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Insulin Enhances the Viability of Doxorubicin-Treated Luminal A Breast Cancer T-47D Cells

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Abstract: Breast cancer continues to pose a significant public health challenge worldwide and ranks among the most commonly diagnosed cancers. Doxorubicin is widely employed as a first-line chemotherapeutic agent for breast cancer treatment. Nevertheless, chemoresistance remains a major challenge in breast cancer chemotherapy. Insulin contributes to chemoresistance through its mitogenic and prosurvival activities. To date, studies evaluating the effect of insulin on the viability of Luminal A breast cancer cells (T-47D) under doxorubicin exposure remain limited. This study investigated whether insulin supplementation influences the viability of doxorubicin-treated Luminal A breast cancer T-47D cells. An in vitro experiment was performed using T-47D cells exposed to varying concentrations of doxorubicin (3.13, 6.25, 12.5, 25, 50, and 100 µg/mL), either in the presence or absence of insulin supplementation. Cell viability was subsequently evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and analysed using a paired-samples t-test. Insulin supplementation consistently resulted in higher T-47D cell viability than the non-insulin condition at all doxorubicin concentrations tested ($p < 0.05$). Furthermore, the relative increase in viability between the two groups tended to rise with increasing doxorubicin concentrations. In conclusion, insulin affects the viability of Luminal A breast cancer T-47D cells exposed to doxorubicin, indicating reduced cellular sensitivity to the cytotoxic effects of doxorubicin.

Keywords: doxorubicin, insulin, breast cancer, T-47D cell, cell viability

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Introduction

Based on GLOBOCAN estimates, cancer accounted for approximately 19,976,499 cases worldwide in 2022. Indonesia contributed 408,661 diagnosed cases. In Indonesia, breast cancer had the highest incidence among all cancer types, reaching 66,271 diagnosed cases reported in 2022, while its mortality reached 22,598 deaths (Globocan, 2024).

Breast cancer refers to a malignant tumour that develops from breast tissue, most commonly originating in the mammary ducts or lobules and supporting tissues of the breast (Ketut & Kartika, 2022). The malignancy level of breast cancer can be determined based on its molecular type. Four major molecular subtypes of breast cancer have been identified, namely Triple-Negative Breast Cancer (TNBC), HER2-enriched, Luminal B, and Luminal A. This classification is determined by presence or absence of estrogen receptor (ER), progesterone receptor (PR), and Human Epidermal growth factor Receptor 2 (HER2) expression. Luminal A represents the

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most common molecular subtype of breast cancer, accounting for approximately 40–45% of all diagnosed cases. This subtype is characterized by positive expression of estrogen receptor (ER) and progesterone receptor (PR), typically with high PR levels, along with the absence of human epidermal growth factor receptor 2 (HER2) overexpression. Although the Luminal A subtype occurs more frequently, this subtype has a slower progression rate compared to other subtypes. This is evidenced by a Ki67 expression percentage of less than 20% compared to the Ki67 expression percentage in other subtypes (Sandhika, 2024; Taurin & Alkhalifa, 2020). Nevertheless, treatment remains essential in the clinical management of Luminal A breast cancer. Luminal A breast cancer is more responsive to endocrine therapy (Menon et al., 2024). However, chemotherapy is commonly considered in advanced-stage disease (Moo et al., 2018).

One chemotherapeutic agent to suppress tumorigenesis, including breast cancer, is doxorubicin (Ebrahimi et al., 2023;Nicoletto & Ofner, 2022). Owing to its demonstrated therapeutic efficacy, doxorubicin has received approval from the Food and Drug Adinistration (FDA) for use in breast cancer treatment (Liaqat et al., 2024). Doxorubicin's cytotoxic effects are mediated through several mechanisms, including DNA intercalation, inhibition of topoisomerase II, mitochondrial dysfunction, and increased production of reactive oxygen species, which can lead to oxidative damage. In addition to activating the mitochondrial apoptotic pathway, doxorubicin may induce various cellular responses and forms of cancer cell death, including senescence, autophagy, pyroptosis, ferroptosis, and necrosis (Kciuk et al., 2023). Nevertheless, as much as 90% of cancer-related deaths are associated with chemoresistance issues (Si et al., 2019). Doxorubicin chemoresistance occurs due to the modulation of microRNA (miR), namely, the modulation of miR-21.

