ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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INVESTIGATION OF URSODEOXYCHOLIC ACID EFFECTS ON SIROLIMUS TREATED ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

URSODEOKSİKOLİK ASİTİN SİROLİMUS UYGULANAN YAĞ DOKUSU KÖKENLİ MEZENKİMAL KÖK HÜCRELER ÜZERİNE ETKİLERİNİN İNCELENMESİ

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Öz

Amaç
Organ nakli sonrası mezenkimal kök hücrelerin (MKH) immünosupresif ilaçlarla birlikte kullanımı klinik uygulamalarda dikkat çekici hale gelmektedir. Bununla birlikte, ilaçlar MKH'leri olumsuz yönde etkilemektedir. Antioksidan bir molekül olan ursodeoksikolik asit (UDKA) bu etkileri tersine çevirebilecektir. Bu çalışmanın amacı, sirolimus ve UDKA'nın bireysel ve kombinasyon olarak uygulanmasının insan yağ dokusu kaynaklı MKH'ler (YDKMKH) üzerindeki etkilerinin incelenmesidir.

Gereç ve Yöntem
Etken maddelerin sitotoksik etkileri zamana ve doza bağlı WST-1 testi ile değerlendirildi. Kombinasyon etkileri, izobologram analizi kullanılarak belirlendi. Apoptoz ve hücre döngüsünün değerlendirilmesi için Muse hücre analizörü kullanıldı. Oksidatif stress belirteçlerinin değişimi biyokimyasal yöntemle ölçüldü.

Bulgular
Sirolimusun IC50 dozu 48. saatte 18.58μM olarak belirlendi. UDKA uygulanan doz aralığında sitotoksik etki belirlenmediği için apoptoz, hücre döngüsü ve oksidatif stres indikatör analizlerine 100 μM güvenli doz ile devam edildi. Sirolimusun, apoptozu teşvik ettiği ve hücre proliferasyonunu inhibe ettiği belirlendi. UDKA'nın antioksidan özelliği ile sirolimusun YDKMKH'ler üzerindeki apoptotik ve antiproliferatif etkilerini azalttığı belirlendi.

Sonuç
Organ ve doku transplantasyonu sonrası immünosupresif tedavi ile kombinasyon halinde UDKA tedavisinin YDKMKH'ler üzerinde olumlu etkileri olabilecektir.

Anahtar Kelimeler: İnsan yağ dokusu kaynaklı mezenkimal kök hücreler; Oksidatif stres; Sirolimus; Ursodeoksikolik asit

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Abstract

Objective
The usage of mesenchymal stem cells (MSC) with immunosuppressive drugs after organ transplantation is becoming remarkable in clinical applications. However, the drugs negatively affect MSCs. Ursodeoxycholic acid (UDCA), which is an antioxidant molecule, may reverse these effects. The study aims that to determine the effects of sirolimus and UDCA on human adipose tissue-derived MSCs (ADMSCs) individually and in combination.

Material and Method
The cytotoxicity of the agents was evaluated by WST-1 test in time and dose-dependent manner. The combinational effects were determined using isobologram analysis. Muse cell analyzer was used for the evaluation of apoptosis and cell cycle. Oxidative stress markers were measured by biochemical methods.

Results
IC50 dose of sirolimus was determined as 18.58μM in the 48th hour. Because no cytotoxic effect was observed at the studied doses of UDCA, the apoptosis, cell cycle, and oxidative stress indicator analyses were continued with a safe dose of 100 μM. Sirolimus promoted apoptosis and inhibited cell proliferation. It was determined that UDCA reduced the apoptotic and anti-proliferative effects of sirolimus on ADMSCs with its anti-oxidant property.

Conclusion
The UDCA treatment in combination with immunosuppressive therapy after organ and tissue transplantation may have positive effects on ADMSCs.

Keywords: Human adipose tissue-derived mesenchymal stem cells; Oxidative stress; Sirolimus; Ursodeoxycholic acid

Introduction

Organ failure that needs transplantation is one of the most important health problems. Although not in autologous transplantation, allogenic grafts may be rejected by the immune system after transplantation (1, 2). Immunosuppressive agents are used to preventing organ rejection in the allogenic transplantation process (3). Sirolimus (rapamycin) which is a macrolide lactone produced by *Streptomyces hygroscopicus*, is an immunosuppressive agent. It demonstrates its immunosuppressive effect by blocking the IL2 which plays a key role in immune response, synthesized from T cells. Sirolimus binds to the specific intracellular receptor, FKBP1A, and inhibits the function of the mTOR pathway (3, 4). MSCs are used in regenerative medicine and tissue repair and they synthesize a large number of cytokines, chemokines, enzymes, and extracellular matrix proteins, to regulate the immune system. The combined usage of mesenchymal stem cells (MSCs) with the immunosuppressive agents to reduce the side effects of the drugs is one of the novel promising strategies (5 – 7). Ursodeoxycholic acid (UDCA) which is a macro cyclic lactone obtained by drying the bile acid of the Chinese Black Bear, is used for the treatment of diseases of liver, kidney, and gallstones. It is also used to prevent hepatic complications in allogenic stem cell transplants (8). UDCA has anti-inflammatory and immunomodulatory properties and it provides resistance against oxidative stress in vitro and in vivo (9). There is no study that includes the effects of UDCA on human adipose tissue-derived MSCs (ADMSCs). The study aims to determine the potential effects of UDCA on the sirolimus-treated ADMSCs.

