Adıyaman University Journal of Science

https://dergipark.org.tr/en/pub/adyujsci

DergiPark AKADEMİK

ISSN 2147-1630
e-ISSN 2146-586X

Investigation of Apoptotic Effect of Klotho Protein on Human Colorectal Cancer Cells via TRAIL Death Receptors

Sibel GÜNEŞ[1,2]*, Onur UYSAL[1,2], Merve Nur SOYKAN[1,2], Ayla Eker SARIBOYACI[1,2]

[1]*Eskisehir Osmangazi University, Cellular Therapy and Stem Cell Production Application and Research Centre, ESTEM, 26040, Eskisehir, Türkiye*

[2]*Eskisehir Osmangazi University, Institute of Health Sciences, Department of Stem Cell, 26040, Eskisehir, Türkiye*

gunesibel@gmail.com, ORCID: 0000-0003-0846-1170
ouysal1971@gmail.com, ORCID: 0000-0001-6800-5607
mervesykn@gmail.com, ORCID: 0000-0003-1231-9791
aylaekersariboyaci@yahoo.com, ORCID: 0000-0003-4536-9859

Received: 30.11.2022 **Accepted:** 23.12.2022 **Published:** 30.12.2022

Abstract

Klotho is a transmembrane protein whose deficiency has pleiotropic effects in a number of aging-related disease processes and various cancers. In some recent studies about klotho protein disorders, it has been shown that klotho is effective in various cancers including lung, liver, breast, kidney, and colon. TNF-related apoptosis-inducing ligand (TRAIL) a TNF family molecule, is a cytokine that stimulates apoptosis through death receptors in many cancer types and therefore attracts attention for tumor therapies in various preclinical models. Interaction with TRAIL death receptors create an apoptotic effect in cancer treatments by reducing the proliferation of cancer cells. In this study, it was aimed to investigate the effects of exogen klotho administration on cell viability and apoptosis on TRAIL death receptors (TRAIL1 and TRAILR2) in human healthy colon cells (CCD 841 CoN) and TRAIL-resistant human colorectal cancer cells (caco2). For this purpose, cells were treated with different concentrations

* Corresponding Author DOI: 10.37094/adyujsci.1212028

of klotho for 24 and 48 hours. To determine the cell viability, proliferation, and death receptors effects of klotho protein on cancer and healthy cells evaluations with WST-8 and real-time qRT-PCR analysis were performed. Our results showed that the increase in klotho protein concentration did not have a significant effect on cell viability in healthy colon cells, whereas it decreased cell viability and proliferation in apoptosis-resistant human colorectal cancer cells. Relative gene expression levels of TRAIL1 and TRAILR2 death receptors increased with klotho applied to the apoptosis-resistant colorectal cancer cell line Caco-2. Therefore, targeting TRAIL death receptors by klotho protein can be considered as a potential therapeutic approach for colorectal cancer therapy.

Keywords: Klotho; Cell viability; Colorectal cancer; Antitumor effect; TRAIL; Apoptosis.

Klotho Proteinin Insan Kolorektal Kanser Hücreleri Üzerindeki Apoptotik Etkisinin TRAIL Ölüm Reseptörleri Üzerinden İncelenmesi

Öz

Klotho, eksikliği bir dizi yaşlanma ile ilişkili hastalık sürecinde ve çeşitli kanserlerde pleiotropik etkilere sahip olan bir transmembran proteindir. Klotho protein bozuklukları ile ilgili son zamanlarda yapılan bazı araştırmalarda klothonun akciğer, karaciğer, meme, böbrek ve kolon dahil çeşitli kanserlerde etkili olduğu gösterilmiştir. Tümör nekroz faktör ilişkili apoptoz indükleyici ligand (TRAIL), birçok kanser türünde ölüm reseptörleri aracılığıyla apoptozu uyaran bir sitokindir ve bu nedenle çeşitli preklinik modellerde tümör terapileri için dikkat çekmektedir. TRAIL ölüm reseptörleri ile etkileşim, kanser hücrelerinin çoğalmasını azaltarak kanser tedavilerinde apoptotik bir etki yaratır. Bu çalışmada, eksojen klotho uygulamasının insan sağlıklı kolon (CCD 841 CoN) ve TRAIL dirençli insan kolorektal kanser hücreleri (Caco-2) üzerindeki hücre canlılığı ve apoptoz üzerine etkilerinin TRAIL ölüm reseptörleri (TRAIL1 ve TRAILR2) aracılığı ile araştırılması amaçlanmıştır. Bu amaçla hücreler, 24 ve 48 saat boyunca farklı konsantrasyonlarda klotho ile muamele edildi. Klotho proteinin kanser ve sağlıklı hücreler üzerindeki hücre canlılığı, proliferasyonu ve ölüm reseptörlerinin etkilerini belirlemek için WST-8 ve real-time qRT-PCR analizi ile değerlendirmeler yapıldı. Sonuçlarımız, klotho protein konsantrasyonundaki artışın sağlıklı kolon hücrelerinde hücre canlılığı üzerinde önemli bir etkiye sahip olmadığını, apoptoza dirençli insan kolorektal kanser hücrelerinde ise hücre canlılığını ve proliferasyonunu azalttığını gösterdi. Apoptoza dirençli kolorektal kanser hücre dizisi Caco-2'ye uygulanan klotho ile TRAIL1 ve TRAILR2 ölüm reseptörlerinin göreceli gen ekspresyonu arttı. Sonuç olarak, TRAIL ölüm reseptörlerinin klotho

proteini ile hedeflenmesi kolorektal kanser tedavisi için potansiyel bir terapötik yaklaşım olarak kabul edilebilir.