Modulation of miR-21 triggers chemoresistance through the insulin signaling pathway. Overexpression of miR-21 in cancer cells enhances the activity of the phosphatidylinositol 3-kinase (PI3K) pathway within insulin signaling, resulting in suppression of apoptotic processes and promotion of cellular proliferation (Liu & Liu, 2021; Si et al., 2019; Ulfa, 2024). Research by Lykhova *et al*. (2023) demonstrated that MCF-7 cells incubated in a culture medium containing insulin and induced with the cytotoxic agent doxorubicin had higher cell viability compared to cells incubated in a culture medium without insulin (Lykhova et al., 2023). Besides MCF-7, there are other cell lines with more progressive protein expression, namely T-47D cells (Aka & Lin, 2012). MCF-7 and T-47D are commonly used Luminal A breast cancer cell lines in *in vitro* studies. Compared with MCF-7 cells, T-47D cells exhibit differences in the expression of cell-cycle regulatory proteins, including higher expression of cyclin D3, which is involved in the G1/S transition and prohibitin, which contributes to the regulation of cell proliferation (Aka & Lin, 2012). These characteristics may contribute to enhanced cellular survival and reduced susceptibility to apoptosis, potentially affecting doxorubicin sensitivity and supporting the use of T-47D as a relevant model for investigating chemoresistance. The findings of this study suggest that insulin may modulate the response of T-47D cells to doxorubicin, providing a basis for further investigation of the potential role of insulin signalling in chemotherapy response.

Materials and Methods

Experimental Design

An *in vitro* analytical experimental study employing a randomized post-test control group design was conducted at the Integrated Research Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. T-47D breast cancer cells were divided into insulin-supplemented and non-insulin groups, followed by doxorubicin treatment at serial concentrations of 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL. The selected doxorubicin concentration range (3.13–100 µg/mL) was based on a previously published *in vitro* study using T-47D breast cancer cells, in which doxorubicin was employed as a positive control across serial two-fold dilutions to evaluate dose-dependent cytotoxicity (Milliana et al., 2025). Recombinant human insulin was administered at a concentration of 0.2 U/mL, based on concentrations used in previous studies investigating the effects of insulin on T-47D cells (Annisa et al., 2022). Each treatment was performed in triplicate. This study received ethical clearance from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (Approval No. KE/FK/1214/EC/2024). An overview of the study procedure is depicted in **Figure 1.**

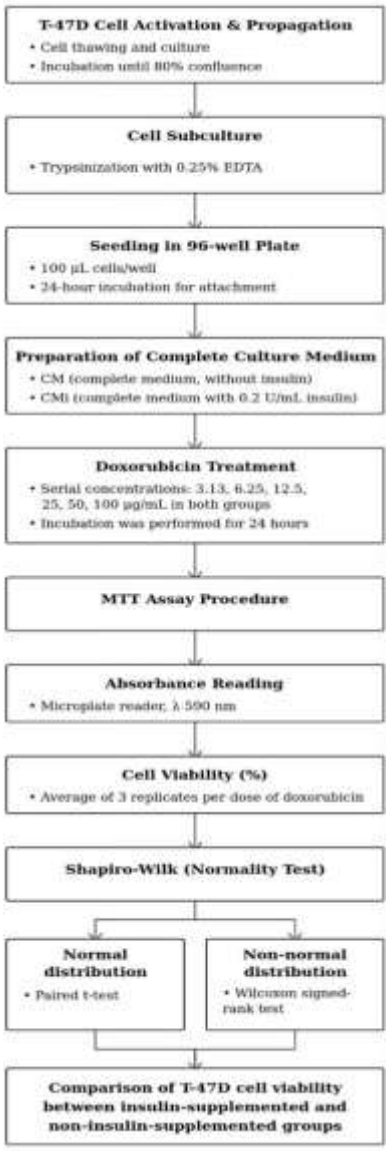

Figure 1. **Schematic overview of the experimental procedure.**

Materials

T-47D cell line was obtained from Integrated Research Laboratory of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. Other materials used in this study included doxorubicin, recombinant human insulin, RPMI 1,640 medium (ATCC Modification), penicillin–streptomycin, fungizone, foetal bovine serum (FBS), phosphate-buffered saline (PBS), 0.25% trypsin-EDTA, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent.

