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Comparative study between irisin and donepezil (Anticholinesterase) on cognitive functions in cases of scopolamine induced Alzheimer's disease: Like condition in male albino rats

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***Abstract*---**Introduction: Alzheimer's disease (AD) is a progressive neurological disorder. Donepezil hydrochloride is a cholinesterase inhibitor that is selective. Irisin is a 112-amino acid glycosylated protein-hormone. Objective: to compare the effect of irisin and donepezil (anticholinestrase) on cognitive function in cases of scopolamine induced Alzheimer's disease like condition in male albino rats. Materials and methods: Fifty albino male rats of a local strain, weighing between 150 and 200 g, enrolled in this study. At room temperature, with 12-hour light/dark cycles, and with free access to running tap water and pelleted laboratory chow, the rats were kept in separate cages (5 rats per cage) for the duration of the experiment. Results: Donepezil administration achieved significant decrease in A che level in donepezil treated group, in relation to AD group, (AD+D). Irisin administration caused insignificant change in A che level in Irisin treated group in relation to AD group (AD+I). Conclusions: Irisin can reduce scopolamine-induced AD manifestations through its antiinflammatory, antioxidant and antiapoptotic effects, which were evaluated both biochemically and behavioural. Donepezil enhances memory as it is anti cholinestrase, also it has anti-inflammatory

effect. Donepezil and irisin treatment potentiate each other as they act synergestically by different mechanisms to counrtact memory loss.

***Keywords*---**Alzheimer's disease, donepezil, irisin, scopolamine.

Introduction

Alzheimer's disease (AD), the most prevalent subtype of dementia, is a progressive neurological disorder marked by memory loss, dysfunction of cognition, attention problems, and a gradual reduction in language functions (Huang, Zhang, & Chen, 2016). Many factors might cause AD, including genetic defects, appearance of neurofibrillary tangles, mitochondrial defects, deficiency of neurotropic factors, trace element neurotoxicity, energy metabolism deficit, and oxidative stress (Huang et al., 2016). By boosting amyloid formation and consequent synaptic and neuronal loss, oxidative stress contributes significantly to the etiology of AD. The link between oxidative stress and Alzheimer's disease shows that oxidative stress is a necessary component of the pathogenic process, and antioxidants may be beneficial in the treatment of AD (Mazur-Bialy, Kozlowska, Pochec, Bilski, & Brzozowski, 2018).

A muscarinic cholinergic receptor antagonist, named Scopolamine, is used in experimental animals to induce cognitive impairment. Scopolamine injection (intraperitoneal) affects cognition and memory in rats through causing cholinergic dysfunction (Bhuvanendran, Kumari, Othman, & Shaikh, 2018). Donepezil hydrochloride is a cholinesterase inhibitor that is selective. Acetylcholinesterase inhibitors (AChE-Is) limit the breakdown of residual acetylcholine in the brain and are the most effective pharmacological therapy for mild to moderate AD patients (Galimberti & Scarpini, 2010; L, Y, & Y, 2011). Currently, AChE-Is are utilized as a symptomatic therapy to restore or maintain central cholinergic function (D'Amelio & Rossini, 2012).

Irisin is a 112-amino acid glycosylated protein-hormone. Irisin is a myocyte-secreted hormone that is thought to act as a link between metabolic and exercise homeostasis. The level of irisin positively correlates with the amount of muscle mass (Huh et al., 2012). Irisin influences hippocampal neurogenesis. Because of the positive effect of exercises on neurogenesis and the fact that hippocampus is considered as the main region affected by neurodegenerative diseases (Alzheimer's or Parkinson's disease), irisin is regarded as the molecular messenger between exercise and brain function (Erickson, Weinstein, & Lopez, 2012) The aim of the current work was to compare the effect of irisin and donepezil (anticholinestrase) on cognitive function in cases of scopolamine induced Alzheimer's disease like condition in male albino rats.

