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Analyzing Methotrexate's Impact on Oxidative Stress Indicators in Breast Cancer Cells

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What is already known on this topic?

- *Methotrexate (MTX) is a chemotherapeutic agent that acts by blocking the enzyme dihydrofolate reductase, which is essential for DNA synthesis.*
- *Methotrexate has been shown to increase oxidative stress in cancer cells.*
- *The available literature on the subject of the effects of MTX on healthy breast cells is limited.*

What does this study add on this topic?

- *Methotrexate reduces cell viability in healthy cells as well as cancer cells.*
- *Methotrexate increases oxidative stress markers and decreases antioxidant enzyme levels in both cancer and healthy cell lines.*
- *These findings suggest that future therapeutic strategies should also take into account the oxidative damage that MTX may cause in non-cancerous tissues.*

Abstract

Objective: Methotrexate (MTX) is one of the most commonly used antiproliferative chemotherapeutic agents. In addition to its antiproliferative effect, MTX may also play a role in the induction of oxidative stress. This study investigates the effects of MTX on cell cytotoxicity and oxidative stress markers (superoxide dismutase [SOD] activity, malondialdehyde [MDA] levels), and total thiol content) in both human healthy breast cells (hTERT-HME1) and human breast cancer cells (MCF-7).

Methods: The hTERT-HME1 and MCF-7 cell lines were divided into untreated control and MTX groups. The cell viability assay was carried out using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Superoxide dismutase, MDA, and total thiol levels were measured spectrophotometrically.

Results: The effects of MTX on cell viability were observed after treatment of hTERT-HME1 and MCF-7 for 24, 48, and 72 hours. In comparison to MCF-7 cells, hTERT-HME1 cells exhibited increased cell survival and half-maximum inhibitory concentration 50 values after MTX treatment. Methotrexate administration also significantly decreased SOD and total thiol levels and increased MDA levels in both cell types.

Conclusion: This study suggests that MTX has high anticancer activity through its effects on cell viability and oxidative stress. However, observation of similar effects in healthy cells requires the development of various combination therapies or targeted drug delivery systems to reduce the side effects of MTX.

Keywords: MCF-7 cells, malondialdehyde, oxidative stress, superoxide dismutase, thiol

Introduction

Breast cancer is the most common cancer in females and remains one of the major causes of cancer mortality worldwide, underlining the ongoing demand for selective and effective treatment strategies.[1] Breast cancer, with its high incidence and mortality rates, remains a global health issue and therefore warrants research into more selective and less harmful methods of treatment.

Among various chemotherapeutic agents, methotrexate (MTX), a folate antagonist, has long been incorporated into standard treatment protocols for breast cancer.[2] Methotrexate, a competitive inhibitor of the enzyme dihydrofolate reductase (DHFR), functions primarily by inducing a decline in the synthesis of tetrahydrofolate cofactor, consequent to the inhibition of this enzyme.[3,4] This cofactor is important for purine and thymidylate synthesis, specifically for DNA synthesis and repair.[5] The absence of this cofactor results in impaired DNA synthesis, which consequently leads to cell cycle arrest, apoptosis, and reduced tumor growth.[6] Although the antiproliferative effects of MTX via folate antagonism in this way are well known, it is argued that it may also exert cytotoxic effects via the induction of oxidative stress.[7,8]

Oxidative stress can be defined as a state of disequilibrium between reactive oxygen species (ROS) and antioxidant defense systems within the cell.[9] The cancer cells contain high levels of ROS due to the increased metabolic rate, mitochondrial dysfunction, and oncogene activation. High levels of

