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BASIC MEDICAL SCIENCES / TEMEL TIP BİLİMLERİ

Anti-Aging Effects of *Hibiscus sabdariffa* on Immortalized Human Ovarian Epithelial Cells

Hibiscus sabdariffa'nın Ölümsüz İnsan Over Epitel Hücreleri Üzerindeki Yaşlanma Karşıtı Etkileri

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Abstract

Objectives: The aim of this study is to examine the anti-aging effect of *Hibiscus sabdariffa* (HS) against oxidative stress induced by hydrogen peroxide (H_2O_2) on immortalized human ovarian epithelial cells (IHOECs).

Materials and Methods: The obtained IHOECs were combined with 12.5 mg/mL HS extracted by adding 1mL of distilled water (dH_2O) and different concentrations of H_2O_2 for oxidative effect. IHOECs cell viability was increased with HS extract and determined by MTT test assay. Real-time polymerase chain reaction was conducted to amplify and detect apoptosis-related BAX and BCL_2, stem cell markers SOX_2 and OCT_4, and antioxidative *nuclear factor erythroid 2-related factor 2* (*NRF2*) gene expressions. For the statistical analysis Kruskal-Wallis analysis and Mann-Whitney U test were used in SPSS program.

Results: The increase in HS in cell proliferation was 14%. While there is no significant increase of cell viability in the combined group with HS and H_2O_2, there were only statistically significant groups are 200, 800, 900 and, 1,000 µl. It was indicated that HS has apoptotic, stem cell promotion and antioxidative effects on high H_2O_2 concentrations of these groups ($p<0.05$).

Conclusion: In combined groups with both HS and H_2O_2 induced oxidative stress, HS increased the expression of proapoptotic gene *BAX* and decreased antiapoptotic gene BCL_2. Although, the remaining cells which are abundantly stem cells expressed genes OCT_4 and SOX_2 remarkably decreased, at high H_2O_2 concentration opposite effects were observed. NRF_2 gene expression also increased in this high oxidative stress.

Keywords: *Hibiscus sabdariffa*, oxidative stress, apoptosis, stem cells

Öz

Amaç: Bu çalışmanın amacı, *Hibiscus sabdariffa*'nın (HS) hidrojen peroksitin (H_2O_2) ölümsüzleştirilmiş insan yumurtalık epitel hücreleri (IHOEC) üzerinde neden olduğu oksidatif strese karşı yaşlanma karşıtı etkisini incelemektir.

Gereç ve Yöntem: Elde edilen IHOEC'ler, oksidatif etki için 1 mL distile su (dH_2O) ve farklı konsantrasyonlarda H_2O_2 eklenerek ekstrakte edilen 12,5 mg/mL HS ile kombine olarak denendi. IHOEC'lerin hücre canlılığının, HS ekstraktı ile arttığı MTT testi ile belirlendi. Apoptozla ilişkili BAX ve BCL_2'yi; kök hücre belirteçleri olan SOX_2 ve OCT_4'ü; antioksidatif belirteç olan *nükleer faktör eritroid 2 ile ilişkili faktör 2* (NRF_2) gen ifadelerini belirlemek için gerçek zamanlı polimeraz zincir reaksiyonu yöntemi kullanıldı. İstatistiksel analiz için SPSS programında Kruskal-Wallis analizi ve Mann-Whitney U testi kullanıldı.

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Bulgular: Hücre proliferasyonunda HS'deki artış %14 idi. HS ve H_2O_2 ile kombine edilen grupta hücre canlılığında anlamlı bir artış görülmezken, sadece 200, 800, 900 ve 1,000 µl'lik gruplar istatistiksel olarak anlamlıydı. HS'nin bu grupların yüksek H_2O_2 konsantrasyonları üzerinde apoptotik, kök hücre teşvik edici ve antioksidatif etkilere sahip olduğu belirtildi ($p<0,05$).

Sonuç: Hem HS hem de H_2O_2 kaynaklı oksidatif stresin olduğu kombine gruplarda HS, proapoptotik gen BAX'ın ekspresyonunu artırdı ve antiapoptotik gen *BCL*$_2$'yi azalttı. Geriye kalan kök hücre bakımından zengin kültürde *OCT*$_4$ ve SOX_2 genlerini ekspresyonu azalırken, yüksek H_2O_2 konsantrasyonunda tersine bir etki gözlendi. Bu yüksek oksidatif streste *NRF*$_2$ gen ekspresyonu da artış gösterdi.

