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RESEARCH

The effects of hydrogen peroxide and mitochondria on human breast cancer cells

Hidrojen peroksit ve mitokondrinin insan meme kanseri hücrelerine etkisi

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Abstract

Purpose: It was aimed to evaluate the effect of hydrogen peroxide (H_2O_2), mitochondria isolated from human breast cancer cells (MCF-7) and human mesenchymal stem cells (IMSCs) on MCF-7 cell proliferation in this study.
Materials and Methods: Mitochondria of IMSCs and MCF-7 cells were isolated according to the protocols specified in the kit and the protein amount of mitochondria was measured by the bicinchoninic acid method (BCA). H_2O_2 doses (0-1000 µM), mitochondria obtained from MCF-7 and IMSCs were transferred to MCF-7 cells and the viability was evaluated by tetrazolium salt (MTT) method. The cell percentages were calculated by measuring absorbance of the samples at 570 nm with a plate reader.
Results: It was found that, ATP level of the mitochondria isolated from IMSCs was higher than MCF-7s. MCF-7 cell viability decreased significantly in 30 µM (%19), 40 µM (%26), 50 µM (%39), 75 µM (%39), 100 µM (%36), 200 µM (%28), 400 µM (%23), 800 µM (%22) and 1000 µM (%23) H_2O_2 doses. The transfer of IMSCs mitochondria caused a tendency to decrease in cell proliferation, but no significance was detected. But, it was found that healthy mitochondria transfer with 10 µM and 1000 µM H_2O_2 reduced the proliferation of MCF-7s (respectively %14 and % 8).
Conclusion: It was determined that the transfer of healthy mitochondria isolated from IMSCs and H_2O_2 to MCF-7 is associated with proliferative processes, however transplantation of the healthy mitochondria into cancer cells is thought to be a promising new method in the treatment of the disease.

Keywords: Stem cell, culture, proliferation

Öz

Amaç: Bu çalışmada, hidrojen peroksit (H_2O_2), insan mezenkimal kök hücreleri (İMKH) ve insan meme kanseri hücrelerinden (MCF-7) izole edilen mitokondrilerin, MCF-7 hücre proliferasyonuna etkisinin değerlendirilmesi amaçlanmıştır.
Gereç ve Yöntem: İMKH ve MCF-7 hücrelerinin mitokondrileri kitte belirtilen protokollere göre izole edildi ve protein miktarı bikinkoninik asit yöntemiyle (BCA) ölçüldü. H_2O_2 dozları (0-1000 µM), MCF-7 ve İMKH'lerden elde edilen mitokondrilerin, MCF-7 hücrelerine transferi sonrasında hücre canlılığı tetrazolyum tuzu (MTT) yöntemiyle değerlendirildi. Örneklerin absorbans değerleri (570 nm) plate okuyucuyla ölçülerek hücre yüzdeleri hesaplandı.
Bulgular: İMKH'lerden izole edilen mitokondrilerin ATP düzeyinin, MCF-7'lerden daha yüksek olduğu bulundu. MCF-7 hücrelerinin canlılığı H_2O_2'nin 30 µM (%19), 40 µM (%26), 50 µM (%39), 75 µM (%39), 100 µM (%36), 200 µM (%28), 400 µM (%23), 800 µM (%22) ve 1000 µM dozunda %23 oranında anlamlı olarak azaldı. İMKH'lerin mitokondrilerinin tek başına transferinin hücre proliferasyonunda azalma eğilimine neden olduğu belirlenirken, herhangi bir anlamlılık tespit edilmedi. Ancak, 10 µM ve 1000 µM H_2O_2'yle birlikte yapılan sağlıklı mitokondri transferinin MCF-7'lerin çoğalmasını azalttığı belirlendi (sırasıyla %14 ve %8).
Sonuç: H_2O_2 ve İMKH'lerden izole edilen sağlıklı mitokondrilerin MCF-7 hücrelerine transferinin proliferatif süreçlerle ilişkili olduğu görülürken, kanser hücrelerine sağlıklı mitokondri naklinin, hastalığın tedavisinde umut vadeden yeni bir yöntem olabileceği düşünülmektedir.

