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COX-mediated Regulation of Multiple Organ Damage by Betulin Treatment in Okadaic Acid-induced Alzheimer Rat Model

Okadaik Asitle İndüklenen Alzheimer Sıçan Modelinde Betulin Tedavisi ile Çoklu Organ Hasarının COX Aracılığıyla Düzenlenmesi

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ABSTRACT

Objective: Alzheimer's Disease (AD) is a progressive neurodegenerative disease. Cyclooxygenases (COXs) are essential in the inflammatory and regenerative processes of AD. This study aims to show that Betulin, a natural phytochemical (triterpene), is a candidate for COX-mediated correction of multiple organ damage of AD.

Materials and Methods: In this study, the effects and treatment potential of Betulin were investigated in the kidney, heart, and small intestine tissue in genetic, and histological contexts in an okadaic acid-induced rat AD model. A total of 36 Wistar albino male rats were included in the study. Cyclooxygenase 1 (COX-1) and Cyclooxygenase 2 (COX2) gene expressions were investigated by quantitative real-time PCR (qRT-PCR) in kidney, heart, and small intestine tissues. COX-1 and COX-2 proteins in tissues were analyzed by immunohistochemistry.

Results: COX-1 and COX-2 genes were detected to be overexpressed in the AD model. The expression of both genes was increased in the AD model and decreased after betulin treatment. Histological scores showed a strong positive effect of Betulin on the kidney, while it was relatively less effective on the heart and small intestine tissue.

Conclusions: In treating organ damage in AD, COXs can be inhibited by Betulin and may be effective in functional recovery.

Keywords: Alzheimer's disease, Cyclooxygenase 1, Cyclooxygenase 2, betulin, organ damage

ÖZ

Amaç: Alzheimer Hastalığı (AH) ilerleyici bir nörodejeneratif hastalıktır. Siklooksijenazlar (COX'ler), AH 'nin inflamatuar ve rejeneratif süreçlerinde gereklidir. Bu çalışma, doğal bir fitokimyasal (triterpen) olan Betulin'in, AH 'nin çoklu organ hasarının COX aracılı düzeltilmesi için aday olduğunu göstermeyi amaçlamaktadır.

Materyal ve Metot: Bu çalışmada okadaik asit ile indüklenen sıçan AH modelinde betulin'in böbrek, kalp ve ince bağırsak dokusundaki genetik ve histolojik bağlamdaki etkilerini ve tedavi potansiyeli araştırılmıştır. Çalışmaya 36 adet Wistar albino erkek sıçan dahil edildi. Böbrek, kalp ve ince bağırsak dokularında Cyclooxygenase 1 (COX1) ve Cyclooxygenase 2 (COX2) gen ekspresyonları kantitatif gerçek zamanlı PCR (qRT-PCR) ile araştırılmıştır. Dokulardaki COX-1 ve COX2 proteinleri immünohistokimya ile analiz edildi.

Bulgular: AH modelinde COX1 ve COX2 genlerinin aşırı eksprese edildiği tespit edildi. Her iki genin ekspresyonu AH modelinde artmış ve betulin tedavisinden sonra azalmıştır. Histolojik skorlar Betulin'in böbrek üzerinde güçlü bir olumlu etkisi olduğunu, kalp ve ince bağırsak dokusu üzerinde ise nispeten daha az etkili olduğunu gösterdi.

Sonuç: AH'de organ hasarının tedavisinde COX'lar betulin tarafından inhibe edilebilir ve fonksiyonel iyileşmede etkili olabilir.

Anahtar Kelimeler: Alzheimer hastalığı, Cyclooxygenase 1, Cyclooxygenase 2, betulin, organ hasarı

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INTRODUCTION

Alzheimer's Disease (AD) is an increasingly common neurodegenerative disease that generally affects older people and impairs the quality of life of patients.[1] In today's world, with the aging population, there is also an increase in age-related, non-communicable diseases such as dementia and its most common cause, Alzheimer's.[2] AD initially appears with memory loss and is accompanied by other cognitive functional impairments such as orientation difficulties and speech disorders. There are many questions about the etiopathogenesis of this disease.[3] Betulin is a pentacyclic triterpene metabolite and is found in large amounts in the outer bark of birch trees (Betula, Betulaceae).[4] Recent studies conducted with Betulin also show that it has various properties useful in treating metabolic, cardiovascular, and neurological disorders.[5] Betulin also reduces diet-induced obesity by inhibiting cholesterol and fatty acid biosynthesis. It also reduces the size of atherosclerotic plaques and increases their stability.[6] COX-1 and COX-2 enzymes play a role in maintaining many physiological functions in living things, as well as appearing in pathological events.[7] COXs are expressed in at least two isoforms: COX1 is expressed in most tissues, while COX2 is primarily an inducible enzyme. COX2 expression increases rapidly in many tissues in response to tissue damage or the presence of proinflammatory cytokines.[8] In the gastrointestinal tract, it has led to the idea that COX1 is critical for the cleansing action in the gastrointestinal mucosa. Therefore, COX2 appears to be responsible for inflammation.[9] In light of this information, determining the activity of two enzymes and possible treatment in case of multiorgan dysfunction may pave the way for systemic recovery.

In this study, the potential of Betulin to improve systemic organ dysfunctions and delay/prevent the occurrence of AD by regulating COX1 and COX2 enzymes that contribute to the inflammatory processes of AD was evaluated with genetic and immunohistochemical markers.

MATERIALS AND METHODS

Ethics Committee Approval: The study protocol was approved by Gaziantep University Animal Experiments Local Ethics Committee (Decision Number: 2023/29, Protocol Number: 323).