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ROS may add to cell proliferation and survival, while toxic levels of ROS production can push the cells beyond their oxidative threshold to apoptotic cell death. This dual role of ROS makes oxidative stress a good candidate target for various therapeutic options in cancer treatment.[10] Several biomarkers, reflecting the antioxidant and oxidant capacity in the cells, can be used for assessing the influence of MTX on the oxidative stress. Superoxide dismutase (SOD), a significant indicator of antioxidant capacity, is a critical antioxidant enzyme that catalyzes the process of converting superoxide radicals into hydrogen peroxide, thereby constituting the primary line of defense within cells against ROS.[11] Cysteinylglycine, cysteine, homocysteine, glutathione, γ-glutamylcysteine and proteins such as albumin show antioxidant properties by reducing unstable free radicals as a result of the thiol (–SH) groups in their structure acting as electron donors. The total thiol level refers to the total amount of thiol groups in these molecules and is used as an indicator of cellular redox balance.[12] Malondialdehyde (MDA) is a widely utilized marker of lipid peroxidation, defined as the specific reaction of ROS with lipids and oxidative stress.[13]

Methotrexate has been shown to improve quality of life and induce durable remission in breast cancer patients with leptomeningeal metastasis when administered intravenously at high doses.[2] Despite its antitumor potential and positive effects on quality of life, anemia, hypokalemia, mucositis, transient elevations in liver enzymes, and thrombocytopenia as a result of MTX toxicity have also been reported in breast cancer patients.[14] In addition, some studies have reported that MTX is an effective and inexpensive drug that can disrupt malignant growth without irreversible damage to normal tissues.[15] Several previous studies have increasingly focused on the oxidative stress mechanisms underlying MTX-induced cytotoxicity in breast cancer cells.[16,17] Although these redox imbalances contribute to the drug's cytotoxic effects on malignant cells, the extent to which non-tumorigenic breast epithelial cells respond to MTX-induced oxidative stress remains poorly understood.

One of the ways in which the anticancer effect can be assessed is through inducing oxidative stress. Therefore, in this study, the aim was to comparatively evaluate SOD activity, MDA levels, total thiol content and cellular viability in breast cancer cells (MCF-7) and healthy breast epithelial cells (hTERT-HME1) after MTX treatment.

Methods

Since this study is conducted on cell cultures and does not involve materials obtained from humans, ethics committee approval was not required, and no application was submitted. Additionally, informed consent was not necessary for this study.

Cell Culture

The commercially available cell lines hTERT-HME1 (ME16C, The American Type Culture Collection; ATCC, Manassas, VA, USA) and MCF-7 (HTB-22, ATCC) cell lines were cultured at 37°C with 10% fetal bovine serum (FBS, Euroclone S.p.A, Italy), high glucose Dulbecco modified Eagle's medium (Gibco, USA) with 5% CO and 95% humidity, and 1% penicillin-streptomycin (10 000 U/mL, Sigma-Aldrich, USA). hTERT-HME1 and MCF-7 cells, which reached 80%-90% density, were washed with Dulbecco's phosphate buffer saline (Euroclone S.p.A, Italy) and removed with 0.25% Trypsin-EDTA (T4049, Sigma-Aldrich, USA). The trypsin-EDTA-cell mixture was centrifuged at 1200 rpm for 3 minutes. After centrifugation, the supernatant was removed, and fresh medium was added. Cells were seeded in 96-well plates (1×10^4 cells/well) and incubated.

MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test is used to measure the metabolic activity of cells as an indicator of cell viability, proliferation, and cytotoxicity. To evaluate the effect of MTX (Abdi İbrahim; Submex, Istanbul, Türkiye) on cell viability in hTERT-HME1 and MCF-7 cell lines, an MTT (Sigma-Aldrich, USA) test was performed by applying MTX at different concentrations and times. The cell group to which the MTX was not applied was accepted as the control group. The cells were initially seeded at a density of 1×10^4 cells/well on 96-well plates. After 24h incubation, cells were treated with 6 different concentrations (2,5-5-10-25-50 and 100 μM) MTX for 24, 48, and 72 hours in 5% CO_2 atmosphere at 37°C. At the end of the experiment period, the medium was removed from all wells, and 100 μL of medium and 20 μL (5 mg/mL) MTT solution were added to the wells. After 3 hours of incubation, 100 μL DMSO (Sigma-Aldrich, USA) was added to each well, and approximately 5 minutes later, absorbance values were measured at 570 nm in a microplate reader (Multiskan GO-Thermo, Waltham, MA, USA). The MTT assays were performed 3 times with 6 replicates for each group. The percentage of cell survival relative to the control (untreated sample) was calculated and used to determine relative viability from the MTT plots. For both cell lines, the dose (IC_{50}) values that reduced cell viability by 50% were calculated for 24, 48, and 72 hours using the GraphPad Prism 9 (California, USA) program.[18,19]

