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Effect of doxorubicin on Caspase-1/GSDMD-mediated pyroptosis in MCF-7 breast cancer spheroids

Doksorubisinin MCF-7 meme kanseri sferoidlerinde Caspase-1/GSDMD aracılı piroptoz üzerine etkisi

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ABSTRACT

Aim: Doxorubicin, a widely used anthracycline antibiotic, exerts its antitumor effects through multiple mechanisms, including DNA intercalation, inhibition of topoisomerase II, and the induction of oxidative stress. This study aimed to evaluate its potential to activate the inflammatory cell death pathway, pyroptosis via Caspase-1 and Gasdermin D (GSDMD), as well as its impact on spheroid morphology in a three-dimensional (3D) cell culture model.

Materials and Methods: Spheroids were formed using 3D cell culture techniques and treated with increasing concentrations of doxorubicin (0.1–100 μM) for 24 and 48 hours. Spheroid number and diameter were analyzed using ImageJ v1.47. Caspase-1 and GSDMD protein levels were quantified by ELISA to assess the activation of pyroptotic pathways.

Results: Doxorubicin treatment led to a dose-dependent reduction in both spheroid number and size. At 100 μM, spheroid formation was completely inhibited and replaced by disorganized cell clusters. Caspase-1 and GSDMD protein levels increased significantly with higher doxorubicin concentrations, with peak expression observed at 25 μM; suggesting activation of inflammatory cell death pathways. No significant differences were found between 24- and 48-hour treatments.

Conclusion: Doxorubicin disrupts 3D spheroid structure and induces the expression of pyroptosis-related proteins in a concentration-dependent manner. These findings highlight its dual role in both impairing tumor architecture and activating inflammatory cell death mechanisms, providing insights into its therapeutic potential in solid tumors.

Keywords: Breast cancer, doxorubicin, spheroid morphology, cytotoxicity, pyroptosis.

ÖZ

Amaç: *Doksorubisin, yaygın olarak kullanılan bir antrasiklin antibiyotiği olup antitümör etkilerini DNA interkalasyonu, topoizomeraz II inhibisyonu ve oksidatif stresin indüklenmesi gibi çeşitli mekanizmalar aracılığıyla gösterir. Bu çalışma, doksorubisinin Kaspaz-1 ve Gasdermin D (GSDMD) aracılığıyla inflamatuar hücre ölümü yolu olan piroptozu aktive etme potansiyelini ve üç boyutlu (3B) hücre kültürü modelinde sferoid morfolojisi üzerindeki etkisini değerlendirmeyi amaçlamaktadır.*

Gereç ve Yöntem: *Sferoidler 3B hücre kültürü teknikleriyle oluşturulmuş ve farklı dozlardaki doksorubisin (0,1–100 μM) ile 24 ve 48 saat süreyle tedavi edilmiştir. Sferoid sayısı ve çapı ImageJ*

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v1.47 yazılımı ile analiz edilmiştir. Kaspaz-1 ve GSDMD protein düzeyleri, piroptotik yolların aktivasyonunu değerlendirmek için ELISA yöntemiyle ölçülmüştür.

Bulgular: *Doksorubisin uygulaması, sferoid sayısı ve çapında doz bağımlı azalmaya yol açmıştır. 100 µM'de, sferoid oluşumu tamamen inhibe edilmiş ve dağılmış hücre kümeleri gözlenmiştir. Kaspaz-1 ve GSDMD protein düzeyleri, yüksek doksorubisin konsantrasyonları ile anlamlı şekilde artmış, maksimum ekspresyon 25 µM'de belirlenmiştir; bu da inflamatuar hücre ölüm yollarının aktive olduğunu göstermektedir. 24 ve 48 saatlik tedavi süreleri arasında anlamlı bir fark saptanmamıştır.*