Animals: The animals were obtained from the Gaziantep University Experimental Animals Research and Application Center. All procedures respected the Guidelines of the European Union (86/609/EU). A total of 35 male Wistar Albino rats (8-12 weeks old, 250-300 gr) were included in the study.

Experimental groups:

1. Control Group (C) (n=6); The control group was not treated.

2. DMSO Group (n=5): Dimethyl Sulfoxide (DMSO) is a Betulin solvent and was applied intraperitoneally (i.p.) once a day (between 9.00-11.00) for 4 weeks as 5 mg/ml (Thermo Fisher, D12345).

3. *Betulin Group (n=6):* The treatment agent Betulin (Selleckchem, Sylvanfield Drive, Houston, TX 77014 USA, Cat no. S4754) was dissolved in DMSO according to the commercial protocol. It was administered as 20 mg/kg/day i.p. every day for 4 weeks.[1]

4. *Phosphate Buffer Saline (PBS) Group (n=6):* The animals were placed in a stereotaxic chamber, and a total of 10 µl of PBS was administered under anesthesia, 5 µl to one side of the brain and 5 µl to the other side.

5.Okadaic Acid (OKA) Group (n=6): Animals in this group were placed in the stereotaxic chamber under anesthesia. OKA was dissolved in DMSO according to the manufacturer's instructions. Animals received 5ml bilateral OKA once (200 ng/kg) (BioVision, Waltham, MA, USA, 78111-17-8in PBS[2] and were kept for 30 days for the AD model to form. According to the bregma coordinates (0.8mm posterior to bregma, 1.8mm lateral, and 3.6mm beneath the cortical surface), bilateral holes were drilled (OmniDrill35, 124 World Precision Instrument, Hertfordshire, UK) into the skull (ICV).

6. Okadaic Acid+Betulin Group (n=6): Animals were placed in the stereotaxic chamber under anesthesia. They received 5 ml bilateral OKA once (200 ng/kg) and were kept for 30 days for the model to form. At the end of the period, Betulin was administered once a day i.p.[3]

Tissue Preparation: The kidney, heart, and small intestine tissues of the animals were removed under xylazine + ketamine (5 mg/kg and 75 mg/kg) anesthesia, washed with PBS, divided into two, and stored in 10% formalin (for immunohistochemistry analyses) and RNA solution (for gene expression analyses).

Gene Expression Differences: Total RNA isolation from a 50 mg tissue sample was performed using the Triazole Method.[10] RNA concentration was measured (260 nm) with a spectrophotometer (MultiSkan® Go, Thermo ScientificÔ), and samples were diluted (10 ng of RNA in 10 ml of complementary DNA (cDNA) reaction). Reverse Transcriptase PCR (RT-PCR) reactions were prepared according to the commercial kit (Abm Good, G236) protocol. The cDNA samples were incubated in a thermal cycler (Veriti, Thermo Fisher) (16°C, 30 minutes, 1 cycle; 42°C, 30 minutes, 1 cycle; 85°C, 5 minutes 1 cycle). At the end of the process, the products were immediately placed on ice and stored at -80°C until analysis. A 20 ml PCR reaction was prepared from

this cDNA library to analyze the expression changes of *COX-1* (Qiagen, QT00187859) and *s* (Qiagen, QT02486701) genes. The reaction contained 2X SYBR Green Reaction Mix (Qiagen), 10X Gene Expression Assay, and ddH_2O. Expression data were normalized to the rat endogenous control beta-actin (ACTB) gene (Qiagen, QT00193473) and a universal rat reference RNA (Thermo Fisher, QS0641). The samples were incubated in the Real-Time PCR (Qiaegen, Rotor-Gene Q) device under two-step incubation conditions (95°C, 15 min, 1 cycle; 94°C-15 sec, 60°C-30 sec, 40 cycles). All measurements were analyzed in triplicate. The results were analyzed for DDCt. Fold change values (Fc) were calculated with $2^{-\Delta\Delta Ct}$ [11].

Immunohistochemical Analysis: Tissues were removed from 10% formalin, dehydrated, embedded in paraffin, cooled, and 3 mm thick sections were cut. Samples were inhibited with endogenous peroxidase solution and then treated with antigen retrieval (Abcam, ab970). They were then kept in a normal blocking solution and blocked with avidin-biotin (Santa Cruz, sc-516217), incubated for 24 hours for primary antibody staining for COX-1 (Elabscience, E-AB-61656) and COX-2 (Elabscience, E-EL-R0792). In the next step, they were incubated with a biotin-labelled secondary antibody at the end of the incubation, and the samples were kept in streptavidin-HRP (Abcam ab7403). Finally, they were stained with a DAB Substrate Kit (Abcam, ab64238) and observed under a light microscope (Primo Star, Zeiss). In evaluating COX-1 and COX-2 in each tissue, scoring was done based on the staining intensities and percentages of the stained area.[12]

Statistical Analysis: All analyses were performed in SPSS 22.0 (Release 22.0, SPSS Inc, Chicago, IL, USA). The normality of the groups was tested with Shapiro-Wilk. Tukey was used for post-hoc analyses, and One-Way ANOVA was used for the differences between the groups. The direction of significance was determined by descriptive statistics and multiple comparative analyses. Correlations were analyzed for the expression of the genes in each tissue. The Kruskal-Wallis Test was used to analyze the variances. $p < 0.05$ was considered statistically significant.

