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The Role of Hyperthermia in Enhancing Anti-tumor Efficacy of Pemetrexed and Altering *hsa-MiR-548c-3p* Expression Profile in A549 Cell Line (Human Lung Cancer)

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ABSTRACT

Lung cancer remains a major cause of cancer-related mortality worldwide, with current treatments such as surgery, chemotherapy, and immunotherapy facing limitations, including severe side effects and high costs. Hyperthermia (H) has emerged as a promising strategy to enhance tumor sensitivity to treatments and reduce toxicity. This study investigates the effects of H in enhancing the anti-tumor efficacy of pemetrexed (PEM) and altering the expression profile of *hsa-MiR-548c-3p* (tumor suppressor) in the A549 cell line.

A549 cells were cultured in DMEM medium and divided into four groups: Control, and treatment with H, PEM, and a combination of H and PEM. Subsequently, cell viability, apoptosis percentage, release rate of LDH, production of ROS, and the expression level of *hsa-MiR-548c-3p*, *CASP 8* and 9, and *TYMS* genes were measured.

Results revealed that the combination of H and PEM treatment had greater antitumor effects compared to the other groups. The combination of H and PEM significantly reduced cell viability, increased the percentage of apoptosis, LDH release, and ROS production, and upregulated *hsa-MiR-548c-3p*, *CASP 8*, and 9, while downregulating *TYMS*.

The findings suggest that H enhances the chemotherapeutic efficacy of PEM by upregulating *hsa-MiR-548c-3p* expression and promoting apoptosis in lung cancer cells, making it a promising complementary approach for overcoming drug resistance.

Keywords: Apoptosis; *hsa-MiR-548c-3p*; Hyperthermia; Lung cancer; Pemetrexed

INTRODUCTION

Lung cancer remains one of the leading causes of cancer-related mortality worldwide. Despite recent advances in therapeutic options, the efficacy of current treatments for lung cancer patients remains suboptimal.[1] The primary treatment strategies for lung cancer depend on the cancer type and stage but generally include

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surgery, chemotherapy, radiotherapy, and targeted therapies. In early-stage non-small cell lung cancer (NSCLC), surgery is the mainstay treatment. In more advanced stages, combinations of these modalities are typically employed.[2] For instance, according to the National Cancer Institute (NCI), surgery is recommended for patients with resectable tumors. However, in cases of metastasis or unresectable tumors, adjunctive treatments such as chemotherapy and radiotherapy are often recommended.[3] Pemetrexed (PEM), a chemotherapy drug, is particularly used in the treatment of NSCLC and melanoma. As an antimetabolite, it targets enzymes involved in nucleotide synthesis, thereby inhibiting tumor cell division.[4] Nevertheless, PEM is associated with significant side effects, including cytopenia, gastrointestinal disturbances such as nausea and vomiting, and dermatologic reactions.[5] One of the primary challenges in lung cancer treatment lies in the inadequate patient response to chemotherapy and radiotherapy, particularly in advanced disease stages.[6] In addition to severe side effects, the development of treatment resistance further limit the effectiveness of existing therapeutic approaches. Chemotherapy drugs, while effective in targeting rapidly dividing cells, can also affect normal, fast-growing healthy cells due to their systemic action.[7] Given the limitations of conventional therapies in reducing lung cancer-related mortality, physicians are exploring complementary approaches to enhance treatment efficacy and shorten the duration of therapy. One such innovative approach is hyperthermia (H), which has shown significant promise in sensitizing cancer cells to chemotherapeutic agents, overcoming drug resistance, and inhibiting tumor growth.[8] H is almost invariably employed in conjunction with other cancer treatment modalities such as radiotherapy and chemotherapy. This technique can augment the sensitivity of certain cancer cells to radiation or damage cells that radiation or target cells that are resistant to destruction by radiation alone.[9] H exerts its anticancer effects through various mechanisms: a) DNA Damage: Elevated temperatures damage the DNA structure in cancer cells, potentially inducing apoptosis.[10] b) Disruption of Structural Proteins: High temperatures alter essential proteins required for maintaining cellular structure and function, impairing cancer cell activity. c) Increased Membrane Permeability: H enhances cell membrane permeability, improving the efficacy of chemotherapeutic drugs.[11] d) Immune Response Activation: Heat can stimulate the immune system, aiding in the recognition and elimination of cancer cells. e) Reduced Tumor Oxygenation: High temperatures may weaken tumor vasculature, thereby reducing oxygen and nutrient supply to cancer cells. Numerous clinical studies have evaluated the combination of H with radiotherapy or chemotherapy in treating various cancers, including sarcomas, carcinomas, melanomas, and cancers of the head and neck, brain, lung, esophagus, breast, bladder, anus, liver, appendix, cervix, and peritoneal lining (mesothelioma).[12–14] One method of assessing the efficacy of novel treatments such as H is by examining changes in microRNA (miR) expression. MicroRNAs (miRs) serve as biomarkers and provide valuable insights into cancer cells to treatment. By binding to the 3′ untranslated regions of messenger RNA, miRs regulate gene expression post-transcriptionally and play a critical role in cancer cell growth, metastasis, and therapeutic resistance.[15] There are two main classes of miRs:tumor suppressors and oncogenes. Chemotherapy drugs have been shown to modulate miR expression.[16] Specifically, *hsa-MiR-548c-3p* has demonstrated a critical role in lung cancer suppression by targeting signaling pathways associated with cell survival and apoptosis. Alterations in *hsa-MiR-548c-3p* expression influence genes regulating apoptosis, metastasis, and drug resistance, highlighting its tumor-suppressive role.[17,18] While the involvement of miRs in cellular processes and therapeutic responses is well-documented, limited research exists on the direct effects of H in combination therapies, such as PEM treatment, on hsa-MiR-548c-3p expression in lung cancer. This study aims to investigate the effects of H in enhancing the anti-tumor efficacy of PEM and modulating the expression profile of *hsa-MiR-548c-3p* known tumor suppressor in the A549 lung cancer cell line.