Experimental Groups

Experimental groups were created according to the IC_{50} value calculated for 48 hours. The experimental groups were composed as follows:

1. Control group: hTERT-HME1 cells that have not been subjected to treatment with MTX.
2. Methotrexate + Control group: MTX-treated hTERT-HME1 cells.
3. MCF-7 group: MCF-7 cells that have not been subjected to treatment with MTX.
4. Methotrexate + MCF-7 group: MTX-treated MCF-7 cells.

MTX Treatment and Cell Lysate Preparation

The cell lines (hTERT-HME1 and MCF-7) were seeded in 6-well plates (3×10^5 cells/well) in a medium composed of 90% DMEM, 9% FBS, and 1% penicillin-streptomycin antibiotic solution. The wells were designated for the untreated control and MTX groups. Following a 24-hour incubation period, the control group was replaced with normal medium, while the MTX groups were exposed to medium containing MTX. All groups were subsequently incubated for an additional 48 hours. At the end of the experimental timeline, cell lysates were prepared using 1 × RIPA lysis buffer and a protease inhibitor cocktail set (Merck KGaA, Darmstadt, Germany) as stated in the previous studies.[18,19] Cell lysates were kept in a deep freezer at –80°C until measurement.

Oxidative Stress Biomarker Analyses

To evaluate oxidative stress in the cultured hTERT-HME1 and MCF-7 cell lines, the following biochemical assays were performed: SOD activity, total thiol content, and MDA levels. Each assay was adapted for use with cell lysates obtained from cultured cells. The assay protocols were adapted for cell culture by adjusting proportionally sample and reagent volumes to match the lower sample quantities and to suit the 96-well plate format and optimized for use in cell culture conditions. All analyses were performed at least in triplicate.

Superoxide Dismutase Activity Assay

Superoxide dismutase activity was measured using a modified version of the method described by Sun et al[20] The assay was adapted for use with cell lysates. Briefly, cells were harvested and lysed using a cold lysis buffer. After centrifugation, the supernatant was collected and incubated with xanthine and xanthine oxidase to generate superoxide radicals. The inhibition of nitroblue tetrazolium (NBT) reduction was measured spectrophotometrically at 560 nm. Results were expressed as a unit (U). All reagents (Na_2CO_3, Na_2EDTA, xanthine, NBT, bovine serum albumin, $CuCl_2.2H_2O$, xanthine oxidase, and $(NH_4)_2SO_4$) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Total Thiol Content Assay

Since total thiol levels reflect the antioxidant capacity of the cell, total thiol levels were determined colorimetrically using the method of Ellman[21] with modifications suitable for cell lysates. After harvesting, cells were lysed, and the supernatant was mixed with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer. The absorbance of the resulting yellow complex was measured at 412 nm. Thiol levels were calculated using the molar extinction coefficient and expressed as μmol/L. All reagents (tris hydrochloride, DTNB, methanol, and l-glutathione reduced) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Malondialdehyde Assay

Malondialdehyde (MDA), a lipid peroxidation index, was measured by the modified thiobarbituric assay method.[22] After cell lysis, the supernatant was reacted with thiobarbituric acid (TBA) and acetic acid and incubated at 95°C for 60 minutes. After cooling, absorbance was measured at 532 nm. Malondialdehyde levels were expressed in μmol/L. All reagents (TBA, trichloroacetic acid, and hydrochloric acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Inhibitory concentration 50 values of methotrexate on hTERT-HME1 and MCF-7 cell lines. Data are presented as the mean ± standard error of the mean (n = 6)

Cell Lines	24 hours	48 hours	72 hours
MCF-7 cells	15.01 ± 0.8 μM	10.07 ± 1.3 μM	8.01 ± 0.9 μM
hTERT-HME1 cells	24.12 ± 0.7 μM	16.09 ± 1.2 μM	12.08 ± 1.1 μM

Statistical Analysis

Shapiro–Wilk tests were employed to confirm the distribution of all analyzed parameters. Subsequently, the Kruskal–Wallis and Dunn's post-hoc tests were employed to compare the groups. Spearman's correlation analysis was used to perform the correlation analysis. A *P*-value less than .05 was considered to be significant. Biochemical analyses were performed using JASP version 0.19.1, while all other statistical evaluations were conducted using GraphPad Prism version 9 (GraphPad Software; California, USA).