Material and Method

Stem Cell Cultivation
The fifth passage ADMSCs (Cat No: SCC038, Millipore, Merck) was cultured via 2% FBS-including RPMI 1640 medium in a cell culture incubator at 37°C, 95% humidity, and 5% CO_2. Light and inverted microscopes, trypan blue dye used for culture follow-up. The cell cultivating and experiments were performed in an UV laminar airflow cabinet (ESCO Class II, Biological Safety Cabinets).

Preparation of Active Substances
Sirolimus (Rapamycin from *Streptomyces hygroscopicus* ≥95%; Sigma, Cat.No: R0395-1MG, MW: 914.17 g/mol) and UDCA (Ursodeoxycholic acid ≥99%; Sigma, Cat.No:U5127-1G, MW: 392.57 g/mol) were dissolved in DMSO (Merck, Cat.No: 276855) and absolute ethanol (Merck, Cat.No:1009831000) to adjust the final concentrations of 25mM, respectively.

Cytotoxicity and Combination Assays
The cytotoxic effects of the sirolimus and UDCA on ADMSCs were determined in time and dose-dependent manner by WST-1 test (Merck, Cat. No: 5015944001). The cells were seeded into 96-well

plate 5x104 cells/ well/ 100 µl medium concentration for 24 h. The medium was removed after the 24 h incubation period. Sirolimus and UDCA were added to wells at the dose range of 1 nM-100 mM and treated cells were incubated for 24, 48, and 72 h. Untreated cells used as the control group. Ten µl of WST-1 solution was added to the wells at the end of each time period and tetrozolium-formazan turnover was quantitated by Multiskan FC microplate reader at 450- and 620 nm. The IC50 values of sirolimus and UDCA to ADMSCs were calculated using CalcuSyn 2.0 (Biosoft) software. To determine the potential combinational effects of sirolimus and UDCA on ADMSCs as synergistic, additive and antagonistic, isobologram analysis was performed. Cells were seeded as in cytotoxicity experiments and evaluated with WST-1 assay after the treatment of active substance at the time and dose determined in the cytotoxicity experiment.

Apoptosis Assay

Annexin V & Dead Cell Kit (Merck, Cat.No: 637362) and Muse Cell Analyzer (Millipore, Merck) were used to determine the apoptotic effects of the sirolimus and UDCA. The cells were seeded into 6-well plate 1x105 cells/ well/ 3 ml medium concentrations for 24 h. The medium was removed after the 24 h incubation period. Sirolimus and UDCA were added to wells at the dose range of 1 nM-100 mM and treated cells were incubated for 48h. Untreated cells used as the control group. After the incubation period apoptosis assay was performed the manufacturer's instructions. With Muse cell analyzer, dead cell percentage, apoptosis/ dead cell percentage, early apoptosis percentage, live percent cell results were obtained. Continuous variables; mean, standard deviation and error, minimum and maximum values, and 95% confidence intervals for averages are presented. Firstly, 2x2 factorial ANOVA was applied. However, since the interaction between the two factors was significant, four groups were created and one-way analysis of variance was applied. For binary analysis of the groups after multiple comparisons Dunnett T3 method was used. All hypothesis controls were carried out at the significance level of 0.05.

Cell Cycle Tests

To determine the effects of sirolimus, UDCA and the combination on cell cycle of the ADMSCs, Muse Cell Cycle kit (Millipore, Cat. No: MCH100106) based on the PI binding florescent correlation to chromosomal DNA density was used. The cells were seeded into 6-well plate 1x105 cells/ well/ 3ml medium concentration for 24 h. The medium was removed after the 24 h incubation period. Following the cells were treated with sirolimus, UDCA and the combination. Untreated cells used as the control group. After the 96 h incubation period, the supernatant was discarded, cells washed with PBS and trypsinized. After the washing with PBS, 200 µl cold 70% ethanol was added to each group and incubated at -20oC for 3h at dark. After washing with PBS, 200 µl MuseCell Cycle Solution was added to each group and incubated at room temperature for 30 min at dark. After the incubation period, Muse Cell Analyzer was used for analysis.

Oxidative Stress Marker Assays

The effect of the sirolimus, UDCA, and the combination on the lipid peroxidation (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) activities oxidative stress markers of ADMSCs was determined using the MDA Assay Kit (BioVision, Cat. No: K739-100), GPX Activity Colorimetric Assay Kit (BioVision, Cat. No: K762-100), CAT Colorimetric/ Fluorometric Assay Kit (BioVision, Cat. No: K773-100), and SOD Activity Assay Kit (BioVision, Cat. No: K335-100), according to the manufacturer's instructions respectively. The oxidative stress marker assay results were evaluated by IBM SPSS 20.00 Statistics (Statistical Package for Social Sciences) by one-way analysis of variance and Bonferroni methods at 95% confidential interval.