Anahtar Kelimeler: Klotho; Hücre canlılığı; Kolorektal kanser; Antitümör etki; TRAIL; Apoptoz.

1. Introduction

Klotho is a transmembrane beta-glucuronidase protein known for its anti-aging, anti-inflammatory, and antitumor properties [1, 2, 3]. Klotho has been shown to reduce the cell viability of tumor cells and induce apoptosis [4, 5]. Klotho protein deficiency plays a role in acute and chronic kidney disease [6], cancers [7], and hypertension [8]. Klotho is expressed in the renal proximal tubule, parathyroid gland, ovary, testis, and placenta [9, 10, 11, 12, 13]. In some studies, it has been reported that the klotho protein level is low in many cancer types, including colon cancer, compared to normal tissues [14, 15, 16, 17]. Studies showing the relationship of the klotho protein, which has known antitumor activity in colorectal cancer, with the apoptosis-inducing ligand TRAIL signaling pathway and death receptors are limited [18,19].

TRAIL, also known as Apo2L, stands out with its ability to activate both pathways of apoptosis by binding to death receptors (TRAILR1 and TRAILR2) on the cell surface. TRAILR1 and TRAILR2 are types I transmembrane proteins that recruit apoptosis signaling molecules to induce cell death [20]. TRAILR1 and TRAILR2 located in the cell membrane receive cellular death signals produced by TRAIL and initiate programmed cell death. These receptors act as regulators of the TRAIL death pathway [21]. TRAIL death receptors (TRAILR1 and TRAILR2) are regulated by the p53 transcription factor. TRAILR1 and TRAILR2 receptors not only trigger apoptosis in TRAIL-sensitive cells but also activate survival pathways in tumor cells that resist induction of cell death when exposed to TRAIL [22]. In a study conducted in three different colon cancers (SW948, Caco-2, and Colo320), the TRAIL sensitivities of the cells were analyzed. It was determined that there are more TRAILR2 receptors than TRAILR1 in colon cancer cell lines where the TRAIL signaling pathway is effective. Similar levels of TRAILR1 and TRAILR2 receptors were found to be expressed in resistant colon cancer cell lines [23, 24, 25].

Klotho protein plays an important role in relation to anticancer treatments. There are limited studies of recombinant klotho protein causing cytotoxic effects on human colorectal adenocarcinoma or healthy colon cells. We aimed to investigate the anticancer effects of klotho protein against the human colorectal adenocarcinoma cells and the selectivity using healthy colon cells by adding exogen klotho protein to the medium of Caco-2 cancer and the CCD 841

CoN healthy cell lines and analyzing its effect on cell viability and apoptosis process. Additionally, we aimed to evaluate the effects of klotho protein as a potential antiproliferative and an apoptotic inducer factor on TRAIL death receptors (TRAILR1 and TRAILR2).

2. Materials and Methods

2.1. Cell culture and morphological analysis

Colorectal cancer (ATCC® HTB-37) and healthy colon cells (ATCC® CRL-1790™) were purchased commercially (ATCC, Washington DC, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM ; Biochrom, Holliston, MA, USA) and incubated at 37°C in a humidified atmosphere containing 5%CO_2 and 5%O_2. The medium was changed every other 3 days over a 5-6-day period. For each passage, the cells were plated similarly and grown to a confluency of 70%.

2.2. WST-8 cell viability assay

Human recombinant klotho protein was purchased from Sigma (Sigma-Aldrich, Darmstadt, Germany, catalog no: SRP3102-20UG). The effect of klotho protein on the viability of Caco-2 and CCD 841 CoN cells was tested with (WST)-8 (AAT Bioquest, California, USA) [26]. Recombinant klotho protein was treated in a doses range 0.04, 0.15 and 0.3 µg/ml [2, 27, 28].

After 24 and 48h, 10 µl/well of WST-8 solution was added, subsequently, cells were incubated at 37°C in a humidified atmosphere containing 5%CO_2 for 3 days. Then the absorbance values were measured at 460 nm in a microplate reader with a monochromator system (BIOTEK ELx808IU, Vermont, USA). Next, the viability of the klotho-treated cells was calculated, assuming the viability of untreated cells to be 100%. The following formula was used to calculate the percent viability:

[(OD klotho-treated cells-OD blank)/(OD untreated cells-OD blank)] × 100.

The OD refers to the optical density.

2.3. Analysis of death receptors by real-time qRT-PCR

Real-time qRT-PCR analysis was performed in order to determine the gene expression changes of TRAIL death receptors (TRAILR1 and TRAILR2) in CCD 841 CoN and Caco-2 cells. Total RNA was isolated using the extraction total RNA kit (Blirt S.A., Gdańsk, Poland) in accordance with the manufacturer's protocol. Complementary DNA (cDNA) was obtained from

the obtained mRNAs by using a cDNA synthesis kit (Transcriptome RNA kit, Blirt S.A., Gdansk, Poland). The obtained cDNA was stored at -20°C.

After obtaining cDNA, gene amplification in real-time qRT-PCR device (One-Step Thermo Scientific™ Carlsbad, USA) using probe-primers suitable for target genes (Table 1) and SYBR Green RT-PCR kit (Blirt S.A., Gdansk, Poland). The target genes were amplified and analyzed.