Anahtar Kelimeler: *Hibiscus sabdariffa*, oksidatif stres, apoptoz, kök hücreler

Introduction

A wild tropical plant in the Malvaceae family *Hibiscus sabdariffa* (HS) is also known as roselle. There are various historical uses for HS in many cultures and established benefits of HS that are as follows: antihypertensive, anti-inflammatory, anti-obesity qualities; nephron-, hepato- and cardio- protective effects etc (1). Although further investigations are needed on its anti-aging properties, studies of this medicinal plant are still ongoing.

Immortalized human ovarian epithelial cells (IHOECs) were derived from human ovarian surface epithelium, which contains stem cells and expresses *SOX*$_2$, *OCT*$_4$ and *nuclear factor erythroid 2-related factor 2* (*NRF*$_2$) genes (2,3). We aim to investigate if and how we can use HS to slow down aging in IHOECs and improve ovarian health.

A number of the body's primary processes gradually deteriorate with age, making aging an extremely complicated process. Hereditary factors, lifestyle choices, and environmental factors including xenobiotic pollutants, infectious agents, UV radiation, diet-borne chemicals, and so forth all have an impact on this unavoidable process. Aging and senescence are accompanied by a number of internal and exterior signs and symptoms, such as wrinkles and dry skin, diabetes, atherosclerosis, cancer, and neurological diseases. The most prevalent and deadly kind of ovarian cancer is epithelial and was linked to aging in many studies. In animal models, the removal of aging cells slows or stops the aging process. It has been suggested that a reduction in mitochondrial activity causes aging because it increases reactive oxygen species (ROS) generation and macromolecule damage (4,5).

The imbalance between oxidants and antioxidants leads to oxidative stress, which is one of the primary initiating elements of aging-related damages that culminate in apoptosis. Free radicals have the ability to harm proteins, DNA, and fatty tissues in human body when their numbers exceed the antioxidants' capacity to keep them in balance. Antioxidants can prevent these processes lowering the level of oxidative stress (6).

The previous works support the scientific hypothesis that HS plants enriched with bioactive constituents play an imperative role in the management of degenerative and chronic diseases, lipid metabolism (anti-cholesterol), anti-diabetic and anti-hypertensive effects that are associated with oxidative stress (7).

Within the overall antioxidant defense strategy, the function and efficacy of the first line defensive antioxidants, such as catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD), are critical and vital. Our body's defense system against aging and oxidation is antioxidants like SOD, CAT and GPX can be enhanced by HS (8).

In this study we aimed to define the antiaging effect of HS on the IHOECs through some mechanisms and genes like *BAX*, *BCL*$_2$, *SOX*$_2$, *OCT*$_4$ and *NRF*$_2$.

In determining the survival of a cell there are many factors. There are two main routes that regulate apoptosis: the intrinsic (mitochondrial pathway) and extrinsic (death receptor pathway). The B-cell lymphoma 2 (BCL-$_2$) protein family orchestrates and regulates the intrinsic pathway. This protein family can be categorized based on their functions as either anti-apoptotic, including BCL-$_2$, BCL-x, BCL-w, MCL-$_1$, and A$_1$/BFL-$_1$, or pro-apoptotic, such as BAX, BAK. In addition, recent studies show BAX and BCL$_2$ contribute also to the cell cycle and apoptosis regulation (9).

SOX$_2$ and *OCT*$_4$ genes are embryonic stem cell markers that maintain pluripotency and regulate differentiation of stem cells. The upregulation of these genes under oxidative stress conditions with the addition of HS aqueous extract will indicate that the stem cell population is preserved. When cells transition away from a pluripotent state, SOX$_2$ signaling becomes vital for the development of various endodermal and ectodermal tissues during fetal development (10).

OCT$_4$ belongs to the Oct family of POU transcription factors. It holds a crucial position in controlling both the pluripotency and differentiation of stem cells. Considering the stem cell's role in tissues we can relate the expression of these genes to a tissue aging, the ability to regenerate and preservation (11).

NRF$_2$'s protective function extends to averting chemical- and radiation-induced carcinogenesis by facilitating the enzymatic modification and elimination of carcinogens, as well as using gene transcription to repair oxidative damage and suppress ROS. Consequently, NRF$_2$ is widely regarded as a cytoprotective transcription factor, pivotal for cellular defense mechanisms and survival. Its deficiency renders organisms more susceptible to

carcinogenesis and metastasis, highlighting its role as a tumor suppressor NRF's significance in maintaining cellular integrity and adaptation to environmental stressors (12,13).