Sonuç: *Doksorubisin, 3B sferoid yapısını bozarak ve piroptozla ilişkili proteinlerin ekspresyonunu doz bağımlı şekilde artırmaktadır. Bu bulgular, doksorubisinin tümör yapısını bozan ve inflamatuar hücre ölüm mekanizmalarını aktive eden iki yönlü etkisini vurgulayarak, solid tümörlerdeki terapötik potansiyeline dair önemli ipuçları sunmaktadır.*

Anahtar Sözcükler: *Meme kanseri, doksorubisin, sferoid morfolojisi, sitotoksisite, piroptoz.*

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women globally and represents a significant public health concern due to its high incidence and mortality rates (1). Despite advancements in early detection and targeted therapies, chemotherapy remains a cornerstone in the management of breast cancer, particularly in aggressive and advanced subtypes (1, 2). Among chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, has demonstrated broad-spectrum antitumor activity and is frequently included in standard treatment regimens. While traditionally known to induce apoptosis, growing evidence suggests that doxorubicin may also activate alternative forms of regulated cell death, including pyroptosis, which could influence therapeutic efficacy and immune modulation in the tumor microenvironment (2-5).

Pyroptosis is a highly inflammatory type of programmed cell death that differs from apoptosis in both its molecular mechanisms and immunological consequences. It is typically initiated by inflammasome activation, leading to the cleavage of pro-Caspase-1 into its active form. Activated Caspase-1 then processes Gasdermin D (GSDMD), cleaving it into an N-terminal fragment that forms pores in the plasma membrane. These pores result in cell swelling, membrane rupture, and the release of pro-inflammatory cytokines such as IL-1β and IL-18. While pyroptosis has been extensively studied in immune cells and infectious diseases, its role in cancer, particularly in response to chemotherapy, is still emerging (6, 7).

Recent studies have suggested that induction of pyroptosis in tumor cells may enhance anti-tumor immune responses and contribute to tumor regression. In this regard, the expression and activation of Caspase-1 and GSDMD are considered crucial indicators of pyroptotic activity (8-10). Some chemotherapeutic agents have been shown to trigger pyroptosis under certain conditions, suggesting a potential dual mechanism of action that involves both direct cytotoxicity and immune system activation (6, 9). However, the specific relationship between doxorubicin-induced stress and the activation of pyroptotic pathways in breast cancer cells remains to be fully elucidated (3, 8, 11).

Three-dimensional (3D) cell culture models, such as spheroids, offer a more physiologically relevant system compared to conventional two-dimensional (2D) monolayer cultures (12). Spheroids mimic the complex architecture of solid tumors, including gradients of nutrients, oxygen, and cellular proliferation, making them valuable tools for studying drug penetration, resistance, and mechanisms of cell death. Moreover, 3D models better replicate the tumor microenvironment and are particularly suitable for evaluating morphological and molecular responses to treatment (13-16).

In this study, we aim to investigate the effects of doxorubicin on breast cancer spheroids, with a specific focus on spheroid morphology and the induction of pyroptosis through Caspase-1 and GSDMD expression. By employing a 3D culture system, we seek to gain a deeper understanding of how doxorubicin influences non-apoptotic cell death pathways and to explore the potential contribution of pyroptosis to the overall therapeutic response in breast cancer. Understanding these mechanisms may not only improve our knowledge

of doxorubicin's multifaceted action but also open new avenues for combination therapies targeting inflammatory cell death pathways.

MATERIALS AND METHODS

Cell Culture

The MCF-7 human breast cancer cell line was purchased from Şap Institute of Ministry of Agriculture and Forestry, Ankara, Turkey. They were cultured in RPMI-1640 (22400-089, New York, USA) media containing 10 % fetal bovine serum (S1810, Biowest, France), 200mM L-glutamine (K0100-670, Cegrogen Biotech, Stadtallendorf, Germany) and 100UI/ml. penicillin/streptomycin (P0100-790, Cegrogen Biotech, Stadtallendorf, Germany) at 37^0 C, 5 % CO_2 and 70% humidified atmosphere. When 70-80% confluency was reached, cells were passaged to form spheroids.