Results

Sirolimus has a cytotoxic effect on ADMSCs but not ursodeoxycholic acid

Although IC_{50} dose of sirolimus was calculated as 18.59µM on 48^{th} h (R^2=0.97, p<0.05), the cytotoxic effect of UDCA was not determined up to 100µM on 24^{th}, 48^{th} and 72ndh incubation periods on ADMSCs (Figure 1 and 2). Because UDCA was not shown a cytotoxic effect, isobologram analysis was performed the combinations which include different ratios of sirolimus (10- 100µM) with a constant UDCA concentration (100 µM) on 48^{th}.The combination did not show an effect as synergistic, additive or antagonistic and IC50 dose of the sirolimus was also calculated as 18.51 uM (R2=0.98, p<0.05) on 48^{th} h in combination with UDCA (Figure 3).

Ursodeoxycholic acid alleviates the apoptotic effect of sirolimus on ADMSCs

Dead and live cell percentage were determined as 1.03% and 98.97% in the control group, respectively. Similarly, these ratios were 2.59% and 97.41% in UDCA group. In the sirolimus group, the rate of early apoptosis was 7.69%, the rate of late apoptosis was 4.31%, dead cell 8.10% and live cell 79.90%. In the sirolimus and UDCA combination results, it

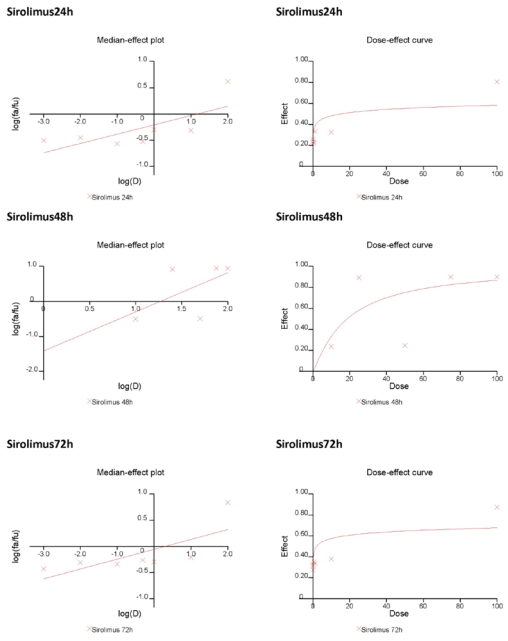

Figure 1
Cytotoxic effect of sirolimus on ADMSCs at 24th, 48th, and 72nd h.

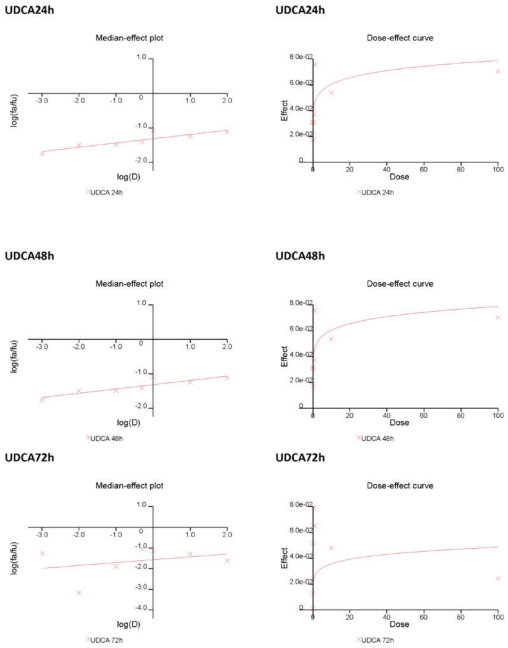

Figure 2
Cytotoxic effect of UDCA on ADMSCs at 24th, 48th, and 72nd h.

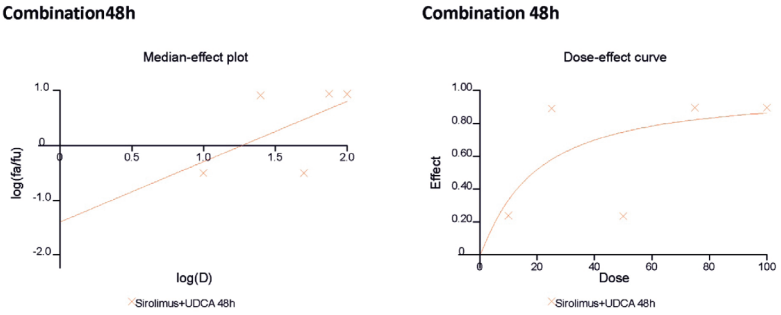

Figure 3
Cytotoxic effect of sirolimus and UDCA combination on ADMSCs at 48th h.