Table 1: Sense and antisense primers for qRT-PCR analysis

Gene	Primer (5' – 3')	
	Forward	Reverse
Klotho (KL)	ACT CCC CCA GTC AGG TGG CGG TA	TGG GCC CGG GAA ACC ATT GCT GTC
TRAILR1 (TNFRSF10)	CTG AGC AAC GCA GAC TCG CTG TCC AC	TCC AAG GAC ACG GCA GAG CCT GTG CCA T
TRAILR2 (TNFRSF10B)	CAC CAC GAC CAG AAA CAC AG	AAT CAC CGA CCT TGA CCA TC
GAPDH	CAC CCT GTT GCT GTA GCC ATA TTC	GAC ATC AAG AAG GTG GTG AAG CAG

2.4. Statistical Analysis

Statistical analyses were performed using SPSS software 20.0. Categorical and continuous variables were displayed as means ± standard deviation (SD) and percentages, respectively. $p<0.05$ were considered to be statistically significant.

3. Results

3.1. Morphological analysis of cells

During the culture period, CCD 841 CoN and Caco-2 cells were examined and photographed daily by phase-contrast microscopy (Fig. 1).

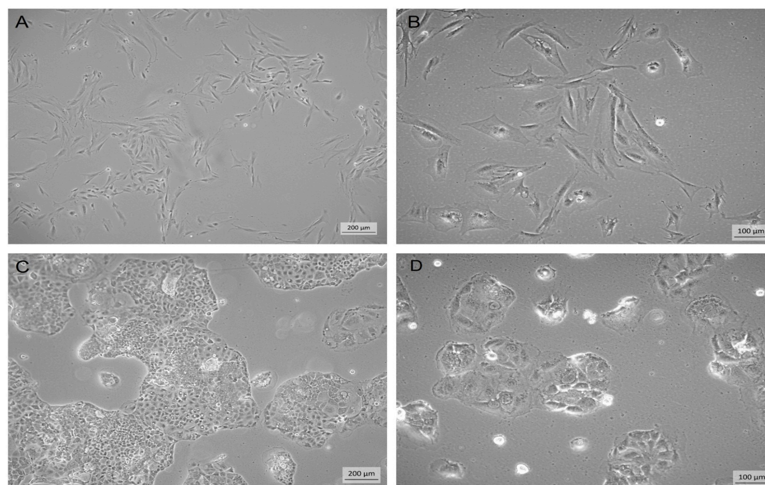

Figure 1: Phase-contrast microscopic views of healthy colon and colorectal cancer cells in early and late passages in culture (A-D) (Scale bars: A-C 200 µm; B-D 100 µm)

In microscopic examinations, CCD 841 CoN cells were attached to culture flasks as clusters, and most cells displayed a fibroblast-like, spindle-shaped morphology during the days of incubation. These cells began to proliferate in approximately 4–5 days and gradually grew as in diffuse monolayer morphology (Figure 1A and B). Caco-2 cells attached to culture flasks sparsely, and most cells displayed an epithelial-like, polygonal-shaped morphology during the days of incubation. These cells began to proliferate in approximately 6–7 days and gradually grew as non-homogenized and monolayer islets to form wide and big colonies (Figure 1C and D). Throughout 15 passages, no changes in their morphology, growth patterns, or immunophenotype were observed.

3.2. Determination of cell viability with WST-8

As a result of the doses of exogen klotho protein treated to healthy colon cells (CCD841 CoN) for 24 hours, cell viability was detected in the cells at the rates of 95%, 97% and 88%, respectively ($p>0.05$). At the doses of 0.04 µg/mL, 0.15 µg/mL, and 0.3 µg/mL klotho applied to healthy colon cells for 48 hours, the viability rates of the cells were 89%, 105%, and 89%, respectively ($p>0.05$) (Table 2, Fig. 2).

Table 2: Viability values of CCD 841 CoN cells (%)

	Cell viability (%)			
	24 hours		48 hours	
Klotho (µg/mL)	mean±SD	*p value*	mean±SD	*p value*
0.04 µg/mL	95,3±1,8	$p>0.05$	89,7±1,9	$p>0.05$
0.15 µg/mL	97,0±2,4	$p>0.05$	105,2±3,7	$p>0.05$
0.3 µg/mL	88,8±0,5	$p>0.05$	89,8±2,6	$p>0.05$

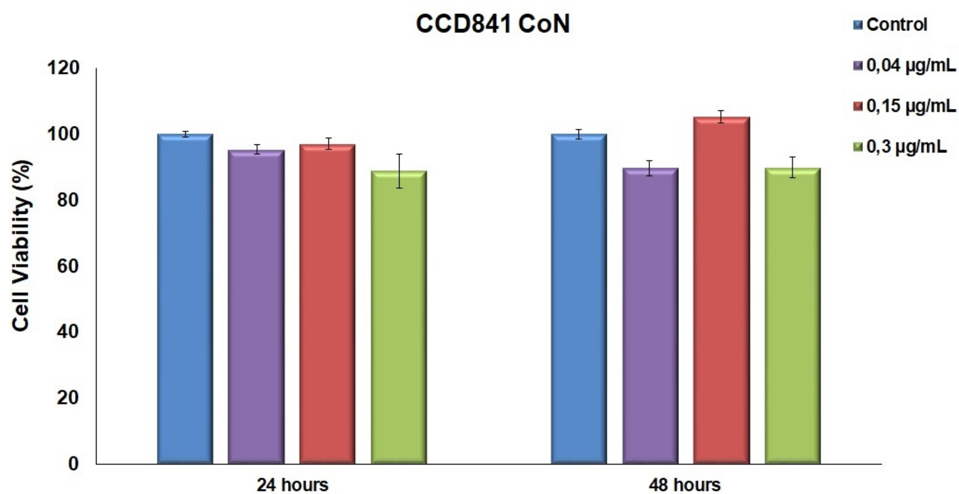

Figure 2: Detection of the effect of klotho on CCD841 CoN cells by WST-8 cell viability assay (n=3)

