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# Analysis of Acetylcholinesterase Gene Expression in Male Mice Exposed to Sublethal Dose of Endosulfan Pesticide

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**Abstract**

The aim of the current study was to investigate endosulfan effects on the Acetylcholinesterase (AChE) enzyme and gene in albino mice. Thirty selected male albino mice were randomly divided into 3 groups. The first group was control group (G1), while the other two treated groups were injected intraperitoneally twice per week with two doses of endosulfanG2 (3 mg/kg) and G3 (17 mg/kg) for 21 and 45 days respectively. The results recorded a significant decrease in AChE enzyme in the group treated with 17 mg/kg b.wt. (3986.67 ±170.32 U/L),compared to the control (5584.33 ±140.35 U/L)and treatment group with 3 mg/kg b.wt.(5556.00 ±341.01) U/L for 21 days. Also, there was a significant decrease in the enzyme level between the two treated groups (4688.00 ±221.49 U/L)and (3413.67 ±305.42 U/L) for the duration of 45 days of the dose, compared with the control group (5584.33 ±140.35 U/L), and increased expression of AChE gene in the treated groups.Gene expression fold in all treated groups was higher than in the control group withthe highest recorded value of 14.320at 17 mg/kg for 45 days, compared with the control group.

**Keywords: AChE gene, Acetylcholinesterase, Pesticide, LD50**

# تحليل التعبير الجيني في ذكور الفئران المعرضه للتراكيز تحت القاتله من مبيد الاندوسلفان

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**الخلاصة**

الهدف من الدراسة الحالية هو دراسة تأثير الإندوسلفان على إنزيم أستيل كولين أستراز (AChE) وجين *AChE* للفئران البيضاء .تم تقسيم ثلاثين من الذكور بشكل عشوائي إلى 3 مجموعات لمعاملتها بمبيد الإندوسلفان. المجموعة الاولى هي مجموعة السيطرة بينما المجموعتين المتبقيتين هما المجموعتين العلاجيتين اللاتي تم حقنهما مرتين في الأسبوع بجرعتين من الإندوسلفان (3 ملغرام\ كغم) و (17 ملغرام\ كغم) لمدة 21 و 45 يومًا على التوالي. سجلت النتيجة انخفاضاً معنوياً في إنزيم AChE في المجموعة المعالجة بمقدار 17 ملغرام / كغم من وزن الجسم (3986.67U/L ± 170.32 ) مقارنة بمجموعة التحكم (5584.33U/L ± 140.35) والمجموعة المعاملة بـ 3 ملغرام / كغم من وزن الجسم (5556.00U/L ± 341.01) لمدة 21 يومًا. كذلك تم تسجيل انخفاض معنوي في مستوى الانزيم بين المجموعتين المعالجتين (4688.00 U/L ± 221.49 ) و ( 3413.67U/L ± 305.42 ) لمدة 45 يوم من الجرعة مقارنة مع المجموعة السيطرة (5584.33 U/L ± 140.35) ، فضلا عن زيادة التعبير عن جين *AChE* في المجموعات المعاملة. كان التعبير الجيني في جميع

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مجموعات المعاملة أعلى منها في المجموعة الضابطة. وكانت أعلى قيمة (14.320) في 17 ملغرام \ كغم لمدة 45 يوم مقارنة مع مجموعة السيطرة.

## Introduction

Endosulfan (EN) (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodio-3-oxide) is a pesticide of a broad-spectrum organochlorine that is used to protect crops in pest management systems [1]. It has a half-life of more than 14 days in water and 60 to 800 days in soil [2]. Endosulfan is comprised of two isomers, α and β, and is ubiquitous in the environment and vegetation [3-6]. Due to their persistence in nature, endosulfan residues polluting the environment undergo bioaccumulation and biomagnification in the food chain, causing deleterious consequences for non-target creatures, including humans [7]. Endosulfan is a highly lipophilic chemical that is quickly absorbed and spreads in tissues, particularly the CNS. Despite the short half-life of endosulfan, it is gradually released from lipophilic depots into circulation [8]. Endosulfan's toxic effects on multiple systems, including the CNS, hepatic, immunological, and cardiovascular systems, have been shown in studies to pose acute, subacute and chronic dangers to human health [9].AChE is a cholinergic enzyme, mainly present in postsynaptic neuromuscular junctions. It degrades AChE into acetic acid and choline promptly[10].AChE's main function is to stop neuronal transmission and signalling between synapses, preventing AChE from spreading and activating neighbouring receptors. Furthermore, AChE suppression action has been widely employed as a biomarker for pesticide harming evaluation [11,12].Several environmental pollutants, such as organophosphate (OP) and carbamate insecticides, have similar effects on AChE and suppress its enzymatic activity irreversibly[13].Endosulfan is also known to inhibitAChE activity, resulting in high levels of acetylcholine (AChE) and behavioural problems [14]. Acetylcholinesterase (AChE) is one of the utmost commonly utilized biomarkers for nervous system toxicity (AChE, EC 3.1.1.7).AChE is a single-gene product [15].