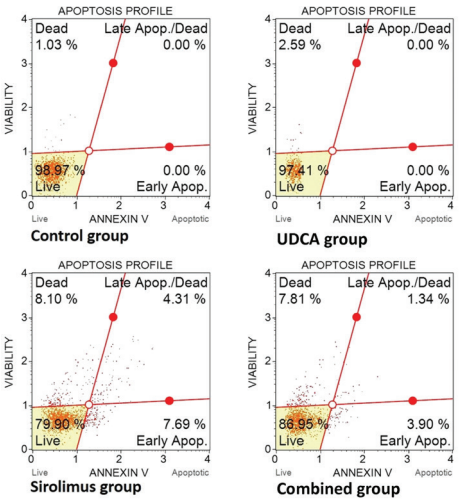

Figure 4
Apoptotic effects of the sirolimus, UDCA and the combination groups.

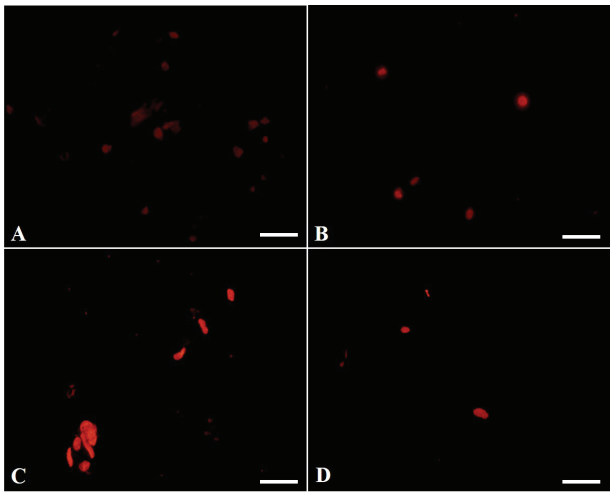

Figure 5
Immunofluorescence imaging with annexin V. A.Control group. B.UDCA group C.Sirolimus group. D. Combination group. Bar scale=50µM.

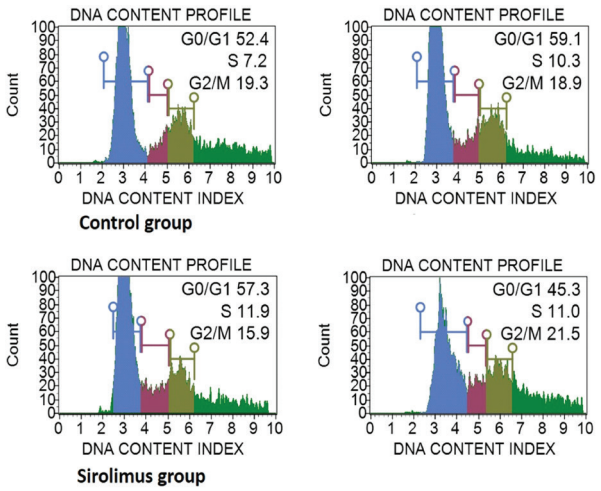

Figure 6
Cell cycle results of control, sirolimus, UDCA and the combination groups.

was determined that the rates of early apoptosis, late apoptosis, and dead cells decreased to 3.90%, 1.34%, and 7.81%, respectively, and the percentage of living cells increased to 86.95% (Figure 4 and 5; Table 1).

Ursodeoxycholic acid improves the mitotic activity which is reduced by the sirolimus

The G2/M phase percentages which indicate the mitotic activity, in the control and UDCA group were determined as 19.3% and 18.9%, respectively. In the sirolimus group, this ratio decreased to 15.9%. In the combined group G2/M phase was increased to 21.5%, thusly the mitotic activity reduction was reversed by the UDCA in the combination (Figure 6).

Oxidative stress indicators

MDA level is significant between UDCA-combination group ($p<0.001$). Not all differences between other binary groups are statistically significant. Between control-UDCA groups GSH level is not statistically significant ($p=0.085$). All other binary groups are significant ($p<0.001$). CAT level is significant between control-UDCA, control-combination, UDCA-sirolimus, and UDCA-combination groups ($p<0.001$). All differences between other binary groups are not statistically significant. All of the binary differences between all groups are statistically significant ($p <0.001$) in SOD levels [Table 2].

Discussion

To reduce graft rejection which is the most important challenge in the organ transplantation process, researches have focused on immunosuppressive agents application (10). Sirolimus which is a well-known immunosuppressor suppresses the immune system by blocking the *IL2* synthesized from T cells and prevents the function of the *mTOR* pathway (11, 12). Because immunosuppressive drugs are associated with toxicity, different doses and combinations are frequently applied in treatment processes (13). Unlike its analogue tacrolimus, sirolimus does not inhibit calcineurin, therefore it does not have an acute nephrotoxic profile of calcineurin inhibitors (10). Although initially the positive effects were observed in patients whose other immunosuppressive agent therapy was replaced with sirolimus, it was later revealed that sirolimus may be associated with in vitro cytotoxicity, delayed graft function, potentiated nephrotoxicity (14-17). To reduce the side effects of immunosuppressive drugs, MSCs attracted the attention of researchers with their immunomodulatory properties and migration abilities (18). Then a new period has started with treatments which includes the combinationally use of MSCs with immunosuppressive agents in organ transplants (19). At this point, the interaction between the immunosuppressive agents and MSCs has become an important topic that needs to be illuminated. In this study, the effects of the sirolimus on adipose tissue-derived MSCs (ADMSCs) and the potential effects of UDCA whose antioxidative properties are known, on sirolimus-related toxicity were evaluated. In a study of Buron et al. bone marrow-derived MSC (BMSC) and five immunosuppressive drugs (cyclosporine, tacrolimus, sirolimus, mycophenolic acid, dexamethasone) interactions evaluated. BMSC

