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Acetylcholine Esterase Gene Expression in Salivary Glands of Albino Rats after Treatment with amitriptyline or/and Ashwagandha

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ABSTRACT

Acetylcholinesterase is required as an enzyme to counteract the effects of acetylcholine. The aim of the study is to assess how amitriptyline and Ashwagandha affect the acetylcholinesterase gene in rat salivary glands. Forty healthy albino rats were divided randomly into four equal groups: Group I (control) received distilled water for 30 days. Group II received amitriptyline (10mg/kg) for 30 days. Group III received ashwagandha watery root extract (200mg/kg) orally for 30 days and Group IV received the combination of amitriptyline orally and ashwagandha root extract orally for 30 days. Rats in each group were sacrificed after day 30 and salivary glands were dissected for measurement of the acetylcholinesterase gene using a Polymerase Chain Reaction technique (PCR). Acetylcholinesterase gene measurements reveal an increase in groups treated with amitriptyline alone (1.55±0.11) and in the group treated with a combination of amitriptyline with Ashwagandha (1.92±0.16), in comparison with the control group. There were no discernible differences between the Ashwagandha treated group (1.073± 0.25) compared to the control group (0.76±0.19).In conclusion**,** Amitriptyline alone and, when combined with Ashwagandha cause transcription of the acetylcholinesterase gene.

Keywords: Acetylcholinesterase gene, Amitriptyline, Ashwagandha, Salivary glands.

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INTRODUCTION

One gene encodes the enzyme Acetylcholinesterase (AChE), which is necessary for the termination of acetylcholine's action. Alternative mRNA processing results in the development of three different carboxyl-terminated enzyme forms. These structural variations control the expressed enzyme's cellular location but do not impact its catalytic activity **(Taylor, 2011)**. A 6 kb gene with several transcriptions starts sites codes for AChE **(Bronicki and Jasmin, 2012)**. The promoter includes a multitude of regulatory components, such as Sp-1, Egr-1, and AP2 binding sites **(Rotundo, 2020).** A number of other heat shock components bring on induction of AChE transcription following heat shock (**Chen *et al.*, 2010).** Separation has also been proposed as a regulator of AChE expression **(Layer *et al.*, 2013).**

Alternative splicing occurs in up to 90% of human genes, and AChE is no exception. In the brain, muscle, and erythropoietic tissue, alternative splicing of a single AChE gene in the 5' region results in the production of isoforms with tissue-specific expression patterns. **(Tapial *et al.*, 2017)**. For instance, it has been demonstrated that the brain isoform uses a higher upstream transcriptional start point **(Li *et al.*,1993)**. At the 3' end, AChE pre-mRNA is also prone to alternative splicing. Three transcripts are produced as a result of this, reading through (AChE R), hydrophobic (AChE H), and synaptic (AChE S) or tail (AChE T). AChE H is present in erythrocytes as glycosylphosphatidylinositol (GPI)-anchored dimers **(Massoulie *et al.*, 2005; Meshorer and Soreq, 2006)**.

However, depending on whether the 5' donor site downstream of E4 splices to the acceptor site upstream of E5 or E6, alternative splicing in neurons results in either AChET or AChER. By splicing to the distal E6 splice site and integrating E6 into the mRNA, the synaptic AChET is produced **(Bronicki and Jasmin, 2012)**. Nevertheless, although AChET often predominates, cell stress encourages the upregulation

of AChER. **(Shaked *et al.*, 2008)**. In neuronal cell lines, APP can suppress the transcription of AChE. When acting, APP has a contractual partner, possibly ITGA5. Increased levels of total Akt and phospho-Akt result from this interaction's signal transduction, which may involve FAK. The transcription of AChE is repressed due to this activation of Akt **(Gunn *et al.*, 2022).** Since amitriptyline causes anticholinergic side effects including xerostomia. So, this study to assess the amitriptyline or/and ashwagandha effect on the acetylcholinesterase gene in rat salivary glands and the possibility of overcoming anticholinergic side effects of amitriptyline by Ashwagandha.