Formation of Spheroid

To generate spheroid with MCF-7 cells, the hanging drop method was employed. Cells were harvested via trypsin/EDTA (SH30042.01, Hyclone Cytiva, Utah, USA) and then counted using trypan blue exclusion dye. 20 µl droplets containing 5000 cells were added to the inner surface of the 100 mm petri dish lid. To prevent the droplets from evaporation, sterile phosphate buffer solution (PBS) was added to the bottom of the petri dish, and the lid was closed to ensure the droplets remained suspended. Spheroid formation was monitored morphologically under an inverted microscope (Carl Zeiss, Germany) (12).

Drug Treatment

Doxorubicin (Saba, Turkey) treatment was applied along with droplet seeding during the spheroid formation phase. This allowed for the investigation of doxorubicin's effect on cell viability and pyroptosis in spheroid formation from the beginning. For this purpose, five different doxorubicin concentrations (0.1, 1, 5, 25, and 100 µM) were selected (17), and 6 groups were formed including the control group. Doxorubicin treatment was performed for 24 and 48 hours. At the 24th and 48th hours, photographs of the spheroids were taken, the spheroid numbers were counted, and their diameters were measured in area of 160x160 µm, using the ImageJ v1.47 software (Figure-1). Also, CCK-8 cell viability and ELISA tests were performed.

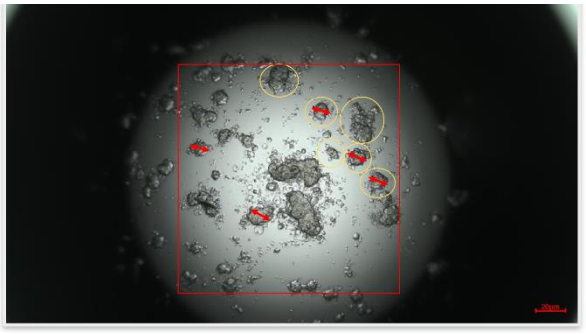

Figure-1. The evaluation of spheroid morphology using ImageJ v1.47 software. Square: Counting area (160x160 µm). Circles: Spheroid formation, Arrows: Spheroid diameter. Scale bar: 20 µm.

CCK-8 Assay

After spheroid formation in the presence of doxorubicin, cell viability was determined using Cell Counting Kit-8 (CCK-8, ab228554, Abcam). Spheroids treated with doxorubicin were collected and transferred to 96-well cell culture plates (50 spheroids/100 µL medium/well). To each well, 100 µL of fresh medium was added, followed by 10 µL of CCK-8 solution, and incubated for 4 hours at 37^0C, 5 % CO_2 conditions. At the end of the incubation, absorbance changes were detected at 460 nm using a wavelength-selective microplate reader spectrophotometer (EPOCH2, BioTek, USA). The experiment was repeated thrice.

ELISA Assay

After treatments, Caspase-1 (EH0595, Fine Test) and Gasdermin D (GSDMD) (EH8956, Fine Test) protein levels were analyzed in the cells using commercially available Enzyme-Linked Immunoassay (ELISA) kits prepared according to the Sandwich method. As a result of the treatment durations, the spheroids were collected, and cell lysates were obtained according to the instructions of the ELISA kits. Following the protocols, protein levels were measured based on the color change resulting from the reactions, which were detected at a wavelength of 450 nm using a microplate reader. Differences between the groups were plotted as absorbance values, and statistical analysis was performed.

Statistical Analysis

The obtained data were analyzed using the repeated-measures ANOVA test in GraphPad software. To determine the differences between groups, the Tukey-Kramer multiple comparisons test was used. The results are presented as mean

± standard deviation. A P-value of < 0.05 was considered statistically significant (18).

