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ORIGINAL ARTICLE

Does Acteoside Have Effects on Colon Cancer Stem Cells Via Inflamation or Apoptosis?

Kolon Kanseri Kök Hücrelerinde Akteosid'in Inflamasyon yada Apoptoza Etkisi Var mıdır?

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ABSTRACT

Aim: Colon cancer is one of the leading health problems worldwide. Cancer stem cells (CSCs) are referred to as tumor-initiating cells involved in tumor heterogeneity and dormancy. CSCs can cause drug resistance, metastasis, and recurrence of primary and metastatic cancers. Colon cancer stem cells may be an alternative route for effective treatment. In our study, we aimed to evaluate the effects of acteoside on stem cell properties, apoptotic and inflammatory processes in non-metastatic (HCT-116) and metastatic (Colo-741) colon cancer cells and CSCs.
Materials and Methods: CSCs were obtained from both types of colon cancer cell lines with the MINIMACS system using the anti-CD133 reagent. After Oct-4 and CD 133 expressions specified, metastatic COLO-741 and non-metastatic HCT-116 CD133+ cancer stem cells and CD133- cancer cells were cultured with or without Acteoside for 48 hours. Immunohistochemical expression of Caspase-3, Bcl-2, Bax, and Fas-L for apoptosis, and IL-1β, TNF-a, IL-6, IL8 and IL-10 proteins for inflammation were statistically analyzed by performing H-Score. Changes in stem cell properties and cell morphology of the cells were determined comparatively before and after the application.
Results and Conclusion: Expression of Oct-4 and CD 133 significantly decreased after actoside administration in both metastatic and non-metastatic colon cancer cells. In addition, it was observed that the Bax/Bcl ratio changed in both COLO-741 CD133+ and HTC-116 CD133+ cells. It was determined that acteoside did not affect inflammation in cells immunohistochemically. It has been observed that acteoside can affect the stem cell properties and apoptotic process of colon cancer stem cells.

Keywords: Colon cancer stem cells, COLO-741, HCT-116, apoptosis, inflammation, acteoside.

ÖZ

Amaç: Kolon kanseri dünya çapında sağlık problemlerinin başında gelmektedir. Kanser kök hücreleri (CSC'ler), tümör heterojenitesi ve dormansisinde yer alan tümör başlatan hücreler olarak adlandırılır. CSC'ler ilaç direncine, metastaza ve primer ve metastatik kanserlerin nüksetmesine neden olabilir. Kolon kanser kök hücreleri etkili tedavi için alternatif bir yol olabilir. Çalışmamızda non-metastatik (HCT-116) ve metastatik (Colo-741) kolon kanseri hücreleri ve CSC'lerinde, akteosidin kök hücre özelliği, apoptotik ve inflamatuar sürece etkilerini değerlendirmeyi amaçladık.
Gereç ve Yöntem: CSC' ler, anti-CD133 belirteci kullanılarak MINIMACS sistemi ile her iki tip kolon kanseri hücre hattından elde edildi. Oct-4 ve CD 133 ekspresyonlarına bakılarak, metastatik COLO-741 ve non-metastatik HCT-116 CD133+ kanser kök hücreleri ve CD133- kanser hücreleri, 48 saat boyunca akteosid olmadan veya akteosid ile kültüre edildi. Apoptoz için Kaspaz-3, Bcl-2, Bax, ve Fas-L, inflamasyon için IL-1β, TNF-a, IL-6, IL8 ve IL-10 proteinlerinin immunohistokimyasal ifadesi H-Score yapılarak istatistiksel olarak analiz edildi. Hücrelerin kök hücre özellikleri ve hücre morfolojisindeki değişiklikler uygulama öncesi ve sonrası karşılaştırmalı olarak belirlendi.
Bulgular ve Sonuç: Oct-4 ve CD 133 ifadesinin hem metastatik hem de metastatik olmayan kolon kanser hücrelerinde akteosid uygulamasından sonra anlamlı derecede azaldığı görüldü. Ayrıca COLO-741 CD133+ ve HTC-116 CD133+ hücrelerin her ikisinde de Bax/Bcl oranının değiştiği görüldü. Akteosidin hücrelerde inflamasyona immunohistokimyasal olarak etki etmediği tespit edildi. Akteosidin kolon kanseri kök hücrelerinin kök hücre özelliğini ve apoptotik sürecini etkileyebileceği görülmüştür.

Anahtar Kelimeler: Kolon kanseri kök hücreleri, COLO-741, HCT-116, apoptoz, inflamasyon, acteosit.