Exposing HS, an antioxidant that uses the cascade mechanism, to the cell at an optimal level will delay the aging of the IHOECs and will extend healthy longevity. As a main result, it will prolong the reproductive age.

Materials and Methods

The cells used in the study are commercially available so ethics committee approval is not required. The study protocol was appropriate in accordance with the Declaration of Helsinki.

1. Cell Culture

IHOECs were obtained from American Type Culture Collection. IHOECs were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 2.5mM L-glutamine (Bio-Ind, USA), 15mM HEPES, 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma, St Louis, MO, USA) and 10% fetal bovine serum and incubated at 37 °C in humidified 5% CO_2 incubator. Every 48 hours, the growth medium was changed, and when the cells achieved 75-85% confluence, 0.25% trypsin was used to separate them.

2. HS Extract Preparation and Applying

The flower part of HS were collected and identified by the Department of Biochemistry at Ankara University Faculty of Medicine. The 12.5 mg/mL of HS were dried, mashed and extracted by adding 1 mL of dH_2O. Before applying the 12.5 mg/mL extract underwent filtration a mesh with apertures measuring 0.22 mm in diameter. Subsequently, an extract of HS was added to the cell plates and incubated for 24 hours.

3. MTT Assay

To determine the viability of IHOECs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Cells were inoculated onto 96-well plates as $2x10^4$ cells per well and expected to adhere overnight. The cell groups cells were incubated with 12.5 mg/mL HS extract for 24 hours. Then they were treated with solutions of H_2O_2 within a concentration range of 200 to 800 µM and waited for 3 hours. Following this the 10 µM (5 mg/mL) of MTT reagent was introduced to each cell group for 2 hours at 37 °C. Later, 100 µL detergent reagent was added. The color change was detected by using a spectrophotometric plate reader (Biotek, USA), each sample's optical density was measured at 570 nm, using 690 nm as a reference.

4. Real-time Polymerase Chain Reaction

IHOECs cell lines being already treated were cultured in 6-well plates with seeding density of $5×10^5$ cells/well. The total RNA was isolated by Trizol reagent (Invitrogen, USA). Complementary DNA (cDNA) synthesis was carried out using reverse transcriptase (iScript cDNA synthesis kit; Biorad). To amplify and detect apoptosis-related BAX and BCL_2, stem cell marker SOX_2, OCT_4 and antioxidative NRF_2 genes Real-time polymerase chain reaction (RT-PCR) was conducted using the CFX Connect RT-PCR Detection System (Biorad, CA, USA). To normalise the expression level of mRNAs, the expression of GAPDH mRNA was used as an endogenous control. This system uses the SYBR Green PCR Master Mix (iTaq Universal SYBR Green Supermix, Biorad, CA). The amplification parameters were heating to 95 °C for 10 min, followed by 50 cycles of each denaturation to 95 °C for 30 s, annealing to 60 °C for 30 s, and extension to 72 °C for 30 s.

Statistical Analysis

The results were expressed as the mean ± standard deviation (mean ± SD) and were analysed at SPSS Statistics using Kruskal-Wallis analysis and Mann-Whitney U test. Differences with values of $p<0.05$ were considered statistically significant.

Results

1. Optimization of HS Concentration and Cell Viability Relation

By applying HS at different concentrations without exposing IHOECs to any oxidative effect, the most appropriate concentration that provided 14% maximum cell proliferation for our experiment was found to be 12.5 mg/mL HS extract, and this concentration was used in all experiments. Cell viability of IHOECs in DMEM F12 medium for 24 hours was analysed by MTT test assay. The quantitative data are presented as mean ± SD in 3 repetitive experiments ($p<0.05$) (Figure 1).