After 24-hour application of different doses of recombinant klotho protein to colorectal cancer cell line (Caco-2), the viability values of the cells were 81%, 68%, and 60%, respectively (*p*<0.001). When klotho was applied to Caco-2 cells at doses of 0.04 µg/mL, 0.15 µg/mL, and 0.3 µg/mL for 48 hours, the cells were viable at 92%, 83%, and 75%, respectively (*p*<0.001) (Table 3, Fig. 3).

Table 3: Viability values of Caco-2 cells (%)

Klotho (µg/mL)	Cell viability (%)			
	24 hours		48 hours	
	mean±SD	*p value*	mean±SD	*p value*
0.04 µg/mL	81,7±1,6	****p*<0.001	92,6±1,9	*p*>0.05
0.15 µg/mL	68,8±1,4	****p*<0.001	83,8±2,2	****p*<0.001
0.3 µg/mL	60,1±0,9	****p*<0.001	75,2±1,4	****p*<0.001

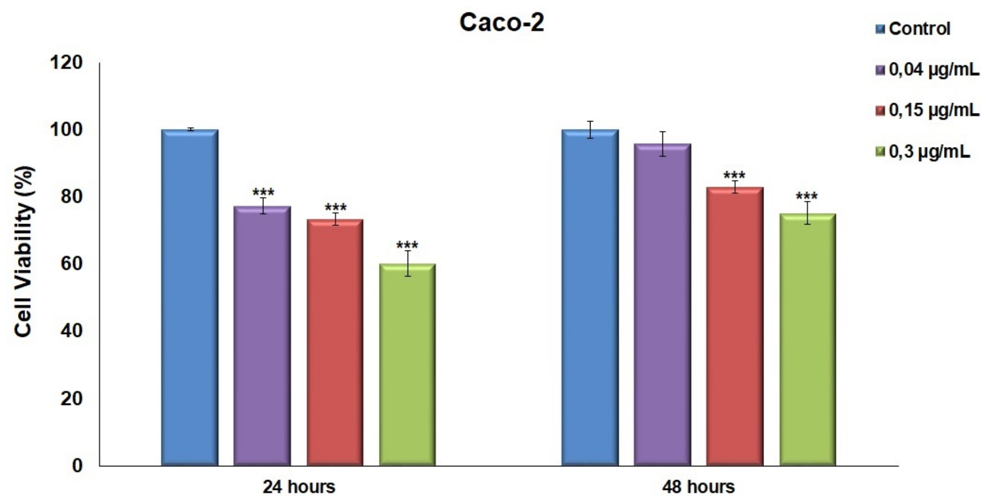

Figure 3: The results of the viability effect of recombinant klotho protein on Caco-2 cells by WST-8 analysis (n=3, ****p*<0.001)

3.3. Real-Time qRT-PCR analysis of TRAIL receptors

Real-time qRT-PCR analysis was performed in order to determine the gene expression changes of TRAIL death receptors (TRAILR1 and TRAILR2) in cells. When CCD841 CoN cells were treated with recombinant klotho for 24 hours, there were changes in TRAILR1 (44.91 fold) and TRAILR2 (30.49 fold) gene expressions compared to the control group (CCD841 CoN cells not treated with klotho) (*p*<0.001). When CCD841 CoN cells were treated with exogen klotho for 48 hours, there was a change in TRAILR1 (34.45-fold) and TRAILR2 (32.51-fold) gene expressions compared to the control group (*p*<0.001) (Fig. 4 and Fig.5).

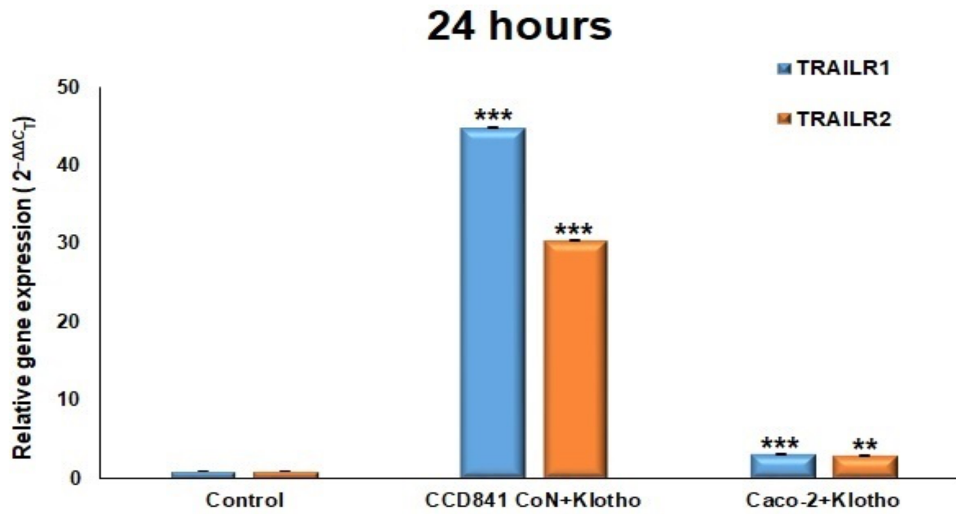

Figure 4: The effect of klotho protein (0.3 µg/mL) applied to healthy and colorectal cancer cells for 24 hours on TRAILR1 and TRAILR2 gene expression changes (n=3, **$p<0.01$, ***$p<0.001$)