MATERIALS AND METHODS

Cell Line Preparation: A549 Cells

A549 cells (ATCC CCL-185™) were obtained from the Pasteur Institute of Iran and cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, at 37°C and 5% CO_2. Cells were divided into four groups: the negative control group (NC), the hyperthermia (H) treatment group, the group exposed to the pemetrexed (PEM) chemotherapy drug, and the combination therapy group treated simultaneously with hyperthermia and pemetrexed (PEM+H).

Pemetrexed

PEM, marketed as Alimta, is a chemotherapy drug used to treat pleural mesothelioma and non-small cell lung cancer. To determine the half-maximal inhibitory concentration (IC_{50}) of PEM on A549 cells, twelve concentrations of PEM ranging from 1 to 12 µM were administrated for 24 hours.

Hyperthermia Induction

To induce H, an appropriate number of cells were plated in suitable wells (depending on the assay type) and incubated for 24 hours under standard culture conditions. The plates were then transferred to an incubator set at 42°C for one hour, maintaining similar humidity and CO_2 levels as the initial incubation except for the elevated temperature. After one hour, the plates were returned to their initial incubation conditions.[19]

MTT Assay

After 24 hours of treating 10^4 A549 cells with H, PEM, and H+PEM, the culture medium was carefully removed from each well, and 90 µL of fresh culture medium was added along with 10 µL of MTT solution at a concentration of 5 mg/mL (final concentration of 0.5 mg/mL) to each well. The plates were incubated for 4 hours. After confirming the formation of formazan crystals under an inverted microscope, the medium was gently removed, and 100 µL of DMSO (Sigma Aldrich, USA) was added to each well. The plates were incubated for 30 minutes to dissolve the crystals, and the purple color was measured using an ELISA reader at a wavelength of 570 nm. All concentrations were performed in triplicate to ensure accuracy.[19]

Apoptosis Assay: Qualitative and Quantitative

Fluorescent dyes acridine orange and ethidium bromide were used to stain cell nuclei. Acridine orange is capable of permeating live cell membranes, whereas ethidium bromide penetrates only dead cell membranes. In this assay, viable cells appear green, while apoptotic cells show a shift from orange to red. A suspension of 10^4 cell in DMEM was distributed in 24-well plates (500 µL per well) and incubated for 24 hours. After a 24-hour incubation period, the wells were treated according to the study design. After 24 hours, the treatment solutions were discarded, and 250 µL of a mixture of acridine orange and ethidium bromide (concentration of 10 µg/mL) was added for 2 minutes. The cells were then observed under fluorescence microscopy.[20]