Anahtar kelimeler: Kök hücre, kültür, proliferasyon

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INTRODUCTION

Breast cancer is the type of cancer that causes the most of deaths among women, and it is reported that millions of women are newly diagnosed every year around the world[1]. It is a disease characterized by the uncontrolled proliferation of breast cells due to epigenetic and genetic factors. In the treatment of breast cancer; conventional methods such as surgery, radiotherapy and chemotherapy are used[2]. The human breast cancer cell line (MCF-7) is frequently used in studies on breast cancer[3,4]. The MCF-7 cell line is also important in that it has estrogen, progesterone and glucocorticoid receptors. These cells were first isolated from pleural effusion in metastasized breast adenocarcinoma by Doctor Soule at Michigan Cancer Institute in 1970[5]. With the rapid increase in clinical studies on these cells, it makes significant contributions to cancer researches and the lives of cancer patients.

Reactive oxygen products (ROS) are among the causes of cancer cell proliferation and angiogenesis. In addition, increased ROS is associated with cell growth. It has been reported that there is an increase in the amount of ROS in some cancer types[6]. The reason for this situation is shown as oncogenic stimuli increasing the ROS level and triggering metabolic activity, DNA and mitochondrial damage. However, ROS can cause opposite and different cellular effects in terms of causing both cancer formation and inhibition. Superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radical are ROS produced by metabolism endogenously in our body, and it is stated that their production increases in cancer[6,7]. Low amounts of ROS cause DNA damage, cell proliferation, increased Warburg effect, while excessive amounts of ROS activate mechanisms such as apoptosis, autophagy and necroptosis, creating anti-cancer effects (inhibition of cell proliferation)[8]. H_2O_2 is a physiological ROS and is used medically in the treatment of cancer[9]. However, it has also been reported that ROS are potential carcinogens, causing cancer formation and progression[10].

In addition to the mitochondria being the energy production center of the cells; it has many important functions in intracellular physiology (growth, proliferation, necrosis, apoptosis, autophagy, etc.)[11,12]. It has been reported that there is dysfunction in the mitochondria of cancerous cells and that damaged mitochondria are responsible for cancer progression, metastasis and drug resistance[13]. It is also known that cancerous cells can control their own mitochondria. Moreover, they have the ability to terminate the function of their own mitochondria at any time and it is seen that mtDNA (mitochondrial DNA) copy number decreases in cancer types such as stomach and liver cancer[14]. It is thought that the transfer of mitochondria (mesenchymal stem cells) obtained from healthy cells will prevent the progression of cancer by changing the remodeling and microenvironment in cancer cells[15]. On the other hand, the effect of mitochondria transfer (enhancer or suppressor) on metastasis of tumor cells is not very clear[16].

In this study, it was aimed to evaluate the effect of transfer of the mitochondria isolated from MCF-7 and IMSCs, and H_2O_2 administration on proliferation of MCF-7 cells. In addition, transplantation of unhealthy mitochondria of MCF-7 cancer cells and healthy mitochondria isolated from IMSCs is thought to be an important study that will contribute to the literature in which the effects of breast cancer cell cytotoxicity are evaluated.

MATERIALS AND METHODS

Cell culture and applications

In this study, IMSCs and MCF-7 cell lines were used and were obtained from University of Health Sciences Gülhane Stem Cell Research Center. Ethics committee approval is not required as the cells used are commercially available cell lines. MCF-7 cells were grown in 90% RPMI-1640 (with 1% L-glutamine, 25M HEPES, Corning, VA, USA), 10% FBS (Fetal bovine serum, Capricorn Scientific) and 1% PSA (Penicillin streptomycin antibiotic, P4333-100mL), Sigma-Aldrich) and IMSCs were grown in Dulbecco's Modified Eagle's Medium (DMEM, D6046, Sigma-Aldrich), 10% FBS and 1% PSA. Cell media were changed every 72 hours.