Cell Culture

T-47D cells were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 1% penicillin–streptomycin, and 0.5% fungizone. Following rapid thawing at 37°C, the cells were maintained under standard culture conditions (37°C, 5% CO_2) until reaching approximately 80% confluence and were subsequently harvested using 0.25% trypsin-EDTA and seeded into 96-well plates for subsequent experiments (Gertz, 2010).

Cytotoxicity Assay

A total of 1×10^4 T-47D cells suspended in 100 µL of complete medium were seeded into each well of a 96-well plate and incubated overnight at 37°C in 5% CO_2 to allow cell adherence.

Following overnight incubation, the culture medium was removed and replaced with complete medium either without insulin (CM) or supplemented with 0.2 U/mL recombinant human insulin (CMi). Cells in each condition were subsequently treated with doxorubicin at serial concentrations of 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL (Annisa et al., 2022; Lykhova et al., 2023). Cell control wells contained untreated cells, whereas media control wells contained culture medium without cells. All treatments were performed in triplicate and incubated for 24 hours in a humidified incubator maintained at 37°C and 5% CO_2, with incubation extended for an additional 24 hours if no cytotoxic effect was observed (Tunjung & Sayekti, 2019).

Following treatment, the medium was aspirated, and the cells were gently rinsed once or twice with 200 µL of phosphate-buffered saline (PBS). Subsequently, each well was supplemented with 50 µL of serum-free medium and 50 µL of MTT reagent, followed by incubation at 37°C in 5% CO_2 for 3 hours until purple formazan crystals were formed. After incubation, 150 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solvent was added to each well, followed by incubation at room temperature for 15 minutes. Absorbance was determined at 450 nm using a microplate Enzyme-Linked Immunosorbent Assay (ELISA) reader, where viable cells appeared purple and non-viable cells appeared yellow. The absorbance values obtained were subsequently converted into percentage cell viability relative to the control group using the equation below:

$$(\%)\ Cell\ Viability = \frac{(At\ -\ Ab)}{(Ac\ -\ Ab)} \text{x}\ 100\%$$

Notes: The absorbance in the treatment group is represented by At, the absorbance in the cell control group by Ac, and the absorbance in the blank medium by Ab.

Statistical Analysis

Experiments were conducted in triplicate, and data are expressed as mean ± SD. Group comparisons were analysed using a paired-samples t-test. Statistical

significance was defined as a p-value < 0.05. Data processing and statistical analyses were carried out using IBM SPSS Statistics for Windows version 24.0.

Results

The MTT assay was used to measure the impact of insulin supplementation on the viability of T-47D cells treated with doxorubicin. **Table 1** shows the proportion of viable cells in the insulin-treated group. On the other hand, **Table 2** displays the proportion of viable cells in the cells that were not treated with insulin. As shown in **Table 1** and **Table 2**, cell viability decreased with increasing concentrations of doxorubicin (3.13, 6.25, 12.5, 25, 50, and 100 µg/mL), indicating a dose-dependent cytotoxic effect. Despite this trend, the insulin-treated group consistently demonstrated higher percentages of cell viability compared to the non-insulin group across all tested concentrations. Morphological observations of T-47D cells following treatment are shown in **Figure 2**.