LDH Release Assay

Lactate dehydrogenase (LDH) is an enzyme released as a marker of cell damage, and cytotoxicity assays are based on its release. LDH is found in eukaryotic cells, and upon cell damage or death, it is released from the plasma membrane. LDH was measured using a Kiazist kit (KLDH96). A total of 5,000 cells per well in a 96-well plate were incubated for 24 hours. Treatment groups were applied, and 20 µL of Permi solution was added to each well. The plate was incubated for one hour at room temperature. For LDH measurement, 50 µL from each well was transferred to a new plate, and 50 µL of working buffer was added. After incubation at 37°C in darkness for 30 minutes, absorbance was read at 570 nm.

ROS Production Analysis

Reactive oxygen species (ROS) are chemically reactive oxygen-containing substances that are naturally generated endogenously within the mitochondria or through exposure to external agents. These radicals can damage cellular cellular macromolecules, leading to tissue and cellular disorders, including the development of cancer. Various types of ROS include superoxide radicals, hydroxyl radicals, and peroxides. In this assay, 2′,7′ dichlorofluorescein diacetate (DCFDA) dye was used to detect ROS. This dye is cell-permeable and is de-esterified by intracellular enzymes within live cells. Upon oxidation by specific ROS, it is converted into a fluorescent compound. Cells were plated in a 96-well plate, treated, and washed with ROS Buffer. Then, 100 µL of working solution DCFDA was added to each well and incubated in the dark at 37°C for 45 minutes. Fluorescence was measured at wavelengths 485/528 nm.[20]

RNA Extraction

RNA extraction from cell cultures was performed using the RNX Plus kit. For this purpose, 1 mL of RNX Plus solution was added to 10^6 cells and transferred to a microtube. Then, 200 µL of cold chloroform was added, and the sample was shaken and centrifuged for 15 minutes at 13 000 rpm at 4°C. The clear supernatant was transferred to a new microtube, and 500 µL of isopropanol was added and centrifuged for 15 minutes at 13 000 rpm at 4°C. RNA was observed as a white precipitate. One milliliter of 75% ethanol was added and centrifuged for 10 minutes at 8500 rpm at 4°C. The supernatant alcohol was discarded, and after drying, 50

μL of RNase-free DEPC water was added to it. The quality and quantity of RNA was assessed using Nano Drop. RNA was stored at -70°C.

cDNA Synthesis

The Add Bio cDNA synthesis kit was used for cDNA synthesis. For this purpose, 1 μg of DNase I-treated RNA was added to a 0.2 mL microtube, along with 1 μL of RT enzyme, 2 μL of dNTP, 4 μL of buffer, and 3 μL of a mix of Oligo dT and random hexamer primers. The volume was brought to 20 μL with DEPC water. The resulting mixture was incubated for 60 minutes at 50°C and 10 minutes at 80°C. The synthesized cDNA was stored at −21°C until the Real Time PCR step. For miR cDNA synthesis, the specific primer designed in Supplementary Table 1 was used instead of Oligo dT and random hexamer.

Real Time PCR

To evaluate gene expression changes, Real time PCR was performed using the Cybergreen method according to the Parstus kit protocol. Ten microliters of Cybergreen Mastermix were added into a special Real time PCR microtube. Then, 1 μL of the forward and reverse primer mixture of the desired gene at a concentration of 10 μM was added (Supplementary Table 1 and 2). One microliter of the prepared cDNA was added, and the final volume was adjusted to 20 μL with distilled water.

Statistical Analysis

Statistical analysis and figure plotting for cellular data were performed using GraphPad Prism, version 8.2.1. A one-way ANOVA was used to compare different groups. In all analyses, a $p<0.05$ was considered statistically significant, and data were presented as Mean ± SEM. The relative expression of genes was assessed using the Relative Expression Software Tool (REST), version 2.0.13. The results from REST were interpreted alongside the cellular data to ensure comprehensive analysis.