Table 1 Percentage of dead cells statistical graph

MultipleComparisons							
Bonferroni							
DependentVariable			MeanDifference	Std. Error LowerBound	Sig. UpperBound	95% ConfidenceInterval	
Deadcellpercentage	Control	UDCA	-1,545000*	0,042131	0,000	-1,74938	-1,34062
		Sirolimus	-7,025000*	0,042131	0,000	-7,22938	-6,82062
		Combination	-6,760000*	0,042131	0,000	-6,96438	-6,55562
	UDCA	Control	1,545000*	0,042131	0,000	1,34062	1,74938
		Sirolimus	-5,480000*	0,042131	0,000	-5,68438	-5,27562
		Combination	-5,215000*	0,042131	0,000	-5,41938	-5,01062
	Sirolimus	Control	7,025000*	0,042131	0,000	6,82062	7,22938
		UDCA	5,480000*	0,042131	0,000	5,27562	5,68438
		Combination	,265000*	0,042131	0,020	0,06062	0,46938
	Combination	Control	6,760000*	0,042131	0,000	6,55562	6,96438
		UDCA	5,215000*	0,042131	0,000	5,01062	5,41938
		Sirolimus	-,265000*	0,042131	0,020	-0,46938	-0,06062
Lateapoptosis/ Deadpercentage	Control	UDCA	0,000000	0,027386	1,000	-0,13285	0,13285
		Sirolimus	-4,290000*	0,027386	0,000	-4,42285	-4,15715
		Combination	-1,300000*	0,027386	0,000	-1,43285	-1,16715
	UDCA	Control	0,000000	0,027386	1,000	-0,13285	0,13285
		Sirolimus	-4,290000*	0,027386	0,000	-4,42285	-4,15715
		Combination	-1,300000*	0,027386	0,000	-1,43285	-1,16715
	Sirolimus	Control	4,290000*	0,027386	0,000	4,15715	4,42285
		UDCA	4,290000*	0,027386	0,000	4,15715	4,42285
		Combination	2,990000*	0,027386	0,000	2,85715	3,12285
	Combination	Control	1,300000*	0,027386	0,000	1,16715	1,43285
		UDCA	1,300000*	0,027386	0,000	1,16715	1,43285
		Sirolimus	-2,990000*	0,027386	0,000	-3,12285	-2,85715
Earlyapoptosispercentage	Control	UDCA	0,000000	0,061033	1,000	-0,29607	0,29607
		Sirolimus	-7,615000*	0,061033	0,000	-7,91107	-7,31893
		Combination	-3,945000*	0,061033	0,000	-4,24107	-3,64893
	UDCA	Control	0,000000	0,061033	1,000	-0,29607	0,29607
		Sirolimus	-7,615000*	0,061033	0,000	-7,91107	-7,31893
		Combination	-3,945000*	0,061033	0,000	-4,24107	-3,64893
	Sirolimus	Control	7,615000*	0,061033	0,000	7,31893	7,91107
		UDCA	7,615000*	0,061033	0,000	7,31893	7,91107
		Combination	3,670000*	0,061033	0,000	3,37393	3,96607
	Combination	Control	3,945000*	0,061033	0,000	3,64893	4,24107
		UDCA	3,945000*	0,061033	0,000	3,64893	4,24107
		Sirolimus	-3,670000*	0,061033	0,000	-3,96607	-3,37393
Live cellpercentage	Control	UDCA	1,545000*	0,096047	0,001	1,07908	2,01092
		Sirolimus	18,930000*	0,096047	0,000	18,46408	19,39592
		Combination	12,005000*	0,096047	0,000	11,53908	12,47092
	UDCA	Control	-1,545000*	0,096047	0,001	-2,01092	-1,07908
		Sirolimus	17,385000*	0,096047	0,000	16,91908	17,85092
		Combination	10,460000*	0,096047	0,000	9,99408	10,92592
	Sirolimus	Control	-18,930000*	0,096047	0,000	-19,39592	-18,46408
		UDCA	-17,385000*	0,096047	0,000	-17,85092	-16,91908
		Combination	-6,925000*	0,096047	0,000	-7,39092	-6,45908
	Combination	Control	-12,005000*	0,096047	0,000	-12,47092	-11,53908
		UDCA	-10,460000*	0,096047	0,000	-10,92592	-9,99408
		Sirolimus	6,925000*	0,096047	0,000	6,45908	7,39092

*. The mean difference is significant at the 0.05 level.