Table 1. Percentage of T-47D cell viability with insulin supplementation

Doxorubicin Concentration (µg/mL)	T-47D Cell Viability (%)			
	R1 (%)	R2 (%)	R3 (%)	Mean ± SD (%)
3.13	97.94	98.52	98.31	98.26 ± 0.29
6.25	96.86	96.53	96.47	96.62 ± 0.21
12.5	92.54	92.78	93.77	93.03 ± 0.65
25	84.62	86.71	86.23	85.85 ± 1.09
50	74.02	74.86	75.94	74.94 ± 0.96
100	61.27	61.94	62.95	62.05 ± 0.85

R: replicate; SD: standard deviation; µg/mL: microgram per mililiter.

Table 2. Percentage of T-47D cell viability without insulin supplementation

Doxorubicin Concentration (µg/mL)	T-47D Cell Viability (%)			
	R1 (%)	R2 (%)	R3 (%)	Mean ± SD (%)
3.13	90.18	88.62	85.71	88.17 ± 2.27
6.25	87.56	79.22	75.81	80.86 ± 6.04
12.5	66.25	62.62	59.69	62.85 ± 3.29
25	53.18	57.26	53.51	54.65 ± 2.27
50	49.45	46.50	40.31	45.42 ± 4.66
100	31.81	27.69	21.97	27.16 ± 4.94

R: replicate; SD: standard deviation; µg/mL: microgram per mililiter.

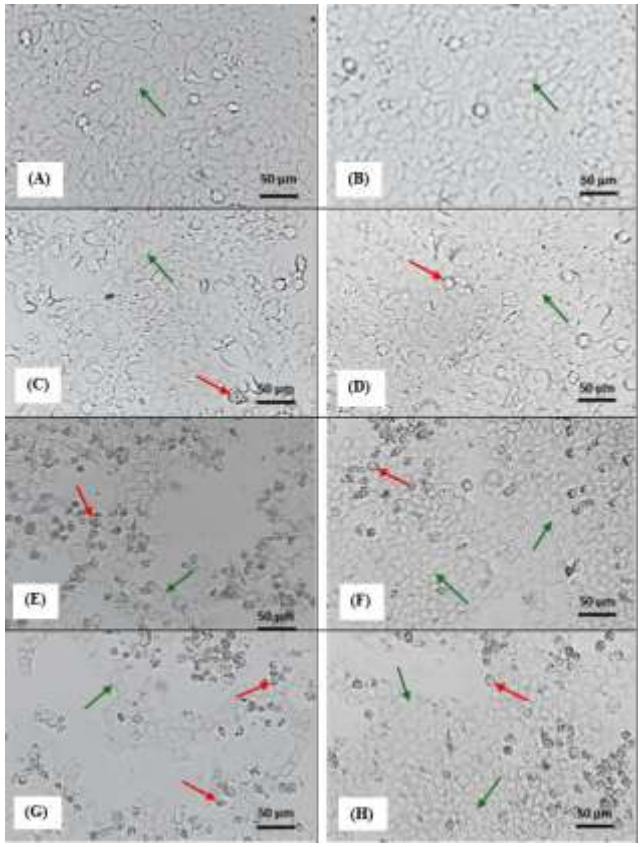

Figure 2. Morphology of T-47D cells after 24 hours of doxorubicin induction without or with insulin supplementation. Non-insulin control **(A);** Insulin control **(B)**; Non-insulin group + dox (6.25) **(C)**; Insulin-treated group + dox (6.25) **(D);** Non-insulin group + dox (25) **(E)**; Insulin-treated group + dox (25) **(F)**; Non-insulin group + dox (100) **(G)**; Insulin-treated group + dox (100**) (H**). Cell morphology was observed using an inverted microscope at 200× magnification. Viable cells are marked by green arrows, while cells showing apoptosis-like morphological changes are indicated by red arrows.

Microscopic observations (**Figure 2**) showed that control cells maintained normal morphology, characterised by a polygonal shape, intact cell membranes, preserved cell to cell adhesion, and firm attachment to the culture plate. Following exposure to doxorubicin for 24 hours, apoptosis-related morphological alterations, such as cell rounding and increased cellular density (darker appearance), and detachment from the plate surface, were observed. These apoptotic features were more prominent in the non-insulin group and increased with higher

concentrations of doxorubicin. In contrast, a higher number of viable cells was observed in insulin-treated cells.