Introduction

Among the cells found in tumor cancerous tissues, there are many subpopulations such as connective tissue cells, inflammatory cells, vascular cells and neoplastic cells. While many of these cells are responsible for vital activities such as the physical structure and nutrition of tumor tissue, a small subpopulation defined as cancer stem cells (CSCs) is capable of infinite division (1). CSCs are a subpopulation of tumor cells with properties of high tumorigenic and drug resistance, which lead to recurrence and poor prognosis like normal stem cells. These features come from the resistant structure, self-renewal and proliferation characteristics that normal stem cells have, and it is known that both stem cells and cancer stem cells share the Oct-4 gene, which can be associated with these features (2). The cell surface proteins CD133 and variant forms of CD44 are considered putative markers for CSC populations of many types of cancers, including colon cancer (3). In 2018, colon cancer accounted for 10.2% of all cancer cases around the world and 9.2% of all cancer cases that resulted in death were caused by colon cancer (1). Primary colorectal cancers are commonly treated by surgical resection, in addition, other treatment strategies such as chemotherapy, radiotherapy, immunotherapy etc. are chosen if the tumor has metastasized. The main reasons for unsuccessful treatment or the recurrence

of cancer are still unknown; however, CSCs may be responsible for this. Therefore, an effective treatment strategy could involve the elimination of CSCs to inhibit tumor reformation and metastasis. Dysregulation of CSCs survival and their cross-talk with other tumors and other cells that are in the microenvironment of the tumor tissue may be an alternative method of treating colorectal cancer.

Inflammation is an important factor in cancer progression and malignant transformation (4,5). Inflammatory response and pro-inflammatory cytokines (IL-1a, IL-1β, IL-2, IL-6, and IL-10) are related to tumor development, cancer growth, metastasis, apoptosis and angiogenesis. Interleukin-8 is a pro-angiogenic chemokine, which stimulates cancer progression by promoting tumor cell migration and invasion, and increases in many types of solid tumors such as gastric, pancreatic, melanoma, ovarian, bladder, and prostate. IL-10 is associated with poor prognosis in cancer and is known to be effective in avoiding the microenvironment response of melanoma, lung cancer and lymphoma cancer cells by showing an immunosuppressive effect (6). Tumor necrosis factor (TNF)-a is another key inflammatory cytokine in tumor progression and an important effector molecule in cell-mediated killing of certain tumors (7). Especially in colon cancer, inflammatory factors such as interleukin-1β (IL-1β), IL-6, IL-8, IL-10 are often over-expressed. Therefore, inflammatory process cannot be ruled out in colon cancer treatment and may be considered to be an approach that can be used for cancer treatment (8,9). It was emphasized that many natural phytochemicals are used as anti-inflammatory and for regulate cellular process and prevent carcinogenesis (5).

Apoptosis is a type of programmed cell death that is regulated by caspase proteins in the cell. Activation of Caspase-3 indicates that cell death has begun and is now irreversibly progressing. Caspase-3 is activated in the apoptotic cell by both extrinsic (via death ligand such as Fas-L) and intrinsic (mitochondrial) pathways (10,11). Fas-L is one of the extracellular and apoptotic cell death regulatory molecules. Death signals coming from outside the cell activate Fas-L, activate caspase-8 and then caspase-3 in the cell and initiate an irreversible cell death signal. The regulation of the intracellular apoptotic pathway is regulated by the expression of anti-apoptotic proteins such as Bcl-2 or the downregulation or mutation of pro-apoptotic proteins such as Bax. Apoptotic death of cancer cells is triggered by death signals coming from outside the cell or by the activation of intracellular proteins by factors such as stress. In recent studies, treatment strategies targeting the apoptosis of cancer stem cells have been considered and developed.

In recent studies, many phytochemicals such as caffeic acid phenyl ester, curcumin, resveratrol, cyclopamine and piperine have been used in cancer treatment by targeting CSCs (12,13). Acteoside is an antioxidant phenylethanoid glycoside, was extracted from a herb (syringa vulgaris) (14). A number of studies have reported the in vitro and in vivo inhibition effects of acteoside in leukemia, melanoma, glioma and lung cancer. In alternative therapies, the roots of the syringa vulgaris plant are used for the treatment of epilepsy and the leaves are used for the healing of wounds and caries (15). Recently, acteoside has been shown to have a very important hepatoprotective effect in immunological liver damage in mice via antioxidant properties, immunoregulatory functions, and hepatic apoptosis regulation (16). A study has shown that combined treatment of acteoside with temozolomide in glioblastoma G6 cells alters caspase-3 and LC3 levels and increases apoptosis and autophagy. In a study by Liao et al (2012), it was shown that acteoside suppresses invasion and migration in MCF-7 cells (17). According to these studies, because of the acteoside effects cell proliferation, differentiation and cell cycle, it has the potential to control cancer cell homeostasis and the progression.