RESULTS

Spheroid Morphology

The number of spheroids and their morphology were evaluated using ImageJ v1.47 software, and the results were given in Table 1. The highest number of spheroids was observed in the control group. A concentration-dependent decrease in spheroid number was detected with increasing doses of doxorubicin. A statistically significant reduction was particularly noted at the 1 µM concentration (P<0.05). At 100 µM, cellular organization was disrupted, and spheroid formation was completely inhibited (Figure-2 and Figure-3). When comparing the effects of doxorubicin at 24 and 48 hours, no statistically significant differences were observed.

Table-1. Morphological parameters of MCF-7 spheroids in all concentrations of doxorubicin.

	Spheroid number		Spheroid diameter (µm)	
Concentrations	*24 h*	*48 h*	*24 h*	*48 h*
Control	24.5 ± 5.5 [a]	25.1 ± 5.0 [a]	35.7 ± 8.8 [a]	48.8 ± 12.1 [a]
0.1 µM	22.13 ± 3.8 [a]	23.2 ± 3.4 [a]	32.6 ± 8.2 [a]	39.5 ± 11.2 [b]
1 µM	15.8 ± 3.1 [b]	16.4 ± 3.5 [b]	26.4 ± 7.5 [b]	28.6 ± 8.7 [b]
5 µM	9.6 ± 1.7 [b]	10.9 ± 2.0 [b]	21.7 ± 7.6 [b]	19.7 ± 4.1 [b]
25 µM	5.1 ± 1.4 [b]	6.6 ± 1.7 [b]	17.2 ± 6.3 [b]	12.6 ± 2.7 [b]
100 µM	1.0 ± 0.0 [b]	1.0 ± 0.0 [b]	5.0 ± 0.0 [b]	4.0 ± 0.0 [b]

Data are expressed as mean± standard deviation. The same letters in the same row indicate similarity between groups and different letters indicate difference between groups. P < 0.001.

Measurement of spheroid diameters revealed that in accordance with the reduction in spheroid numbers, increasing drug concentrations led to a decrease in spheroid size (Table-1). As the drug concentration increased, spheroid diameters decreased, correlating with the reduction in spheroid number. In the control group, the mean spheroid diameter was 35.7 ± 8.8 µm at 24 hours and 48.8 ± 12.1 µm at 48 hours, suggesting that cells continued to aggregate and grow over time. Following treatment with 1 µM doxorubicin, a noticeable decrease in spheroid size was observed. At the highest concentration (100 µM), instead of well-defined spheroids, only disorganized cell clusters were present (Figure-2 and Figure-3).

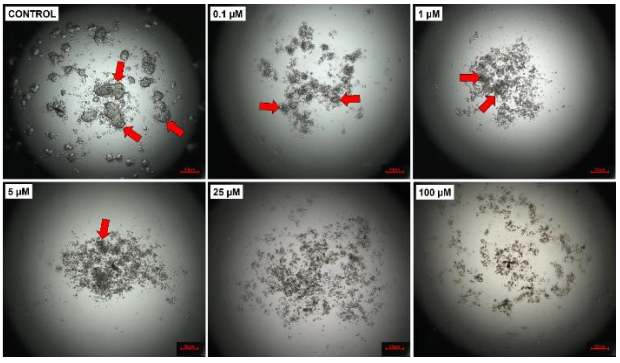

Figure-2. Effects of doxorubicin on spheroid number, diameter, and morphology at 24 hours. Arrows: Spheroids. Scale bars: 20 µm.

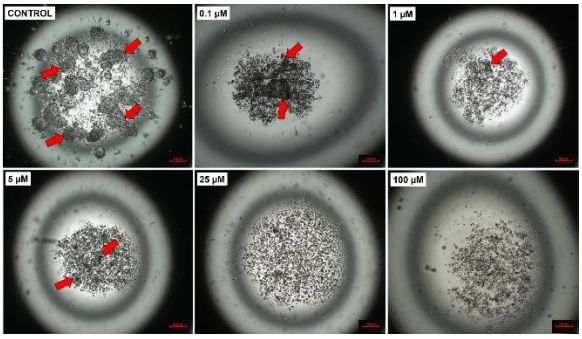

Figure-3. Effects of doxorubicin on spheroid number, diameter, and morphology at 48 hours. Arrows: Spheroids. Scale bars: 20 µm.