MATERIALS AND METHODS

Experimental substances

This research used forty healthy albino rats that were 8-10 weeks old and weighed 200-250g. They were obtained from the Faculty of Veterinary Animal House at Mosul University, Iraq. They were kept in rodent plastic cages with wire mesh covers. The animals were kept at a room temperature of 22±2C° with 12 hours of light and darkness and unrestricted access to food and water *ad-libitum*. All procedures followed the guidelines of the Faculty of Dentistry's institutional animal research ethics committee in the College of Dentistry, University of Mosul, Iraq(UOM. Dent/A.L.56/22).

Ashwagandha root extract was available in powder obtained from Naturalaya Kimya company/Antalya /Turkey. (Fresh aqueous solution of Ashwagandha was prepared and administered orally every day. (Rats treated orally by oral gavage needle with 0.5 ml aqueous of Ashwagandha root extract (100 ml water +5000mg plant) at a dose of 200 mg \ kg body weight (50 mg/rats) for 30 days **(Mahmoud *et al.*, 2022).** Amitriptyline was available in the form of tablets from accord company United Kingdom. (Fresh solution of amitriptyline was prepared and administered orally every day (Fig.1).

Fig.1: Amitriptyline tablet used in this study

Experimental design

Forty rats were randomly divided equally into four groups as follows:

Group I (Control n=10): rats were daily received distilled water at (1-2 ml/kg) for 30 days experiment

Group II (Amitriptyline group n=10): rats were given Amitriptyline 10mg/kg orally using a gavage needle daily, from the first day to the last day of the experiment.

Group III(Ashwagandha group n=10): rats were given Ashwagandha aqueous root extract 200mg/kg orally daily using an oral gavage needle, with 0.5 ml from the first day to the last day of the experiment.

Group IV(combination group n=10): rats were given a combination of Amitriptyline 10mg/kg and Ashwagandha watery root extract 200mg/kg orally using a gavage needle from the start of the trial until its conclusion.

Salivary glands tissue preparation

Salivary glands were removed to measure the tissue's acetylcholine esterase gene using Polymerase Chain Reaction (PCR) machine (fig 2). Salivary gland samples were put in a buffered phosphate solution for analysis. After 30 days of the administration, two hours following the last treatment, the animals in each group were put under light ether anesthesia and sacrificed.

Fig.2: Polymerase Chain Reaction PCR device used in the study

Gene expression analysis

Tissue extraction protocol

1. Place up to 20 mg of tissue that has been cut into smaller pieces in a 1.5 ml microcentrifuge tube with 200 µl of Lysis Solution.

2. The sample tube was filled with 20 µl of Proteinase-K-solution (20 mg/ml), proper mixing by vortexing, and then incubated at (56 °C) until the tissue was lysed. To ensure that it is distributed evenly during incubation, you can also put the sample tube in a vibrating water bath or on a platform that rocks. The amount of lysis time depends on the kind

of tissue that is being treated. Overnight lysis had no impact on the preparation.

3. The tube was rotated downward to clear any droplets from the sample tube cap.

4. (Optional DNase A treatment) If RNA is required, add the 20 µl of DNase A Solution (10 mg/ml, not supplied).

5. After adding 200 µl of the binding solution to the sample tube, thoroughly mix it with a pulsing vortex for 15 seconds.

6. Incubate for 10 minutes at 56 °C. Longer incubation times have no impact on the quantity or caliber of the purified RNA.

7. (200 µl) of absolute ethanol was filled up and mixed thoroughly using a pulsing vortex for (15) seconds. Once you've finished doing this, To remove the drops caught on the lid of the container, quickly spin it.

8. using a 2.0 ml collection tube, delicately transfer the lysate into the upper reservoir of the spin column without saturating the rim.