RESULTS

Cell Viability

As shown in Figure 1A, the concentration of PEM required to inhibit the growth of 50% of A549 cells after 24 hours was equal to 4.766 μM. Also, Figure 1B demonstrated that all treatments (PEM, H, PEM+H) significantly reduced viability compared to the control group ($p<0.05$). Specifically, the combination therapy (PEM+H) group exhibited the most significant reduction, while the H group showed the least significant decrease in viability compared to the control group ($p<0.05$). Notably, PEM+H outperforms PEM alone.

Apoptosis

Figure 2 presents a comparison of apoptosis percentages among the different treatment groups. The control group (untreated) shows the lowest apoptosis levels, as no external factors were applied. H resulted in a slight, non-significant increase in apoptosis compared to the control ($p>0.05$). In contrast, PEM significantly increased apoptosis, showing a significant difference from the control group ($p<0.0001$). The highest apoptosis percentage was found in the combination therapy group (PEM + H) and PEM, with a significant increase ($p<0.0001$). However, no statistically significant difference was observed between the combination therapy group and the PEM group ($p>0.05$).

LDH Assay

As shown in Figure 3, the level of LDH release significantly increased in all treatment groups compared to the control group ($p<0.05$), except for the H group Although the H group demonstrated an increase in LDH release, this difference was not statistically significant when compared to the control group ($p> 0.05$). compared to the control group ($p>0.05$). Furthermore, the combination therapy (PEM+H) group exhibited a significant increase in LDH release compared to the single treatment (PEM) group ($p<0.0001$).

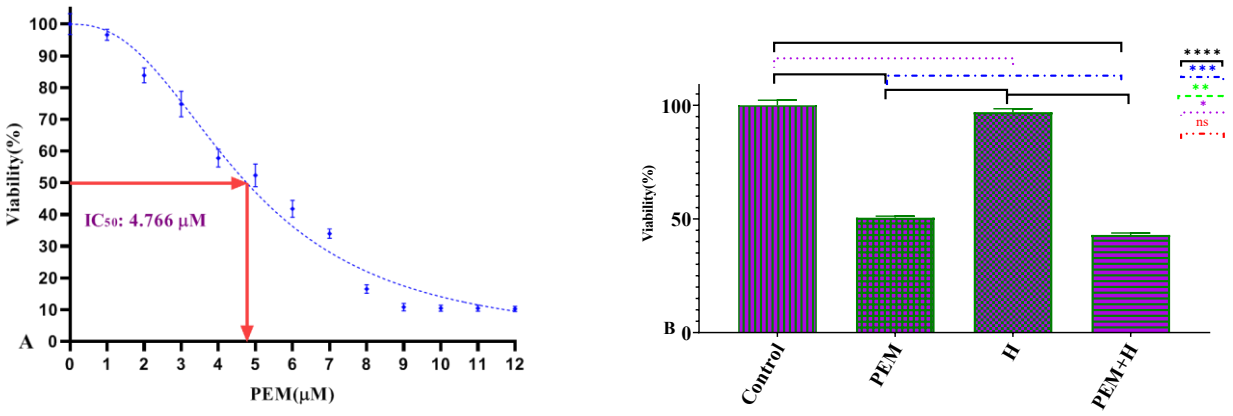


Figure 1. A) The percentage of viability and the calculation of IC_{50} for A549 cells after 24 hours of treatment with PEM. B) Comparing the cell viability of A549 cells after treatment with the study groups (* indicates $p<0.05$, ** indicates $p<0.01$, * indicates $p<0.001$, **** indicates $p<0.0001$, ns indicates non-significant).**

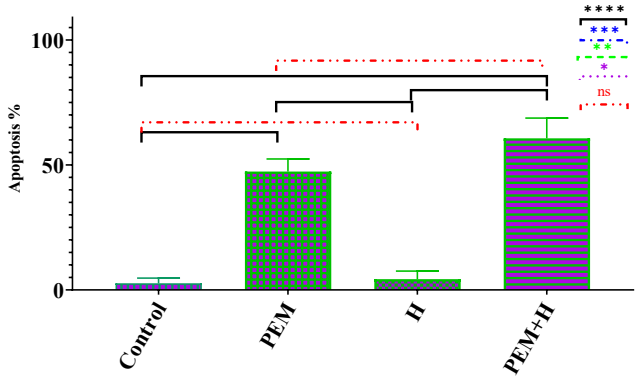


Figure 2. Comparing the apoptosis percentage of A549 cells after treatment with the study groups (* indicates $p<0.05$, ** indicates $p<0.01$, * indicates $p<0.001$, **** indicates $p<0.0001$, ns indicates non-significant).**