RESULTS

In kidney tissue analysis, the difference between the groups was statistically significant for COX-1 (df (5.29), MS=204.294, F=31.612, P<0.05) and COX-2 (df (5.29), MS=133.187, F=28.218, P<0.05). Two genes were increased in AD rats, and they decreased after the Betulin treatment. For heart tissue, COX-1 (df (5.29), MS=163.943, F=55.585, P<0.05) and COX-2 (df (5.29), MS=164.509, F=123.060, P<0.05) expressions were found to be increased in AD and decreased in treatment. Small intestinal tissue was also statistically significant for COX-1 (df (5.29), MS=117.409, F=96.362, P<0.05) and COX-2 (df (5.29), MS=118.809, F=43.899, P<0.05) genes.

Table 1. Descriptive statistics for COX-1 and COX-2 gene expressions in three tissues.

Tissues	Genes	Experimental Groups	n	Mean	SD	SE	95% CI for Mean	P-value
Kidney	COX-1	Control	6	37.787	2.066	0.843	35.619-39.954	**0.001***
		DMSO	5	36.288	3.864	1.728	31.490-41.086	
		Betulin	6	22.888	0.307	0.126	22.566-23.211	
		PBS	6	35.675	0.844	0.344	34.790-36.560	
		OKA	6	37.907	4.040	1.650	33.666-42.147	
		OKA+ Betulin	6	37.510	2.034	0.830	35.375-39.645	
	COX-2	Control	6	38.283	1.116	0.456	37.112-39.455	**0.001***
		DMSO	5	34.552	1.264	0.565	32.983-36.121	
		Betulin	6	25.896	2.197	0.897	23.591-28.202	
		PBS	6	35.452	3.859	1.575	31.402-39.501	
		OKA	6	38.472	0.372	0.152	38.081-38.862	
		OKA+ Betulin	6	37.215	2.235	0.913	34.869-39.561	
Heart	COX-1	Control	6	39.467	0.326	0.133	39.124-39.809	**0.001***
		DMSO	5	35.468	0.478	0.214	34.874-36.062	
		Betulin	6	25.208	0.714	0.292	24.459-25.958	
		PBS	6	36.580	2.788	1.138	33.654-39.506	
		OKA	6	38.730	1.270	0.519	37.397-40.063	
		OKA+ Betulin	6	37.337	2.631	1.074	34.576-40.098	
	COX-2	Control	6	39.015	2.301	0.939	36.601-41.429	**0.001***
		DMSO	5	33.232	0.337	0.151	32.814-33.650	
		Betulin	6	24.510	0.365	0.149	24.127-24.893	
		PBS	6	35.043	0.692	0.283	34.317-35.770	
		OKA	6	37.462	0.339	0.138	37.106-37.817	
		OKA+ Betulin	6	37.057	1.282	0.523	35.712-38.402	

*: P<0.05; SD: Standard deviation; SE: Standard error; CI: Confidence interval; DMSO: Dimetil sülfoksit; OKA: Okadaik asit; COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; PBS: Phosphate Buffer Saline.

Table 1. Continue.

Small Intestine	COX-1	Control	6	35.352	0.921	0.376	34.385-36.319	**0.001***
		DMSO	5	33.942	0.554	0.248	33.254-34.630	
		Betulin	6	25.140	0.720	0.294	24.385-25.895	
		PBS	6	35.783	1.598	0.653	34.106-37.461	
		OKA	6	35.468	0.298	0.121	35.156-35.781	
		OKA+ Betulin	6	37.505	1.905	0.778	35.506-39.504	
	COX-2	Control	6	35.683	1.365	0.557	34.251-37.116	**0.001***
		DMSO	5	32.938	0.511	0.228	32.304-33.572	
		Betulin	6	25.110	1.080	0.441	23.977-26.243	
		PBS	6	35.583	2.380	0.972	33.085-38.081	
		OKA	6	36.387	0.979	0.400	35.359-37.415	
		OKA+ Betulin	6	36.875	2.416	0.986	34.340-39.410	

*: P<0.05; SD: Standard deviation; SE: Standard error; CI: Confidence interval; DMSO: Dimetil sülfoksit; OKA: Okadaik asit; COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; PBS: Phosphate Buffer Saline.

Descriptive statistics for gene expressions in each tissue are given in Table 1.

Differences in group averages were tested with the Independent-samples Kruskal Wallis Test. Genes were detected as significant for all 3 tissues (Figure 1).

Multiple comparative analysis of groups is given in Table 2.