We aimed to morpholojically and immunohistochemically examine the effect of acteoside administration as well as the effect on molecules involved in the apoptosis and inflammation process and on the stem cell properties of the cells in primary (HCT-116) and metastatic (COLO-741) colon cancer cells.

Materials and Methods

Ethics committee approval

The cells used in the study are commercially available so ethics committee approval is not required.

Cell Culture

Metastatic colon cancer cell line COLO-741 (ECACC), which are adherent cells with fibroblast-like morphology, and primary colon cancer cell line HCT-116 (ATCC), which are adherent cells with epithelial morphology, were used. Mycoplasma tests of cell lines were done by the companies purchased. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Capricorn scientific), 1% penicillin-streptomycine (Gibco™) and 1% L-glutamine (Gibco™) in an incubator at 5% CO2 and 37°C.

Cancer stem cell isolation by miniMACS system

The CSCs from the COLO-741 and HCT-116 cell lines were obtained using CD133 antibody conjugated to magnetic beads by MiniMACS kit according to the manufacturer's instructions (Miltenyi Biotech, Germany).

Firstly, cells were harvested by trypsinization (total number of cells to be passed through MACS system 2x108/ml). After centrifugation, the supernatant was discarded and cells were resuspended by adding 60 µl buffer (Miltenyi Biotec). Secondary, 20 ml blocking reagent (Miltenyi Biotec) and 20 ml antibody-labeled

CD133 microbeads (Miltenyi Biotec) were added and the cells and incubated for 15 min. with stirring on ice. After incubation, 1ml buffer was added and centrifuged for 10 minutes at 300g. The supernatant was discarded and 500 µl buffer was added and resuspended. In the magnetic field, the column was placed and washed with 500 µl buffer and cells were passed through the column. In the positive separation, CD133+ cells remained in the column. Undesirable (negative separation) accumulated in the bottom tube. Unwanted cells were drained with 3x500 µl buffer. The column was separated from the magnetic field and CD133+ cells were collected in another tube. After centrifugation 300 g for 10 min. cells were washed once with medium and were then cultured. After the MiniMACS separating procedure CD133+, CD133- cells were obtained from COLO-741 and HCT-116 cell lines.

Acteoside Administration and Groups

To determine the IC50 dose, the MTT (3-(4,5-methylthiazol-2-yl)-2,5-diaphenyltetrazolium-bromide) test was performed according to the manufacturer's instructions. Cells were spread in 96 well culture dishes with 2x104 cells per well and 25, 50, 75, 100 and 125 µmol acteoside was applied to the cells for 48 hours. After the IC50 dose of 100 µmol was determined, the cells were divided into groups and the application was made. Acteoside (biopurify phytochemicals) was dissolved in culture media 100 µmol /ml. HCT-116 or COLO-741 CD133+ or CD133- cells treated or untreated with acteoside for 48 hours (18). After incubation, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in the all groups.

Immunocytochemical Analyses

Caspase-3, Bcl-2, Bax, IL-1β, TNF-a, IL-6, IL8 and IL-10 distributions were analyzed using an indirect immunocytochemical method. After fixation, the cells were incubated on ice for 15 minutes in 4% Triton X100 solution for permeabilization and then 3% H2O2 was applied for 5 minutes to inhibit cell endogenous peroxidase. Cells were washed with PBS for 5 minutes three times and then treated with a blocking solution for 1 hour. After removal of the blocking solution, they were incubated with the primary antibodies against CD133, Caspase-3, Bcl-2, Bax, IL-1β, TNF-a, IL-6, IL8 and IL-10 overnight at 4 0C. After washing with PBS for 5 minutes, biotinylated secondary antibodies were added and incubated for 30 minutes followed by PBS washing (3x) for 5 min. Horseradish Peroxidase (HRP) Streptavidin complex was added to the cells and they were then incubated for 30 min at room temperature. After washing with PBS, Diaminobenzidine was added to the cells and they were incubated for 5 min. The DAB solution was washed with distilled water and counterstained with Mayer's hematoxylin for 10 min. They were then mounted with a mounting medium and examined under a light microscope (Olympus BX40). Cells were classified as low (+), moderate (++) and strong (+++) according to their immunoreactivity intensities, and 300 cell counts were made for H-score analysis (19) (Tablo1 and 2).

Statistical Analysis

Data were expressed as mean ± SD. The results were analyzed using Statistical Package for the Social Sciences 20.0 (SPSS 20.0). Mann–Whitney U test was used for differences among groups. A P value less than 0.05 was considered statistically significant.