9. Remove the flow-through and connect the 2.0 ml collecting tube to the spin column after a minute of centrifuging at 13,000 rpm.

10. For 1 minute, centrifuge at 13,000 rpm while adding 500 µl of washing one solution using a collection tube to connect the spin column: Drain the flow through first, then insert the 2.0 ml collection tube into the spin column.1. Centrifuge for one minute at 13,000 rpm. 11. 500 µl of the Washing 2 Solution should be added. Remove the flow through and put the 2.0 ml collecting tube into the spin column.

12. The spin column was dried by running a further 1 minute of 13,000 rpm centrifugation to remove any remnant ethanol.

13. Use the new 1.5 ml microcentrifuge tube and insert it inside the spin column.

14. Pour 100 to 200 µl of the elution buffer solution into the spin column within the micro-centrifuge tube, and then leave it alone for at least a minute.

15. Centrifuge at 13,000 rpm for 1 minute to elute the genomic RNA.

Primer design for genes (forward and reverse primer sequence)

One of the most important aspects of quantitative real-time PCR (qPCR) analyses' performance and quality is the design of the primers since effective primer design is essential for accurate and reliable quantification. To locate possible primers for certain qPCR assays, primer design should follow several criteria. The GAPDH gene was used as a housekeeping gene. Acetylcholinesterase gene primers were designed through the use of well-known website software which is (NCBI); the primer sequences as in the following (Table 1).

Table1. Primer sequences of acetylcholine

Genes	The forward sequence of primers	The reverse sequence of primers
GAPDH	ACATGCACAGGGTACTTCGA	TTACCCCAGCCTTCTCCATG
Acetylcholinesterase gene	TTGCGTAGAACGGAGAGCTG	GTCAGTTCTTCCCTGCCGAG

GoTaq® qPCR Master Mix:

The second kit is for quantitative RNA detection, which is (GoTaq qPCR Master Mix), from (Promega Corporation USA). The quantitative PCR (qPCR) reagent system GoTaq® qPCR Master Mix (a,b). This system includes a fluorescent RNA-binding dye (BRYT Green® Dye) that binds to double-stranded RNA (dsRNA) and exhibits higher fluorescence amplification. All necessary components for qPCR are included in the easy-to-use, stable 2X formulation known as (GoTaq® qPCR Master Mix) except (sample RNA, primers and water).

This formula includes GoTaq® Hot Start Polymerase, $MgCl_2$, dNTPs, a custom reaction buffer, a proprietary dsRNA-binding dye, and a low concentration of carboxy-X-rhodamine (CXR) reference dye (identical to ROXTM dye) yields the best results in qPCR tests. For use with instruments that need more reference dye than what is in the GoTaq® qPCR Master Mix, a separate bottle of CXR Reference Dye is provided.

Quantitative measurement of acetylcholine esterase gene

Includes a quantitative measurement of acetylcholine esterase RNA materials, RNA extracted as described above from submandibular gland tissues using AddPrep Genomic RNA Extraction Kit. The acetylcholine esterase genomic material was determined by (qPCR) by using the (Go-Taq-qPCR master mix) produced by Promega and PCR max Eco machine. Replication reactions of the goal gene and household genes were performed for the samples. ΔΔCt calculated for comparison of genes between samples. Replication reactions were done for the genes of the study and household genes were done for all samples. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping genes were used as a control to calculate the ΔCT value. ΔΔCT calculated for comparison of the results of gene expression between samples. The ΔCT value was calculated for each sample as the difference in CT between the gene of interest and the household gene. ΔΔCT was measured as the difference between the ΔCT values of the study sample and the control sample. The acetylcholinesterase genes in this study were expressed as ΔΔCT (mean ±SD).

ΔCT (Sample) = CT AchE gene – CT GAPDH

ΔCT (control) = CT control – CT GAPDH

ΔΔCT = ΔCT (Sample) – ΔCT (control).