Results

Cell Viabilty and Cytotoxicity

The effect of MTX applied at different concentrations (2.5,10, 25, 50, and 100 μM for 24, 48, and 72 hours) on cell viability in healthy breast and breast cancer cell lines was investigated. Cell viability investigations were performed using MTT. The IC_{50} values of MTX in hTERT-HME1 cells were calculated as 24.12 ± 0.7 μM for 24 hours, 16.09 ± 1.2 μM for 48 hours, and 12.08 ± 1.1 μM for 72 hours. Also, IC_{50} values of MTX in MCF-7 cells were calculated as 15.01 ± 0.8μM for 24 hours, 10.07 ± 1.3 μM for 48 hours, and 8.01 ± 0.9 μM for 72 hours. (Table 1). It indicates that MTX showed

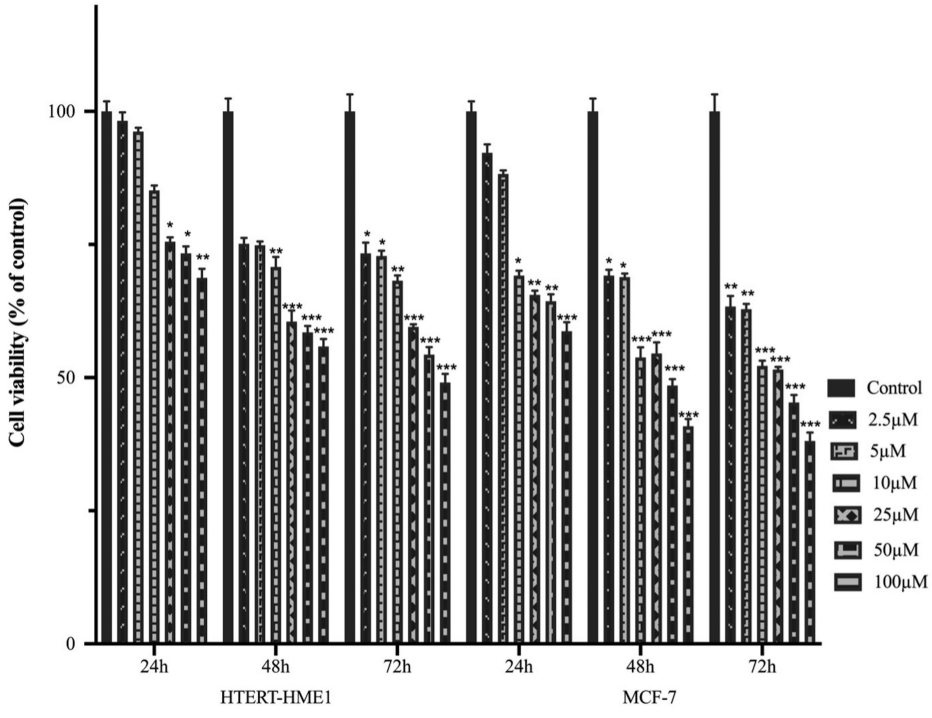

Figure 1. Effects of methotrexate (MTX) on the viability of hTERT-HME1 and MCF-7 cells. The MTX groups were treated with methotrexate as detailed in the Methods. Data are shown as the mean ± SD from 3 independent experiments. The *, **, and *** symbols represent the significant difference between the treated cells and untreated control at statistical levels of $P < .05$; $P < .01$; $P < .001$ respectively (n:6).