Cell Viability

Cell viability decreased in a concentration- and time-dependent manner following treatment with doxorubicin (Figure-4). In the control group, cell viability was maintained at 100% at both 24 and 48 hours. Exposure to 0.1 µM doxorubicin resulted in minimal cytotoxicity, with cell viability values of 96.36% and 95.66% at 24 h and 48 h, respectively. At 1 µM, a notable reduction in viability was observed, particularly at 48 hours (73.36% at 24 h vs. 57.39% at 48 h), indicating a time-dependent increase in cytotoxic effects. A more pronounced decrease in viability was observed at 5 µM, with viability dropping to 37.11% at 24 h and 28.83% at 48 h (***P<0.001). A significant reduction in cell viability was observed at higher concentrations, consistent with a dose-dependent cytotoxic effect. At 25 µM,

values decreased to 17.21% (24 h) and 14.89% (48 h), and at 100 µM, viability was reduced to 14.38% and 12.24%, respectively (***P<0.001). These findings demonstrate that doxorubicin induces a strong cytotoxic response in a dose- and time-dependent manner, with significant reductions in cell viability observed at concentrations ≥1 µM.

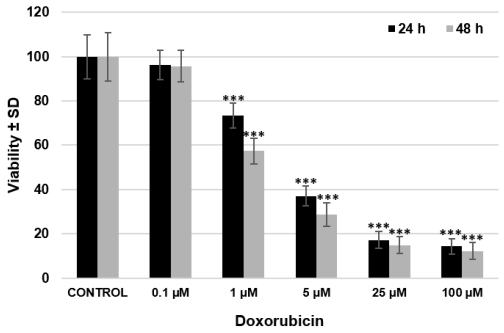

Figure-4. The viability levels of MCF-7 spheroids in the presence of doxorubicin at 24th and 48th hours via CCK-8 assay. ***P<0.001.

Caspase-1 and GSDMD Protein Levels

Caspase-1 protein levels, expressed in ng/mL, increased in a dose-dependent manner following doxorubicin treatment (Figure-5A). In the control group, Caspase-1 levels were 0.40 ng/mL at 24 hours and 0.39 ng/mL at 48 hours, showing minimal fluctuation over time. Treatment with 0.1 µM doxorubicin resulted in a slight increase (0.55 ng/mL at 24 h and 0.58 ng/mL at 48 h), which indicates early activation of Caspase-1. At higher concentrations, Caspase-1 levels continued to rise. Notably, at 1 µM, levels reached 0.85 ng/mL at 24 h and 0.87 ng/mL at 48 h. A more pronounced elevation was observed at 5 µM (1.05 ng/mL at 24 h and 1.12 ng/mL at 48 h), and the highest expression was detected at 25 µM (1.45 ng/mL at 24 h and 1.35 ng/mL at 48 h) (***P<0.001). These data suggest that doxorubicin induces Caspase-1 expression in a concentration-dependent manner, with peak activation observed at 25 µM. The consistent increase in Caspase-1 levels over time and with increasing drug concentrations indicates a potential role for doxorubicin in promoting inflammatory or pyroptotic pathways.