All applications of cell culture were performed under sterile conditions in a laminar airflow cabinet (NUVE MN120). Cells were passaged when they covered 80% of the flask and cell morphologies were examined under an invert microscope (Nikon Eclipse TS100). The phenotypic morphologies of the cell cultures are shown in Figure 1. Total cell number, healthy and dead cell ratio were determined by Trypan Blue method. The cells stained in blue were considered dead, and cells that were transparent and unstained were considered healthy. Culture viability

was calculated as ~95-97% (Live cell count/Total cell count)[17].

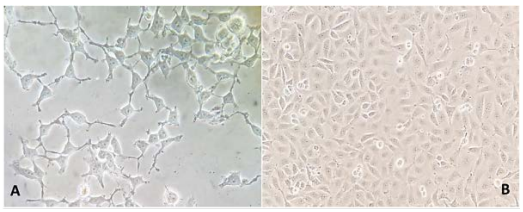

Figure 1. A) Invert microscope image of MCF-7 cell line at x400 magnification, B) Invert microscope image of IMSCs at x200 magnification.

In this study, H_2O_2 doses (H1009-100 mL, Sigma Aldrich, USA) and mitochondria isolated from MCF-7 and IMSCs were applied to MCF-7 cells according to the specified experimental groups, and cell viability was evaluated by MTT method. In the 24-hour study of doses of H_2O_2 between 0-1000 μM; Control (C), H_2O_2 10 μM, H_2O_2 20 μM, H_2O_2 30 μM, H_2O_2 40 μM, H_2O_2 50 μM, H_2O_2 75 μM, H_2O_2 100 μM, H_2O_2 200 μM, H_2O_2 400 μM, H_2O_2 800 μM, and H_2O_2 1000 μM. There are 12 groups in total. In the 24-hour study of the combined application of 10, 100 and 1000 μM doses of mitochondria (IMSCs-Mito and MCF-7-Mito) and H_2O_2 isolated from IMSCs and MCF-7 cells; Control (C), H_2O_2 10 μM, H_2O_2 100 μM, H_2O_2 1000 μM, IMSCs-Mito, MCF-7-Mito, H_2O_2 10 μM+IMSCs-Mito, H_2O_2 100 μM+IMSCs-Mito, H_2O_2 1000 μM+IMSCs-Mito, H_2O_2 10 μM+MCF-7-Mito, H_2O_2 100 μM+MCF-7-Mito and H_2O_2 1000 μM+MCF-7-Mito.

Cell viability and MTT method

In this method, dehydrogenase enzymes in living cells convert yellow colored MTT into purple colored formazan crystals. According to the protocols specified in the Cell-Quant MTT Cell Proliferation Assay kit (A015, ABP, Biosciences), 100 μL of cell suspension was seeded with a multichannel pipette, with approximately $10x10^3$ MCF-7 cells per well. After MCF-7 cells were incubated for 24 hours to adhere to the flask base and proliferate, the cell medium on them was removed and H_2O_2 doses were applied to the wells. At the end of the 24-hour incubation, the mitochondria isolated from MCF-7 and IMSCs were administered at a dose of 5 μg/mL. After 24 hours, the chemicals in the wells were removed and 100 μL of fresh cell medium was added. 10 μL of reagent A was added to each well and incubated for 4 hours. Then, 100 μL of reagent B was added and waited for 2-4 hours again and it was measured with a plate reader at a 570 nm absorbance value (Molecular Devices Filter Max F5).

Mitochondria isolation

Mitochondria isolation was performed according to the procedure of mitochondria isolation kit (Biovision, K288-50). IMSCs and MCF-7s were produced up to ~20 million cells. Isolated mitochondria were incubated for ~1 hour in ice at +4 °C until transplantation.

Total protein amount measurement

Total protein amount of the mitochondria obtained from MCF-7 and IMSCs was measured with BCA kit (Thermo Fisher Scientific Inc., 23227, MA, USA). Standards and samples were placed in the wells according to the experimental procedure specified in the kit. By measuring the absorbance values with a plate reader at 562 nm wavelength, a standard curve was drawn and the protein amount of mitochondria was calculated.