Table 2 MDA, GSH, CAT, and SOD levels statistical graph

MultipleComparisons							
Bonferroni							
DependentVariable			MeanDifference	Std. Error	Sig.	95% ConfidenceInterval	
				LowerBound	UpperBound		
MDA (nmol/mg)	Control	UDCA	0,006014	0,010192	1,000	-0,04343	0,05546
		Sirolimus	-0,033077	0,010192	0,189	-0,08252	0,01636
		Combination	-,106183*	0,010192	0,003	-0,15562	-0,05674
	UDCA	Control	-0,006014	0,010192	1,000	-0,05546	0,04343
		Sirolimus	-0,039092	0,010192	0,111	-0,08853	0,01035
		Combination	-,112197*	0,010192	0,002	-0,16164	-0,06276
	Sirolimus	Control	0,033077	0,010192	0,189	-0,01636	0,08252
		UDCA	0,039092	0,010192	0,111	-0,01035	0,08853
		Combination	-,073105*	0,010192	0,012	-0,12255	-0,02366
	Combination	Control	,106183*	0,010192	0,003	0,05674	0,15562
		UDCA	,112197*	0,010192	0,002	0,06276	0,16164
		Sirolimus	,073105*	0,010192	0,012	0,02366	0,12255
GPX (mU/ml)	Control	UDCA	,057507*	0,009557	0,023	0,01114	0,10387
		Sirolimus	-1,785473*	0,009557	0,000	-1,83184	-1,73911
		Combination	,385016*	0,009557	0,000	0,33865	0,43138
	UDCA	Control	-,057507*	0,009557	0,023	-0,10387	-0,01114
		Sirolimus	-1,842980*	0,009557	0,000	-1,88934	-1,79662
		Combination	,327509*	0,009557	0,000	0,28115	0,37387
	Sirolimus	Control	1,785473*	0,009557	0,000	1,73911	1,83184
		UDCA	1,842980*	0,009557	0,000	1,79662	1,88934
		Combination	2,170489*	0,009557	0,000	2,12413	2,21685
	Combination	Control	-,385016*	0,009557	0,000	-0,43138	-0,33865
		UDCA	-,327509*	0,009557	0,000	-0,37387	-0,28115
		Sirolimus	-2,170489*	0,009557	0,000	-2,21685	-2,12413
Catalase Activity	Control	UDCA	-4,730556*	0,232784	0,000	-5,85979	-3,60132
		Sirolimus	-5,507778*	0,232784	0,000	-6,63702	-4,37854
		Combination	1,291112*	0,232784	0,031	0,16187	2,42035
	UDCA	Control	4,730556*	0,232784	0,000	3,60132	5,85979
		Sirolimus	-0,777222	0,232784	0,173	-1,90646	0,35202
		Combination	6,021667*	0,232784	0,000	4,89243	7,15090
	Sirolimus	Control	5,507778*	0,232784	0,000	4,37854	6,63702
		UDCA	0,777222	0,232784	0,173	-0,35202	1,90646
		Combination	6,798889*	0,232784	0,000	5,66965	7,92813
	Combination	Control	-1,291112*	0,232784	0,031	-2,42035	-0,16187
		UDCA	-6,021667*	0,232784	0,000	-7,15090	-4,89243
		Sirolimus	-6,798889*	0,232784	0,000	-7,92813	-5,66965
SOD (inhibition %)	Control	UDCA	7,212500*	0,444912	0,001	5,05423	9,37077
		Sirolimus	134,767500*	0,444912	0,000	132,60923	136,92577
		Combination	3,895500*	0,444912	0,006	1,73723	6,05377
	UDCA	Control	-7,212500*	0,444912	0,001	-9,37077	-5,05423
		Sirolimus	127,555000*	0,444912	0,000	125,39673	129,71327
		Combination	-3,317000*	0,444912	0,010	-5,47527	-1,15873
	Sirolimus	Control	-134,767500*	0,444912	0,000	-136,92577	-132,60923
		UDCA	-127,555000*	0,444912	0,000	-129,71327	-125,39673
		Combination	-130,872000*	0,444912	0,000	-133,03027	-128,71373
	Combination	Control	-3,895500*	0,444912	0,006	-6,05377	-1,73723
		UDCA	3,317000*	0,444912	0,010	1,15873	5,47527
		Sirolimus	130,872000*	0,444912	0,000	128,71373	133,03027

*. The mean difference is significant at the 0.05 level.