When 0.3 µg/mL klotho was treated to colorectal cancer cells (Caco-2) for 24 hours, there was a change in TRAILR1 (3.22 fold) and TRAILR2 (3.01 fold) gene expressions compared to the control group (Caco-2 cells without klotho protein treatment) ($p<0.01$). When Caco-2 colorectal cancer cells were treated with klotho for 48 hours, there was a change in TRAILR1 (0.93 fold) and TRAILR2 (0.74 fold) gene expressions compared to the control group ($p<0.01$) (Fig. 4 and Fig. 5).

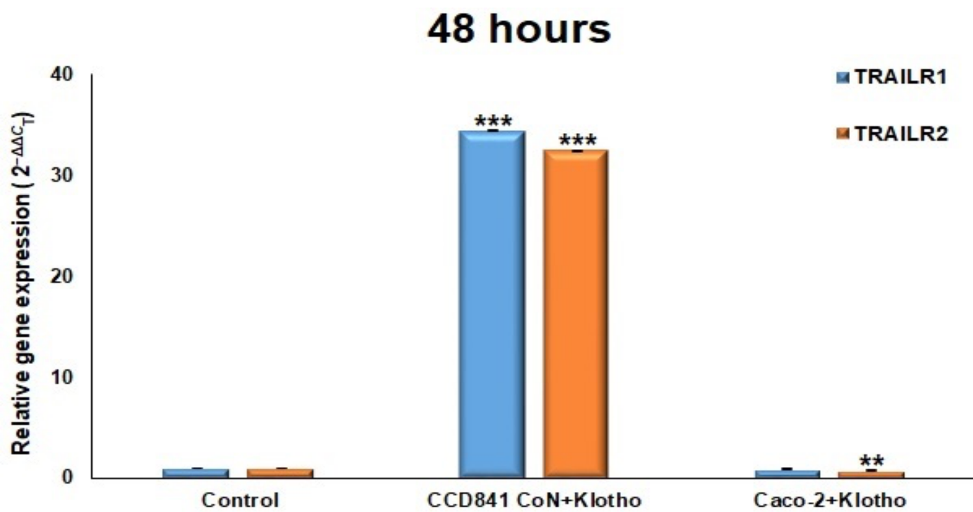

Figure 5: The gene expression changes of TRAILR1 and TRAILR2 gene expression levels after treatment of klotho protein on healthy colon and colorectal cancer cells for 48 hours (n=3, **$p<0.01$, ***$p<0.001$)

4. Discussion

Klotho protein has many well-known functions such as anti-aging, anti-inflammatory, and antitumor. Alterations in klotho gene expression affect cellular events such as cell viability and programmed cell death. While klotho protein function contributes to apoptotic cell death in normal cells, it acts as an inducer of apoptosis in cancer cells in combination with multiple signaling pathways [17, 29, 33]. Studies have shown that klotho deficiency may be a marker that can be used in the early diagnosis of colorectal cancer [5, 16]. The aim of this study was to evaluate the apoptotic effects of recombinant klotho in chemotherapeutic-resistant human adenocarcinoma colorectal cancer cell line (Caco-2) and healthy colon cells (CCD841 CoN). It has been shown that in vitro application of recombinant klotho in cancer cells reduces cell viability by stopping the cell cycle and leads to cell death [30]. Although the role of klotho in these mechanisms is not clear yet, studies have shown that it has a tumor-suppressive property [5]. It has been shown that klotho significantly suppresses cell proliferation and invasion in hepatocellular carcinoma cells and induces apoptosis of cells or autophagy [31]. Klotho protein levels have been found to be decreased in colorectal cancer [33, 34]. In studies with various colorectal cancer cells, it was found that klotho significantly decreased the viability of cells and induced cell death [35].

In our study, we found that the use of klotho protein in cells has a dose-dependent cytotoxic effect and the most effective dose in apoptosis was 0.3 µg/ml. As a result of klotho treatment to healthy cells for 24 and 48 hours, the viability values of the cells at all doses decreased compared to the control group, although it was not statistically significant ($p>0.05$). It was determined that klotho significantly reduced cell viability and stimulated apoptosis in cancer cells treated with klotho protein ($p<0.001$). These results indicated that klotho induced apoptosis in resistant Caco-2 cells and contributed to the normal apoptotic process in healthy colon cells.

The relationship between TRAILR1 and TRAILR2 death receptors is crucial in the activation of the TRAIL signaling pathway for the death of colorectal cancer cells. In cells, TRAILR1 and TRAILR2 death receptors are initiators of TRAIL-induced programmed cell death [36]. In studies conducted with TRAIL sensitivity in colon cancer cells, it was found that TRAIL-sensitive cell line SW948 expressed more TRAILR2 than TRAILR1 on the cell membrane, while two relatively resistant cell lines, Caco-2 and Colo320, expressed similar levels of TRAILR1 and TRAILR2 [37]. It has been shown that the majority of colorectal cancer cells are resistant to TRAIL. Therefore, Caco-2 cells, which are highly resistant to TRAIL-induced cell death, were selected in this study. In a study on the elimination of cancer cells of

TRAIL death receptors (TRAILR1 and TRAILR2), TRAILR2 was shown to be more effective for caspase activation than TRAILR1 [38]. In our study, we found that the relative gene expression levels of TRAILR2 in colorectal cancer cells were significantly increased at 24 and 48 hours after klotho administration. This result can be interpreted as that klotho activates the cell death receptor TRAILR2 and thus can cause apoptosis of cancer cells by causing caspase activation.