Resources and Procedures

Fifty albino male rats of a local strain, weighing between 150 and 200 g, were used in this study. At room temperature, with 12-hour light/dark cycles, and with free access to running tap water and pelleted laboratory chow, the rats were kept in separate cages (5 rats per cage) for the duration of the experiment. All rats

were fed a standard diet consisting of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture, and 5% vitaminized starch as protein (Zowail, Awwad, Khater, & Nafie, 2018). It was obtained from El Gomhoureya Company for Trading Drugs & Medical Supplies.

All operations were carried out in accordance with the ethical guidelines established by the Tanta University Faculty of Medicine's ethics committee. After two weeks of acclimatization, the animals were randomly separated into five different groups (10 rats each). For 15 days, rats in group 1 (control group) received daily intraperitoneal (i.p.) injections of 1 ml saline. In group 2 (Alzheimer's group) the animals were injected i.p. with scopolamine single dose (16 mg/kg) then saline 1 ml i.p. once daily for 2 weeks (El-Marasy, Abd-Elsalam, & Ahmed-Farid, 2018). In group 3 (Donepezil treated AD group) the animals were injected with scopolamine single dose (16mg/kg) on the first day, then donepezil (Aricept; Pfizer Inc) i.p. once daily with a dosage of 1 mg/kg daily for 2 weeks (Meunier, Ieni, & Maurice, 2006). In group 4 (Irisin treated AD group), the animals were injected with scopolamine single dose (16mg/kg) at first day i.p then Irisin i.p. at a dose 15 μg/kg once daily for 2 weeks (Asadi, Gorjipour, Behrouzifar, & Vakili, 2018; J. Zhang et al., 2017). In group 5 (Donepezil and Irisin treated AD group), the animals were injected with scopolamine single dose (16mg/kg) at first day i.p then both Irisin (15μg/kg) and donepezil (1 mg/kg) were injected once daily i.p for 2 weeks.

Materials and Methods

Procedures

Cervical dislocation was used to euthanize rats in all groups, the hippocampus was dissected quickly and carefully out from the isolated brain to avoid mechanical trauma and then in ice cold phosphate buffer saline (PBS) (pH 7.4) or in specific buffer, the specimens were weighed and homogenized. Homogenates samples were centrifuged according to parameter and the clear supernatants were separated in a clean storage plastic test tubes and stored at -80oc (Jangra et al., 2015) for estimation of brain derived neurotrophic factor (BDNF), which was determined using the Binder and Scharfman method (Binder & Scharfman, 2004), caspase-3 assay kit which was measured according to the method described by Kamada et al. (Kamada et al., 1998), determination of glutathione level (GSH) which was measured according to the method described by Beutler and Kelly (Beutler & Kelly, 1963), malondialdehyde (MDA) level: which was measured according to the method described by Zhao et al. (Zhao et al., 2014), superoxide dismutase (SOD) which was measured according to the method described by Strålin et al. (Strålin, Karlsson, Johansson, & Marklund, 1995), acetylcholinesterase enzyme (AChE) which was measured according to Magnotti et al. method (Magnotti, Eberly, Quarm, & McConnell, 1987) and serum tumor necrosing factor alpha (TNF-α) by enzyme-linked immunosorbent assay (ELISA) Maskos et al. (Maskos et al., 1998).

Passive avoidance test is a behavioral test to evaluate memory function in all groups in which rats with normal memory spent longer time until forget danger in dark and enter the dark than rats with abnormal memory and remembered

danger in dark compartment and spent shorter time in dark than rats with abnormal memory. T-Maze Test (Conde, Costa, & Tomaz, 1999) was used to assess learning and memory features in mice. The euthanized animals were packaged in a specific packaging with safety and infection control procedures in mind and transported to the hospital as a biohazard.

Statistical Analysis

DSPSS 22.0 was used to analyze ata (SPSS, Chicago, USA) The mean and standard deviation were used to describe the quantitative data (SD). The sum of all observations divided by the number of observations is the mean value. When it comes to the standard deviation [SD], it measures how widely distributed the mean values are across different samples. Tests for analysis of variance [ANOVA]: In the case of a comparison involving more than two means, an ANOVA is used. The Post Hoc test was employed to compare multiple variables at once.