Statistical analysis

SPSS program version 21 for Windows was used for the statistical analysis. Mean and standard deviation are two ways to express descriptive statistics of data (SD). Statistical tests such as one-way analysis of variance (ANOVA)followed by (Duncan's post-hock) were used to compare the differences between the four study groups. **(Ali and Bhaskar 2016).** P≤0.05 was the significance level.

RESULTS

Our results showed a significant increase in the acetylcholinesterase gene in the group treated with amitriptyline alone ΔΔCT value (1.55±0.11), and a combination of amitriptyline with ashwagandha ΔΔCT value (1.92±0.16) in comparison with control group ΔΔCT (0.76±0.19) at p-value ≤0.05 but no significant difference between group treated with Ashwagandha alone ΔΔCT value (1.073± 0.25) in comparison with the control group.

In Ashwagandha treated alone, an increase in acetylcholine esterase gene expression was found but not as shown when given amitriptyline alone or in combination with amitriptyline with Ashwagandha, which shows a significant increase in the transcription of acetylcholinesterase gene when compared to the control group (Table 2 and Fig.3).

Table 2: The mean and standard deviation of Acetylcholinesterase gene expression in salivary glands

Groups	ΔΔCT of Acetylcholinesterase gene	P- value
Control	0.76±0.19	0.001*
Amitriptyline alone	1.55±0.11	0.001*
Ashwagandha alone	1.073± 0.25	0.077
Combination (Ashwagandha with amitriptyline)	1.92±0.16	0.000*

* meaning significant difference at p< 0.05.

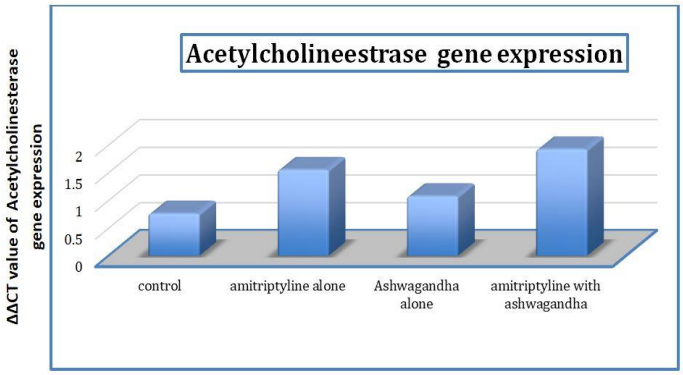

Fig.3: The histogram of the statistical analysis of the Acetylcholinesterase gene level after 30 days.

DISCUSSION

A hydrolytic enzyme called acetylcholinesterase (AChE) has been connected to the pathophysiology of many diseases like Alzheimer's disease (AD) **(Wang and Zhang, 2018)**.

This study showed an increase in acetylcholinesterase gene expression at day 30 when combining amitriptyline with Ashwagandha as a result of ashwagandha agonist action on muscarinic receptors, whatever, drugs that act as serotonin, noradrenaline dopamine and muscarinic agonist can stimulate Egr1 expression in different cell types of M1AchE with the muscarinic agonist **(Konaret *et al.*, 2019)**, The results were consistent with prior research, demonstrating that the muscarinic agonist carbachol significantly increases the transcription of the Egr-1, Egr-2, Egr-3, and Egr-4 transcription factors. The AChE gene has binding sites for the transcription factors Spl, Egr-I, and AP2. Experiments showed that Spl and Egr-1 sites are necessary for activating AChE gene expression,

whereas AP2 suppressed it. According to a prior study, treatment with the cholinesterase inhibitor tacrine for 12 months dramatically raised AChE activity in CSF by 50% compared to baseline. This study demonstrated an increased ChE gene expression following 30 days of dosing **(Darreh–Shori, *et al.*, 2002)**.