Statistical analysis

IBM SPSS 21.0 package program (Chicago, IL, USA) was used in the statistical analysis of the data obtained and expressed as mean ± standard error (mean ± S.E). While the normal distribution was determined by the Shapiro-Wilk test, the homogeneity of the group variances was demonstrated by the Levene test. Differences between the groups were determined by one-way analysis of variance (ANOVA) which was a parametric test. While the comparison of H_2O_2 doses (10-1000 μM) with the control group was made with the post hoc Dunnett test and Tukey test was used in the pairwise comparison of the groups in combined applications. The level of statistical significance was determined as *** $p<0.001$, ¶¶¶ $p<0.001$, $^{\delta\delta}$ $p<0.01$, $^{\#\#}$ $p<0.01$, †† $p<0.01$.

RESULTS

Effect of H_2O_2 doses on MCF-7 cell viability

MCF-7 cell viability was evaluated 24 hours after administration of H_2O_2 doses (0-1000 μM). The images under an inverted microscope are shown in Figure 2.

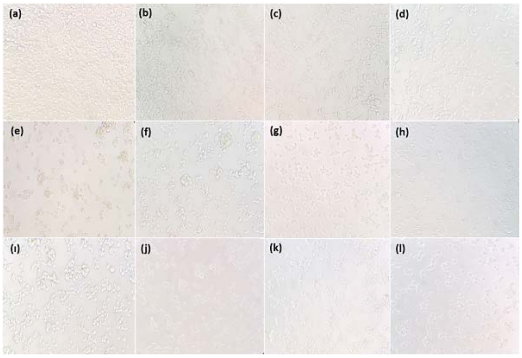

Figure 2. Invert microscope images of the 24-hour effect of H_2O_2 doses (0-1000 µM) on MCF-7 cells at x200 magnification, (a) Control, (b) 10 µM, (c) 20 µM, (d) 30 µM, (e) 40 µM, (f) 50 µM, (g) 75 µM, (h) 100 µM, (i) 200 µM, (j) 400 µM, (k) 800 µM, (l) 1000 µM.

When H_2O_2 dose applications were compared with the control group, it was determined 30 µM (19%), 40 µM (26%), 50 µM (39%), 75 µM (39%), 100 µM (36%), 200 µM (28%), 400 µM (23%), 800 µM (22%) and 1000 µM (23%) significantly reduced the number of MCF-7 cells (p=0.000). Although a decrease in cell viability was observed at the doses of H_2O_2 10 µM (8%, p=0.262) and H_2O_2 20 µM (7%, p=0.298), but significant difference wasn't detected (Figure 3).

Effect of mitochondria isolated from IMSCs and MCF-7 cells and H_2O_2 doses on MCF-7 cell viability

Combined application doses of H_2O_2 10, 100 and 1000 µM, IMSCs-Mito and MCF-7-Mito were performed for 24-hour. When the H_2O_2 10 µM, H_2O_2 100 µM and H_2O_2 1000 µM groups were compared with the control group, cell viability was found to be significantly reduced (p=0.000). When the H_2O_2 1000 µM+IMSCs-Mito group was compared with the H_2O_2 1000 µM group, it was observed that the cell number decreased significantly by 8% (p=0.002); but H_2O_2 10+IMSCs-Mito and 100 µM+IMSCs-Mito groups are compared with the H_2O_2 10 and 100 µM groups; no difference was determined (p=0.070 and p=0.321, respectively). In addition, there was a significant 8% increase in cell number in H_2O_2 10 µM+MCF-7-Mito group compared to the H_2O_2 10 µM group (p=0.002), However, H_2O_2 100 µM+MCF-7-Mito and H_2O_2 1000 µM+MCF-7-Mito groups were compared with H_2O_2 100 µM and H_2O_2 1000 µM groups, no difference was found (p=0.392 and p=1.000, respectively). Further, H_2O_2 10 µM+IMSCs-Mito group was compared to the H_2O_2 10 µM+MCF-7-Mito group, a 14% reduction was observed, and H_2O_2 1000 µM+IMSCs-Mito group is compared with the H_2O_2 1000 µM+MCF-7-Mito group, a significant decrease in cell viability was detected by 8% (p=0.000 and p=0.002, respectively), (Figure 4).