Results

Table 1 showed that in AD group statistically significant decrease occurred in BDNF in relation to control group. Donepezil administration achieved significant increase in BDNF in donepezil treated group compared to AD group. Irisin administration achieved significant increase in BDNF in irisin treated group compared to AD group (AD+I). Donepezil and Irisin administration achieved significant increase in BDNF In Donepezil and Irisin treated group compared to AD group (AD+D+I).

Table 2 showed that a statistically significant increase occurred in Serum Tumor necrozing factor alpha in AD group in relation to control group. Donepezil administration achieved significant decrease in Serum Tumor necrozing factor alpha in donepezil treated group in relation to AD group, (AD+D). Irisin administration achieved significant decrease in Serum Tumor necrozing factor alpha in Irisin treated group in relation to AD group (AD+I). Donepezil and Irisin administration achieved significant decrease in Serum Tumor necrozing factor alpha in Donepezil and Irisin treated group in relation to AD group, (AD+D+I).

Table 3 showed that a statistically significant increase occurred in AD group in relation to control group. Donepezil administration achieved significant decrease in A che level in donepezil treated group, in relation to AD group, (AD+D). Irisin administration caused insignificant change in A che level in Irisin treated group in relation to AD group (AD+I). Donepezil and Irisin achieved significant decrease in A che level in Donepezil and Irisin treated group in relation to AD group, (AD+D+I) Donepezil and Irisin achieved significant decrease in A che level in Donepezil and Irisin treated group in relation to AD group, (AD+D+I).

Table 4 showed that a statistically significant increase occurred in time in the dark in AD group in relation to control group. Donepezil administration achieved significant decrease in time in the dark in donepezil treated group in relation to AD group, (AD+D). Irisin administration achieved significant decrease in time in the dark in Irisin treated group in relation to AD group, (AD+I). Donepezil and Irisin administration achieved significant decrease in time in the dark in

Donepezil and Irisin treated group in relation to AD group, (AD+D+I) and this decrease was insignificant in relation to control group, but significant in relation to Donepezil treated group and Irisin treated group.

Table 5 showed that a statistically significant increase occurred in latency period in AD group in relation to control group. Donepezil administration achieved significant decrease in latency period in donepezil treated group in relation to AD group, (AD+D). Irisin administration achieved significant decrease in latency period in Irisin treated group in relation to AD group, (AD+I). Donepezil and Irisin administration achieved significant decrease in latency period in Donepezil and Irisin treated group in relation to AD group, (AD+D+I) and this decrease was insignificant in relation to control group, but significant in relation to Donepezil treated group and Irisin treated group.

Table 6 showed that a statistically significant decrease occurred in number of correct trials in AD group in relation to control group Donepezil administration achieved significant increase in number of correct trials in Donepezil treated group in relation to AD group (AD+D). Irisin administration achieved significant increase in number of correct trials in Irisin treated group in relation to AD group, (AD+I). Donepezil and Irisin administration achieved significant in number of correct trials in Donepezil and Irisin treated group in relation to AD group, (AD+D+I). Donepezil and Irisin administration achieved significant in number of correct trials in Donepezil and Irisin treated group in relation to AD group, (AD+D+I).

Table 7 showed that a statistically significant decrease occurred in step through latency in AD group in relation to control group. Donepezil administration achieved significant increase in Step through latency in donepezil treated group in relation to AD group, (AD+D). Irisin administration achieved significant increase in Step through latency in irisin treated group in relation to AD group, (AD+I). Donepezil and Irisin administration achieved significant increase in Step through latency in Donepezil and Irisin treated group in relation to AD group, (AD+D+I) but this increase was insignificant in relation to control group, but significant in relation to Donepezil treated group and Irisin treated group.