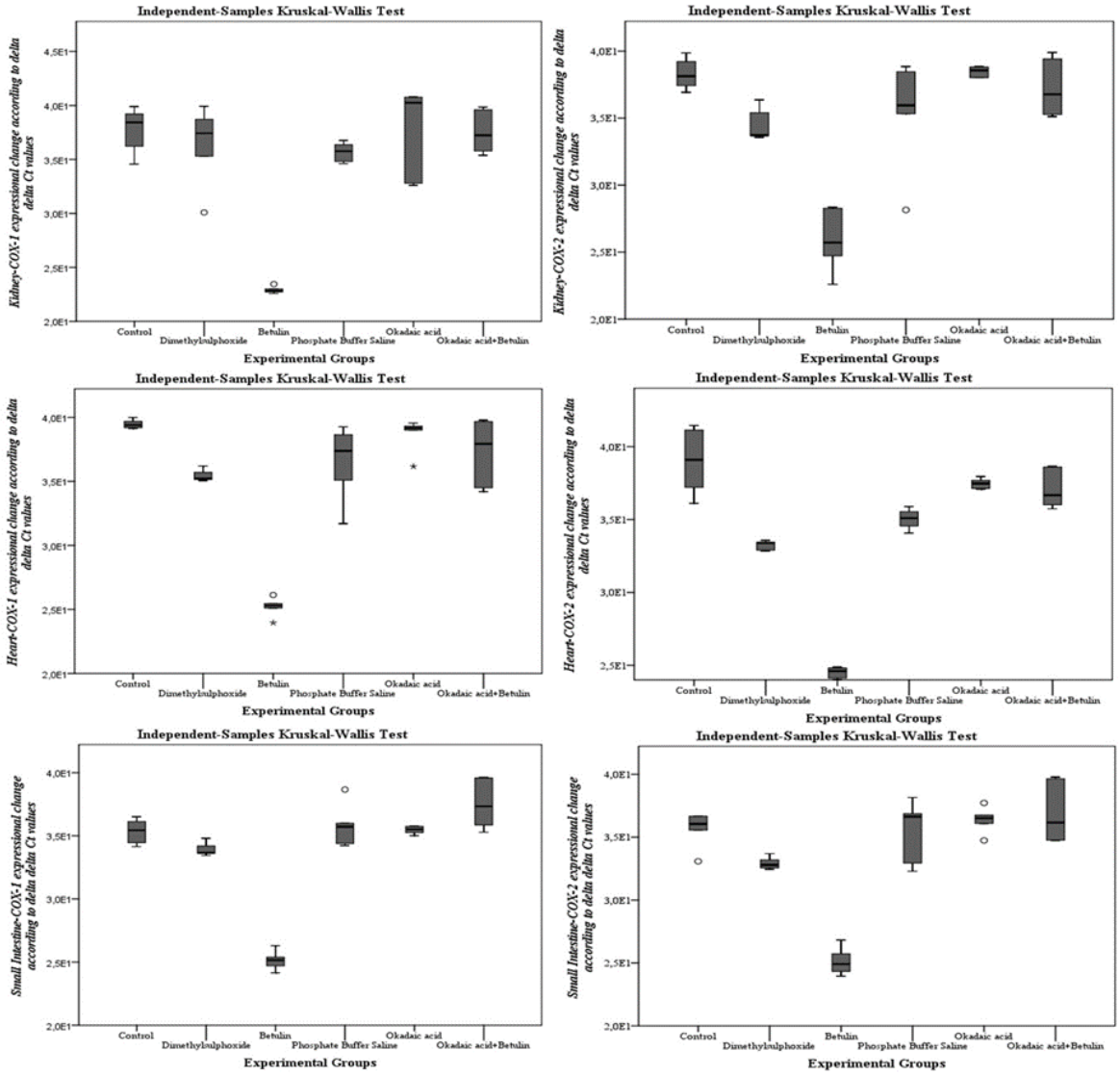

Figure 1. The box-plot graph represents gene expression differences between the three tissues of group means. Comparative analyses were performed using the Independent-Samples Kruskal-Walli's test.

Table 2. Multiple comparative analyses for groups in different tissues.

Tissues	Genes	Experimental Groups (I)	Experimental Groups (II)	Mean	SE	95% CI for Mean	P-value
Kidney	COX1	Control	DMSO	1.499	1.539	-3.194-6.191	0.923
			Betulin	14.898	1.468	10.421-19.327	**0.001***
			PBS	2.112	1.468	-2.363-6.586	0.704
			OKA	-0.120	1.468	-4.594-4.354	1.000
			OKA+ Betulin	0.277	1.468	-4.198-4.751	1.000
		DMSO	Control	-1.499	1.539	-6.191-3.194	0.923
			Betulin	13.400	1.539	8.707-18.093	**0.001***
			PBS	0.613	1.539	-4.080-5.306	0.999
			OKA	-1.619	1.539	-6.311-3.074	0.896
			OKA+ Betulin	-1.222	1.539	-5.915-3.471	0.966
		Betulin	Control	-14.898	1.468	-19.373--10.424	**0.001***
			DMSO	-13.400	1.539	-18.092--8.707	**0.001***
			PBS	-12.787	1.468	-17.261--8.312	**0.001***
			OKA	-15.018	1.468	-19.493--10.544	**0.001***
			OKA+ Betulin	-14.622	1.468	-19.096—10.147	**0.001***
		PBS	Control	-2.111	1.468	-6.586-2.363	0.704
			DMSO	-0.613	1.539	-5.305-4.080	0.999
			Betulin	12.787	1.468	8.312-17.261	**0.001***
			OKA	-2.232	1.468	-6.706-2.247	0.654
			OKA+ Betulin	-1.835	1.468	-6.309-2.639	0.809
		OKA	Control	0.120	1.468	-4.354-4.594	1.000
			DMSO	1.619	1.468	-3.074-6.311	0.896
			Betulin	15.018	1.468	10.544-19.492	**0.001***
			PBS	2.231	1.468	-2.243-6.706	0.654
			OKA+Betulin	0.397	1.468	-4.078-4.871	1.000
		OKA+Betulin	Control	-0.277	1.468	-4.751-4.197	1.000
			DMSO	1.222	1.539	-3.471-5.914	0.966
			Betulin	14.622	1.468	10.147-19.095	**0.001***
			PBS	1.835	1.468	-2.639-6.309	0.809
			OKA	-0.397	1.468	-4.871-4.077	1.000
	COX2	Control	DMSO	3.731	1.315	-0.279-7.741	0.080
			Betulin	12.387	1.254	8.563-416.210	**0.001***
			PBS	2.832	1.254	-0.992-6.655	0.955
			OKA	-0.188	1.254	-4.012-3.635	1.000
			OKA+ Betulin	1.068	1.254	-2.755-4.892	0.955
		DMSO	Control	-3.761	1.315	-7.741-0.279	0.080
			Betulin	8.655	1.315	4.645-12.666	**0.001***
			PBS	-0.899	1.315	-4.910-3.111	0.982
			OKA	-3.920	1.315	-7.930-0.091	0.058
			OKA+ Betulin	-2.663	1.315	-6.673-1.247	0.354
		Betulin	Control	-12.387	1.254	-16.211--8.563	**0.001***
			DMSO	-8.656	1.315	-12.666--4.645	**0.001***
			PBS	-9.555	1.254	-13.379--5.731	**0.001***
			OKA	-12.575	1.254	-16.399--8.751	**0.001***
			OKA+ Betulin	-11.318	1.254	-15.143--7.495	**0.001***
		PBS	Control	-2.832	1.254	-6.655-0.992	0.244
			DMSO	0.900	1.315	-3.111-4.910	0.982
			Betulin	9.555	1.254	5.731-13.379	**0.001***
			OKA	-3.020	1.254	-6.846-0.803	0.187
			OKA+ Betulin	-1.763	1.254	-5.587-2.060	0.723
		OKA	Control	0.188	1.254	-3.635-4.012	1.000
			DMSO	3.919	1.315	-0.091-7.930	0.058
			Betulin	12.575	1.254	8.752-16.399	**0.001***
			PBS	3.020	1.254	-0.804-6.843	0.187
			OKA+Betulin	1.257	1.254	-2.567-5.080	0.914
		OKA+Betulin	Control	-1.068	1.254	-4.892-2.755	0.955
			DMSO	2.663	1.315	-1.347-6.673	0.354
			Betulin	11.318	1.254	7.495-15.142	**0.001***
			PBS	7.763	1.254	-2.060-5.587	0.723
			OKA	-1.257	1.254	-5.080-2.567	0.914