It has been reported that some clinically used drugs make cancer cells sensitive to TRAIL by increasing the protein level of TRAILR1 and TRAILR2 [39]. Previous studies have shown that DAT1, one of the diaminothiazole drugs known to have antimitotic effects on colon cancer cells (HCT-116), activates the MEK/ERK signaling pathway, increasing the expression of the TRAILR2 receptor independent of p53. In another study, it was found that Azithromycin, which is widely used in the clinic, increases the expression of TRAILR1 and TRAILR2 in HCT116 and SW480 colon cancer cells and sensitizes cancer cells to TRAIL [40]. In another study, the chemotherapeutic drug gemcitabine was shown to increase the expression of death receptors in HCT116 [41].

5. Conclusion

In our findings, it was observed that klotho protein decreased cell viability in cancer cells and had a cytotoxic effect by inducing apoptotic cell death via receptors. The fact that klotho protein increases the numbers of TRAILR1 and TRAILR2 death receptors can be interpreted as klotho may sensitize colorectal cancer cells to TRAIL. In conclusion, we think that exogen klotho protein can be used as a dose dependent adjuvant therapy in the treatment of human colorectal adenocarcinoma. In addition, we think that extrinsic and intrinsic apoptotic caspases and proapoptotic protein levels should be investigated in order to demonstrate the apoptotic effects of exogenous klotho on death receptors.

Funding

This study was supported by grants Eskisehir Osmangazi University, Scientific Research Projects (ESOGU-BAP, Code: 202046A113).

Author Contributions

Sibel Gunes is the main author in this study and contributed to the study conception and design. Material preparation, data collection and analysis were performed by Sibel Gunes and Merve Nur Soykan. The first draft of the manuscript was written by Onur Uysal and Ayla Eker

Sariboyaci. All authors commented on previous versions of the manuscript. Corresponding author read and approved the final manuscript.

Ethical statement

This study is not approval required for the ethics committee.

Declaration of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Zhou, X., Wang X., *Klotho: a novel biomarker for cancer*. J Cancer Res Clin Oncol. 2015;141(6):961-9. doi: 10.1007/s00432-014-1788-y.

[2] Tang, X., Wang, Y., Fan, Z., Ji, G., Wang, M., Lin, J., Huang, S., Meltzer, SJ., *Klotho: a tumor suppressor and modulator of the Wnt/β-catenin pathway in human hepatocellular carcinoma*. Lab Invest. 2016;96(2):197-205. doi: 10.1038/labinvest.2015.86.

[3] Shu, G., Xie, B., Ren, F., Liu, DC., Zhou, J., Li, Q., Chen, J., Yuan, L., Zhou, J., *Restoration of klotho expression induces apoptosis and autophagy in hepatocellular carcinoma cells*. Cell Oncol (Dordr). 2013;36(2):121-9. doi: 10.1007/s13402-012-0118-0.

[4] Chen, B., Wang, X., Zhao, W., Wu, J., *Klotho inhibits growth and promotes apoptosis in human lung cancer cell line A549*. J Exp Clin Cancer Res. 2010;29(1):99. doi: 10.1186/1756-9966-29-99.

[5] Li, XX., Huang, LY., Peng, JJ., Liang, L., Shi, DB., Zheng, HT., Cai, SJ., *Klotho suppresses growth and invasion of colon cancer cells through inhibition of IGF1R-mediated PI3K/AKT pathway*. Int J Oncol. 2014;45(2):611-8. doi: 10.3892/ijo.2014.2430.

[6] Hu, MC., Shi, M., Zhang, J., Quiñones, H., Griffith, C., Kuro-o, M., Moe, OW., *Klotho deficiency causes vascular calcification in chronic kidney disease*. J Am Soc Nephrol. 2011;22(1):124-36. doi: 10.1681/ASN.2009121311.

[7] Gunes, S., Soykan, MN., Sariboyaci, AE., Uysal, O., Sevimli, TS., *Enhancement of Apo2L/TRAIL signaling pathway receptors by the activation of Klotho gene with CRISPR/Cas9 in Caco-2 colon cancer cells*. Med Oncol. 2021;38(12):146. doi: 10.1007/s12032-021-01595-7.

[8] Zhou, X., Chen, K., Lei, H., Sun, Z., *Klotho gene deficiency causes salt-sensitive hypertension via monocyte chemotactic protein-1/CC chemokine receptor 2-mediated inflammation*. J Am Soc Nephrol. 2015;26(1):121-32. doi: 10.1681/ASN.2013101033.

[9] Matsumura, Y., Aizawa, H., Shiraki-Iida, T., Nagai, R., Kuro-o, M., Nabeshima, Y., *Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein*. Biochem Biophys Res Commun. 1998;242(3):626-30. doi: 10.1006/bbrc.1997.8019.

[10] Shiraki-Iida, T., Aizawa, H., Matsumura, Y., Sekine, S., Iida, A., Anazawa, H., Nagai, R., Kuro-o, M., Nabeshima, Y., *Structure of the mouse klotho gene and its two*

transcripts encoding membrane and secreted protein. FEBS Lett. 1998;424(1-2):6-10. doi: 10.1016/s0014-5793(98)00127-6.