Discussions

Although AD is the most prevalent kind of dementia, with a high rate of prevalence rise, there are currently no conclusive medicines available to address this illness directly. AD is a neurodegenerative condition that mostly impairs memory and has no known treatment. Numerous underlying processes have been hypothesized to account for the pathophysiology of AD (Lourenco, Ferreira, & De Felice, 2015). There is substantial evidence that memory impairment in AD is caused by synaptic failure and loss (Lepeta et al., 2016). As a result, interventions focused at reestablishing or maintaining synaptic function and cognition are highly needed.

In the present work, scopolamine administration reduced BDNF, this is may be due to accumulation of Aß bodies that cause down regulation of BDNF mRNA transcripts as Aβ decreased phosphorylated cAMP responsive element binding

protein (CREB) cellular transcription factor. It interacts to certain DNA sequences known as cAMP response elements (CRE), boosting BDNF transcription as illustrated by Ng et al. (Ng, Ho, Tam, Kua, & Ho, 2019). In agreement with this, Konar et al. (Konar et al., 2011), concluded that i.p administration of scopolamine cause down regulation of BDNF expression in dose dependent manner.

Chen et al. (Chen et al., 2018) has also reported that BDNF depletion by scopolamine in mice cause reduction in myelin basic protein immunoreactivity in the hippocampus and subsequent memory impairment. While in irisin treated group and in donepezil treated group there was significant increase in BDNF, but this increase was more significant in combined administration of irisin and donepezil. Irisin is thought to be a critical regulator of survival of neurons in degenerative disorders such as AD as it causes proliferation of hippocampal cells, although it was first confined to physical fitness and browning (Moon, Dincer, & Mantzoros, 2013).

The expression of BDNF, particularly in the hippocampus, enables FNDC5/irisin to play a role in learning and memory. This demonstrates the critical significance of FNDC5/irisin in preventing brain disorders such as AD (Rabiee et al., 2020). These results are in agreement with Jodeiri Farshbaf and Alviña, (Jodeiri Farshbaf & Alviña, 2021) who postulated that irisin administration increase BDNF. Moreover, increasing BDNF improves the memory by suppressing the neuron's cytotoxic response to Aβ toxicity in AD (L. Zhang et al., 2015). Another mechanism that facilitats BDNF's neuroprotective effect is that it promotes brain regeneration and adult neurogenesis by modulating synaptogenesis and synaptic plasticity, as well as increasing cell survival and decreasing apoptosis. As a result, BDNF is helpful at rehabilitating neuronal skills and capacities related with learning, memory and perceptual motion (Tu, Peng, & Jiang, 2020).

BDNF has a high affinity for the tropomyosin receptor kinase B (TrkB) and a low affinity for the neurotrophin receptor NTR. The positive benefits of BDNF signaling are primarily mediated by TrkB receptor through well-described signaling cascades involving phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR), or extracellular signal-regulated (ERK) (de Freitas, Lourenco, & De Felice, 2020). Irisin administration is believed to have neuroprotective role and memory enhancement effect through increasing a variety of proteins such as cAMP, PKA and CREB (Lourenco et al., 2019). Irisin inhibits the binding of amyloid-β oligomers (AβO) to neurons and inhibits AβO-induced eukaryotic initiation factor 2α (eIF2α) and protein synthesis inhibition. These steps are required for synaptic and memory dysfunction in AD models, indicating a possible downstream mechanism by which irisin protects memory (Lourenco et al., 2015).

Exercise can stimulate the central expression of BDNF as well as other neuroprotective and memory-enhancing genes. As a result, De Pesce and colleagues concluded that exercised mice had less cognitive and memory deficits, which were connected with increased brain levels of irisin, FNDC5, PGC-1, and BDNF. Interestingly, when exercised rats were treated with an anti-FNDC5 antibody, exercise's beneficial benefits on cognition and memory were reversed (Pesce et al., 2021). Another hypothesis is that persistent high-intensity exercise

significantly boosts critical plasma hormones, proteins, or miRNAs associated with exercise's positive effects in humans and mice (Amaro Andrade et al., 2018). On the other hand, BDNF Choi et al., 2018 has demonstrated that Over-expression of BDNF alleviated neuro-degeneration and rescued the impaired memory (Choi et al., 2018).