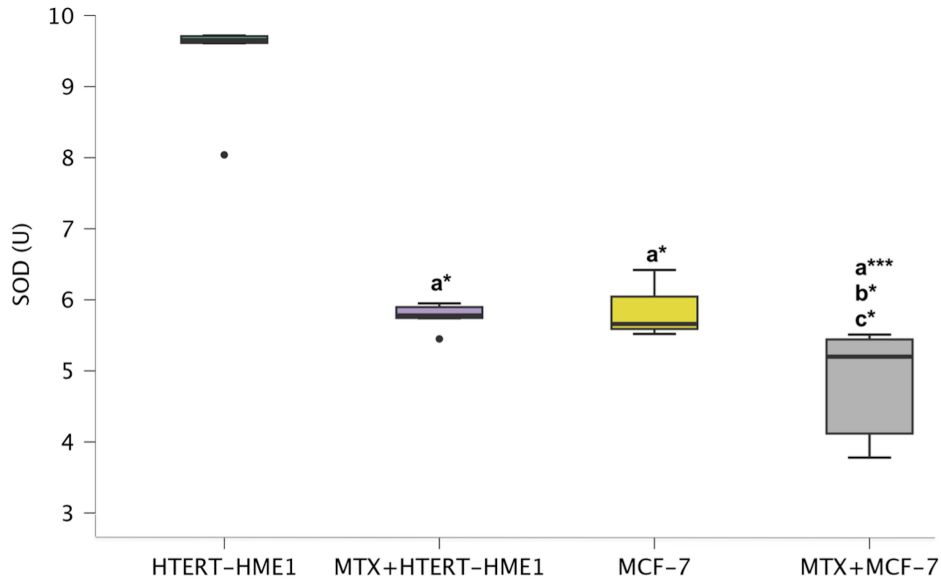

Figure 2. Effects of methotrexate (MTX) on the SOD activity of hTERT-HME1 and MCF-7 cells. "a" symbol means compared to hTERT-HME1 group, "b" symbol means compared to MCF-7 group, and "c" symbol means comparred to MTX+hTERT-HME1 group. The *, **, and *** symbols represent the significant difference between the treated cells and untreated control at statistical levels of $P < .05$; $P < .01$; $P < .001$ respectively (n:6).

a dose-dependent cytotoxic effect on both cells after 24, 48 and 72 hours of incubation on hTERT-HME1 and MCF-7 cells (Figure 1).

Superoxide Dismutase Activity

When healthy cells were evaluated, SOD activity was found to be significantly reduced in the MTX-treated hTERT-HME1 cell line (5.77 ± 0.18) compared to the untreated hTERT-HME1 cells (9.40 ± 0.67, $P = .027$). Similarly, among cancer cells, MTX treatment led to a notable decrease in SOD activity in the MCF-7 cell line (4.83 ± 0.82) compared to untreated MCF-7 cells (5.83 ± 0.37, $P = .041$). In addition, SOD activity was found to be decreased in MTX-treated MCF-7 cells (4.83 ± 0.82) compared to MTX-treated hTERT-HME1 cells (5.77 ± 0.18, $P = .034$) (Figure 2 and Table 2).

Malondialdehyde Levels

When healthy cells were evaluated, MDA levels were found to be significantly increased in the MTX-treated hTERT-HME1 cell line (5.74 ± 1.48) compared to the untreated hTERT-HME1 cells (3.16 ± 0.27, $P = .022$). Similarly, among cancer cells, MTX treatment significantly increased MDA levels in the MCF-7 cell line (13.17 ± 3.57) compared to untreated MCF-7 cells (5.57 ± 1.70, $P = .022$). In addition, MDA levels were higher in MTX-treated MCF-7 cells (13.17 ± 3.57) compared to both the untreated hTERT-HME1 cell line (3.16 ± 0.27, $P < .001$) and the MTX-treated hTERT-HME1 cell line (5.74 ± 1.48, $P = .034$). Furthermore, MDA levels were significantly increased in untreated MCF-7 cells (5.57 ± 1.70) compared to untreated hTERT-HME1 cells (3.16 ± 0.27, $P = .034$) (Figure 3 and Table 2).