Results

Cell morphology

HCT-116 cells had epithelial-like morphology that grow as a monolayer (figure 1-A, B. COLO-741 also had fibroblast-like cell morphology that grow as a monolayer (figure E, F). CD133+ and CD133− cells were detected after separation in HCT-116 and COLO-741 cells. CD133+ and CD133− cells were shown to have similar morphology from both HCT-116 (figure 1A, B) and COLO-741 (figure E, F) cells. After 48 hours of acteoside treatment, while HCT-116 CD133+ (figure 1 C) and CD133− (figure 1 D) cells still had epithelioid characteristics, COLO-741 CD133+ (figure 1-G) and CD133− (figure 1-H) cells were smaller and floating.

Characterization of Colon Cancer Stem Cells

After cell separation by the MiniMACS system, immunocytochemical characterization of the cells was performed for the CD133 positivity. The ratio of CD133+ cells was about 85% in HCT-116 (figure 2-A) and COLO-741 cells (figure 2-C) after separation. The ratio of CD133- cells was 15 % in HCT-116 and COLO-741. The CD 133 negative cells of HCT-116 and COLO-741 were non-stem cancer cells (figure 2-B, D). There were no immunoreactivities in negative control groups of immunocytochemical stainings of HCT-116 (figure 2-E, F) and COLO-741 (figure 2-G, H).

The Stemness of the Colon Cancer Stem Cells After Acteoside Treatment

While HCT-116 CD133+ cells were strong positive for CD133 (figure 3-A) and Oct-4 (figure 3-E) immunoreactivities in acteoside untreated groups, both CD133 and Oct-4 immunoreactivities were slightly decreased to weak after acteoside treatment [(figure 3-B, F, respectively) (Table 1)] p=0,067.

In acteoside untreated COLO-741, CD133+ cells were strong positive for CD133 and moderate after acteoside treatment p=0,048. Oct-4 immunoreactivities (figure 3-C, G) of COLO-741 cells were strong but after 48 hours of acteoside treatment, were weak [(figure 3-D, H (Table 1)] p=0,059.

Relation of Apoptosis and Acteoside on colon CSCs

After immunocytochemical analyses, Bax and Bcl-2 immunoreactivities were similar and weak

in the untreated HCT-116 CD133+ (figure 4- A, B) and untreated CD133- (figure 4-E, F) cells. But after acteoside treatment were strong compared to the untreated HCT-116 CD133+ and CD133- cells for Bax and Bcl-2 p=0,009 (figure 4- I, J and M, N). The caspase 3 immunoreactivity was weak in HCT-116 CD133+ untreated group (figure 4 C), but was moderate in HCT-116 CD133- cells (figure 4 G). After acteoside treatment, while caspase 3 immunoreactivity was moderate in HCT-116 CD133- acteocide treated cells (figure 4 O), it was strong in HCT-116 CD133+ acteocide treated cells p=0,008 (Figure 4 K, Table 2, 3).

In COLO-741 CD133+ cells before acteoside treatment Bax immunoreaktivities were moderate after treatment were strong p=0,008 (figure 5-A, I). In COLO-741 CD133- it was moderate before and after treatment (figure 5-E, M). COLO741 CD133- and COLO741 CD133+ cells Bcl-2 immunoreactivities were moderate before (figure 5- B, F) and after (figure 5-J, N) treatment (Table 2). Strong caspase 3 immunoreactivity was detected in COLO-741 CD133+ untreated (figure 5-C) and acteoside treated groups (figure 5-K), but this immunoreactivity was moderate in COLO-741 CD133- cells before (figure 5-G) and after acteoside treatment [(Figure 5-O), (Table 2, 3)].

In HCT-116 CD133+ and HCT-116 CD133- cells the immunostainings of Fas-L were weak in acteoside treated and untreated groups p>0,05. (figure 4-D, H), p>0,05. (figure 4- L, P). Similar to that, Fas-L immunoreactivities were weak in both COLO-741 CD133+ (figure 5-D, H) p>0,05. (figure 5-L, P) and COLO-741 CD133- cells acteoside untreated and treated groups p>0,05. (Table 2).

Table 1. Immunostaining intensity of stem cell markers on HCT-116 and COLO -741 cancer stem and cancer cells treated and untreated.

	UNTREATED GROUPS		TREATED GROUPS	
	HCT-116	COLO-741	HCT-116	COLO-741
CD133	+++	+++	+	+/++
Oct-4	+++	++	+	+

Table 2. Immunostaining intensity of cell survival markers on HCT-116 and COLO -741 cancer stem and cancer cells after 48 hours of acteoside treatment.