Technique of real-time quantitative reverse transcription PCR (qRT-PCR) is commonly used to track changes in gene expression [16] and the approach is regarded to be sensitive, specific, repeatable and accurate [17].

## Materials and Methods

### Animals Housing and Chemicals

Adult male albino mice aged 6-8 weeks on average weighing approximately (25±5 g),used in the current studywere obtained from the Biotechnology Research Centre, Al-Nahrain University in Baghdad City. All mice were left for one week to acclimatize to the laboratory conditions before starting the experiments. The animals were housed in polypropylene cages in a specially controlled environment with 12±2 hours of light/dark cycles, with 25±5°Ctemperature and a relative humidity of 50-60%. The nutrition sources available were ad libitum. Commercial product of endosulfan pesticide that complies with European Union standards, was purchased by the Agricultural Pesticides Office in Al-Sinak District, Baghdad Governorate. The active ingredient of 35% endosulfan in 1 L, was used in the experimental study in liquid form.The molecular formula of endosulfan is C9H6Cl6O3S and its molecular weight of 406.9 g/mol with 106°C melting point [18] and EN 1.745 g/cm³ density at 20°C[19]. Ethical approval statement, Ref.: CSEC/1021/0077 was issued on the 5th of October, 2021 by the Ethics Committee/ Department of **Biology/ College of Science at the University of Baghdad.**

**Determination of $LD_{50}$ of Endosulfan Pesticide**

Albino male mice were intraperitoneally injectedby (3.5mg/kg, 7mg/kg, 14mg/kg, 28mg/kg, and 56mg/kg) of endosulfan to determine the $LD_{50}$. The mortality was recorded within 24 hours. The estimated lethal dose of endosulfan in mice is 34 mg /kg.

**In vivoStudy**

To determine the toxicological effects of endosulfan, 30 male albino mice were randomly divided r into 3 groups: control group and two treated groups with EN (G1, G2 and G3). One group was taken as the control, while the other two groups were injected intraperitoneally twice per week with two doses of endosulfan, G2 (3 mg/kg)and G3(17 mg/kg).

Measuring Acetylcholinesterase (AChE) Acetylcholine assay kit is based on the enzyme driven reaction that detects acetylcholine via acetylcholinesterase enzyme and choline oxidase. Acetylcholinesterase first hydrolyzes acetylcholine into choline and acetic acid. Choline is then oxidized by choline oxidase to produce hydrogen peroxide. The hydrogen peroxide can then be detected with a highly specific colorimetric probe. For this study, the absorbance measured was sat 540-570 nm range.

**Primer**

Primers used in the study were according to their reference sequence in the molecular diversity preservation international (MDPI)as shown in Table 1.

**Table 1:** Primer sequences used in the current study

| Primer | Sequence (5′→3′) |
|---|---|
| **AChE** | |
| **Forward** | CCTGGATCCCTCGCTGAA |
| **Reverse** | CCTGTGCGGGCAAAATTG |
| **GAPDH** | |
| **Forward** | CGGGTTCCTATAAATACGGACTG |
| **Reverse** | CCAATACGGGCCAAATCCGTTC |

**RNA Extraction, cDNA Synthesis, and Real-time PCR**

RNA was extracted from the brain tissue using *TransZol* Up Plus RNA Kit (TranGen Biotech). NanoDrop2000c (Thermo Fisher Scientific, USA) was used to assess the isolated RNA concentration to detect the goodness of samples for further assessment in RT-qPCR.

The cDNA synthesis was performed following the protocol of EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix. Real-time PCR using TransStart® Top Green qPCR SuperMix was used to measure the relative expression levels of the mRNAs of the target genes in the brain tissue, with GAPDH as an internal reference.

**Statistical Analysis**

Statistical analysis system- SAS (2012) was used to detect the effects of different factors on the study parameters. Least significant difference –LSD test in the context of the analysis of variance-one way (ANOVA) was used to compare between means in this study.