*: P<0.05; DMSO: Dimetil sülfoksit; OKA: Okadaik asit; COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; PBS: Phosphate Buffer Saline.

Table 2. Continue.

Tissues	Genes	Experimental Groups (I)	Experimental Groups (II)	Mean	SE	95% CI for Mean	P-value
		Control	DMSO	3.400	1.040	0.828-1.469	**0.007***
			Betulin	14.258	0.991	11.236-17.281	**0.001***
			PBS	2.887	0.991	-0.136-5.909	0.068
			OKA	0.737	0.991	-2.286-3.759	0.975
			OKA+ Betulin	2.130	0.991	-0.893-5.153	0.292
		DMSO	Control	-3.999	1.039	-7.169--0:825	**0.007***
			Betulin	10.260	1.039	7.089-13.430	**0.001***
			PBS	-1.112	1.039	-4.282-2.058	0.889
			OKA	-3.262	1.039	-6.432--0.092	**0.041***
			OKA+ Betulin	-1.869	1.039	-5.039-1.301	0.483
		Betulin	Control	-14.258	0.991	-17.281--11.236	**0.001***
			DMSO	-10.260	1.039	-13.430--7.089	**0.001***
			PBS	-11.372	0.991	-14.394--8.349	**0.001***
			OKA	-13.522	0.991	-16.544--10.499	**0.001***
			OKA+ Betulin	-12.128	0.991	-15.151--9.106	**0.001***
	COX1	PBS	Control	-2.887	0.991	-5.909-0.136	0.068
			DMSO	1.112	1.040	-2.059-4.282	0.899
			Betulin	11.372	0.991	8.349-14.394	**0.001***
			OKA	-2.150	0.991	-5.173-0.873	0.283
			OKA+ Betulin	-0.757	0.991	-3.779-2.266	0.972
		OKA	Control	-0.737	0.991	-3.759-2.286	0.975
			DMSO	3.262	1.040	-0.872-5.172	**0.041***
			Betulin	13.523	0.991	10.499-16.5442	**0.001***
			PBS	2.150	0.991	-0.873-5.173	0.283
			OKA+Betulin	1.393	0.991	-1.629-4.416	0.724
		OKA+Betulin	Control	-2.130	0.991	-5.153-0.893	0.292
			DMSO	1.869	1.040	-1.301-5.040	0.483
			Betulin	12.128	0.991	-.106-15.151	**0.001***
			PBS	0.757	0.991	-2.266-3.779	0.972
			OKA	-1.393	0.991	-4.416-1.629	0.724
		Control	DMSO	5.783	0.700	3.649-7.917	**0.001***
			Betulin	14.505	0.667	12.470-16.540	**0.001***
			PBS	3.972	0.667	1.937-6.007	**0.001***
			OKA	1.553	0.667	-0.482-3.588	0.216
			OKA+ Betulin	1.958	0.667	-0.077-3.993	0.065
		DMSO	Control	-5.783	0.700	-7.917--3.649	**0.001***
			Betulin	8.722	0.700	6.588-10.856	**0.001***
			PBS	-1.811	0.700	-3.946--2.095	**0.001***
			OKA	-4.230	0.700	-5.959--1.690	**0.001***
			OKA+ Betulin	-3.825	0.700	-5.959-1.690	**0.001***
		Betulin	Control	-14.505	0.667	-16.540--12.470	**0.001***
			DMSO	-8.722	0.700	-10.856--6.588	**0.001***
			PBS	-10.533	0.667	-12.568--8.498	**0.001***
			OKA	-12.952	0.667	-14.897--10.971	**0.001***
Heart			OKA+ Betulin	-12.547	0.667	-14.582--10.512	**0.001***
	COX2	PBS	Control	-3.972	0.337	-6.007--1.937	**0.001***
			DMSO	1.811	0.700	-0.323-3.946	0.133
			Betulin	10.533	0.667	8.498-12.568	**0.001***
			OKA	-2.418	0.667	-4.453--0.383	**0.013***
			OKA+ Betulin	-2.013	0.667	-4.048-0.022	0.054
		OKA	Control	-1.553	0.667	-3.588-0.482	0.216
			DMSO	4.230	0.700	2.095-6.364	**0.001***
			Betulin	12.952	0.667	10.917-14.987	**0.001***
			PBS	2.418	0.6670	0.383-4.453	**0.013***
			OKA+Betulin	0.405	0.667	-1.630-2.244	0.990
		OKA+Betulin	Control	-1.958	0.667	-3.993-0.076	0.065
			DMSO	3.825	0.700	1.690-5.959	**0.001***
			Betulin	12.547	0.667	10.512-14.582	**0.001***
			PBS	2.013	0.667	-0.022-4.048	0.054
			OKA	-0.405	0.667	-2.440-1.630	0.990