	UNTREATED GROUPS				TREATED GROUPS			
	CD133+		CD133-		CD133+		CD133-	
	HCT-116	COLO-741	HCT-116	COLO-741	HCT-116	COLO-741	HCT-116	COLO-741
Bax	+	++	+	++/+++	+++	+++	+++	++
Bcl-2	+	++	+	+/++	+++	++	+++	++
Caspase 3	+	+++	++	++	+++	+++	++	++
Fas-L	+	+	++/+	+	++/+	+	+	+

Relation of Inflammation and Acteoside on colon CSCs

The TNF-a immunostaining was moderate in untreated HCT-116 CD133+ and HCT-116 CD133- cells before and after acteoside treatment. Similar to that in COLO-741 CD133+ and COLO-741 CD133- treated and untreated groups it was moderate too p>0,05. The IL-1β immunoreactivitiy was strong for all groups of treated and untreated cells p>0,05.

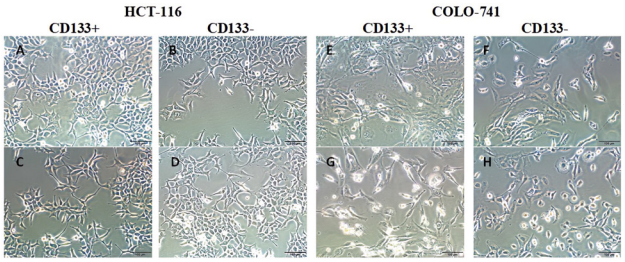

Fig. 1: Acteoside untreated (A, B, E, F) and treated (C, D, G, H) HCT-116 (A-D) and COLO-741 (E-F) CD133+ (A, C, E, G) and CD133- (B, D, F, H) cells. Scale bars= 100 µm

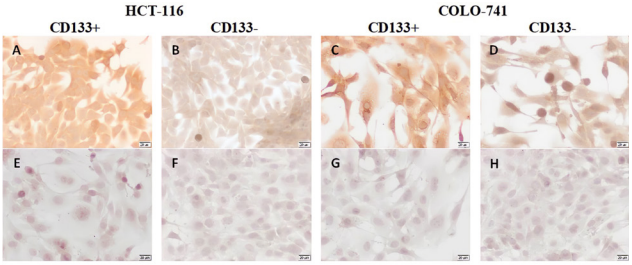

Fig. 2: Immunocytochemical staining of CD133+ (A, C) and CD133- (B, D) cells that were obtained from HCT-116 (A, B) and COLO-741 (C, D) cells after isolation of cancer stem cells by miniMACS system. The control groups of immunocytochemical staining in both cells (E, F, G, H). Scale bars= 20µm

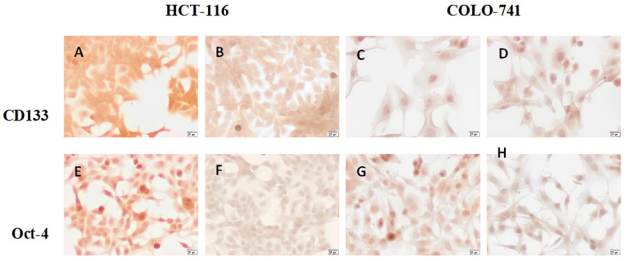

Fig. 3: CD133 and Oct-4 immunoreactivities in CD133+ cells from acteoside untreated (A, C, E, F) and treated (B, D, F, H) of HCT-116 (A, B, E, F) and COLO-741 (C, D, G, H) cells. Scale bars= 20 µm

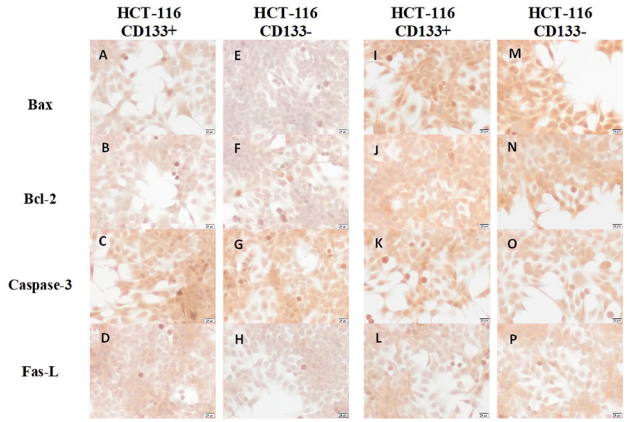

Fig. 4: Bax, Bcl-2, caspase 3 and Fas-L immunoreactivities in CD133+ (A, B, C, D) and CD133- cells (E, F, G, H) from acteoside untreated and acteoside treated CD133+ (I, J, K, L) and CD133- cells (M, N, O, P) of HCT-116. Scale bars= 20 µm