Assessment of data distribution Showed that every variable satisfied the normalcy assumption ($p > 0.05$). As a result, a paired-samples t-test was used to examine group differences. The findings showed statistically significant variations in cell viability between insulin-treated and untreated cells across all tested concentrations of doxorubicin ($p < 0.05$) (**Table 3).**

Table 3. The paired-samples t-test results

Paired-Samples t-Test	
Group	**Sig**
[Dox (3.13) + insulin] & Dox (3.13)	.019
[Dox (6.25) + insulin] & Dox (6.25)	.043
[Dox (12.5) + insulin] & Dox (12.5)	.006
[Dox (25) + insulin] & Dox (25)	.001
[Dox (50) + insulin] & Dox (50)	.012
[Dox (100) + insulin] & Dox (100)	.009

Sig: significance

Furthermore, the mean percentage of cell viability in both groups is illustrated in **Figure 3**. The viability of T-47D cells was evaluated by MTT assay, and the results are presented as mean ± standard deviation (SD). The insulin-treated group consistently showed higher mean viability values compared to the non-insulin group.

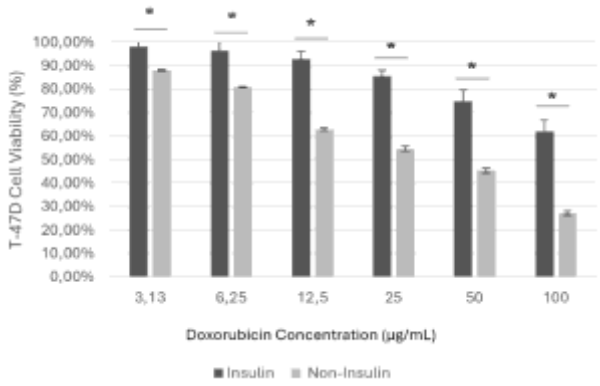

Figure 3. **Mean percentage of T-47D cell viability in insulin and non-insulin groups**. *$p < 0.05$, demonstrating a significant difference between the insulin and non-insulin groups based on a paired-samples *t*-test.

Discussion

Chemotherapeutic resistance persists a major obstacle to successful breast cancer management (Si et al., 2019). One of the factors contributing to chemoresistance is insulin. Physiologically, insulin is secreted by pancreatic β-cells and serves an essential role in glucose metabolism. Beyond its metabolic function, insulin exhibits mitogenic properties and is capable of activating several intracellular pathways that contribute to cellular growth and differentiation (Rahman et al., 2021). Previous *in vitro* investigations employing the MCF-7 breast cancer cell line have demonstrated that insulin can induce anti-apoptotic signalling and enhance cell survival capacity (Szablewski, 2024).

Effect of Insulin Supplementation on T-47D Cell Viability

This research demonstrates that insulin supplementation influences the response of T-47D cells to doxorubicin. As shown by the MTT assay findings **(Figure 3)**, T-47D cells exposed to insulin supplementation exhibited greater viability than cells cultured without insulin across all tested concentrations of doxorubicin. Despite these effects, cell viability declined progressively in both groups as the concentration of doxorubicin increased, indicating that the cytotoxic effect of doxorubicin increased with concentration. This pattern is compatible with the established mechanism of action of doxorubicin as a topoisomerase II inhibitor, which induces DNA damage and intracellular oxidative stress, ultimately activating apoptotic pathways and reducing cell viability (Muna & Maulidina, 2022; Si et al., 2019).

Although both groups exhibited decreased viability, the insulin-supplemented group consistently maintained higher viability values at all tested concentrations of doxorubicin. The difference is statistically significant (**Table 3**) and shows a comparison of the increasing multiples of viability, namely 1.1; 1.2; 1.5; 1.6; 1.6; up to 2.3 times at 3.13; 6.25; 12.5; 25; 50; and 100 µg/mL of doxorubicin. At lower concentrations of doxorubicin, the difference in viability between groups was relatively small, likely due to limited cell death. Nevertheless, with increasing doxorubicin concentrations, cell viability decreased more markedly in the non-insulin group, while insulin-treated cells consistently exhibited greater viability. These observations indicate that insulin may contribute to preserving cell viability through prosurvival mechanisms under doxorubicin induced cytotoxic stress.