An interesting research established that FNDC5/irisin is activated in the brain via PGC-1α and enhances BDNF expression (J. Zhang et al., 2017). The present work recorded significant rise of inflammatory marker TNF-α in the AD induced animals. Irisin administration and donepezil administration significantly reduced this elevated TNF- α. But this decrease was more significant in irisin treated group. On the other hand, Cutuli et al. (Cutuli et al., 2013) and Guo et al., (Guo et al., 2015) showed that donepezil increased learning and memory capacities by inhibiting microglial activation and reducing the release of anti-inflammatory cytokines such as IL-1β and TNF-α. Furthermore, donepezil boosted the expression of IDE, one of the enzymes that degrades Aβ, and lowered Aβ levels (Pattanashetti, Taranalli, Parvatrao, Malabade, & Kumar, 2017).

Irisin treatment decreased pro-inflammatory cytokine TNFα (which is secreted by macrophages) which suggests that irisin may act through an anti-inflammation mechanism to reduce the neurological disorders (Wang et al., 2019). Other research corroborates this idea which showed that intravenous irisin reduced microglia activation and neuroinflammation in mice (Li, Li, Yuan, Qu, & Wang, 2017). Additionally, irisin pretreatment resulted in a significant decrease in Myeloid differentiation primary response protein 88 (MyD88) levels and Toll-like receptor (TLR4), as well as decreased nuclear factor-κB (NFκB) phosphorylation, thereby limiting the release of critical pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), as well as monocyte chemotactic protein (MCP-1) and keratinocyte chemoattractant (KC) (Rabiee et al., 2020). Wang and his followers had concluded that there were significant changes of the phosphorylation level of p38 and signal transducer and activator of transcription 3 (STAT3). STAT3 is a critical regulator of a variety of cytokine cascades, including those involving IL-6, IL-10, and TNFα (Wang et al., 2019).

Concerning the role of AChE activity in AD, memory impairment is caused in AD by disruption of cholinergic system as evidenced by increase in acetyle choline esterase enzyme activity (enzyme that catalyze hydrolysis of acetylcholine) resulting in a reduction in the brain's acetylcholine levels. Donepezil administration revealed decrease in AchE activity in treated group. This is consistent with El-Marasy et al. (El-Marasy et al., 2018)who stated that scolpolamine cause elevation of AChE activity. Elevated AChE activity induce memory impairment by increase formation of Aß plaques which in turn activates astrocytes and upregulate glial fibrilatory acid protein (GFAP) which cause neuroinflammation (Singh et al., 2013) (Ghumatkar, Patil, Jain, Tambe, & Sathaye, 2015).

Scopolamine's mechanism of increasing AChE activity may be caused by increase the expression noncatalytic activity protein of acetylcholineesterase by oxidative stress induced by scopolamine, so AChE activity increase and subsequent memory impairment occurs (Rahimzadegan & Soodi, 2018). Memory improvement

caused by significant reduction in the level of AchE, which enhanced the availability of acetylcholine for memory improvement. This may be due to increase NOS expression after donepezil administration and enhance levels of NO which maintain long term potentiation in hippocampal synapse and promotes Ach release and enhance memory as illustrated by Zhou et al., 2016 (Zhou et al., 2016).

Donepezil's molecular structure is unique in that it inhibits both active and peripheral anionic sites AchE simultaneously (Sharma, 2019). In agreement with this, Pattanashetti et al. (Pattanashetti et al., 2017) and Shin et al. (Shin et al., 2018), recorded that donepezil improve scopolamine memory impairment in mice. Our results were confirmed by behavioral tests, passive avoidance test and T-maze learning. The passive avoidance model has been used to investigate the acquisition of knowledge and memory in response to a stressful event. The technique is based on rats' natural preference for the dark area of the apparatus and its suppression following exposure to an inescapable shock; that is, passive avoidance performance is an adaptive reaction to a stressful event that acts as a measure of learning and memory (Tsuji, Takeda, & Matsumiya, 2003).