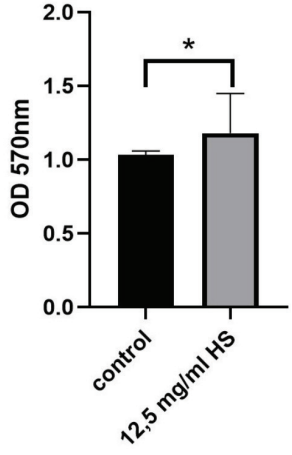

Figure 1: IHOECs cell viability without and with HS

Control: IHOECs + water; 12.5 mg/mL HS: IHOECs + 12.5 mg/mL HS

IHOECs: Immortalized human ovarian epithelial cells, HS: *Hibiscus sabdariffa*

According to Shapiro-Wilk test results, only 200, 800, 900 and 1,000 µL combined experiment results provide a test of normality (p=0.013). IHOECs were fed with HS were under oxidative influence, it was expected that a high rate of cell proliferation would be maintained due to the anti-oxidative effect of HS. Contrary to the expected results, the predicted increase in cell viability in MTT assay was not observed. MTT results indicated that cell viability in H_2O_2 and HS combined group had no significant effect on cell proliferation to change HS's anti-aging effect (p≥0.05) (Figure 2).

It has been determined that H_2O_2 at concentrations of 200 µL, 800 µL, 900 µL, 1,000 µL with HS may have a negligible antioxidant effect (p=0.05). H_2O_2 concentrations of 200 µL and 800 µL with HS showed a minor antioxidant effect and were linked to decreased cell viability, suggesting HS may alter this (Figure 2). These concentrations were further studied for their slight contribution to proliferation and potential apoptotic effects in IHOECs. They are key to demonstrating HS's impact on the expression of *SOX*$_2$, *OCT4*, *BAX*, *BCL*$_2$, and *NRF2* genes.

2. HS Treatment Protects Stem Cell Marker Gene Expressions

In the control HS group, OCT_4 and SOX_2, which are stem cell markers of survived cells, are extremely abundant. Cell lines contain a high amount of stem cell gene expressions also has been demonstrated with this experiment even without any treatment.

HS does not have an increasing effect on stem cell marker gene expression in 200 µL H_2O_2 concentrations but an increase in stem cell marker gene expressions was observed at high 800 µL concentration of H_2O_2 (Figure 3).

Although the expected anti-oxidative effect was not observed, it was observed that HS increased the *SOX*$_2$ and *OCT*$_4$ gene expression at a concentration of 800 µL, where the cells started to receive the most oxidative damage.

In the HS + 800 µL H_2O_2 group, a 4-fold increase was observed for the *OCT*$_4$ gene expression and a 5-fold increase for the *SOX*$_2$ genes under the excessive oxidative stress.

3. HS Treatment Increase the Apoptosis of Damaged Cells

Apoptosis is referred to as programmed cell death because it occurs due to biochemical instructions in the cell's DNA, is a type of protection mechanism of cells under stress (14). Apoptosis signaling is controlled by certain genes within the cell, and *BAX* (Figure 4a) is one of these apoptotic genes whereas *BCL*$_2$ is one of the anti-apoptotic genes (Figure 4b).

According to the RT-PCR reults while *BAX* gene expression increased, *BCL*$_2$ gene expression decreased. There was a strong positive correlation between HS and BAX/BCL_2 ratio. Between HS + 200 µL H_2O_2 and HS + 800 µL H_2O_2, 180-fold difference in the ratio of *BAX/BCL*$_2$ genes (Figures 4a and 4b).

This fold increase in BAX/BCL_2 proved to us that the cell was using the apoptotic pathway. The death type of cells leads that HS might protect IHOECs which under the high concentration of oxidative stress via apoptosis. In the high oxidative conditions, induction of apoptosis by the HS in the damaged cells causes clearence of vulnerable IHOECs.

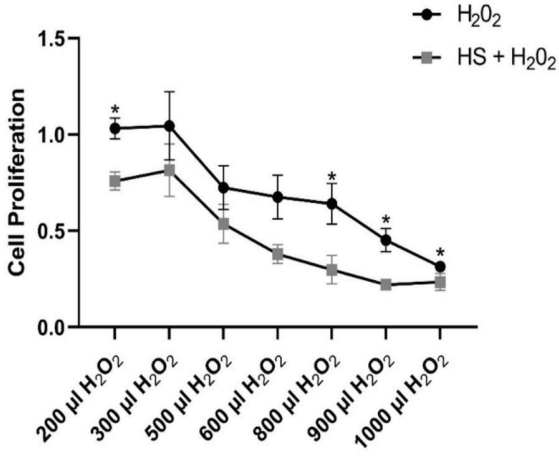

Figure 2: IHOECs proliferation in combinations of HS and different concentrations of H_2O_2