were first given to T lymphocyte cells, and then drug was administered with BMSC. In the experimental group where sirolimus and BMSC were given together, the increase in the proliferation of T cells was evaluated as the negative effect of sirolimus on the stem cell (17). In another study, it was found that sirolimus induces adipogenic differentiation, and this was associated with hyperlipidemia, which is a side effect of sirolimus (16). There are also studies showing the use of sirolimus to prevent GVHD after allogenic stem cell transplants (20, 21). Several randomized studies with kidney transplantation have shown that the immunosuppressive regimen established with the addition of sirolimus is associated with allograft survival and long-term renal function after transplantation (21). In these studies, the relationship between BMSCs and sirolimus with each other has not been investigated, and this there is a knowledge gap in the literature, regarding co-effect of immunosuppressive drugs and MSCs on MSCs. Considering cell culture studies, in the study of Biray Avci et al. determined the IC50 doses of sirolimus at DU145, PC3 and LNCaP cell lines at 72 h as 11.08, 50.80 and 1.25 nM, respectively (22). Hoogduijin et al. determined that sirolimus causes significant decrease in MSC viability at a concentration of 50 ng/ml at 7 days incubation periods (16). Although there are studies on the effects of sirolimus on the viability of ADMSCs, there is no IC50 value determined in the literature. In our study, we determined the IC50 dose of sirolimus on ADMSC as 18.58 µM at 48th h with WST-1 test.

UDCA is a macrocyclic lactone derived from the bile acid of the Chinese Black Bear for the first time (8, 23). In addition to its use in the treatment of diseases such as liver, kidney, gallstone dissolving in the clinic, it is also used to prevent hepatic complications in allogenic stem cell transplants (24-26). In recent years, studies have shown that UDCA has anti-inflammatory, immunomodulatory properties and gives resistance to oxidative stress in vitro and in vivo (8, 27). Poupon et al. showed that UDCA did not cause damage to the cell membrane in hepatic cells up to 500 µmol/l (28). When we look at the literature regarding stem cells, there is a study where UDCA is used in the clinic in order to prevent liver GVHD, which develops frequently after allogenic hematopoietic stem cell transplantation (24, 25). It has been reported that with the use of UDCA; liver GVHD, intestinal GVHD, acute and chronic GVHD and disease recurrence rate decrease and the survival rate is high (25). However, in the literature data examined, no study has been found evaluating the effects of UDCA on stem cells. At Qi et al.'s paper, experimental studies were established with a dose of 500 µmol/l of UDCA, whose cytotoxic effect was not observed (29). In our study, WST-1 test was performed in order to determine IC50 value of UDCA on human ADMSC line and it was observed that there was no cytotoxic effect on cells up to 100 µM dose. In the literature, there is no data regarding the IC50 value of UDCA on MSC, there are studies showing that there are no cytotoxic effects in therapeutic doses and there was no cytotoxic effect up to 100 µM in the WST-1 test we done, so experiments have been established at the dose of 100 µM.

WST-1 test were used to investigate the possible interactions between sirolimus and UCDA combination. In this test, a fixed dose of UDCA of 100 µM was used in doses of varying between 10-100 µM in the dose range of the sirolimus, and it was determined that the cytotoxic effect of the combination was the same as the effect of the sirolimus, and the IC50 dose in combination was the same as the dose in the individual administration. All subsequent experiments were performed with untreated (control), sirolimus (18.58uM), UDCA (100uM), combination (sirolimus 18.58uM and UDCA 100uM) groups.

It is known mTOR, which is the target protein of the sirolimus in mammals, regulates critical cellular functions and cell death by inhibiting *TP53, BCL2, BAD, CDKN1A (p21), CDKN1B (p27)* and *MYC* molecules via the *PI3K/AKT/mTOR* pathway and in the presence of sirolimus it can not lead to this control and leads the cell to apoptosis (20, 22, 30). UDCA prevents apoptosis by preventing mitochondrial dysfunction and cytochrome C release in hepatocytes (23, 27). In Rodrigues et al. study with rats, they observed that deoxycholic acids induce apoptosis, whereas UDCA inhibits apoptosis 50-100% which caused by proapoptotic stimulants *FASLG*, *TGFB1* or ethanol in vitro and in vivo (31). It has been observed that UDCA regulates *TP53* and *BAX* signal molecules in hepatocyte cells and inhibits apoptosis caused by deoxycholic acids (32, 33). In a study Koga et al., they observed an increase in the expression of *BCL2*, an apoptosis-inhibiting protein used as a marker in the increase of apoptosis, by using UDCA (34). There are studies reporting that UDCA prevents apoptosis by modulating cytochrome C release, *PTP, BAX* translocation and mitochondrial membrane disruption, as well as through cAMP, *AKT, PI3K* and *NFKB, MAPK* pathways (23, 35). Qiao et al. showed that UDCA stimulates apoptosis in hepatocytes when the MAPK and PI3K pathways are inhibited (36). Rodrigues et al. reported in another study that UDCA prevents apoptosis by inhibiting mitochondrial depolarization and protecting cells by reducing reactive oxygen (8,

27). In their review study Roma et al. showed that apoptotic cell death induced by xenodeoxycholic acid can be prevented by UDCA affecting proteins such as *EGFR, ROS1, BID, CASP12, TP53* (8).

The apoptotic effects of sirolimus, UDCA, and combination were determined by Annexin V test and Muse Cell Analyzer. It was determined that sirolimus treatment induced apoptosis. In line with the literature, UDCA did not cause significant apoptosis induction. These results support the findings in the literature and observed in this study with the WST-1 test that sirolimus has a toxic effect on the cells, while the UDCA has no such effect. In the results of the apoptosis assay of combination group increased the percentage of live cells, this result indicated that in the combination UDCA reverses the effect of sirolimus (38, 39). In the statistical evaluation of cell viability analysis, the percentage of dead cells was statistically significant between other groups except for the sirolimus and the combined group ($p < 0.0001$).