*: P<0.05; DMSO: Dimetil sülfoksit; OKA: Okadaik asit; COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; PBS: Phosphate Buffer Saline.

Table 2. Continue.

Tissues	Genes	Experimental Groups (I)	Experimental Groups (II)	Mean	SE	95% CI for Mean	P-value
		Control	DMSO	1.410	0.706	-0.743-3.562	0.368
			Betulin	10.212	0.673	8.159-12.264	**0.001***
			PBS	-0.432	0.673	-2.484-1.620	0.987
			OKA	-0.117	0.673	-2.169-1.935	1.000
			OKA+ Betulin	-2.153	0.673	-4.205—0.101	**0.035***
		DMSO	Control	-1.410	0.706	-3.562-0.743	0.368
			Betulin	8.802	0.706	6.650-10.954	**0.001***
			PBS	-1.841	0.706	-3.994-0.311	0.127
			OKA	-1.526	0.706	-3.679-0.626	0.286
			OKA+ Betulin	-3.563	0.706	-5.715—1.411	**0.001***
		Betulin	Control	-10.212	0.673	-12.264—8.159	**0.001***
			DMSO	-8.802	0.706	-10.954—6.650	**0.001***
			PBS	-10.643	0.673	-12.695—8.591	**0.001***
			OKA	-10.328	0.673	-12.380—8.276	**0.001***
			OKA+ Betulin	-12.365	0.673	-14.417—10.312	**0.001***
	COX1	PBS	Control	0.432	0.673	-1.620-2.483	0.987
			DMSO	1.841	0.706	-0.311-3.994	0.127
			Betulin	10.643	0.673	8.591-12.696	**0.001***
			OKA	0.315	0.673	-1.737-2.367	0.997
			OKA+ Betulin	-1.722	0.673	-3.774-0.330	0.141
		OKA	Control	0.117	0.673	-1.935-2.169	1.000
			DMSO	1.526	0.706	-0.626-3.679	0.286
			Betulin	10.328	0.673	8.276-12.380	**0.001***
			PBS	-0.315	0.673	-2.367-1.737	0.997
			OKA+Betulin	-2.0367	0.673	-4.089-0.015	0.053
		OKA+Betulin	Control	2.153	0.673	0.101-4.205	**0.001***
			DMSO	3.563	0.706	1.411-5.715	**0.001***
			Betulin	12.365	0.673	10.312-14.417	**0.001***
			PBS	1.722	0.673	-0.330-3.774	0.141
			OKA	2.037	0.673	-0.015-4.089	0.053
		Control	DMSO	2.745	0.996	-0.291-5.782	0.094
			Betulin	10.573	0.950	7.678-13.469	**0.001***
			PBS	0.100	0.950	-2.795-2.995	1.000
			OKA	-0.703	0.950	-3.599-2.192	0.975
			OKA+ Betulin	-1.192	0.950	-4.087-1.704	0.806
		DMSO	Control	-2.745	0.996	-5.782-0.291	0.094
			Betulin	7.820	0.996	4.791-10.865	**0.001***
			PBS	-2.645	0.996	-5.682-0.391	0.116
			OKA	-3.449	0.996	-6.485--0.485	0.019
			OKA+ Betulin	-3.937	0.996	-6.974--0.900	**0.006***
		Betulin	Control	-10.573	0.950	-13.469--7.678	**0.001***
			DMSO	-7.828	0.996	-10.865--4.791	**0.001***
			PBS	-10.473	0.950	-13.369--7.578	**0.001***
			OKA	-11.277	0.950	-14.172--8.381	**0.001***
			OKA+ Betulin	-11.765	0.950	-14.660--8.869	**0.001***
*Small Intestine	COX2	PBS	Control	-0.100	0.950	-2.995-2.795	1.000
			DMSO	2.645	0.996	-0.391-5.682	0.116
			Betulin	10.473	0.950	7.578-13.369	**0.001***
			OKA	-0.803	0.950	-3.699-2.092	0.956
			OKA+ Betulin	-1.292	0.950	-4.187-1.604	0.750
		OKA	Control	0.703	0.950	-2.192-3.599	0.975
			DMSO	3.449	0.996	0.7412-6.485	**0.019***
			Betulin	11.277	0.950	8.381-14.172	**0.001***
			PBS	0.803	0.950	-2.092-3.699	0.956
			OKA+Betulin	-0.488	0.950	-3.384-2.407	0.995
		OKA+Betulin	Control	1.192	0.950	-1.704-4.087	0.806
			DMSO	3.937	0.950	0.900-6.974	**0.006***
			Betulin	11.765	0.950	8.869-14.660	**0.001***
			PBS	1.292	0.950	-1.604-4.187	0.750
			OKA+Betulin	0.488	0.950	-2.407-3.384	0.995

*: P<0.05; DMSO: Dimetil sülfoksit; OKA: Okadaik asit; COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; PBS: Phosphate Buffer Saline.