H_2O_2: IHOECs only with H_2O_2 at different concentrations; HS+ H_2O_2: IHOECs with HS and H_2O_2 at different concentrations; Mann-Whitney U test; p values ≥ 0.05 were considered statistically non-significance. Cell viability was not homogeneously distributed

IHOECs: Immortalized human ovarian epithelial cells, HS: *Hibiscus sabdariffa*

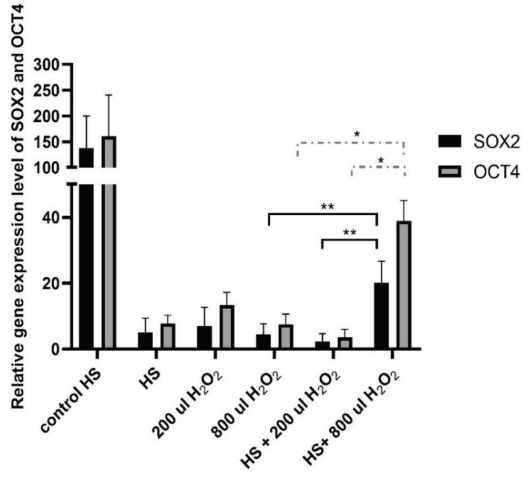

Figure 3: The expression of OCT_4 and SOX_2 genes in IHOECs in HS and different concentrations of H_2O_2, was measured by RT-PCR

control HS: IHOECs + water; HS: IHOECs + HS; 200,800 µL H_2O_2: IHOECs + H_2O_2; HS + H_2O_2: IHOECs + HS + H_2O_2; n=4; mean of 4 independent experiments are shown in the graph. All data are presented as mean ± SD. Statistical significance: *p≤0.005 (OCT4), **p≤0.001 (SOX_2)

IHOECs: Immortalized human ovarian epithelial cells, HS: *Hibiscus sabdariffa*, RT-PCR: Real-time Polymerase Chain Reaction, SD: Standard deviation

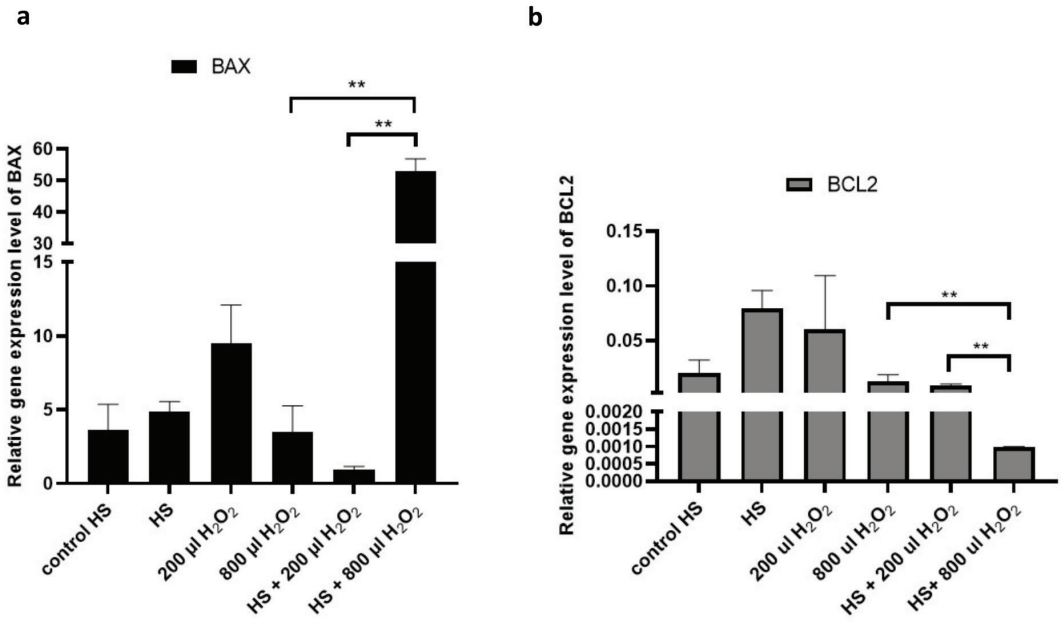

Figure 4a, b: The expression of BAX and BCL_2 genes in IHOECs in combinations of HS and different concentrations of H_2O_2, was measured by RT-PCR

n=2; mean of 2 independent experiments are shown in the figure. All data are presented as mean ± SEM (error bars). Statistical significance: *p≤0.001

IHOECs: Immortalized human ovarian epithelial cells, HS: *Hibiscus sabdariffa*, RT-PCR: Real-time polymerase chain reaction, SEM: Standard error of the mean

4. HS Treatment Increase Cellular Defence and NRF_2 Expressions in High Concentrations

The transcription factor NRF_2 controls the production of genes involved in drug detoxification and the oxidative stress response, which in turn protects cells against toxic and oxidative assaults. Cells become resistant to inflammatory stimuli and chemical carcinogens when NRF_2 is activated (15).