[11] Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-Iida, T., Nishikawa, S., Nagai, R., Nabeshima, YI., *Mutation of the mouse klotho gene leads to a syndrome resembling ageing*. Nature. 1997;6;390(6655):45-51. doi: 10.1038/36285.

[12] Imura, A., Tsuji, Y., Murata, M., Maeda, R., Kubota, K., Iwano, A., Obuse, C., Togashi, K., Tominaga, M., Kita, N., Tomiyama, K., Iijima, J., Nabeshima, Y., Fujioka, M., Asato, R., Tanaka, S., Kojima, K., Ito, J., Nozaki, K., Hashimoto., N, Ito., T, Nishio., T, Uchiyama., T, Fujimori., T, Nabeshima., Y., *alpha-Klotho as a regulator of calcium homeostasis*. Science. 2007; 316(5831):1615-8. doi: 10.1126/science.1135901.

[13] Hu, MC., Shi, M., Zhang, J., Pastor, J., Nakatani, T., Lanske, B., Razzaque, MS., Rosenblatt, KP., Baum, MG., Kuro-o, M., Moe, OW., *Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule*. FASEB J. 2010;24(9):3438-50. doi: 10.1096/fj.10-154765.

[14] Urakawa, I, Yamazaki, Y, Shimada, T, Iijima, K, Hasegawa, H, Okawa, K, Fujita, T, Fukumoto, S, Yamashita, T., *Klotho converts canonical FGF receptor into a specific receptor for FGF23*. Nature. 2006;444(7120):770-4. doi: 10.1038/nature05315.

[15] Ben-Dov, IZ., Galitzer, H., Lavi-Moshayoff, V., Goetz, R., Kuro-o, M., Mohammadi, M., Sirkis, R., Naveh-Many, T., Silver J., *The parathyroid is a target organ for FGF23 in rats*. J Clin Invest. 2007;117(12):4003-8. doi: 10.1172/JCI32409.

[16] Ritter, CS., Zhang, S., Delmez, J., Finch, JL., Slatopolsky, E., *Differential expression and regulation of Klotho by paricalcitol in the kidney, parathyroid, and aorta of uremic rats*. Kidney Int. 2015;87(6):1141-52. doi: 10.1038/ki.2015.22.

[17] Pan, J., Zhong, J., Gan, LH., Chen, SJ., Jin, HC., Wang, X., Wang, LJ., *Klotho, an anti-senescence related gene, is frequently inactivated through promoter hypermethylation in colorectal cancer*. Tumour Biol. 2011;32(4):729-35. doi: 10.1007/s13277-011-0174-5.

[18] Ligumsky, H., Rubinek, T., Merenbakh-Lamin, K., Yeheskel, A., Sertchook, R., Shahmoon, S., Aviel-Ronen, S., Wolf I., *Tumor Suppressor Activity of Klotho in Breast Cancer Is Revealed by Structure-Function Analysis*. Mol Cancer Res. 2015;13(10):1398-407. doi: 10.1158/1541-7786.MCR-15-0141.

[19] Refaat, A., Abd-Rabou, A., Reda, A., *TRAIL combinations: The new 'trail' for cancer therapy (Review)*. Oncol Lett. 2014;7(5):1327-1332. doi: 10.3892/ol.2014.1922.

[20] Corazza, N., Kassahn, D., Jakob, S., Badmann, A., Brunner, T., *TRAIL-induced apoptosis: between tumor therapy and immunopathology*. Ann N Y Acad Sci. 2009;1171:50-8. doi: 10.1111/j.1749-6632.2009.04905.x.

[21] Ma, L., Wei, HL., Wang, KJ., Meng, XY., Ni, SQ., Zhou, C., Li, Y., Yu, R., Ma, Q., *Rhein promotes TRAIL-induced apoptosis in bladder cancer cells by up-regulating DR5 expression*. Aging (Albany NY). 2022;14(16):6642-6655. doi: 10.18632/aging.204236.

[22] R, Mishra DP., *Trailing TRAIL Resistance: Novel Targets for TRAIL Sensitization in Cancer Cells*. Front Oncol. 2015;5:69. doi: 10.3389/fonc.2015.00069.

[23] Yildiz, Y., Yaylim-Eraltan, I., Arikan, S., Ergen, H.A., Küçücük, S., Isbir, T., *Is there any correlation between TNF-related apoptosis-inducing ligand (TRAIL) genetic variants and breast cancer?* Arch Med Sci. 2010;6(6):932-6. doi: 10.5114/aoms.2010.19304.

[24] van Geelen, CM., Pennarun, B., Le, PT., de Vries, EG., de Jong, S., *Modulation of TRAIL resistance in colon carcinoma cells: different contributions of DR4 and DR5*. BMC Cancer. 2011;11:39. doi: 10.1186/1471-2407-11-39.

[25] Li, X.B., Deng, Y.G., Hu, J.P., Wang, Z., Xie, R.Z., Luo, H., Hu, X.Y., *Resveratrol overcomes TRAIL resistance in human colon cancer cells*. Bangladesh Journal of Pharmacology, 2015;10(3), 568-576. doi: 10.3329/bjp.v10i3.21208.

[26] Stoddart, MJ., *WST-8 analysis of cell viability during osteogenesis of human mesenchymal stem cells*. Methods Mol Biol. 2011;740:21-5. doi: 10.1007/978-1-61779-108-6_4.