Table 3:

Antibody	Groups	Cells	H_SCORE N	Std. Deviation	Mean	Median
BAX	treated	HCT-116 CD 133+	5	1.517	250.60	250.00
		HCT-116 CD 133-	5	2.510	250.40	250.00
		COLO-741 CD 133+	5	2.550	493.00	493.00
		COLO-741 CD 133-	5	1.789	250.80	251.00
		Total	20	107.707	311.20	251.50
	untreated	HCT-116 CD 133+	5	3.435	103.40	102.00
		HCT-116 CD 133-	5	3.701	103.80	103.00
		COLO-741 CD 133+	5	1.871	251.00	250.00
		COLO-741 CD 133-	5	4.980	251.40	250.00
		Total	20	75.792	177.40	177.50
	Total	HCT-116 CD 133+	10	77.622	177.00	178.50
		HCT-116 CD 133-	10	77.322	177.10	178.50
		COLO-741 CD 133+	10	127.563	372.00	371.00
		COLO-741 CD 133-	10	3.542	251.10	250.50
		Total	40	114.196	244.30	250.00
BCL_2	treated	HCT-116 CD 133+	5	2.510	251.40	250.00
		HCT-116 CD 133-	5	2.191	250.40	251.00
		COLO-741 CD 133+	5	2.775	250.20	251.00
		COLO-741 CD 133-	5	5.701	251.00	252.00
		Total	20	3.323	250.75	251.00
	untreated	HCT-116 CD 133+	5	3.050	104.60	105.00
		HCT-116 CD 133-	5	2.280	104.80	105.00
		COLO-741 CD 133+	5	3.847	251.40	251.00
		COLO-741 CD 133-	5	4.062	251.00	251.00
		Total	20	75.217	177.95	177.00
	Total	HCT-116 CD 133+	10	77.415	178.00	178.50
		HCT-116 CD 133-	10	76.767	177.60	177.00
		COLO-741 CD 133+	10	3.225	250.80	251.00
		COLO-741 CD 133-	10	4.667	251.00	251.50
		Total	40	64.192	214.35	249.50
CASPASE_3	treated	HCT-116 CD 133+	5	4.062	485.00	486.00
		HCT-116 CD 133-	5	5.357	250.20	250.00
		COLO-741 CD 133+	5	6.458	490.20	491.00
		COLO-741 CD 133-	5	5.431	251.00	251.00
		Total	20	121.694	369.10	368.50
	untreated	HCT-116 CD 133+	5	3.975	104.40	103.00
		HCT-116 CD 133-	5	3.493	251.20	251.00
		COLO-741 CD 133+	5	5.630	489.80	489.00
		COLO-741 CD 133-	5	6.269	250.60	249.00
		Total	20	141.868	274.00	250.00
	Total	HCT-116 CD 133+	10	200.630	294.70	294.50
		HCT-116 CD 133-	10	4.296	250.70	250.50
		COLO-741 CD 133+	10	5.715	490.00	490.00
		COLO-741 CD 133-	10	5.534	250.80	250.50
		Total	40	139.065	321.55	254.50
FAS_L	treated	HCT-116 CD 133+	5	3.362	108.40	109.00
		HCT-116 CD 133-	5	5.263	105.20	103.00
		COLO-741 CD 133+	5	3.847	107.40	109.00
		COLO-741 CD 133-	5	3.362	104.60	107.00
		Total	20	4.031	106.40	107.00
	untreated	HCT-116 CD 133+	5	3.701	105.80	105.00
		HCT-116 CD 133-	5	3.578	104.60	106.00
		COLO-741 CD 133+	5	3.271	106.20	106.00
		COLO-741 CD 133-	5	3.286	105.40	106.00
		Total	20	3.236	105.50	106.00
	Total	HCT-116 CD 133+	10	3.604	107.10	108.00
		HCT-116 CD 133-	10	4.254	104.90	104.50
		COLO-741 CD 133+	10	3.425	106.80	108.00
		COLO-741 CD 133-	10	3.162	105.00	106.50
		Total	40	3.637	105.95	106.50
Total	treated	HCT-116 CD 133+	20	138.632	273.85	250.00
		HCT-116 CD 133-	20	64.587	214.05	249.50
		COLO-741 CD 133+	20	168.664	335.20	366.50
		COLO-741 CD 133-	20	65.134	214.35	249.50
		Total	80	126.608	259.36	250.00
	untreated	HCT-116 CD 133+	20	3.379	104.55	104.00
		HCT-116 CD 133-	20	65.290	141.10	106.00
		COLO-741 CD 133+	20	141.248	274.60	250.50
		COLO-741 CD 133-	20	64.834	214.60	248.50
		Total	80	105.907	183.71	109.00
	Total	HCT-116 CD 133+	40	129.298	189.20	108.50
		HCT-116 CD 133-	40	73.983	177.58	178.00
		COLO-741 CD 133+	40	156.590	304.90	252.50
		COLO-741 CD 133-	40	64.146	214.47	249.00
		Total	160	122.380	221.54	248.50