In this four repetitive *NRF_2* gene expression determination in different concentration of H_2O_2 (200 µL H_2O_2 and 800 µL H_2O_2) experiment, there is any effect of HS in terms of cell proliferation in the control group or cellular defence against 200 µL H_2O_2 applied group (Figure 5).

Also, there is a higher expression rate in HS + 800 µL H_2O_2 than HS + 200 µL H_2O_2. These results indicate that HS may be more effective in terms of anti-oxidative and cellular defence effects in high concentration compared to low concentrations. P<0.001 indicates there was strong significance difference (Figure 5).

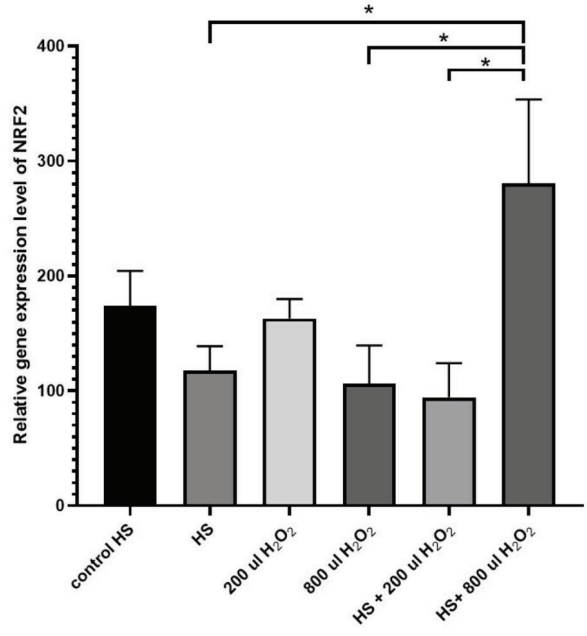

Figure 5: The expression of NRF_2 in IHOECs in combinations of HS and different concentrations of H_2O_2, was measured by RT-PCR

n=4; mean of 4 independent experiments are shown in figure. All data are presented as mean ± SD. Statistical significance: *p≤0.001 (NRF_2)

IHOECs: Immortalized human ovarian epithelial cells, HS: *Hibiscus sabdariffa*, RT-PCR: Real-time polymerase chain reaction, SD: Standard deviation, NRF_2: Nuclear factor erythroid 2-related factor 2

Discussion

HS is a rich source of bioactive compounds which makes it a significantly rewarding topic of research. In recent literature, it is found to have many effects including antiseptic, diuretic, antioxidant and antimutagenic properties (16).

The anti-aging and antitumoral effects of HS were investigated on different types of cells including fibroblasts of skin (17), breast cells (18) and to name few.

However, IHOECs cell lines were not commonly studied until our study, although we see many examples of similar cell lines such as immortalized human ovarian surface epithelial cells.

The expected anti-oxidative effect of HS was not observed when the cells were under oxidative influence. Despite the anticipation that HS would maintain a high rate of cell proliferation due to its anti-oxidative effect, an increase in cell viability was not observed. This suggests that while HS may have some antioxidant properties, its effectiveness may be limited to concentrations of oxidative stress.

None of the previous studies in the literature examined stem cell properties in IHOECs. With this study, we examined the basal levels of *SOX*$_2$ and *OCT*$_4$ genes which have embryonic stem cell pluripotency in IHOECs for the first time and showed with our experiments that these cells contain stem cells.

It is known that ovarian epithelial cells are an important source of stem cells. Before being used therapeutically, it must have the proliferative potency and the capacity to maintain stem cells and progenitor cells without undergoing genetic modification during *ex vivo* culture (19).