Maze learning is a gold standard for evaluating the effects of cholinergic and glutaminergic modulation on cognitive performance in animals. Numerous results concerning the impact of new chemicals or therapeutic treatments on spatial memory and spatial working memory are based on maze learning paradigm performance (Snyder, Bednar, Cromer, & Maruff, 2005). The T-maze test is based on rats' proclivity to explore novel environments. Normal animals will choose to explore an arm of the maze that is different from the one they visited previously. This test is primarily used to measure working memory that is hippocampal-dependent. As a result, an animal with a memory deficiency will be unable to recollect which arm of the labyrinth it just visited, resulting in a reduced proportion of alternation.

In the present study, after scopolamine administration, there was increase in latency period and decrease percentage of alteration (number of correct trials) in T maze rest while there was decrease in step through latency period, increase time in darkness in passive avoidance test. Decrease percentage of alteration (decreased number of correct trials), step through latency period and increase time in darkness is caused by working memory impairment by scopolamine. Similar to this study, Gacar et al., (Gacar et al., 2011) and Seifhosseini et al., (Seifhosseini, Jahanshahi, Moghimi, & Aazami, 2011) showed that scopolamine significantly shortened step through latency period.

Memory improvement occurred in donepezil treated group and irisin trated group, as in T maze test, there was significant increase percentage of alteration (increase number of correct trials) and significant decrease in latency period. While in passive avoidance test there was significant increase in step through latency period and significant decrease time in darkness. But after combined administration of irisin and donepezil more significant increase occurred in percentage of alteration and more significant decrease in latency period. These results are in agreement with Zhou et al. (Zhou et al., 2016) , Pattanashetti et al.

(Pattanashetti et al., 2017) and Shin et al. (Shin et al., 2018), who recorded that donepezil, improve scopolamine memory impairment in mice.

These significant differences suggest that the treatment of irisin with donepezil may act synergistically to enhance memory and learning in a scopolamine-induced amnesia model by reversing the oxidative stress, decreasing the AchE level and β amyloid level in rat brain which may be the probable mode of actions for its beneficial effect as compared to the individual treatment. Thus, multi-drug therapy would be interesting to get the best response in the treatment of AD.

Recommendation

Irisin can be introduced as a novel therapy in combination with donepezil in treatment of mild to moderate AD patients. Clinical trials are recommended to identify the effective dose of irisin in treatment of AD.

Conclusions

We conclude that irisin has the ability to reduce scopolamine-induced AD manifestations through its antiinflammatory, antioxidant and antiapoptotic effects, which were evaluated both biochemically and behavioural. Donepezil enhances memory as it is anti cholinestrase, also it has anti-inflammatory effect. Donepezil and irisin treatment potentiate each other as they act synergestically by different mechanisms to counrtact memory loss.

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Tables

Table 1: BDNF in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	1358	935	1038	1185	1340
2	1315	758	1253	1245	1255
3	1496	1142	1294	1019	1315
4	1265	985	1469	1475	1545
5	1496	975	1097	996	1650
6	1854	815	1025	1274	1520
7	2130	1315	985	1498	1480
8	2150	1056	1512	1468	2080
9	1765	980	1502	1063	1960
10	1630	995	1059	1245	2120
BDNF	Control	AD	AD+D	AD+I	AD+D+I
Range	1265 – 2150	758 – 1315	985 – 1512	996 – 1498	1255 – 2120
Mean ±	1645.90 ±	995.60 ±	1223.40 ±	1246.80 ±	1626.50 ±

SD	321.22	156.57	210.99	187.75	319.30
F test	12.777				
P value	0.001*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
0.001*	0.001 *	0.001 *	0.862	0.046*	0.029 *	0.001*	0.83 4	0.001 *	0.001 *