Gasdermin D (GSDMD) protein levels increased in a concentration-dependent manner following doxorubicin treatment (Figure-5B). In the control group, GSDMD expression levels were relatively low, measured at 0.25 ng/mL at 24 hours and 0.22 ng/mL at 48 hours. Minimal changes were observed at 0.1 µM doxorubicin (0.28 ng/mL at 24 h; 0.25 ng/mL at 48 h) (**P<0.01), indicating limited activation of GSDMD at this concentration. However, a progressive increase in GSDMD levels was evident at higher concentrations. At 1 µM, protein levels rose to 0.35 ng/mL and 0.37 ng/mL at 24 and 48 hours, respectively. This increase continued with 5 µM treatment, reaching 0.56 ng/mL at 24 h and 0.57 ng/mL at 48 h. The highest expression levels were observed at 25 µM, with 0.70 ng/mL at 24 h and 0.72 ng/mL at 48 h (***P<0.001). These results indicate that doxorubicin induces the upregulation of GSDMD in a dose-dependent manner, suggesting the activation of pyroptotic signaling pathways as part of the cellular response to chemotherapeutic stress.

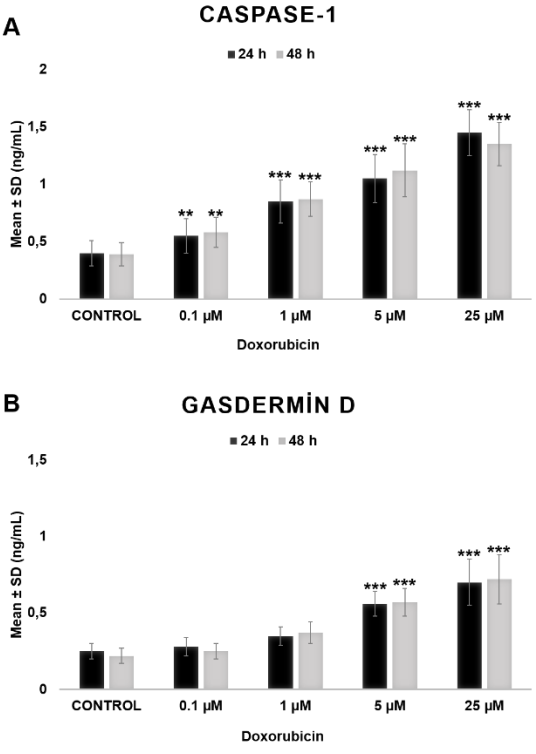

Figure-5. Caspase-1 (A) and Gasdermin D (B) protein levels following doxorubicin treatment. Caspase-1 (A) and Gasdermin D (B) levels were measured at 24 and 48 hours after exposure to increasing concentrations of doxorubicin (0.1–25 µM). Data are shown as mean ± SD. **P < 0.01; ***P < 0.001.

DISCUSSION

This study demonstrates that doxorubicin significantly affects breast cancer spheroid morphology and promotes the expression of pyroptosis-associated proteins Caspase-1 and GSDMD in a dose-dependent manner. The observed decrease in both spheroid number and size with increasing concentrations of doxorubicin indicates a strong cytotoxic effect on cellular aggregation and structural integrity within the 3D tumor model. The complete disruption of spheroid formation and presence of disorganized cell clusters suggest that high-dose doxorubicin may not only inhibit proliferation but also compromise the architecture necessary for spheroid development.

It has been demonstrated that doxorubicin treatment induces epithelial-mesenchymal transition (EMT) related changes in mammary tumor spheroids, characterized by decreased E-cadherin and increased vimentin expression. This phenotypic transition is associated with increased migratory capacity and potential metastatic behavior, yet paradoxically, doxorubicin also reduced spheroid size, viability, and cell migration. These findings suggest that while doxorubicin effectively impairs tumor growth, it may inadvertently promote a more invasive phenotype through EMT induction (19). In contrast, Liu et al. (2016) explored the therapeutic potential of xanthohumol, a prenylated chalcone, in doxorubicin-resistant MCF-7/ADR cells. Their results revealed that xanthohumol significantly reduced both cell viability and cancer stem cell (CSC) markers, suggesting its potential as a chemo sensitizing agent capable of reversing drug resistance, this is particularly significant, given the well-established role of CSCs in mediating resistance and recurrence (20). Another study focused on the metabolic adaptation of MCF-7 spheroids to doxorubicin, particularly the upregulation of phase I drug-metabolizing enzymes. Their findings underscore the importance of cellular metabolism in chemoresistance and point to the dynamic nature of enzyme expression in response to sustained drug exposure. These results emphasize the need for combinatorial approaches that target not only the tumor cells but also their adaptive mechanisms (21). In our study, we observed that doxorubicin adversely affected spheroid morphology, leading to a reduction in both spheroid diameter and number.