According to other studies, Physostigmine, an AChE inhibitor, enhanced AChE gene expression in cerebrospinal fluid **(Vecchio, *et al.*, 2021).** Analysis of the muscarinic receptor subtypes revealed that, in addition to the ml AChR, the m2, m3, and m4AChR could also induce the transcription of the EGR-I gene, albeit to differing degrees **(Nitsch, *et al.*,1998)**. That agrees with the results of the present study since amitriptyline administration for 30 days will lead to an increase in muscarinic receptors expression that is consistent with the inhibition of acetylcholinesterase enzyme caused by Ashwagandha **(Gros, *et al.*, 2021).**

The results of previous studies showed that Ashwagandha administration for 30 days enhanced cholinergic markers by inhibiting acetylcholinesterase enzyme and improving muscarinic receptor binding abilities **(Al-mahmud, *et al.*, 2016)**. Ashwagandha is crucial for correcting the decline in cholinergic indicators including acetylcholine and choline acetyltransferase (ChAT) **(white, *et al.*, 2016; Roy, 2018)**. Ashwagandha inhibited the AChE and BuChE enzyme activity in an in vitro experiment **(Behl, *et al.*,2020).** Therefore, blocking muscarinic acetylcholine receptors increased the amount of AChE released by cells (mAChRs). This pathway, which most likely involves the transcription factor Egr-1, will result in the transcriptional upregulation of AChE by mAChRs **(Mashimo, *et al.*, 2021)**. Although Egr-1 appears to be the primary target of activation of mAChRs through MAPK, Egr-2, -3, and -4 levels at both the protein and mRNA levels have also been demonstrated to be regulated **(Gitenay and Baron, 2009)**.

Elk1 has been intimately associated with Egr-1 activation and AChE control. It has been hypothesized that the aforementioned MAPK activation causes the Elk-1 and SAP-1/-2 families of transcription factors to become active **(Yang, *et al.*, 2013).** Elk-1, a member of the E-twenty six (Ets) family of transcription factors (TFs), activates the Egr-1 promoter by forming a complex with CREB-binding protein (CBP) and SRF **(Besnard, *et al.*, 2011)**. Phosphatases like protein phosphatase 2B prevent Elk-1 signaling **(Zhao, *et al.*, 2021).** A 6 kb gene with several transcriptions start sites codes for AChE **(Bronicki and Jasmin, 2012).** AChE transcription is induced following heat shock in addition to other heat shock components **(Kim, *et al.*, 2021).** At the 3' end, AChE pre-mRNA is also prone to alternative splicing. Three transcripts are produced as a result of this, reading through (AChER), hydrophobic (AChEH), and synaptic (AChET). By splicing to the distal E6 splice site and integrating E6 into the mRNA, the synaptic AChET is produced **(Bronicki and Jasmin, 2012).**

Nevertheless, despite the fact that AChE T typically predominates, cell stress encourages the upregulation of AChER **(Lin and Zhang, 2018).** A mechanical connection between the regulation of AChE expression and the Amyloid precursor protein (APP) was revealed by later research. AChE mRNA levels will decrease in response to the over-expression of APP, a protein that plays a function in cell adhesion and is frequently mediated through protein interactions **(Tang, 2019)**. When APP was knocked down and AChE mRNA increased noticeably, this regulatory link between APP and AChE was validated **(Rump, *et al.*, 2022).** The extracellular, N-terminal E1 domain of APP, and more especially the copper-binding region therein, were necessary for the protein to be able to inhibit AChE transcription. The capacity of APP to suppress AChE's transcription was completely eliminated once these domains were deleted **(Uddin, *et al* ., 2020)**.

CONCLUSION

Amitriptyline when given alone and/or with Ashwagandha induces acetylcholinesterase gene transcription as a potentiation interaction. While giving Ashwagandha alone non-significantly increased acetylcholinesterase gene transcription compared with the control group.

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Conflicts of interest

The authors declared no competing interests.

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