[27] Sun, H., Gao, Y., Lu, K., Zhao, G., Li, X., Li, Z., Chang, H., *Overexpression of Klotho suppresses liver cancer progression and induces cell apoptosis by negatively regulating wnt/β-catenin signaling pathway*. World J Surg Oncol. 2015 Oct 24;13:307. doi: 10.1186/s12957-015-0717-0. PMID: 26499380; PMCID: PMC4619481.

[28] Chen, C.D., Li, H., Liang, J., Hixson, K., Zeldich, E., Abraham, C.R., *The anti-aging and tumor suppressor protein Klotho enhances differentiation of a human oligodendrocytic hybrid cell line*. J Mol Neurosci. 2015 Jan;55(1):76-90. doi: 10.1007/s12031-014-0336-1. Epub 2014 Jun 8. PMID: 24907942; PMCID: PMC5154549.

[29] Sariboyaci, A.E, Uysal, O., Soykan, M.N., Gunes, S. ,*The potential therapeutic effect of klotho on cell viability in human colorectal adenocarcinoma HT-29 cells*. Med Oncol. 2022 Sep 7;39(12):191. doi: 10.1007/s12032-022-01793-x. PMID: 36071274.

[30] Rubinek, T., Wolf, I. (2015). *Klotho Tumor Suppressor*. In: Schwab, M. (eds) Encyclopedia of Cancer. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-27841-9_7219-1

[31] Chen, CD., Li, H., Liang, J., Hixson, K., Zeldich, E., Abraham, C.R., *The anti-aging and tumor suppressor protein Klotho enhances differentiation of a human oligodendrocytic hybrid cell line*. J Mol Neurosci. 2015;55(1):76-90. doi: 10.1007/s12031-014-0336-1.

[32] Sun, H., Gao, Y., Lu, K., Zhao, G., Li, X., Li, Z., Chang, H., *Overexpression of Klotho suppresses liver cancer progression and induces cell apoptosis by negatively regulating wnt/β-catenin signaling pathway*. World J Surg Oncol. 2015;13:307. doi: 10.1186/s12957-015-0717-0.

[33] Zhou, X., Fang, X., Jiang, Y., Geng, L., Li, X., Li, Y., Lu, K., Li, P., Lv, X., Wang, X., *Klotho, an anti-aging gene, acts as a tumor suppressor and inhibitor of IGF-1R signaling in diffuse large B cell lymphoma*. J Hematol Oncol. 2017;10(1):37. doi: 10.1186/s13045-017-0391-5.

[34] Xie, B., Zhou, J., Yuan, L., Ren, F., Liu, DC., Li, Q., Shu, G., *Epigenetic silencing of Klotho expression correlates with poor prognosis of human hepatocellular carcinoma*. Hum Pathol. 2013;44(5):795-801. doi: 10.1016/j.humpath.2012.07.023.

[35] Arbel Rubinstein, T., Shahmoon, S., Zigmond, E., Etan, T., Merenbakh-Lamin, K., Pasmanik-Chor, M., Har-Zahav, G., Barshack, I., Vainer, GW., Skalka, N., Rosin-Arbesfeld, R.,

Varol, C., Rubinek, T., Wolf, I., *Klotho suppresses colorectal cancer through modulation of the unfolded protein response*. Oncogene. 2019;38(6):794-807. doi: 10.1038/s41388-018-0489-4.

[36] Zhang, Z., Li, Z., Wu, X., Zhang, CF., Calway, T., He, TC., Du, W., Chen, J., Wang, CZ., Yuan, CS., T*RAIL pathway is associated with inhibition of colon cancer by protopanaxadiol*. J Pharmacol Sci. 2015;127(1):83-91. doi: 10.1016/j.jphs.2014.11.003.

[37] Zhang, B., Liu, B., Chen, D., Setroikromo, R., Haisma, HJ., Quax, WJ., *Histone Deacetylase Inhibitors Sensitize TRAIL-Induced Apoptosis in Colon Cancer Cells*. Cancers (Basel). 2019;11(5):645. doi: 10.3390/cancers11050645.

[38] Nahacka, Z., Svadlenka, J., Peterka, M., Ksandrova, M., Benesova, S., Neuzil, J., Andera, L., *TRAIL induces apoptosis but not necroptosis in colorectal and pancreatic cancer cells preferentially via the TRAIL-R2/DR5 receptor*. Biochim Biophys Acta Mol Cell Res. 2018;1865(3):522-531. doi: 10.1016/j.bbamcr.2017.12.006.

[39] Thamkachy, R., Kumar, R., Rajasekharan, K.N., Sengupta, S., *ERK mediated upregulation of death receptor 5 overcomes the lack of p53 functionality in the diaminothiazole DAT1 induced apoptosis in colon cancer models: efficiency of DAT1 in Ras-Raf mutated cells*. Mol Cancer. 2016;15:22. doi: 10.1186/s12943-016-0505-7.

[40] Qiao, X., Wang, X., Shang, Y., Li, Y., Chen, S.Z., *Azithromycin enhances anticancer activity of TRAIL by inhibiting autophagy and up-regulating the protein levels of DR4/5 in colon cancer cells in vitro and in vivo*. Cancer Commun (Lond). 2018;38(1):43. doi: 10.1186/s40880-018-0309-9.

[41] Gravett, A.M., Dalgleish, A.G., Copier, J., *In vitro culture with gemcitabine augments death receptor and NKG2D ligand expression on tumour cells*. Sci Rep. 2019;9(1):1544. doi: 10.1038/s41598-018-38190.