P1: Control & AD P2: Control & AD+D P3: Control & AD+I
P4: Control & AD +D+I P5: AD & AD+D P6: AD & AD+I
P7: AD & AD+D+I P8: AD+D & AD+I
P9: AD+D & AD+D+I P10: AD+I & AD+D+I

Table 2: TNF α in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	10.65	25.73	15.28	10.03	10.45
2	7.22	26.65	17.08	8.08	9.04
3	9.03	28.12	18.20	9.34	11.19
4	8.16	29.44	17.14	8.23	8.80
5	9.40	28.60	16.80	9.15	11.09
6	8.77	30.25	15.50	7.84	10.22
7	8.20	28.14	18.34	10.78	9.06
8	9.04	33.07	17.78	8.36	10.27
9	10.56	29.08	16.01	8.94	9.45
10	9.64	30.12	18.76	9.14	8.93
TNF α	Control	AD	AD+D	AD+I	AD+D+I
Range	7.22-10.65	25.73-33.07	15.28-18.76	7.84-10.78	8.80-11.19
Mean ± SD	9.07± 1.07	28.92± 2.04	17.09±1.21	8.99± 0.92	9.85± 0.91
F test	439.211				
P value	0.0002*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
0.007*	0.008*	0.894	0.184	0.002*	0.006*	0.004 *	0.005 *	0.003*	0.14 5

P1: Control & AD P2: Control & AD+D P3: Control & AD+I
P4: Control & AD +D+I P5: AD & AD+D P6: AD & AD+I
P7: AD & AD+D+I P8: AD+D & AD+I
P9: AD+D & AD+D+I P10: AD+I & AD+D+I

Table 3: A che in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	751.2	890.1	875.6	784.3	888.3
2	791.5	994.8	891.2	997.5	888.4
3	810.5	795	625.8	835	694.5
4	894.2	890	658.9	898.3	882.3
5	671.5	1200	890.3	1210	715.2
6	1080.6	1280	794.2	889.5	886.2
7	705.3	885.4	886.3	1220	694.2

8	891.2	947.5	940.2	889.7	623.5
9	790.5	1050.3	1100.5	947.6	1050.3
10	605.3	890.2	884.3	889.6	961.5
A che	Control	AD	AD+D	AD+I	AD+D+I
Range	605.3 – 1080.6	795 – 1280	625.8 – 1100.5	784.3 – 1220	623.5 – 1050.3
Mean ± SD	799.18 ± 134.15	982.33 ± 153.47	854.73 ± 136.23	956.15 ± 147.83	828.44 ± 137.89
F test	5.220				
P value	0.008*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
0.006*	0.387	0.017*	0.647	0.042*	0.682	0.020*	0.047*	0.681	0.042*

P1: Control & AD P2: Control & AD+D P3: Control & AD+I
P4: Control & AD +D+I P5: AD & AD+D P6: AD & AD+I
P7: AD & AD+D+I P8: AD+D & AD+I
P9: AD+D & AD+D+I P10: AD+I & AD+D+I

Table 4: Time in the dark in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	23.4	102.3	24.5	30.9	14.2
2	14.6	75.6	24.5	21.3	14.2
3	15.7	117.3	21.5	24.5	13.2
4	24.5	107.1	38.5	32.7	24.5
5	18.4	103.2	41.2	41.6	26.3
6	16.6	91.6	38.5	34.1	20.9
7	21.9	85.3	31.9	37.5	23.6
8	17.2	80.4	30.9	41.2	19.2
9	14.2	87.5	36.5	39.3	20.8
10	15.9	91.3	31.2	30.3	23.6
Time in the dark	Control	AD	AD+D	AD+I	AD+D+I
Range	14.2 – 24.5	75.6 – 117.3	21.5 – 41.2	21.3 – 41.6	13.2 – 26.3
Mean ± SD	18.24 ± 3.72	94.16 ±13.01	31.92 ± 6.78	33.34 ± 6.85	20.05 ± 4.73
F test	164.012				
P value	0.001*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
0.001*	0.001*	0.001*	0.603	0.001*	0.001*	0.001*	0.683	0.001*	0.001*