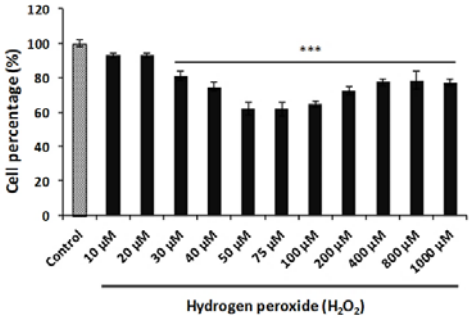

Figure 3. MCF-7 cell viability decreased from 30 µM to 1000 µM in H_2O_2 doses.

Data are expressed as mean ± S.E. *** p<0.001 Compared to control group. One-Way ANOVA, post hoc Dunnett test (n=5-8).

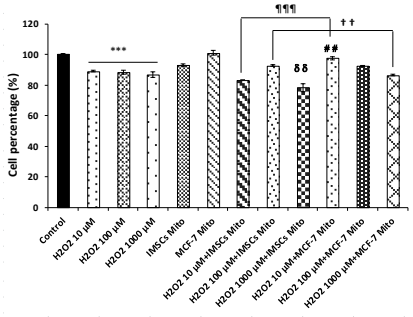

Figure 4. Percentage of MCF-7 cell viability of combined administrations of mitochondria isolated from MCF-7 and IMSCs with H_2O_2 doses.

Data are expressed as mean ± S.E. *** p<0.001 relative to control group, δδ p<0.01 relative to 1000 µM group, ## p<0.01 relative to 10 µM group, ¶¶¶ p<0.001 According to H_2O_2 10 µM+MCF-7-Mito group, †† p<0.01 H_2O_2 1000 µM+MCF-7-Mito group. One-Way ANOVA post hoc Tukey test (n=3).

ATP and protein quantities of IMSCs and MCF-7 cells

ATP and protein amount of the mitochondria isolated from IMSCs and MCF-7 cells were calculated

using standard curve functions (Figure 5). In our study, the protein amount of mitochondria isolated from IMSCs was 0.552 mg/mL, ATP amount was 6.573 nmol/mL, while it was calculated as 11.899 nmol ATP/mg protein. While the protein amount of mitochondria isolated from MCF-7 is 2.475 mg/mL, the amount of ATP is 18.188 nmol/mL; It was calculated as 7.348 nmol ATP/mg protein. As a result, it was determined that the ATP level of mitochondria isolated from IMSCs was higher than that of MCF-7.

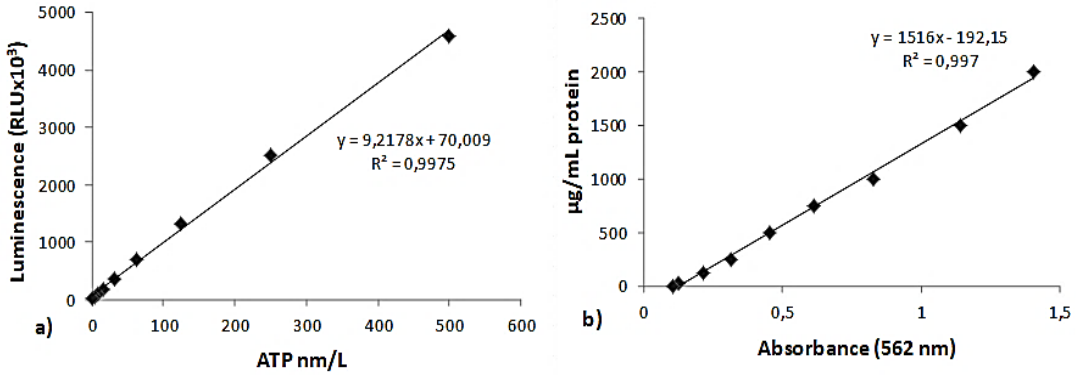

Figure 5. Standard curve of a) ATP and b) protein amount of isolated mitochondria