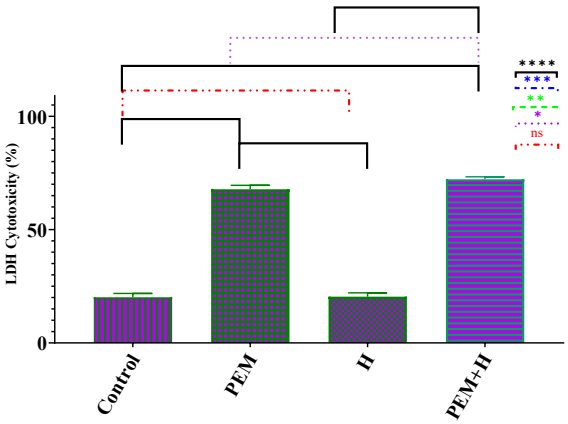


Figure 3. Comparing the LDH release of A549 cells after treatment with the study groups (* indicates $p<0.05$, ** indicates $p<0.01$, * indicates $p<0.001$, **** indicates $p<0.0001$, ns indicates non-significant).**

ROS Production

As illustrated in Figure 4, the production of ROS showed a significant rise in all treatment groups reletive to the control group (p<0.05), with the exception of the H group, which, although increased, did not reach significance relative to the control group (p>0.05). Additionally, it was noted that the combination therapy group (PEM+H) demonstrated a substantial increase in ROS production in comparison to the single treatment group (PEM) (p<0.0001).

Expression of *hsa-miR-548c-3p*, *CASP 8* and *CASP 9*, and *TYMS*

As depicted in Figure 5, the expression levels of *hsa-miR-548c-3p* were significantly increased in all treatment groups (H, PEM, and PEM+H) compared to the control group (p<0.05). The highest expression was observed in the combination therapy (PEM+H) group, whereas the H group demonstrated the lowest relative to the control group (p<0.05). Analysis of caspase 8 expression, representative of the extrinsic apoptosis pathway, and caspase 9, indicative of the intrinsic apoptosis pathway, revealed that both treatment modalities (H and PEM) and combination therapy (PEM+H) stimulate apoptosis through both internal and external pathways (p<0.05). Additionally, the expression levels of TYMS were significantly decreased in all treatment groups compared to the control group (p<0.05). The most substantial reduction was observed in the combination therapy (PEM+H) and PEM groups, while the least reduction occurred in the H group compared to the control group (p<0.05). No significant difference was observed between the combination therapy (PEM+H) and PEM groups (p>0.05).

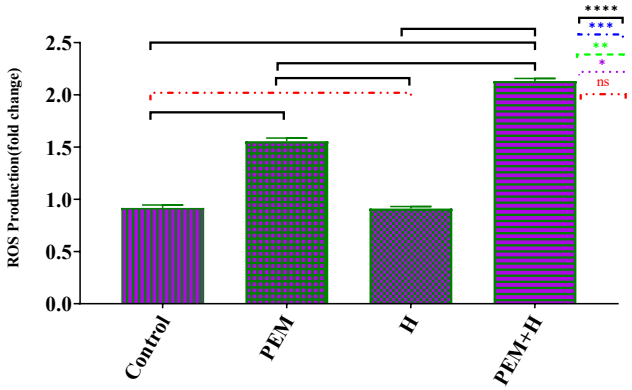
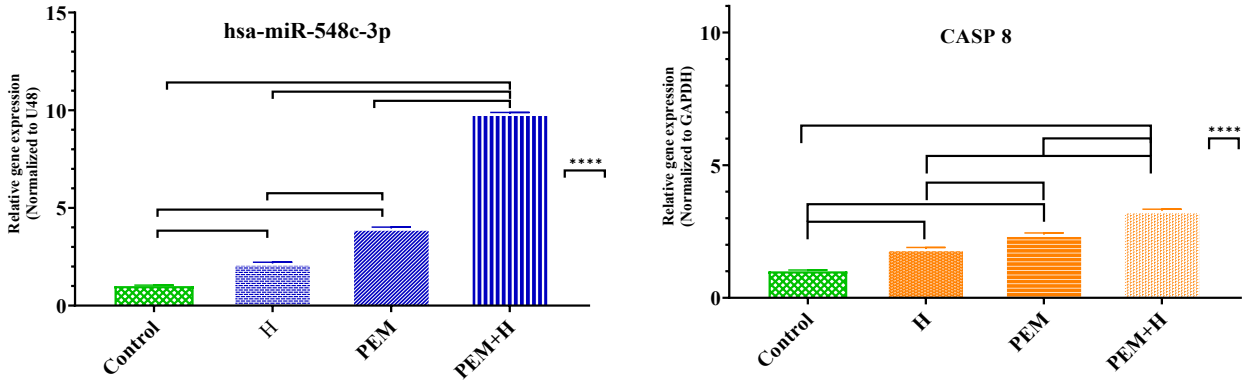