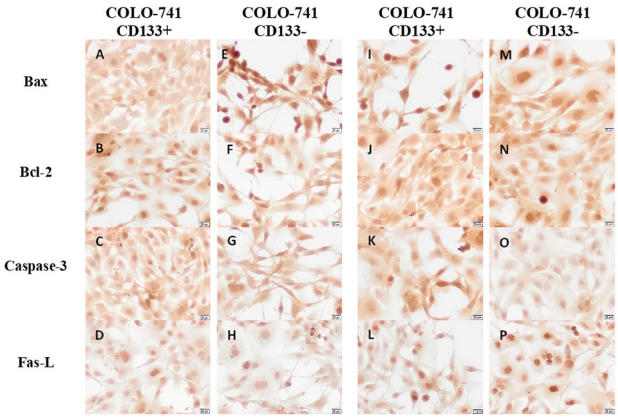

Fig. 5: Bax, Bcl-2, Caspase 3 and Fas-L immunoreactivities in CD133+ (A, B, C, D) and CD133- cells (E, F, D, H) from acteoside untreated and acteoside treated CD133+ (I, J, K, L) and CD133- cells (M, N, O, P) of COLO-741. Scale bars= 20 µm

Discussion

In this study, the effects of acteoside administration for 48 hours on metastatic (COLO-741) and non-metastatic (HTC-116) colon cancer cells were investigated. Cells were separated into two groups as cancer stem cells and cancer cells by magnetic separation method (MINIMACS). The expression of the CD133 and Oct-4 stem cell markers was demonstrated by the immunohistochemical staining METHOD of the dissociated cells. CD133+ cells were accepted as those with stem cell characteristics. Acteoside, whose IC50 dose was determined by MTT method, was applied to CD133+ and CD133- cells for 48 hours. The change in cell morphology was evaluated with the help of an invert microscope. In addition, the expression of cell death proteins and inflammation proteins of acteoside application in metastatic and non-metastatic colon cancer cells were determined immunohistochemically and evaluated statistically.

CSCs are known as immortal cells with high self-regeneration ability, enabling the tumor to reform (20), (21). These cells are similar to stem cells because of their characteristics such as forming different cell types in stem cells, repeating the old tumor and creating a new tumor type (22), a group of 0.2% and 2.2% of the all tumor population and express very low levels of cell surface antigens (1). CSCs are among the main causes of tumor recurrence, drug resistance and malignant progression in cancer patients. These cells escape from treatment due to various known and unknown mechanisms or cause the development of treatment resistance. Therefore, CSCs must be selected as targets in treatment. Due to the heterogeneity of the cell population within the tumor, targeting CSCs in treatment is very important in terms of eliminating the source of cancer development and reducing the risk of recurrence (23).