DISCUSSION

The double-sided effect of ROS on cancer cells is known, it is reported that high levels increase oxidative stress and thus prevent the proliferation of cancerous cells. However, cancer cells try to balance their increased ROS production by increasing their antioxidant capacity[8]. Consistent with the studies in the literature, it was determined that 24-hour H_2O_2 application decreased the number of MCF-7 cells from 30 µM dose to 1000 µM dose in this study. However, although there was a tendency to decrease in cell viability at 10 and 20 µM doses, no significant change was detected. While Abbasi et al. found that cell viability was significantly reduced in 4T1 mouse breast cancer cells at exposure to 100, 200, 500 and 1000 µM H_2O_2 doses for 24 hours; although there was a tendency to decrease in proliferation at 10, 20 and 50 µM doses, no significant difference was detected[8]. In different studies, it is stated that 50-200 µM H_2O_2 application inhibits the proliferation of MCF-7 cells[18], while 1-10 µM doses are reported to increase the proliferation of 7721 hepatoma cells[19]. However, it has been reported that the application of 1-10 µM H_2O_2 increases the proliferation of HT 29 colon cancer cells, while the administration of a very high dose (1000 µM) causes apoptosis[10]. These results in the studies suggest that different H_2O_2 doses may have a bipolar effect on different cancer cell lines. In addition, it reveals cytotoxic and genotoxic effects through stimulate different signaling mechanisms in cancer cells[20,21]. Mesenchymal stem cells are frequently preferred as a source for mitochondria transfer. Considering the extraordinary repair abilities of mitochondria of mesenchymal stem cells, promising results are obtained in mitochondria transplantation researches[22]. In many studies, it has been reported that MSCs-derived mitochondria prevent damage in recipient cells, thus increasing ATP production and re-arranging mitochondrial functions[23]. Contrary to this situation, the mitochondria of cancer cells, it has been reported to have certain problems due to decreased apoptosis, aerobic glycolysis and increased ROS[15,24]. Aharoni-Simon et al. found that mitochondria isolated from BALB/c mouse liver produced ~260 nm/mg protein ATP in the presence of ADP substrate, in the deficiency of ADP, the basal level was determined as ~1-10 nm/mg protein ATP[25].

In this study, while IMSCs were used as a source of healthy mitochondria, MCF-7 cells were used as diseased source and ATP values of isolated mitochondria were measured. It was determined that

the ATP level of mitochondria obtained from IMSCs (11.899 nmol ATP/mg protein) was higher than MCF-7 (7.348 nmol ATP/mg protein).

In the study of Coppi et al., they measured the ATP level in the medium as 7.6 ± 0.57 nM 24 hours after the medium change of IMSCs[26]. In another study, they determined the amount of ATP produced per minute in mitochondria obtained from 12-week-old rat skeletal muscle tissue as 7.29 ± 1.01 nmol mg protein, while they determined it as 3.17 ± 0.99 nmol ATP mg protein in 26-week-old rat mitochondria[27]. When the outcomes of mitochondria ATP level in this current study and the results found in other in vivo studies are evaluated together, it is seen to be compatible with the literature, and it shows the result that sufficient and functional mitochondria are obtained.

Additionally, when the single and co-administration of 10, 100, 1000 μM doses of H_2O_2, and transplantation of IMSCs-Mito 5 μg/mL and MCF-7-Mito 5 μg/mL were examined in current study. After 24-hour application of 1000 μM H_2O_2, the growth-reducing effect of transplantation of healthy mitochondria from IMSCs into MCF-7 cells was observed, whereas in the application with 10 μM H_2O_2 unhealthy mitochondria from MCF-7s into MCF-7 cells showed a proliferation-enhancing effect. However, these significant effects on cell proliferation could not be determined at 100 μM H_2O_2 dose.

In addition to these results, when the transplantation of mitochondria isolated from IMSCs and MCF-7s were compared with each other after 24-hour application of H_2O_2 10 μM and 1000 μM doses, it was observed that healthy mitochondria decreased the viability of MCF-7 cells. However, no change in MCF-7 cell viability was detected in transplantation of healthy mitochondria at a dose of 100 μM H_2O_2.