Table 2. Post-Hoc Comparison ***P***-Values of the Effects of Methotrexate on Superoxide Dismutase, Malondialdehyde, and Total Thiol Levels in hTERT-HME1 and MCF-7 Cell Lines

Post-Hoc Comparison	*P* for SOD	*P* for MDA	*P* for Total Thiol
hTERT-HME1 vs MTX+hTERT-HME1	.027	.022	.003
hTERT-HME1 vs MCF-7	.022	.034	.141
hTERT-HME1 vs MTX+MCF-7	<.001	<.001	<.001
MTX+hTERT-HME1 vs MCF-7	.935	.870	.141
MTX+hTERT-HME1 vs MTX+MCF-7	.034	.034	.141
MCF-7 vs MTX+MCF-7	.041	.022	.003

MDA, malondialdehyde; MTX, methotrexate; SOD, superoxide dismutase.

Total Thiol Concentrations

When the effects of MTX on total thiol were examined in healthy cells, MTX administration significantly decreased total thiol levels

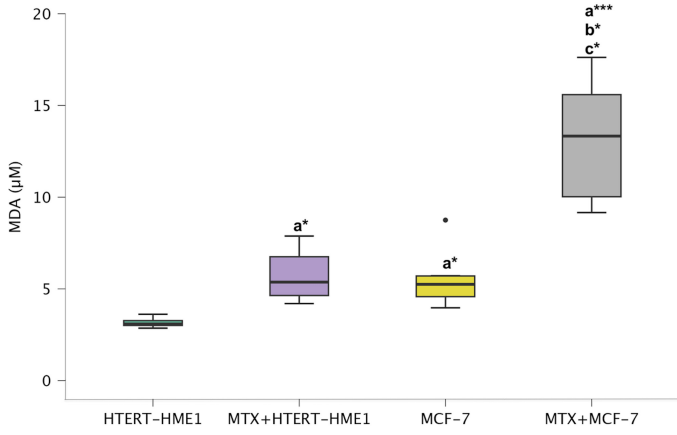

Figure 3. Effects of methotrexate (MTX) on the MDA levels of hTERT-HME1 and MCF-7 cells. "a" symbol means compared to hTERT-HME1 group, "b" symbol means compared to MCF-7 group, and "c" symbol means comparred to MTX+hTERT-HME1 group. The *, **, and *** symbols represent the significant difference between the treated cells and untreated control at statistical levels of $P < .05$; $P < .01$; $P < .001$ respectively (n:6).

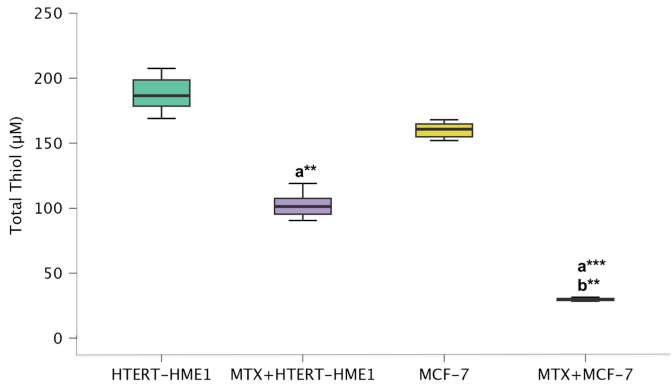

Figure 4. Effects of methotrexate (MTX) on the total thiol levels of hTERT-HME1 and MCF-7 cells. "a" symbol means compared to hTERT-HME1 group, "b" symbol means compared to MCF-7 group, and "c" symbol means compared to MTX+hTERT-HME1 group. The *, **, and *** symbols represent the significant difference between the treated cells and untreated control at statistical levels of $P < .05$; $P < .01$; $P < .001$ respectively (n:6).

in the MTX-treated hTERT-HME1 cell line (102.50 ± 10.40) compared to the untreated hTERT-HME1 cells (188.00 ± 14.74, $P = .003$). Similarly, among cancer cells, MTX treatment diminished total thiol levels in the MCF-7 cell line (29.83 ± 1.17) compared to untreated MCF-7 cells (160.08 ± 6.50, $P = .003$). Furthermore, total thiol levels were significantly decreased in the MTX+MCF-7 cell line (29.83 ± 1.17) compared to the untreated hTERT-HME1 cell line (188.00 ± 14.74, $P < .001$) (Figure 4 and Table 2).