Pyroptosis, a form of inflammatory programmed cell death, has emerged as a novel and promising mechanism for targeting cancer cells, particularly those exhibiting resistance to conventional therapies. This regulated cell death pathway, mediated primarily by the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3)/Caspase-1/GSDMD axis, not only leads to cellular destruction but also enhances anti-tumor immunity through the release of damage-associated molecular patterns (DAMPs) (22-24). The reviewed studies collectively reinforce the potential of exploiting pyroptosis in breast cancer treatment, especially within MCF-7 cell models. It has been demonstrated that photodynamic therapy (PDT) using phthalocyanine zinc induces both apoptosis and pyroptosis in MCF-7 cells via Caspase-1 activation. Their data confirm Caspase-1 as a critical effector molecule that determines the mode of cell death following oxidative stress. Notably, this dual cell death induction may enhance therapeutic outcomes by simultaneously eliminating both proliferative and therapy-resistant cells (10). Another study highlighted the synergistic effects of tetrandrine and adriamycin in reversing multidrug resistance through regulation of the NLRP3/Caspase-1/GSDMD signaling pathway. This combinatorial approach supports the idea that modulation of pyroptosis-related proteins can sensitize resistant cancer cells to chemotherapeutics and potentially prevent relapse (25). TNF-α triggers mitochondrial dysfunction in MCF-7 cells, leading to pyroptosis via the canonical NLRP3 pathway. This finding not only links inflammation to pyroptotic activation but also suggests mitochondrial damage as a critical upstream regulator, broadening the understanding of intrinsic pyroptotic pathway triggers in cancer cells (26).

Together, these studies emphasize the multifaceted potential of pyroptosis as an anticancer mechanism in 2D culture conditions. In our study, the effect of doxorubicin on pyroptosis was investigated using a spheroid model. The morphological changes were paralleled by a significant and concentration-dependent increase in Caspase-1 and GSDMD levels, supporting the hypothesis that doxorubicin can activate pyroptotic pathways in cancer cells. Caspase-1 is a key mediator of inflammasome activation, and its role in the cleavage of GSDMD is well-established as a critical step in initiating pyroptotic cell death. The peak expression of both Caspase-

1 and GSDMD supports a model in which higher doses of doxorubicin may trigger not only apoptotic but also inflammatory forms of cell death. Notably, the consistent rise in protein levels over time, with no significant difference between 24 and 48 hours may reflect a sustained activation of these pathways in response to chemotherapeutic stress.

CONCLUSION

In conclusion, this study provides evidence that doxorubicin not only disrupts spheroid architecture but also activates key components of the pyroptotic pathway in breast cancer cells. Doxorubicin exerts a significant impact on breast cancer spheroid morphology and induces pyroptotic cell death in a dose-dependent manner. Increasing concentrations of doxorubicin led to a marked reduction in spheroid number and size, ultimately disrupting cellular organization at the highest dose. Concurrently, the upregulation of Caspase-1 and GSDMD suggests the activation of pyroptosis as a complementary mechanism to doxorubicin's known cytotoxic effects. These findings highlight the potential role of pyroptotic pathways in enhancing the anti-tumor efficacy of chemotherapy and underscore the importance of further exploring inflammatory cell death as a therapeutic target. Utilizing 3D spheroid models provided a more physiologically relevant approach to evaluating these effects and may contribute to the development of more effective combination strategies in breast cancer treatment.

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