**Results and Discussion**

**Effects onAcetylcholinesterase Enzyme (AChE)**

Regarding acetylcholinesterase enzyme levels, the results showed a significant decrease in the treated group with 17 mg/kg b. wt. (3986.67 ±170.32 U/L), compared to the control

(5584.33 ±140.35 U/L) and treatment group with 3 mg/kg b. wt. (5556.00 ±341.01 U/L) for 21 days.
Also, there was a significant decrease in the enzyme level between the two treated groups (4688.00 ±221.49 and 3413.67 ±305.42 U/L)for 45 days of the dose, compared with the control group (5584.33 ±140.35 U/L).

The results did not show significant differences within the control and treated groups at the first dose of 17 mg/kg b. wt. for 45 days (Table 2).Whereas there were significant differences in the enzyme level within the treated group at the second dose of 3 mg/kg b. wt., (5556.00 ±341.01U/L) for 21 and 45 days (4688.00 ±221.49U/L).
Acetylcholinesterase is one of the most commonly utilized biomarkers for nervous system toxicity. It is a notable AChE inhibitor that results in a prominent increase in the AchE leading to a general decrease in muscular as well as neural control. Moreover, a portfolio of evidence associates the changes in normal behavioural models alongside neurotoxic damages due to pollutant exposure. Similarly, Kim *et al.* [20] reported that EN reduced AChE activity in adult zebrafish.

A study on aquatic invertebrates (Chironomus riparius) by Muñiz-González *et al.*[21] reported that the lowest endosulfan concentration led to increased AChE activity.
Pereira *et al.* [14] discovered after 96 h zebrafish exposed to 2.4 mg endosulfan/L, group presented a significant decrease in AChE activity.
In another study, male Wistar rats were administered endosulfan at a concentration of 5 mg/kg/day for 28 days. EN developed a significant ($p < 0.05$) decrease in AChE activity when measured in the rat brains [22].

**Table 2:** Effectsof endosulfan and period in AChE

| Group | Mean ± SE of AChE(U\L) | | LSD Value |
|---|---|---|---|
| | 21 Days | 45 Days | |
| Control | 5584.33 ±140.35 Aa | 5584.33 ±140.35 Aa | 276.45 NS |
| 3 mg/kg | 5556.00 ±341.01 Aa | 4688.00 ±221.49 Bb | 761.28 ** |
| 17 mg/kg | 3986.67 ±170.32 Ba | 3413.67 ±305.42 Ca | 603.57 NS |
| LSD value | 811.53 ** | 804.24 ** | --- |

**Means with different big letters in the same column and small letters in the same row are significantly different..** (P≤0.01).**

**Expression Profile Study by Quantitative Reverse Transcriptase Real-time PCR**

AChE gene was upregulated in all treated groups (Figure 1). Furthermore, the highest fold change (14.320) was noticed after administrating the animals with 17 mg/kg for 45. Clearly, it can be concluded that the AChE gene had such high expression due to the damage caused by endosulfan on the genetic content.

Kim *et al.* [20] stated that the expression of genes that participated in defence mechanisms was changed after co-treatment as well. For instance,phenanthrene at LC10 and endosulfan at LC50 markedly upregulated the gene encoding superoxide dismutase. Similarly, these concentrations upregulated the gene coding for AChE in zebrafish.

Lee *et al.* [23] mentioned that exposure for 24 h to most biocide led to a decline in the transcriptional expression of *Tigriopus japonicas* (TJ)- AChE genes. Further, exposure to

endosulfan, dimethoate and alachlor significantly decreased the expression of TJ-AChE1. Pereira *et al.* [14] reported a significant decline in AChE activity in zebrafish brain, after 96 h of exposure to 2.4 mg/L of endosulfan. Nevertheless, Preud'homme *et al.* [24] indicated that the endosulfan did not changeAChE mRNA levels in the zebrafish brain. Investigation revealed no significant changes in the expression of the gene coding for brain neurotransmitter receptors (nicotinic α5 AChE) in *Xenopus laevis* the African clawed frog when exposed to low dosedose EN.

