a neurotransmitterthat enhances attention and helps learning processes in physiological conditions. The primary deficiency in AD is disruption of the cholinergic system, particularly brain regions related to learning and memory. In this regard, medications increasing cholinergic transmission are used for the treatment of Alzheimer's disease. Many studies revealed that the disruption in cholinergic functions is in parallel to the disruption of short-term memory, especially. In this regard, blockage of cholinergic muscarinic receptors with scopolamine causes memory impairment similar to AD, and this is one of the most widely used experimental dementia models.[7,8]

Ca^{+2} has significant effects on neurons' survival and functions, and imbalances in calcium signals play an important role in AD pathogenesis. Disruption in the Ca^{+2} balance plays a role in excitotoxicity, synaptic degeneration, and cell death, while reduced Ca^{+2} secretion has a protective effect on neurons.[4,9,10]

Transient receptor potential channels (TRPC) are associated with many pathologic situations in addition to their physiological importance. One of these pathologic situations is neurodegenerative diseases like Alzheimer's and Parkinson'sdisease. Due to regulation of the intracellular Ca^{+2} concentrations, TRPC channels come to the forefront more compared to other subfamilies.[11] TRPC4 was shown to be expressed with TRPC5, which plays an important role in neuronal Ca^{+2} homeostasis. The significant effects of Ca^{+2} on survival and functions of neurons and the imbalance in calcium signals playing an important role in the AD pathogenesis comprise the basis of the Ca^{+2} hypothesis in AD[4,5,9,10].While normal expression of TRPC5 increases neurite extensions, overexpression of TRPC5 inhibits neurite growth.[12]

N-butyl-(1H)-benzimidazole-2-amine (M084) is a molecule inhibiting TRPC4/5. It is thought that inhibition of TRPC4/5 will be effective in regulating the intracellular Ca^{+2} dysregulation. Additionally, studies stated that one of the main causes of symptoms in AD is cholinergic loss. At the same time, it is thought that inhibition of AchE destroying acetylcholine (Ach) with the acetylcholine esterase (AchE) and butyrylcholinesterase enzyme inhibitor M084 may reduce AD symptoms.[13-15]For these reasons, this study was planned with M084, with effects inhibiting TRPC4/5 and AchE.

MATERIALS and METHODS

The study included 48 male BALB/c mice housed in a room with a 12-hour light/12-hour dark cycle at room temperature (22±2 ℃). There were maximum 8 mice in each cage with free access to water and food.

According to the experimental protocol, daily intraperitoneal (ip) injections were given every day at the same time. Scopolamine and M084 doses to be administered to mice have been determined as 3mg/kg by researching the literature.[16-17]Medications were injected after being dissolved in 1 ml 0.9% saline. Groups were created as follows. The control group (C) had 1 ml 0.9% saline solution administered ip for 14 days. The scopolamine group (S) was administered 1 ml 0.9% saline solution ip for the first 7 days and then 3 mg/kg scopolamine ip from days 8 to14. The scopolamine+M084 group (SM) was administered 1 ml 0.9% saline solution ip for the first 7 days and then 3 mg/kg scopolamine i.p. from days 8 to 14 and 20 mg/kg M084 single dose ip from days 10 to 14. In the M084 group (M), subjects were administered 20 mg/kg M084 in a single daily ip dose from days 0 to 14. In the donepezil group (D), the positive control group, subjects were administered 5 mg/kg donepezil ip from days 0 to 14 and 3 mg/kg scopolamine ip from days 8 to 14.

Thirty minutes after administration of the final medication dose on the 14th day, subjects underwent the new object recognition test, passive avoidance test and modified elevated plus maze test as observational tests to investigate the behavior of subjects. Five-minute video recordings taken during these tests were analyzed with the Ethovision-XT program. After each subject, the foreign object recognition test setup was wiped with 20% alcohol and then tapwater and dried.

Brain tissue samples taken from mice were homogenized after being added to 0.01 M pH 7.4 iced phosphate buffer solution with volume 9 times the weight of the sample. The homogenate was centrifuged at 5000 g for 5 minutes, and the supernatant was separated for analysis. The supernatants had BDNF, amyloid β-42, and TAU levels measured using a micro-ELISA reader device (Robonikreadwell touch Thane, India) with ELISA kits (Elabscience, China).

Approval was obtained from the local ethics committee for this research with 3 issues, dated 08.06.2016.