One of the important functions of mTOR, the target protein of sirolimus in mammals, is to regulate mRNA translations of cell cycle proteins (*CCND1, MYC, ODC1*)[40]. At the presence of sirolimus, mTOR cannot perform this function. By forming a complex with the *FKBP1A*, sirolimus prevents activation of the cyclin/CDK complex required for G1/S phase transition in the T cell cycle and prevents T cell activation. Thus, cells cannot be moved from G1 to S phase by being affected in the late G1 phase (23, 27, 41). There are few studies in the literature about the effects of UDCA on the cell cycle. Tsagarakis et al.'s study with *HepG2* hepatocellular carcinoma cells showed that the high concentration of UDCA delays the progression of the cell cycle (42). Cell cycles were calculated with Muse Cell Analyzer using Muse cell cycle kit for four experimental groups consisting of sirolimus, UDCA, combination and control. Although the percentages of the G2/M phases in the control and UDCA groups were similar, sirolimus treatment decreased percentage of this phase. The data show that mitotic activity is reduced in the sirolimus group. In the combination group, mitotic activity was found higher, similar to the control and UDCA group. This result shows that UDCA reverses the effect of sirolimus.

To investigate antioxidative activity of the active substances, experimental groups were enzymatically evaluated with four different oxidative stress parameters: lipid peroxidation, glutathione peroxidase, catalase and superoxide dismutase. There are no studies on the antioxidative effectiveness of UDCA in MSCs in the literature. However, in Akdemir et al.'s study a wound model created by ischemia/reperfusion in rat ovaries, a decrease in edema and malondialdehyde levels (MDA-oxidative stress parameter) observed in the group using UDCA, other oxidative stress parameters, γ -glutaminsisteinsynthetase (γ-Gcs) mRNA and glutathione (GSH) levels have increased (43). Lapenna et al. stated that UDCA showed antioxidant effect by binding to Fe^{+3} and OH- molecules. It showed the best capture at a concentration of 29 mM (9). In their studies, Perez and Briz observed that UDCA showed antioxidative properties directly and indirectly by binding Fe+3 and OH- molecules and affecting catalase, glutathione peroxidase, glutathione S-transferase levels (35). In a study by Qi et al., they observed that oxidative stress created by using selenium was inhibited by UDCA in vitro and in vivo. They observed that UDCA and total antioxidative capacity and other antioxidant enzyme values increased and lipid peroxidation level decreased (29). Lipid peroxidation level was found statistically significant for the UDCA–Combination group at $p=0.001$ significance level ($p=0.031$), while all the differences between the other binary groups were found statistically non significant. The level of glutathione peroxidase is statistically significant for all other binary groups except for the Control-UDCA ($p=0.085$) at $p<0.001$ significance level. Catalase level was found significant for the Control-UDCA, Control-Combined, UDCA-Sirolimus, UDCA-Combined groups ($p<0.001$), whereas it was found insignificant between the Control-Sirolimus, UDCA-Sirolimus and Sirolimus-Combined groups. Considering the level of superoxide dismutase, all of the binary differences between all groups were statistically significant ($p < 0.001$).

In the light of the findings obtained, results and suggestions are as follows. Sirolimus has a cytotoxic effect on adipose tissue derived mesenchymal stem cells (ADMSCs). In the Sirolimus-UDCA combination group, it was found to be closer to the control group in terms of live cell, dead cell, early and late apoptosis percentages. These findings are thought to be influenced by UDCA. Result of cytotoxicity analysis showed the IC50 dose of sirolimus on ADMSCs was determined as 18.59 µM at 48 hours. However, in accordance with the current literature data, it is thought that the use of UDCA in patients who have undergone immunosuppressive therapy and ADMSC application after organ and tissue transplantation, may have positive effects on the MSC population. If successful results are obtained in vitro studies on the subject, it is thought that UDCA has the potential to be used as a new agent in clinical studies includes cellular therapies are applied.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
This article does not contain any studies with human or animal subjects.

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Availability of Data and Materials
Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Authors Contributions
EAN: Conception, Data Collection, Literature Review
BGB: Design, Data Collection and/or Processing, Analysis, Interpretation, Writing
ST: Data Collection and/or Processing, Analysis, Interpretation, Literature Review
GY: Data Collection and/or Processing, Analysis, Interpretation, Literature Review
BG: Data Collection and/or Processing, Analysis, Interpretation, Literature Review
CT: Data Collection and/or Processing, Analysis, Interpretation, Literature Review
TC: Data Collection and/or Processing, Analysis, Interpretation, Literature Review
CBA: Supervision, Analysis, Interpretation
CG: Supervision, Analysis, Interpretation
YU: Conception, Design, Supervision, Analysis, Interpretation, Critical Review

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