Similar findings have been reported in previous investigations showing that MCF-7 cells exposed to doxorubicin in the presence of insulin exhibited higher viability compared to those treated with doxorubicin alone (Lykhova et al., 2023). Comparable observations have been reported in studies involving T-47D breast cancer cells, where insulin was shown to enhance cellular proliferation and differentiation (Ulfa, 2024). Collectively, the available evidence indicates that insulin

alters breast cancer cells' susceptibility to chemoresistance through prosurvival effects that support cellular proliferation and survival. At the molecular level, these effects are primarily mediated through triggering the PI3K/AKT and MAPK signaling cascades (Rose & Vona-Davis, 2012).

Potential Molecular Mechanisms Underlying the Effect of Insulin

The molecular mechanisms underlying the observed effect of insulin on T-47D cell viability were not directly investigated in the present study. Nevertheless, previous studies provide several potential mechanistic explanations for the observed increase in cell viability, which are discussed below in the context of the existing literature.

Phosphatidylinositol 3-kinase (PI3K) is activated when insulin binds to its receptor, starting a signaling network involving insulin receptor substrate (IRS) proteins. Phosphatidylinositol 4,5-bisphosphate (PIP2) is converted by PI3K into phosphatidylinositol 3,4,5-triphosphate (PIP3). This increase in PIP3 attracts and activates 3-phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates Akt. Phosphatase and tensin homolog (PTEN), a phosphatase that reverses PIP3 formation by converting it to PIP2, typically restrain the pathway. In cancer cells, overexpression of miR-21 suppresses PTEN expression, resulting in PIP3 accumulation and persistent Akt activation, thereby enhancing prosurvival signalling (Le et al., 2023).

By suppressing apoptotic signaling, Akt is essential for mediating the pro-survival effects of insulin. Anti-apoptotic proteins like Bcl-2 and Bcl-xL are expressed when Akt is activated. This suppresses intrinsic apoptosis and increases resistance to doxorubicin-induced cell death (Rahman et al., 2021). Moreover, Akt regulates the activity of Forkhead box O (FOXO) transcription factors, leading to reduced transcription of genes involved in apoptosis. Consequently, the suppression of these pro-apoptotic targets supports the maintenance of survival signaling in insulin-stimulated cells (Nigi et al., 2018).

Beyond apoptosis inhibition, Akt activation also induces mTORC1 via inhibition of the TSC1/TSC2 complex. mTORC1 activation subsequently induces phosphorylation of p70S6K and 4E-BP1, causing in increased protein translation and cell growth (Rahman et al., 2021). Phosphorylation of p70S6K also enhances survivin expression, an anti-apoptotic protein that supports cell survival (Chen et al., 2016). Furthermore, PI3K mediated activation of mTORC2 promotes phosphorylation of Akt at Ser473, thereby strengthening anti-apoptotic signalling and reducing FOXO mediated transcription of pro-apoptotic genes (Kazyken et al., 2019).

Insulin signalling via the PI3K/Akt pathway is also linked to nuclear factor-κB (NF-κB) activation. Activated Akt stimulates IκB kinase, resulting in IκB phosphorylation and degradation, which permits NF-κB nuclear translocation and upregulation of genes essential for cell viability and anti-apoptotic processes (Tang et al., 2016).

Apart from the PI3K/Akt signaling pathway, insulin can stimulate the MAPK/Erk cascade through the recruitment of adaptor proteins, including GRB2 and Son of Sevenless (SOS), which subsequently activate Ras. Activated Ras initiates a downstream phosphorylation cascade involving MEK1/2, Erk1/2, and C-Raf. Following phosphorylation, Erk1/2 moves to the nucleus and controls the expression of genes linked to cellular proliferation and cell-cycle progression (Rahman et al., 2021).