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Behavior Tests Used

New Object Identification Test

A novel object recognition test was performed in an open field test setup (L 42 cm, W 42 cm, H 30 cm) to evaluate the visual memory of the animals. In the experiment protocol, mice were placed in an open area with two objects of the same shape and color for a habituation period and then expected to recognize these objects. After 90 minutes, one of the objects was exchanged for a new object with a different shape and color. Mice with disrupted cognitive functions will not notice the difference and will spend similar durations around both objects. Recordings for the novel object recognition test were analyzed using the Etho Vision XT 11 (Noldus Inf. Tech. Netherlands) program.

Passive Avoidance Test

The passive avoidance test setup was used to record affective (emotional) learning and memory functions. The experimental setup consisted of a light room, a dark room (both rooms are the same size: length 14 cm, width 7 cm, height 12 cm), and a door connecting them. The floor of the experimental setup comprised 0.3 cm diameter electrically conductive steel bars. The experiment lasted for 2 days. On the first day, 60 s after subjects were placed in the light chamber, the door was opened and subjects entering the dark chamber were subjected to an electric shock (0.25 mA for 1 s). If the subject did not enter the dark chamber within 5 minutes of the door opening on the first day, they were removed from the experiment. The duration for subjects to pass from the light chamber to the dark chamber was calculated.

Modified Elevated Plus Maze Test

To record cognitive performance, the modified elevated plus maze test setup was used. The setup comprised two open (25 x 5 x 0.5 cm) and two closed (25 x 5 x 16 cm) arms and a central platform (5 x 5 x 0.5 cm). The setup was placed 50 cm above the floor. The experiment lasted two days. On the first day, the micewere placed on the open arms end facing away from the platform and the duration to enter the closed arms was recorded and animals were left for 5 s to discover the closed arm. On the second day, mice were left on the open end in the same conditions and the duration to reach the closed armswere recorded.

Statistical Analysis

Numerical variables are summarized as mean ± standard deviation, median, minimum and maximum values. According to whether the distribution was normal or not, unrepeated measures were analyzed with the Kruskal-Wallis or one-way analysis of variance (ANOVA) post hoc Dunn-Bonferroni test, while repeated measures were analyzed with the Wilcoxon or two-way analysis of variance for repeated measures test. All statistical analyses took $p<0.05$ as statistical significance level. All analyses were performed with SPSS 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) software.

RESULTS

Weight Change in Subjects

Weight change in subjects in group C reduced compared to S, SM, M and D groups, but this difference was notstatistically significant($p>0.05$).

New Object Recognition Test Findings

In terms of learning index values, there were significant differences between S and C, SM, M and D groups. Subjects in group S allocated less time recognizing the novel object ($p<0.001$). The mean and median values for group S were significantly lower than the other groups ($p<0.05$) (Table 1).

Table 1. Novel object recognition test (n=8)

Groups	Mean ± standard deviation	Median [Min - Max]	SSG	p
C	80.775 ± 11.519	79.9 [62.48 - 99.74]	S	p<0.001
S	44.636 ± 15.495	48.6 [69.21 - 64.11]		p>0.05
SM	70.263 ± 14.095	73.03 [47.5 - 87.18]	S	p:0.015
M	68.619 ± 17.200	69.79 [40.22 - 88.58]	S	p:0.028
D	75.192 ± 13.190	79.79 [57.24 - 87.93]	S	p:0.003

C: Control. S: Skopolamine. SM: Skopolamine-M084. M: M084. D: Donepezil. SSG: statisti-cally significant groups

Passive Avoidance Test

When values on the 1st and 2nd day are compared in the same group, there were statistically significant differences identified between the 1st and 2nd day measurements in C, M, and D ($p<0.05$, for all). In each of these groups, 2nd day measurements were very high compared to 1st day measurements.

Results of analysis investigating the differences between the groups for each measurement showed significant differences between the groups only in the 2nd day measurements($p<0.05$). Statistically significant differences were determined between D group with SM and S groups and between SM and C groups ($p<0.05$) (Table 2).

Modified Elevated Plus Maze Test Findings

The variation in t-test values was found to be similar according to the all groups ($p>0.05$).

According to analysis results on a group basis, comparisons of the t-test on the 1st day and 2nd day in separate groups did not identify differences between groups for the t-test 1st day variables ($p>0.05$). There were significant differences between the groups for the t-test 2nd day variable. For the T-test 2nd day variable, there were statistically significant differences between the S group with C and D groups ($p<0.05$) (Table 3).