The distribution of hippocampal COX proteins was tested in the AD rat model. The results showed that Betulin had different histochemical scores in different tissues (H-score = (0 x P_0) + (1 x P_1) + (2 x P_2) + (3 x P_3)). The results showed that the most significant effect of COX1 was in the heart, moderately in the kidney, and to a lesser extent in the small intestine. COX2 showed the most significant effect in the kidney, a moderate impact on the heart, and finally, a low effect in the small intestine tissue. H-scores for AD and treatment group were as follows. In the structural histological evaluation of kidney tissue, a high expression of COX1 was observed in tubular, mesangial cells and podocytes (Figure 2A), and a relatively lower rate of uptake for COX2 was observed (Figure 2G). While protein retention was not observed for COX1 after Betulin treatment (Figure 2B), it was found to decrease for COX2 (Figure 2H). Heart tissue analyses showed focal myonecrosis and degeneration in the AD model for COX1 (Figure 2C) and COX2 (Figure 2I). Low protein levels were detected after Betulin treatment (Figure 2D, 2J). Mild and moderate defects observed in AD heart tissue could not be seen after Betulin treatment. In the small intestine tissue, expression of COX1 and COX2 in the ileum was detected in the AD group (Figure 2E, 2K). A decrease in protein expression

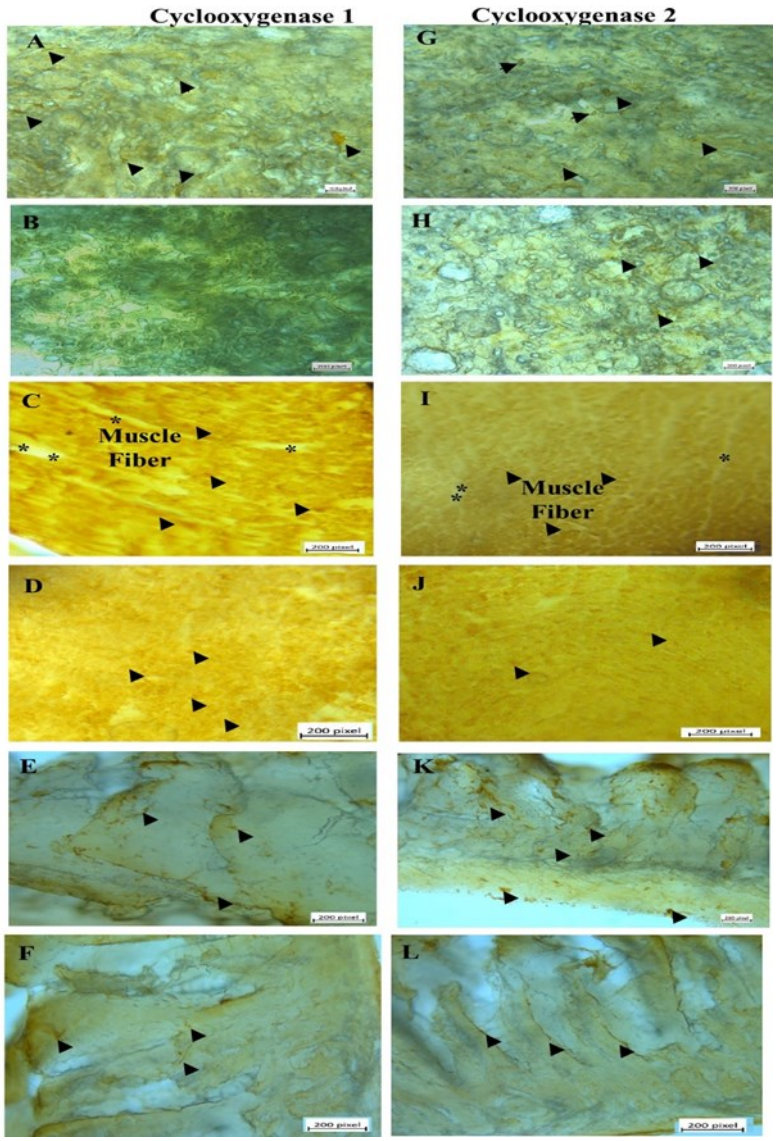

Figure 2. Immunohistochemical staining of COX1 and COX2 in rat kidney, heart, and small intestine (100×). A: Kidney- Okadaic acid showing high expression of COX1 in tubular cell, mesangial cell, and podocytes (black arrow); B: Kidney- Okadaic acid + Betulin group this group showing no expression for COX1; C: Heart- Okadaic acid showing high expression in heart muscle cells for COX1 (black arrow); D: Heart- Okadaic acid + Betulin group after Betulin treatment, COX1 expression was observed in low amounts in heart muscle cells; E: Small intestine-Okadaic acid showing high expression for COX1 in ileum section; F: Small intestine-Okadaic acid + Betulin group, COX1 expression was observed in low amounts in ileum, G. Kidney-Okadaic acid showing high expression of COX2; H: Kidney-Okadaic acid + Betulin group, showing low expression for COX2 in kidney section; I: Heart-Okadaic acid showing high expression in heart muscle cells for COX2; J: Heart- Okadaic acid + Betulin group, COX2 expression was observed in low amounts in heart muscle cells; K: Small intestine-Okadaic acid showing high expression for COX2 in ileum section; L: Small intestine-Okadaic acid + Betulin group, COX2 expression was observed in low amounts in the ileum; MF: muscle fiber; *: mild defect; **: moderate defect.

was observed after Betulin treatment (Figure 2F, 2L).