Discussion

In this study, the cytotoxic effects of methotrexate (MTX) on cell viability and oxidative stress were evaluated comparatively in both healthy (hTERT-HME1) and cancerous breast cell (MCF-7) lines. Methotrexate administration has been demonstrated to result in a reduction of cell viability, exhibiting a dose-dependent response across both cell lines. However, this effect was more pronounced in the MCF-7 cell line.

Although there are studies on MTX in MCF-7 cells, comparative studies with healthy breast cells are insufficient. Manjappa et al[23] determined that the effective dose (IC_{50}) value for MTX in MCF cells was 13.27 ± 2.6 µM for 24 hours and 1.76 ± 0.39 µM for 48 hours.[23] It was shown that in the MCF-7 cell line, the IC_{50} values for MTX (1µg/mL) at 72 hours treatment were 33 µg/mL.[24] In this study, healthy breast and cancer breast cell lines were incubated with different doses of MTX for 24, 48, and 72 hours. The IC_{50} values were calculated, and the effect of MTX on cell viability was evaluated for hTERT-HME1 and MCF-7 cells. The cell viability and IC_{50} value of MTX treatment in healthy breast cells (hTERT-HME) were higher than in MCF-7 cells; methotrexate demonstrated a higher degree of cytotoxicity against MCF-7 cells. Methotrexate significantly reduced cell viability values in hTERT-HME1 and MCF-7 cells.

The best-known feature of MTX is that it inhibits the dihydrofolate reductase (DHFR) enzyme as a folate antagonist. Thus, it blocks thymidine, purine, and DNA synthesis. Cancer cells, on the other hand, are obviously more in need of DNA synthesis and metabolites because they characteristically show rapid proliferation. Therefore, cancer cells may be more affected by this blockade. However, one of the important results of this study is that this decrease in cell viability is also seen in healthy cells. This may explain the possible side effects of MTX treatment.

Cancer cells also undergo metabolic adaptations to maintain the ability to proliferate, and these adaptations result in increased intracellular ROS.[25] In this study, the lower basal (MTX-untreated) superoxide dismutase (SOD) levels and elevated malondialdehyde (MDA) levels in the MCF-7 cell line compared to the hTERT-HME1 cell line support this. Methotrexate application has been shown to increase SOD and total thiol levels in both cell lines, while decreasing MDA levels. The pharmacological mechanism of action of MTX, which is frequently used especially in the treatment of highly proliferative cancers, is still not fully understood, and in recent years, it has been reported that it can cause intracellular oxidative stress and tissue damage in addition to its role as a folate antagonist.[26,27] Anticancer drugs are expected to affect the tumor cell while having minimal effects on the surrounding healthy cells.[25] Therefore, understanding how MTX directs oxidative stress in both cancer cell lines and healthy cell lines will be guiding for the development of future combined therapies.