Table 2. Passive avoidance test (n=8)

Groups	Mean ± standarddeviation		[MedianMin - Max]		2.day SSG	p
	1. day	2. day	1. day	2. day		
C	20.875 ± 15.905	214.5 ± 53.439	17 [4 - 55]	191 [176 - 300]	SM	p:0.032
S	22.25 ± 13.583	86.25 ± 88.783	20.5 [10 - 53]	67 [1 - 222]	D	p:0.003
SM	12.25 ± 6.902	89.125 ± 78.755	9.5 [5 - 25]	108 [3 - 222]	D	p:0.002
M	11.5 ± 6.803	201.625 ± 86.774	11 [2 - 21]	211 [71 - 300]		p>0.05
D	19.75 ± 5.8	256.25 ± 39.536	19 [13 -30]	249 [200 - 300]		p>0.05

C: Control S: Skopolamine. SM: Skopolamine-M084. M: M084. D: Donepezil. SSG: statistically significant groups

Table 3. Modified elevated plus maze test (n=8)

Groups	Mean ± standarddeviation		[MedianMin - Max]		SSG	p
	1. day	2. day	1. day	2. day		
C	36.75 ± 11.973	20.875 ± 7.643	34 [23 - 55]	21 [11 - 31]	S	p:0.036
S	30.375 ± 17.509	34.625 ± 12.317	25 [15 - 68]	35 [12 - 51]		p>0.05
SM	36.875 ± 12.206	26.25 ± 11.158	33.5 [27 - 62]	24 [13 - 50]		p>0.05
M	38.875 ± 14.827	22.875 ± 2.478	38.5 [10 - 57]	22.5 [18 - 26]		p>0.05
D	24.125 ± 6.556	21.125 ± 7.549	25 [14 - 34]	20 [13 - 37]	S	p:0.042

C: Control.S: Skopolamine. SM: Skopolamine-M084. M: M084. D: Donepezil. SSG: statistically significant groups

Biochemical Findings

BDNF measurements were significantly different between the S-C groups, S-D groups and S-M groups (Table 4) ($p<0.05$). Tau measurements were different between M and SM groups ($p<0.05$) (Table 4).

The Aβ42 values were significantly differentiated between groups C and D ($p<0.05$) (Table 4).

Table 4. Biochemical Findings (n=8)

Variable	Groups	Mean ± standarddeviation	[MedianMin - Max]	SSG	p
BDNF (ng/gr doku)	C	1.734± 0.558	1.815[0.85 - 2.43]	S	p:0.021
	S	0.494± 0.343	0.480[0.14 - 1.17]	D	p:0.016
				M	p:0.004
	SM	1.023± 0.755	1.040[0.14 - 1.87]		p>0.05
	M	1.739± 0.889	1.610[0.6 - 2.86]		p>0.05
	D	1.626± 0.496	1.575 [0.71 - 2.28]		p>0.05
Tau(ng/gr doku)	C	0.585 ± 0.380	0.720[0.1 - 0.94]		p>0.05
	S	0.901 ± 0.215	0.855 [0.21 - 1.85]	M	p:0.023
	SM	0.948 ± 0.292	0.890[0.79 - 1.15]		p>0.05
	M	0.65 ± 0.14	0.645[0.55 - 0.78]		p>0.05
	D	0.815 ± 0.068	0.840[0.41 - 1.11]		p>0.05
Aβ42 (ng/gr doku)	C	0.319 ± 0.215	0.265 [0.09 - 0.73]	D	p:0.004
	S	0.761 ± 0.551	0.560[0.21 - 1.85]		p>0.05
	SM	0.661 ± 0.147	0.675 [0.44 - 0.88]		p>0.05
	M	0.543 ± 0.160	0.550[0.29 - 0.8]		p>0.05
	D	0.749 ± 0.140	0.775 [0.44 - 0.89]		p>0.05

C: Control. S: Skopolamine. SM: Skopolamine-M084. M: M084. D: Donepezil. SSG: statistically significant groups