DISCUSSION AND CONCLUSION

The relationship between dementia and acute organ dysfunction threatens the patient's life and affects mortality. In chemically OKA-induced AD, excessive COX1 and COX2 gene expressions in kidney, heart, and small intestine tissues indicate their structural and functional integrity is in danger. The literature mostly mentions the predominant effect of COX2 dysfunction in the kidney. However, there is no definitive comment for COX1. When the function of COX-1 in the kidney is examined, it is seen that it plays a role in hemodynamic regulation.[12] Therefore, afferent and efferent COX1 dysfunction may disrupt renal hemodynamics and unbalance the glomerular filtration rate. The improvement of COX1 and COX-2 levels under the influence of Betulin indicates that this metabolism can be regulated by targeting COXs. The possibility of damage via COXs is supported by a mouse study. The fact that gene expressions can be decreased by Betulin indicates that the COX pathway may be Betulin's target. Ensuring homeostasis related to the absorption of ions in renal functions is regulated by many physiological mechanisms. Studies have shown that protein phosphatase 2 (PP2A) is responsible for maintaining ion channels and homeostasis.[13] In addition to its function in Na-Cl-dependent transporter systems, PP2A accelerates the flow of Na-K-ATPase from the intracellular system to the basal-lateral membrane in human adenocarcinoma cells. In this mechanism, okadaic acid inhibits the increase in Na-K-ATPase activity as a selective inhibitor of PP2A.[14] Two features observed in the initial pathology of chronic kidney disease, glomerulosclerosis, and tubulointerstitial fibrosis, are associated with microvascular endothelial cell dysfunction.[15] Okadaic acid reduces the effect of PP2A on the endothelial cell remodelling process. Thus, okadaic acid harms kidney homeostasis, both structurally and functionally. In our study, it is possible to regulate okadaic acid-induced kidney damage during the AD process by managing COX enzymes with the effect of Betulin. A study supports the accuracy of this idea. The study showed that the left ventricle in AD patients was thicker than in patients without the disease. The reason for this thickness is that Ab plaques in the AD brain accumulate in the same form in the ventricle. Ventricular thickening impedes blood flow, which can result in cardiovascular problems and a higher risk of heart attack and stroke.[16] PP2A function is also present in the heart. It is a phosphatase that modulates Ca^{2+} utilization as a channel regulator. In our study, heart tissue analyses show that cellular COX protein accumulations in the okadaic acid AD model are partially reduced by the effect of Betulin and that damage can be reduced by regulating COX metabolism with Betulin treatment. Therefore, it indicates that partial recovery of heart functions is possible. The most well-known effect of okadaic acid on the intestinal system is that it stimulates the phosphorylation of proteins that control Na release in cells, increases protein phosphorylation due to solute permeability, and therefore causes fluid loss. The data we obtained in our study may indicate that structural differentiation and permeability in intestinal cells may change as a result of overexpression of COXs, causing them to become partially or completely dysfunctional. Reducing the amount of protein with the effect of Betulin can restore this function.

Peripheral inflammation outside the CNS in AD is a risk factor for the disease.[17] The contribution of these processes to the neurology of AD is unclear. However, acute inflammation creates a temporal immune challenge that will cause tissue damage.[18] COXs may have a role in AD's blood-brain barrier and neuro-immune connection.[19,20] The impact of peripheral inflammation on brain function in the neuro-immune connection is associated with increased inflammatory proteins in the blood.[21] These proteins activate endothelial cells, resulting in vascular inflammation.[22] Proinflammatory transcription factors that are subsequently activated cause the expression of the same molecules in the brain.[23] At the same time, peripheral inflammatory proteins can be transported to the brain by age-dependent caveolar transcytosis.[24] The vagus nerve also plays a role in this event.[25] It transmits inflammation signals from the intestine, liver, lung, and other organs to the brain. These signals can activate inflammatory proteins and receptors in glial cells via the solitary nucleus.[17] A sudy shows that sepsis following infection threatens acute organ failure, aggravates AB accumulation and triggers systemic inflammation that causes AD to progress.[26] Damage to the vascular system is associated with an increased risk of AD. Neuroinflammation and systemic overstimulation are among the etiologies of AD and lead to neuronal death due to synaptic dysfunction. There is increasing evidence that infection and organ disorders cause peripheral AB and neurovascular dysfunction.[27]

In conclusion, Betulin may be a COX inhibitor candidate like other potent NSAIDs. Therefore, it may have a role in AD and treating specific damage to each other's organs. Most importantly, it can be mentioned that it has a potential effect on all mechanisms under the COX effect. The idea that organ damage causes AD may change direction towards the idea that AD can also cause organ damage. The limitation of this study is the analysis of structural changes and cellular damage in 3 tissues. Additional studies may help elucidate the extent of textural

damage and its association with genes and histology. In addition, all organ functions are affected by AD, which involves inflammatory processes, and not only is the brain the target of treatment. The COXs expressed in this study have significant potential as therapeutic targets. Although many alternatives exist, gene-level editing tools can be guiding in this regard.

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