The results which show reduced SOD activity, therefore, coincide with previous findings in which MTX has been shown to reduce the levels of antioxidant enzymes, impairing the cellular redox balance while inducing the oxidative stress.[28,29] Considered the first line of defense against ROS accumulation, SOD is a key antioxidant enzyme that catalyzes the dismutation of superoxide anion (O_2^-) to H_2O_2 and molecular oxygen.[11] The mechanism behind MTX's inhibition of SOD activity may be the increase in intracellular ROS levels. In fact, it is suggested that MTX directly increases intracellular ROS levels by impairing the cell's mitochondrial function and oxidative phosphorylation.[30,31] The resulting excess ROS may also cause post-transcriptional modification and deactivation of the SOD enzyme.[32] However, a recent study found that MTX directly mediates the scavenging of free radicals, particularly superoxide.[33] In this context, it is understood that the effects of MTX, which is frequently used especially in cancer treatment, on oxidative stress should be better elucidated. Although this study is not aimed at proving the mechanistic background of this decrease in SOD activity, it is believed that the finding that MTX application increases oxidative stress in healthy cells may provide insight into the possible side effects of MTX for further studies. Supporting the reduction in SOD levels, it was found that total thiol levels, which are important for cellular antioxidant defense, also decreased in both cell types after MTX administration. This depletion in total thiol levels may be due to a general thiol-disulfide imbalance resulting from MTX exposure.[34] Methotrexate, as a DHFR inhibitor, also limits intracellular nicotinamide adenine dinucleotide phosphate (NADPH) supply. NADPH is a cofactor for the glutathione reductase enzyme, which is responsible for reducing glutathione disulfide back to glutathione. It has been reported that the synthesis and recycling of glutathione (the most abundant intracellular thiol) may also be limited due to NADPH deficiency.[35]

In this study, an increase in MDA levels, a marker of lipid peroxidation, was observed simultaneously with the decrease in antioxidant markers. Excessive intracellular ROS production can lead to lipid peroxidation. Malondialdehyde is also a highly reactive and toxic byproduct of lipid peroxidation.[33,36] Lipid peroxidation alters the physical properties of membranes, resulting in increased permeability, loss of lipid symmetry, and accelerated lipid trans bilayer diffusion. These changes lead to altered metabolic pathways, inflammation, and apoptosis.[37] It has been hypothesized that biomarkers of this process, such as MDA, could serve as a potential therapeutic strategy for colorectal cancer.[38] In this previous study, it was also shown that serum MDA levels in breast cancer patients were increased compared to the control group and had high potential as a biomarker.[39] However, MDA levels can

vary depending on diet and eating habits. Therefore, in this study, the finding that basal (MTX-untreated) MDA levels in cancer cells were higher than basal MDA levels in healthy cells supports the previous findings and its promising feature as a biomarker.

There are studies suggesting that MTX also has radical scavenging activity. For instance, Zimmerman et al[33] reported that MTX mediates superoxide radical scavenging, and the study further suggests that MTX inhibits malondialdehyde-acetaldehyde (MAA) adduct formation. Concurrently, acetaldehyde (AA) and MDA have the capacity to interact, resulting in covalent modification of biomolecules, including proteins and lipoproteins. These interactions form highly stable adducts, known as MAA adducts.

Surely, these different results may be due to the differences in study designs. However, further research is necessary to enhance the understanding of the effects of MTX on cells and to elucidate its mechanisms. In this context, there are some limitations of this study. The in vitro design may not reflect the full complexity of the tumor microenvironment or systemic factors that affect the in vivo effects of MTX. Although oxidative stress was evaluated using both antioxidant markers (SOD activity and total thiol levels) and a lipid peroxidation marker (MDA), intracellular ROS levels were not directly measured. Moreover, the measurement of intracellular ROS levels and other oxidative stress markers, such as catalase or glutathione peroxidase, would have further supported the results. Future studies are planned to include ROS-specific assays to complement and strengthen these findings.

In conclusion, this study has shown that MTX has high anticancer activity with its effects on cell viability and oxidative stress. However, observation of similar effects in healthy cells requires the development of various combination therapies or targeted drug delivery systems to reduce the side effects of MTX.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: Ethics Committee approval is not required for cell culture studies.

Informed Consent: Informed consent was not required as this study was conducted on cell cultures.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – S.D., D.D.E., H.U.; Design – S.D., D.D.E., R.G., H.U.; Supervision – S.D., D.E., R.G., H.U.; Resources – S.D., D.E., H.U.; Materials – S.D., D.E., H.U.; Data Collection and/or Processing – S.D., D.E.; Analysis and/or Interpretation – S.D., D.E., H.U.; Literature Search – S.D., D.E., R.G., N.B., H.U.; Writing Manuscript – S.D., D.E., H.U.; Critical Review – S.D., D.E., R.G., N.B., H.U.

Declaration of Interests: The authors have no conflict of interest to declare.

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