DISCUSSION

Studies found that N-butyl-1H]-benzimidazole-2-amine (M084) has functions both as a TRPC4/5 inhibitor and as an acetylcholinesterase and succinylcholine esterase inhibitor.[13,11-22] TRPC channels are associated with many pathologic situations in addition to physiological importance. One of these pathologic situations is neurodegenerative diseases like Alzheimer's.[23] The TRPC channels become more prominent compared to other subfamilies with the regulation of intracellular Ca^{+2} concentration.[24] Studies showed that Ca^{+2} ion imbalances play a role in the pathogenesis of neurodegenerative diseases like Alzheimers.[10,24] TRPC4 was shown to be expressed with TRPC5, which plays an important role in neuronal Ca^{+2} homeostasis in CA1 pyramidal neurons in the hippocampus.[25-27] TRPC4 was shown to have a role in acute and delayed neuronal injury in focal cerebral ischemia. For this reason, inhibition of TRPC4 is thought to be protective against neuronal injury.[25,27,28] Normal expression of TRPC5 increases neurite extension, while overexpression of TRPC5 inhibits neurite growth.[12] For this reason, channels involving TRPC5 may allow greater Ca^{+2} flux than TRPC4/TRPC6 channels which may cause inhibition of neurite growth.[12,28,29] The TRPC4/5 inhibitor of M084 affects TRPC channels and acts as an acetylcholinesterase and succinylcholinesterase inhibitor.[13,18-22] In the new object recognition test measuring visual memory, the SM group had a statistically significantly longer duration examining the new object compared to the S group. This situation shows that the M084 molecule caused an improvement in visual memory. It is thought that this effect may be due to increased cholinergic activity due to inhibition of acetylcholinesterase enzyme, as well as inhibition of TRPC channels and/or regulation of Ca^{+2} balance.

When the modified elevated plus maze test and passive avoidance test findings are investigated, data obtained from the medicated groups found no statistical significance compared to the S group. In the S group, though there were partial differences between the BDNF, Aβ42, and Tau levels between the groups, statistical significance was not present.

In this study, donepezil was used as a positive control as it inhibits acetylcholinesterase enzyme, which plays a role in increasing cholinergic activity. The interest in the novel object in group D was clearly increased compared to group S during the novel object recognition test. For the passive avoidance test, group D had clearly lengthened transition from the light area to the dark area. In the passive avoidance test, group D clearly lengthened the transition time from the light to the dark area. There was no difference observed between the D and S groups for the modified elevated plus maze test. There was a slight fall in Aβ42 levels in the group treated with D compared to S; however, this fall was not statistically significant. Studies stated that donepezil ameliorates Alzheimer's symptoms.[30,31]

Many methods are used on experimental animals in studies to induce Alzheimer-type dementia experimentally. One of these is scopolamine administration. This model inhibits the cholinergic muscarinic receptors playing an important role in learning and memory processes, creating a situation like AD.[32,33] In this study, scopolamine was used to induce AD. In the new object recognition test performed to assess visual memory, subjects in group S had reduced interest in the new object compared to subjects in group C. This situation is compatible with the literature.[16,33] In the passive avoidance test used to assess emotional learning and memory functions, the transition duration from the light area to the dark area was significantly reduced in group S compared to group C. Studies about this topic state that scopolamine administration reduced the transition duration of subjects and this situation showed disrupted emotional learning and memory.[16,35] The modified elevated plus maze test is a test used for spatial learning and memory recording. On the 2nd day of tests, the duration to enter the closed arms was longer in group C compared to group S. These findings are consistent with other studies in the literature.[32]When biochemical parameters are assessed, BDNF levels were observed to be significantly reduced in the scopolamine group compared to group C. Some studies identified that scopolamine administration had similar effects on BDNF level.[32] Although the Aβ42 and Tau levels, which play a role in AD pathogenesis, were higher in group S compared to group C, this finding was not statistically significant. Scopolamine is a compound used to stimulate cortical cholinergic neural loss observed in AD.[36,37] For this reason, the Aβ42 and Tau amounts may not have increased. According to these findings, scopolamine is considered to have induced a successful dementia model.

Limitations of this study are the low number of animal subjects, the M084 drug used in the study being new and not having a standard dose, and the long duration for amyloid and TAU accumulation in the brain, so the 14-day duration of our study may not be sufficient for TAU and amyloid formation.

In conclusion, M084, which inhibits TRPC4/5, acetylcholinesterase, and succinyl cholinesterase, positively affected the novel object recognition test in an AD-like dementia model induced with scopolamine; however, it was ineffective on modified elevated plus maze test and passive avoidance test. We think it will be beneficial to perform this study with more subjects, longer duration and different doses of M084.

Conflict of İnterest

The authors declare that there is not any conflict of interest regarding the publication of this manuscript.

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Authors' Contributions

Concept/Design: AG, HA, EU. Data Collection and/or Processing: AG, HA, EU. Data analysis and interpretation: AG, HA, EU. Literature Search: AG, HA. Drafting manuscript: AG. Critical revision of manuscript: HA, EU. Supervision: HA.

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