Figure 4. Comparing the ROS production of A549 cells after treatment with the study groups (* indicates p<0.05, ** indicates p<0.01, * indicates p<0.001, **** indicates p<0.0001, ns indicates non-significant).**



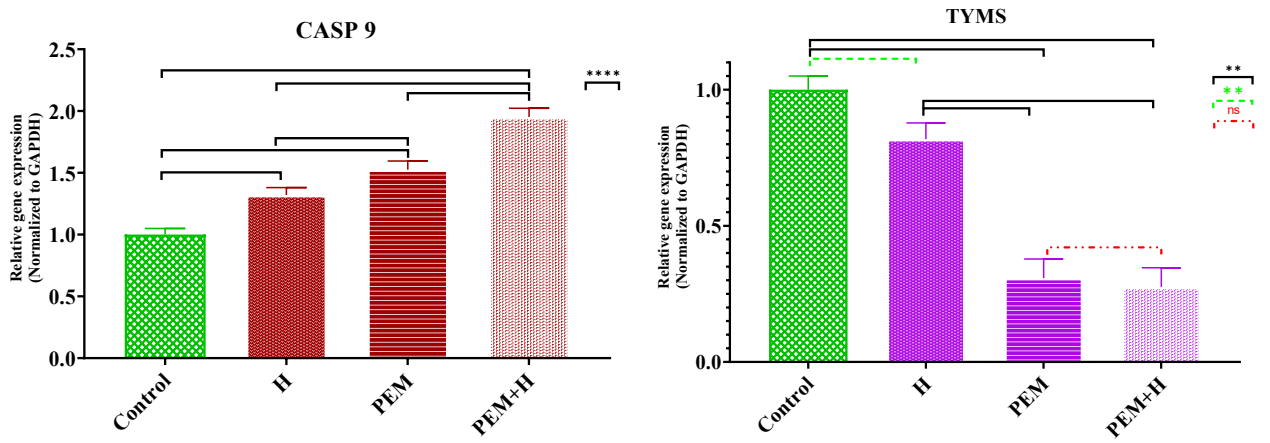


Figure 5. Relative expression of *hsa-miR-548c-3p*, *CASP8*, *CASP9*, and *TYMS*. Relative expression of *hsa-miR-548c-3p* in treated cells versus untreated cells that was normalized to *RNU48*. Relative expression of *TYMS*, *CASP8*, and *CASP9* in treated cells versus untreated cells that was normalized to *GAPDH* (* indicates $p<0.05$, ** indicates $p<0.01$, * indicates $p<0.001$, **** indicates $p<0.0001$, ns indicates non-significant).**