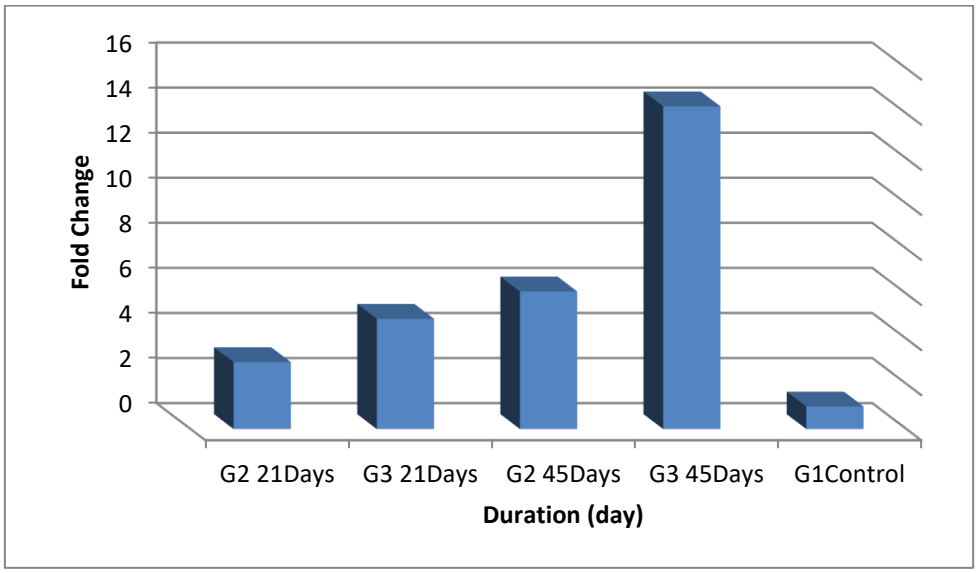


**Figure 1:** Effects of exposure duration of endosulfan on the AChE gene expression in mice.

## Conclusions

This study clarified that endosulfan substantially reduced AChE enzyme activity. Moreover, endosulfan increased AChE gene expression. Hence, AChE can be considered `an important biomarker thatgives the first signs of toxicity.

**Conflicts of Interest**: Authors have no conflicts of interest to declare.

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## References

**[1]** R. Rani, V. Kumar, Z. Usmani, P. Gupta, and A. Chandra, "Influence of plant growth promoting rhizobacterial strains Paenibacillus sp. IITISM08, Bacillus sp. PRB77 and Bacillus sp. PRB101 using Helianthus annuus on degradation of endosulfan from contaminated soil", *Chemosphere*, vol .225, pp. 479-489, 2019.

**[2]** K. Sarma, A.K. Pal, N.P. Sahu, R.S. Dalvi, N. Chatterjee, S.C. Mukherjee, and K. Baruah, "Acute and chronic effects of endosulfan on the haemato-immunological and histopathological responses of a threatened freshwater fish, spotted murrel, Channa punctatus", *Fish physiology and biochemistry*, vol. 38(2), pp. 499-509, 2012.

**[3]** S.Y. Yu, W.J. Liu, Y.S. Xu, Y.Z. Zhao, C.Y. Cai, Y. Liu, and W.X. Liu, " Organochlorine pesticides in ambient air from the littoral cities of northern China: spatial distribution, seasonal variation, source apportionment and cancer risk assessment", *Science of the Total Environment*, vol. 652, pp. 163-176, 2019.

**[4]** Y. Sun, ,X. Chang, L. Zhao, B. Zhou, L. Weng, and Y. Li, "Comparative study on the pollution status of organochlorine pesticides (OCPs) and bacterial community diversity and structure

between plastic shed and open-field soils from northern China", *Science of the Total Environment*, vol. 741, 139620, 2020.

**[5]** R. Sah, A. Baroth, and S.A. Hussain, "First account of spatio-temporal analysis, historical trends, source apportionment and ecological risk assessment of banned organochlorine pesticides along the Ganga River", *Environmental Pollution*, vol. 263, 114229, 2020.

**[6]** C. Olisah, , O.O. Okoh, and A.I. Okoh, " Distribution of organochlorine pesticides in fresh fish carcasses from selected estuaries in Eastern Cape Province, South Africa, and the associated health risk assessment", *Marine Pollution Bulletin*, vol. 149, 110605, 2019.

**[7]** M.M. Milesi, M. Durando, V. Lorenz, M.P. Gastiazoro, and J. Varayoud, " Postnatal exposure to endosulfan affects uterine development and fertility", *Molecular and Cellular Endocrinology*, vol. 511, 110855, 2020.

**[8]** Y. Yavuz, Y. Yurumez, H. Kücüker, Y. Ela, and S. Yüksel, " Two cases of acute endosulfan toxicity", *Clinical Toxicology*, vol. 45(5), pp. 530-532, 2007.

**[9]** D. Xu, T. Liu, L. Lin, S. Li, X. Hang, and Y. Sun," Exposure to endosulfan increases endothelial permeability by transcellular and paracellular pathways in relation to cardiovascular diseases", *Environmental Pollution*, vol. 223, pp. 111-119, 2017.