In the study of Roushandeh et al., they transplanted mitochondria obtained from WI-38 (healthy human fibroblast cells) into HeLa (human cervical cancer) and SAS (human oral squamous cancer). After 6 hours of cisplatin application, transplantation of 5 μg/mL mitochondria obtained from WI-38 cells, it was determined that the number of apoptotic cells increased significantly in both HeLa and SAS cells. In the same study, it was reported that 5 μg/mL mitochondria transfer increased the proliferation of cancerous cells, unlike the application with cisplatin, it was reported that cell proliferation was not affected by this dose but there is an increase in 10 μg/mL mitochondria transfer[16].

However, in a different study by the same researchers, they found that transplantation of the healthy mitochondria from IMSCs to MDA-231 human breast cancer, cell proliferation and invasion increased[14].

Similar to the study of Roushandeh et al., 5 μg/mL mitochondria was transferred in current study. It was also determined that the transfer of mitochondria from IMSCs caused a tendency to decrease in cell proliferation, but no significant difference was detected and no change was observed in cell proliferation in the application of mitochondria obtained from MCF-7s alone. Moreover, it was seen that the transfer of IMSCs to mitochondria together with H_2O_2 decreased the proliferation of MCF-7s. While this result was found to be compatible with the literature, and it suggests that the transfer of healthy mitochondria may cause some intracellular molecular changes related to proliferation in cancer cells.

The limitation of this study is the inability to investigate in detail the mitochondria-associated proliferative intracellular signaling pathways in MCF-7 cells. With the determination of which intracellular mechanisms are associated with both healthy mitochondria and unhealthy mitochondria, it seems to be a very important step in terms of uncovering the pathology of breast cancer as well as new treatment strategies.

As a result, it was determined that the application of H_2O_2 and the transfer of healthy mitochondria are associated with proliferative processes in this study. Thus, the transplantation of both healthy and unhealthy mitochondria seems to be a very important step in terms of identifying the intracellular mechanisms associated with the pathology of breast cancer, as well as revealing new treatment strategies.

It also showed that even more meaningful responses can be obtained when mitochondria transfer is applied together with anti-neoplastic drugs and molecules used in cancer treatment that inhibit the proliferation of cancerous cells. More importantly, it gives hope that exogenous healthy mitochondria may be a new treatment strategy.

Yazar Katkıları: Çalışma konsepti/Tasarımı: ZÇ; Veri toplama: ZÇ; Veri analizi ve yorumlama: ZÇ; Yazı taslağı: ZÇ; İçeriğin eleştirel incelenmesi: ZÇ; Son onay ve sorumluluk: ZÇ; Teknik ve malzeme desteği: ZÇ; Süpervizyon: ZÇ; Fon sağlama (mevcut ise): yok.
Etik Onay: Bu araştırma hücre kültürü çalışması olması sebebiyle, etik kurul onayı alınması gerekmemektedir. Çalışmada kullanılan hücre

kültürü hatları Sağlık Bilimleri Üniversitesi Gülhane Kök Hücre Araştırma Merkezinden temin edilmiştir.
Hakem Değerlendirmesi: Dış bağımsız.
Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.
Finansal Destek: Yazarlar finansal destek beyan etmemişlerdir.
Yazarın Notu: Bu çalışmada kullanılan kimyasallar ve sarf malzemeleri daha önce yapılan proje ve çalışmalardan temin edilmiştir.
Author Contributions: Concept/Design : ZÇ; Data acquisition: ZÇ; Data analysis and interpretation: ZÇ; Drafting manuscript: ZÇ; Critical revision of manuscript: ZÇ; Final approval and accountability: ZÇ; Technical or material support: ZÇ; Supervision: ZÇ; Securing funding (if available): n/a.
Ethical Approval: Since this research is a cell culture study, ethics committee approval is not required. The cell culture lines used in the study were obtained from the Gülhane Stem Cell Research Center of Jul University of Health Sciences.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: Authors declared no financial support
Acknowledgement: The chemicals and consumables used in this study were obtained from previous projects and studies.

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