P1: Control & AD P2: Control & AD+D P3: Control & AD+I
P4: Control & AD +D+I P5: AD & AD+D P6: AD & AD+I
P7: AD & AD+D+I P8: AD+D & AD+I
P9: AD+D & AD+D+I P10: AD+I & AD+D+I

Table 5: Latency Period in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	19	75	35	45	23
2	22	76	32	51	22
3	25	85	39	49	19
4	21	82	42	62	22
5	20	105	51	65	27
6	18	80	56	67	24
7	26	103	54	41	20
8	27	100	39	68	21
9	23	110	52	62	27
10	28	115	45	52	29
Latency Period	Control	AD	AD+D	AD+I	AD+D+I
Range	18 – 28	75 – 115	32 – 56	41 – 68	19 – 29
Mean ± SD	22.90 ± 3.48	93.10 ±15.03	44.50 ± 8.40	56.20 ± 9.74	23.40 ± 3.31
F test	101.045				
P value	0.001*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
0.001*	0.001*	0.001*	0.903	0.001*	0.001*	0.001*	0.006*	0.001*	0.001*

P1: Control & AD
P2: Control & AD+D
P3: Control & AD+I
P4: Control & AD +D+I
P5: AD & AD+D
P6: AD & AD+I
P7: AD & AD+D+I
P8: AD+D & AD+I
P9: AD+D & AD+D+I
P10: AD+I & AD+D+I

Table 6: Trials in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	3	1	2	2	3
2	3	1	2	2	2
3	3	1	2	2	3
4	3	1	2	2	3
5	3	1	2	1	3
6	3	1	1	2	3
7	2	1	2	2	2
8	3	1	2	2	2
9	2	0	1	2	3
10	3	0	2	2	3
Trials	Control	AD	AD+D	AD+I	AD+D+I
Range	2 – 3	0 – 1	1 – 2	1 – 2	2 – 3
Mean ± SD	2.80 ± 0.42	0.80 ± 0.42	1.80 ± 0.42	1.90 ± 0.32	2.70 ± 0.48
F test	37.788				
P value	0.001*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10

0.001*	0.001*	0.001*	0.594	0.001*	0.001*	0.001*	0.593	0.001*	0.001*

P1: Control & AD P2: Control & AD+D P3: Control & AD+I
P4: Control & AD +D+I P5: AD & AD+D P6: AD & AD+I
P7: AD & AD+D+I P8: AD+D & AD+I
P9: AD+D & AD+D+I P10: AD+I & AD+D+I

Table 7: Step through latency in all studied groups

Number	Control	AD	AD+D	AD+I	AD+D+I
1	90.3	25.3	86.2	81.5	79.5
2	76.5	28.6	52.6	52.6	73.5
3	81.3	31.2	61.2	57.5	86.3
4	68.5	34.7	57.9	63.2	87.7
5	96.4	32.8	87.2	64.8	105.6
6	110.3	51.2	63.2	74.3	121.3
7	115.2	48.6	67.5	70.3	109.6
8	105.3	43.6	71.2	79.5	94.5
9	100.3	31.3	82.6	90.7	81.2
10	86.4	34.6	90.1	63.2	82.3
Step through latency	Control	AD	AD+D	AD+I	AD+D+I
Range	68.5 – 115.2	25.3 – 51.2	52.6 – 90.1	52.6 – 90.7	73.5 – 121.3
Mean ± SD	93.05 ±15.16	36.19 ± 8.66	71.97 ±13.59	69.76 ±11.77	92.15 ± 15.35
F test	30.891				
P value	0.001*				

P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
0.001*	0.001*	0.001*	0.879	0.001*	0.001*	0.001*	0.709	0.001*	0.001*

P1: Control & AD P2: Control & AD+D P3: Control & AD+I
P4: Control & AD +D+I P5: AD & AD+D P6: AD & AD+I
P7: AD & AD+D+I P8: AD+D & AD+I
P9: AD+D & AD+D+I P10: AD+I & AD+D+I