**[10]** S. F. McHardy, H. Y. L. Wang, S. V. McCowen, and M. C. Valdez, "Recent advances in acetylcholinesterase inhibitors and reactivators: an update on the patent literature (2012-2015)", *Expert opinion on therapeutic patents*, vol. 27(4), pp. 455-476, 2017.

**[11]** R. Kiran and M. N. Varma, "Biochemical studies on endosulfan toxicity in different age groups of rats", *Toxicology letters*, vol. 44(3), pp. 247-252, 1988.

**[12]** A. O.Alabi, A. M. Ajayi, B. Ben-Azu, A.G. Bakre, and S. Umukoro, "Methyl jasmonate abrogates rotenone-induced parkinsonian-like symptoms through inhibition of oxidative stress, release of pro-inflammatory cytokines, and down-regulation of immnopositive cells of NF-κB and α-synuclein expressions in mice", *Neurotoxicology*, vol.74, pp.172-183, 2019.

**[13]** M. B.Colovic, D. Z. Krstic, T. D. Lazarevic-Pasti, A. M. Bondzic, and V. M. Vasic, "Acetylcholinesterase inhibitors: pharmacology and toxicology". *CurrNeuropharmacol* , vol. 11(3), pp. 315-35, 2013.

**[14]** V. M. Pereira, J. W. Bortolotto, L. W. Kist, M. B. de Azevedo, R. S. Fritsch, R. da Luz Oliveira, and M. R. Bogo, "Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (Danio rerio)", *Neurotoxicology*, vol. 33(3), pp. 469-475, 2012.

**[15]** D. K. Getman, J. H. Eubanks, S. Camp, G. A. Evans, and P. Taylor,"The human gene encoding acetylcholinesterase is located on the long arm of chromosome 7", *American journal of human genetics*, vol. 51(1), pp. 170-177, 1992.

**[16]** M. McMillan, and L. Pereg, "Evaluation of reference genes for gene expression analysis using quantitative RT-PCR in Azospirillumbrasilense", *PLoS One*, vol. 9(5), e98162, 2014.

**[17]** M. Petriccione, F. Mastrobuoni, L. Zampella, and M. Scortichini, "Reference gene selection for normalization of RT-qPCR gene expression data from Actinidia deliciosa leaves infected with Pseudomonas syringaepv. actinidiae", *Scientific reports*, vol. 5(1), pp. 1-12, 2015.

**[18]** H. Goebel, S. Gorbach, W. Knauf, R. H. Rimpau, and H. Huttenbach," Properties, effects, residues, and analytics of the insecticide endosulfan", *Properties, Effects, Residues, and Analytics of the insecticide Endosulfan*, vol. 83, pp. 1-165, 1982.

**[19]** D. R. Lide," CRC Handbook of Chemistry and Physics 89th Edition 2008–2009",2008.

**[20]** K. Kim, H. J. Jeon, S. D. Choi, D. C. Tsang, P. Oleszczuk, Y. S. Ok, and S. E. Lee,"Combined toxicity of endosulfan and phenanthrene mixtures and induced molecular changes in adult Zebrafish (Danio rerio)". *Chemosphere*, vol. 194, pp. 30-41, 2018.

**[21]** A. B. Muñiz-González, M. Novo, and J. L. Martínez-Guitarte, "Persistent pesticides: effects of endosulfan at the molecular level on the aquatic invertebrate Chironomus riparius", *Environmental Science and Pollution Research*, vol. *28*(24), pp. 31431-31446, 2021.

**[22]** M. O. Oyovwi, B. Ben-Azu, E. P. Tesi, A. A. Oyeleke, C. I. Uruaka, R. A. Rotu, and E. O. Aya-Ebi, "Repeated endosulfan exposure induces changes in neurochemicals, decreases ATPase transmembrane ionic pumps, and increased oxidative/nitrosative stress in the brains of rats: Reversal by quercetin", *Pesticide Biochemistry and Physiology*, vol. 175, 104833, 2021.

**[23]** J. W. Lee, B. M. Kim, C. B. Jeong, E. J. Won, J. S. Rhee, and J. S. Lee, "Inhibitory effects of biocides on transcription and protein activity of acetylcholinesterase in the intertidal copepod

Tigriopus japonicus",*Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 167, pp. 147-156, 2015.

**[24]** V. Preud'homme, S. Milla, V. Gillardin, E. De Pauw, M. Denoël, and P. Kestemont, "Effects of low dose endosulfan exposure on brain neurotransmitter levels in the African clawed frog Xenopus laevis", *Chemosphere*, vol. 120, pp. 357-364, 2015.