In the context of IHOECs, in addition to showing high expression in the control group, it was observed that *SOX*$_2$ and *OCT*$_4$ gene expressions decreased dose-dependently only under the influence of H_2O_2. However, when the oxidative stress in the cells was increased following HS treatment application, an increase in the expression of stem cell biomarkers was observed. Cell death was observed in the group in which 200 µL of H_2O_2 was applied together with HS solution and it was stated that stem cell markers decreased. In the group where 800 µL $H_20_2$was applied together with HS solution, although cell death was observed, the expression of stem cell markers increased.

The increase in the expression of SOX_2 and OCT_4 in the presence of high oxidative stress following HS treatment, may provide evidence that HS solution has a protective effect on stem cells in IHOEC.

An increase in stem cell markers could suggest a pre-cancerous effect due to potential DNA damage under high oxidative stress. To verify this, DNA damage should be assessed in these cells under varying oxidative stress levels.

We studied the cell death mechanism in IHOEC due to oxidative stress, a topic not previously explored. We found a significant increase in BAX, a protein that promotes cell death, and a notable decrease in BCL_2, a protein that prevents it. This suggests a high BAX/BCL_2 ratio and indicates apoptosis in the IHOECs.

NRF_2, a cellular oxidative stress sensor, increases the cell's antioxidant capacity by upregulating genes to eliminate ROS (20,21). HS enhances antioxidant defense by reducing ROS and increasing SOD and CAT (22). Our results show NRF_2 expression decreased at low stress but increased at high stress, indicating HS's antioxidant effect at high oxidative stress. Contrary to studies showing NRF_2 upregulates BCL_2 and downregulates BAX (23), our study found an exponential increase in BAX/BCL_2. A study found boric acid's antioxidant and antineoplastic effects on damaged cells by increasing NRF_2 and BAX/BCL_2 (24). Similarly, HS may activate NRF_2 pathway and stimulate apoptosis in stressed IHOEC cells. Parallel to our study, HS may have an antioxidant effect on IHOEC cells by the activation of the NRF_2 antioxidant pathway and stimulation of apoptotic death of the stressed cells.

Nevertheless, in other studies, NRF_2 was found to play an unexpected role in cancer development (25,26). Related to that, an increase in the expression of NRF_2 may be related to the initiation of cancer in the epithelial ovarian cells.

It was found significantly in our study that H_2O_2 application reduced the number of cells. Results can conclude that in the presence of high oxidative stress, HS has the potential to preserve the viability of stem cells while driving normal cells to apoptosis. Since maintaining the viability of stem cells is critical for tissue regeneration and repair, we can say that HS has a positive effect on the general condition of IHOEC.

The effect of HS impact on gene expression and its potential geno-protective effects make it a promising area for further research. Future studies could explore these effects in more detail, potentially uncovering new therapeutic applications for HS. These findings contribute to our understanding of the complex interactions between plant extracts, oxidative-stress, cell viability, stem cell protection and open new avenues for research in this area.

From an alternative perspective, adult-onset ovarian cancer and neo-oogenesis may be caused by stem cells found in IHOECs. This situation may indicate that it controls neo-oogenesis in addition to the pathophysiology of epithelial ovarian cancer. Thus, ovarian cancer and epithelial stem cells are closely related. As a result, a model can be developed to investigate the initial stages of ovarian cancer and produce novel insights.

Conclusion

In conclusion, this study suggests that adding HS to IHOEC during culture has the potential to cause clearance of damaged cells and induce stemness of IHOECs for the first time. It was reported that HS stimulated apoptosis in highly oxidative damaged cells and cause an increase in stem cell markers.

Ethics

Ethics Committee Approval: The cells used in the study are commercially available so ethics committee approval is not

required. The study protocol was appropriate in accordance with the Declaration of Helsinki.

Informed Consent: The cells used in the study are commercially available so informed consent was not obtained.

Footnotes

Authorship Contributions

Laboratory Practices: E.D.K., Y.N.P., Concept: A.S., E.D.K., Y.N.P., Design: A.S., E.D.K., Y.N.P., A.G.B., N.Ö., N.F., Data Collection and/or Processing: Y.N.P., T.Y., M.Ç., D.G., T.Ö., Analysis and/or Interpretation: Y.N.P., T.Y., M.Ç., T.Ö., Literature Search: Y.N.P., T.Y., M.Ç., S.A., H.A., A.I., Writing: E.D.K., Y.N.P., T.Y., M.Ç., S.A.

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

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