Another pathway underlying the prosurvival effects of insulin is the direct control of caspase-9 following PI3K/AKT and MAPK/Erk signaling activation. PI3K/Akt activation results in phosphorylation of caspase-9, inhibiting the caspase cascade and suppressing intrinsic apoptosis. Similarly, MAPK/Erk contributes to caspase-9 phosphorylation through cytosolic substrate modulation. These processes reduce apoptotic activity and further reinforce insulin-mediated survival signalling (Le et al., 2023; Tang et al., 2016).

Taken together, these pathways may contribute to the higher viability observed in insulin-supplemented T-47D cells. However, their involvement cannot be confirmed from the present findings because pathway activation and related molecular changes were not directly assessed.

Biological Characteristics and Chemoresistance of T-47D Cells

Beyond the effects mediated by insulin, the biological characteristics of T-47D cells and the metabolic effects of doxorubicin also contribute to chemoresistance. T-47D cells express mutant p53, which is correlated with decreased therapeutic response to chemotherapy and poorer clinical outcomes (Setiawati et al., 2011). Furthermore, doxorubicin reduces AMP-activated protein kinase (AMPK) activity, inhibiting GLUT-4 translocation and decreasing glucose uptake, which may lead to increased extracellular glucose levels and support cancer cell survival and proliferation (de Lima Junior et al., 2016). The interaction of these factors further enhances resistance of T-47D cells to cytotoxic chemotherapy.

Study Limitations

From a clinical perspective, these findings suggest that insulin may reduce the sensitivity of Luminal A breast cancer cells to doxorubicin. However, the generalisability of these findings is limited by several factors. First, this study employed a single T-47D cell line and evaluated only one insulin concentration, which may not fully represent the heterogeneity of Luminal A breast cancer or the potential effects of different insulin concentrations. Second, the molecular mechanisms underlying the observed effect were not directly investigated. Therefore, the involvement of signaling pathways such as PI3K/Akt, MAPK/ERK, NF-κB, mTOR, FOXO, and caspase-related pathways remains a literature-supported hypothesis rather than a mechanism demonstrated by the present study. Third, cell viability was assessed using a single metabolic assay, and molecular or functional assays were not performed to further validate the observed cellular response. Fourth, quantitative drug-sensitivity parameters, such as the half-maximal inhibitory concentration (IC50), were not determined. In addition, lower doxorubicin concentrations that more closely reflect clinically achievable plasma levels were not included because the primary objective of this study was to evaluate the effect of insulin on cell viability across a dose-dependent cytotoxic range using an established *in vitro* experimental model. Furthermore, the use of relatively high doxorubicin concentrations may limit the direct translation of these findings to clinical settings.

Therefore, further studies are warranted to clarify the role of insulin in doxorubicin response, particularly in Luminal A breast cancer. These should incorporate diverse breast cancer cell lines to better represent tumour heterogeneity, as well as a range of insulin concentrations to characterise dose-dependent effects. *In vivo* models should additionally be considered to validate these findings in a more physiologically relevant context. Complementary cytotoxicity assays and molecular validation of the proposed signaling pathways, including PI3K/Akt, MAPK/ERK, NF-κB, mTOR, FOXO, and caspase-related pathways, are equally needed to substantiate the observed cellular responses. Future investigations would benefit from determining quantitative drug-sensitivity parameters such as IC50.

Conclusions

This study demonstrates that insulin supplementation preserves the viability of Luminal A breast cancer (T-47D) cells following doxorubicin exposure, suggesting reduced cellular susceptibility to doxorubicin-induced cytotoxicity. These findings indicate that insulin-mediated signalling may influence the response of breast cancer cells to chemotherapy and provide further insight into mechanisms potentially involved in chemoresistance. Further studies using clinically relevant drug concentrations, and varying insulin doses are needed to further characterise the dose-dependent effects of insulin on doxorubicin response. *In vivo* studies, complementary cytotoxicity assays, and molecular investigations of the proposed signalling pathways are also warranted to validate these findings and clarify their clinical relevance, including quantitative measures of drug sensitivity such as IC50.

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