DISCUSSION

Resistance to lung cancer treatment is one of the main challenges in managing this disease. Cancer cells may become resistant to chemotherapy and radiotherapy due to genetic changes, the expression of specific proteins, or miRs. Understanding the mechanisms of treatment resistance can aid in the development of new therapeutic strategies.[21] Hyperthermia (thermotherapy) is a treatment method that enhances the effects of anticancer therapies by raising the temperature in cancerous tissues. The temperature rise leads to the production of free radicals and physiological changes in cells, enhancing blood flow and improving drug permeability.[22] This approach can increase the sensitivity of cancer cells to medications, leading to improved treatment outcomes. As a complementary method, hyperthermia has gained attention, particularly in combination with chemotherapy and radiation therapy.[23] This study investigates the effects of H in enhancing the anti-tumor efficacy of PEM and altering the hsa-MiR-548c-3p (tumor suppressor) expression profile in the A549 cell line. The results of this study demonstrated that the combination of H and PEM significantly reduced cell viability, increased cell death, and elevated cytotoxicity, as shown by MTT, apoptosis, and LDH assays, respectively. Additionally, ROS levels were elevated, indicating heightened oxidative stress. Gene expression analysis revealed upregulation of hsa-MiR-548c-3p and activation of both extrinsic and intrinsic apoptotic pathways, including increased activity of caspase-8 and caspase-9. MicroRNAs (miRs) play a crucial role in regulating gene expression and can function as either tumor suppressors or oncogenes. As tumor suppressors, miRs can reduce the expression of genes associated with tumor growth, thereby preventing cancer progression. Conversely, as oncogenes, they can target tumor suppressor genes, promoting the growth and spread of cancer cells.[24] MicroRNAs (miRs) play a significant role in regulating the activity of caspases, particularly caspase-8 and caspase-9. [25] Caspase 8 acts as an initiator in the extrinsic apoptosis pathway and is activated in response to death signals from death receptors. This caspase triggers a cascade of reactions by activating other caspases, leading to cell death.[26] On the other hand, caspase-9 is activated in the intrinsic apoptosis pathway and typically functions in the mitochondria. It promotes the activation of other caspases by forming the apoptosome complex. Precise regulation of these caspases is essential for maintaining the balance between cell survival and death.[27] The TYMS gene (thymidylate synthase) plays a crucial role in lung cancer. This gene is responsible for producing an enzyme essential for the synthesis of DNA and RNA nucleotides. High activity of TYMS can contribute to uncontrolled cell division and tumor growth. In lung cancer, increased expression of TYMS is often associated with resistance to chemotherapy .[28] Mirra et al (2023) investigated the expression of specific lung miRs in lung cancer and chronic obstructive pulmonary

disease (COPD), revealing that changes in miR expression patterns may play a role in the development and progression of these diseases. This study emphasizes the importance of miRs in regulating cellular processes such as inflammation and apoptosis.[29] Carrà et al (2024) demonstrated that changes in miR expression, including their role in metabolic pathways, could be effective in targeted lung cancer therapy.[30] Ghafouri-Fard et al (2021) investigated the effects of various miRs on apoptotic pathways and treatment resistance in lung cancer. This study suggests that certain miRs may have dual roles in lung cancer progression and treatment resistance.[31] Ghorbani et al (2024) examined the effects of PEM chemotherapy on the growth of A549 non-small cell lung cancer cells and showed that PEM inhibited cell growth by upregulating hsa-miR-320a and significantly increased the release of LDH, production of ROS, percentage of apoptosis, and decreased viability.[20] Wang et al (2020) investigated the role of miR-548b-3p as a tumor suppressor in lung cancer, demonstrating that this miR inhibits cancer progression by reducing migration, invasion, and proliferation of cancer cells.[18] Jin et al (2018) demonstrated that H enhances the efficacy of gemcitabine in pancreatic cancer by inducing ROS and activating the JNK pathway, thereby promoting apoptosis.[32] Hwang et al (2014) demonstrated that the combination of PEM and simvastatin led to increased ROS production and oxidative stress in MSTO-211 and A549 cells.[33] Khalili et al (2023) reported that MiR-548c-3p through suppressing *Tyms* and *Abcg2* increases the sensitivity of colorectal cancer cells to 5-fluorouracil.[34]

The findings indicate that H enhances the chemotherapeutic efficacy of PEM by upregulating hsa-MiR-548c-3p expression, which promotes apoptosis in lung cancer cells. This mechanism is crucial for eliminating cancer cells and reducing tumor burden. By increasing hsa-MiR-548c-3p levels, H facilitates a more effective response to PEM treatment. Additionally, H's ability to improve PEM's effectiveness suggests it could be a promising complementary approach to overcome drug resistance in lung cancer therapy. This strategy may lead to better patient outcomes and highlights the need for further clinical investigation.

STATEMENT OF ETHICS

Bushehr University of Medical Sciences's ethical committee confirmed this study's protocols (Ethics: IR.BPUMS.REC.1402.306).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

ZIGAP artificial intelligence (AI) tools used in preparing this manuscript.

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