Apoptosis, a complex but organized molecular network, is one of the most studied mechanisms which is important in cellular development and homeostasis (24). Apoptotic signaling pathways prevent the formation of cancer, but mutations in the normal cells' apoptotic process can cause the cells to transform into cancer-inducing stem cells. Many drugs used for cancer treatment trigger the apoptosis of cells and attack cancer cells. While triggering apoptosis by external drug intervention, mechanisms are used to alter cell signaling pathways, increase expression levels of tumor suppressor oncogene products, and affect proteins that increase other apoptosis (25) (26). The Bcl-2 family proteins regulate the intrinsic apoptotic pathway. Anormal expression levels of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins have been reported to contribute to the life of CSCs. The ratio of pro-apoptotic and anti-apoptotic protein levels also controls the susceptibility of cells to apoptotic drugs by changing their survival balance. Triggering of Bax drags the cell towards apoptosis, increasing the permeability of the mitochondrial outer membrane. Bcl-2 suppresses Bax activity and inhibits apoptosis. Additionally, the Bax/Bcl-2 activity interacts with caspase 3, dragging the cell from the mitochondrial pathway to the apoptosis. The low Bax/Bcl ratio has been associated with poor prognosis and drug resistance in humans, depending on age [29]. Therefore, treatment methods such as the using inhibitors of anti-apoptotic protein family Bcl-2 can have successful results in cancer treatment via targeting CSCs [24]. In our study, the immunostaining of Bax increased in treatment groups of all CD133+ HTC-116 and COLO-741 cells. The Bax/Bcl-2 ratio changed COLO-741 and HTC-116 CD133+ cells after acteoside treatment. According to our results, the acteoside can activate the apoptotic pathway by changing the Bax/Bcl-2 ratio in metastatic COLO-741 CD133+ and nonmetastatic HTC-116 CD133+ cells. Caspase 3 activity also increased in HCT-116 CD133+, but not in HTC-116 CD133- and COLO-741 CD133+ and CD133- cells. These results showed that acteoside can supports the CD 133+ colon cancer cells to go towards the apoptotic process by changing the Bax/Bcl-2 ratio and in non metastatic colon cancer cells changing the caspase 3 protein amonunt. In our study, the change in apoptotic proteins was only seen in colon cancer cells with stem cell characteristics. This difference may be due to the escape of cancer stem cells and cancer cells from apoptosis by using different mechanisms or the resistance of the cells to apoptosis. Acteoside administration seems to affect the apoptosis mechanisms of metastatic and non-metastatic colon cancer stem cells.

Inflammation has been associated with many types of cancer. In the process of cancer, tumor tissue causes inflammatory response in tissues and cells in its microenvironment. During this response, growth factors and interleukins are secreted from the cancer microenvironment, resulting in increased vascular permeability or clot formation. This inflammatory response that develops in the tumor microenvironment contributes to cancer formation and metastasis (27). Proinflammatory cytokines such as IL-6, IL-10, IL-8 secreted from the cells in the microenvironment and tumor tissue in the inflammatory response may

contribute to the proliferation and differentiation of tumor cells into malign tumor cells (28) (29) (30) (6). In our study, there were no changes in the immunostainings of IL-1β, IL-6, IL-8 and IL-10 for all groups. According to our results, the absence of changes in these markers suggests that the inflammatory process is not affected by acteoside treatment for 48 hours. This unresponsiveness in cells may be due to the absence of other cell groups belonging to the microenvironment in the two-dimensional cell culture, and the absence of inflammatory cytokines from the environment. 48 hours of application was used in the study. Changes in the application time or dose may change the inflammatory response of cancer cells or cancer stem cells to acteoside.

TNF-a and Fas-L are activating inflammation and cell survival genes. Activation of TNF-a activates NF-κB and induces growth factors and inflammatory agents, while Fas-L activation triggers the caspase-8 mediated apoptosis (31). In addition, activation of NF-κB significantly induces negative regulators of apoptosis, such as Bcl-2. In present study, no changes in TNF-a and Fas-L immunostaining were detected in any of the groups, suggesting that acteoside treatment was not effective on TNF-a and Fas-L in HCT-116 CD133+, CD133- and COLO-741 CD133+, CD133- cells.

CD133 is expressed in hematopoietic, endothelial progenitors, glial and pediatric and other tumor types and is commonly used for isolation of CSCs (32). Furthermore, the Oct-4 is critically involved in the self-renewal of undifferentiated stem cells. As such, it is frequently used as a marker for undifferentiated cells (33). In the current study, there were significant decreases in both CD133 and Oct-4 immunostaining intensity in HCT-116 and COLO-741 treated groups. Acteoside decreased stem cell markers immunocytochemically in both metastatic and non-metastatic colon cancer cells in our study and this suggests that acteoside may stop the differentiation of cancer cells to CSCs.

In conclusion, the effects of treatment with acteoside for 48 hours were studied on COLO-741 and HCT-116 CD133+ or CD133- cells. Acteoside, by changing the Bax / Bcl-2 ratio in metastatic COLO-741 CD133+ and CD133- cells and non-metastatic HTC-116 CD133+ and CD133- cells, increased caspase 3 activation and helped the cell enter the apoptotic process. In addition, acteoside caused a decrease in the expression of CD113 and Oct-4 stemness markers immunohistochemically in both metastatic COLO-741 and non-metastatic HCT-116 colon cancer cells. This decrease suggests that acteoside could cause CSCs to lose their stemness characteristics.

Author Contrubution

All authors contributed equally.

Compliance with Ethical Standards

No human or animal samples were used in our study. Our study was conducted in vitro with commercially purchased human cell lines. We declare that the approval of the ethics committee is not required and it is not against the ethical rules.

Conflicts of Interest / Competing Interest

The authors did not receive support from any organization for the submitted study.

The authors declare they have no financial interests.

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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