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ORIGINAL ARTICLE

Caffeine Modulates Cell Death and Telomerase Activity in Triple-negative Breast Cancer Cells

Qanita Hana Amira[1], Shadira Anindieta Irdianto[1], Fadilah Fadilah[2], Retno Lestari[3], Fadhillah Fadhillah[3], Anom Bowolaksono[3], Astari Dwiranti[3,4]*

1. Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok Campus, Depok, 16424, Indonesia.
2. Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta, 10440, Indonesia.
3. Cellular and Molecular Mechanisms in Biological System Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok Campus, Depok, 16424, Indonesia.
4. Institute for Advanced Sustainable Materials Research and Technology (INA-SMART), Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok 16424, Indonesia.

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***Corresponding**:
Astari Dwiranti
Address:
Cellular and Molecular Mechanisms in Biological System Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok Campus, Depok, 16424, Indonesia
E-mail:
astari.dwiranti@sci.ui.ac.id

ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive subtype defined by the lack of estrogen receptors (ER), progesterone receptors (PR), and HER2 expression, resulting in limited therapeutic options. Given this challenge, this study explores caffeine, a widely consumed stimulant, as a potential anticancer agent, particularly for TNBC. Although caffeine has demonstrated stimulatory and inhibitory effects on telomerase in other cancer types, its influence on telomerase activity in TNBC remains uncharacterized. This study investigates the impact of caffeine concentrations (10, 15, and 20 mM) on cell viability, proliferation, apoptosis, ultrastructure, and the expression of apoptosis-related genes (*BAX*, *BCL2*, *CASP8*) and telomerase activity (*hTERT*) in MDA-MB-231 cells. Our findings showed that caffeine significantly reduces cell viability and induces early apoptosis with a dose-dependent effect. Morphological changes consistent with early apoptosis were observed, and an increased *BAX/BCL2* ratio indicated the activation of the intrinsic apoptosis pathway. Additionally, caffeine exhibited upregulation of *hTERT* mRNA expression, which may reflect a compensatory response to cellular stress induced by caffeine. These results underscore the multifaceted effects of caffeine on TNBC cells, highlighting its potential not only as an apoptosis inducer but also as a modulator of telomerase activity. Given its accessibility, low toxicity, and established safety profile, caffeine presents an exciting avenue for further research as a complementary or standalone therapeutic strategy for TNBC.

Keywords: Apoptosis, Caffeine, MDA-MB-231, Telomerase, ultrastructure

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Introduction

Triple-negative breast cancer (TNBC) is the most resistant breast cancer subtype to targeted therapies (1, 2). TNBC is a proliferative cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression (3). The lack of expressions of these receptors renders TNBC resistant to conventional targeted treatments for other breast cancer subtypes, including hormonal therapy or anti-HER2 therapy (4). Consequently, TNBC patients face limited treatment options and a less favorable prognosis (5). In light of these challenges, the development of innovative and effective therapies is critically needed. One promising approach is the use of *in vitro* models, for instance, the MDA-MB-231 cell line, which is a commonly used TNBC cellular model due to its characteristics to match the aggressive and proliferative nature of TNBC in clinical practice (6).

While advancements in chemotherapy, drug therapy, and surgery have enhanced the quality of life for TNBC patients, these treatments are frequently associated with adverse toxic effects. In this context, the application of natural compounds has garnered increasing attention as a potential alternative in cancer therapy due to their availability, effectiveness, minimal side effects, and significant roles in both cancer prevention and treatment (7). One such compound is caffeine, a natural alkaloid found in plants, which has been reported to exhibit several antitumor activities, including inhibiting proliferation, inducing apoptosis, and altering the morphology of cancer cells (7, 8). A study by Liu et al. (9) demonstrated that caffeine significantly suppresses cell growth and viability in gastric cancer cell lines through apoptosis induction.

The uncontrolled growth of cells which is the key hallmark of cancer can be caused by the ability to avoid the apoptosis pathway (10). Therefore, targeting the apoptotic pathways with caffeine represents a potential therapeutic approach to counteract the uncontrolled proliferation of cancer cells (11). Apoptosis generally proceeds through two primary pathways: intrinsic and extrinsic. The intrinsic pathway is triggered by an increase in Bcl-2 Associated X-protein (*BAX*) and a decrease in B-Cell Leukemia/Lymphoma 2 (*BCL2*), leading to the release of cytochrome c from the mitochondria, whereas the extrinsic pathway, initiated by the binding of ligands to tumor necrosis factors (TNF) receptors, further activates the initiator of caspases (*CASP8*) and effector caspases (*CASP3* and *CASP7*) (11, 12).

In addition to its role in influencing apoptotic pathways, caffeine has also been reported to inhibit telomerase activity by shortening the length of telomeres in healthy neuronal cells (13). Human telomerase reverse transcriptase (hTERT), a protein-encoding telomerase activity, is recognized for its essential role in regulating cell death. However, limited studies on hTERT regulation in cancer suggest that caffeine's effects on its expression vary by cancer type. For instance, caffeine upregulates hTERT expression and telomere elongation in HeLa, MCF-7, and HepG2 cells (13). In contrast, Steiner et al. reported no significant association between caffeine and telomere length in prostate, lung, and colorectal cancers (14). These discrepancies highlight the need for further investigation into caffeine’s effects on telomerase activity in TNBC.

Through its interactions with BCL2 family and p53 proteins, hTERT influences apoptosis by promoting the oligomerization of pro-apoptotic proteins BCL2-antagonist/killer (BAK) and BAX, leading to the release of cytochrome c, thereby initiating the caspase cascade and leading to cell death (15). Considering that over 90% of cancer cells exhibit elevated telomerase activity, enabling unlimited replication potential, caffeine holds promise as an agent that not only induces apoptosis but also inhibits telomerase activity (16). The activation of these apoptotic pathways, in turn, results in distinct morphological alterations, including membrane blebbing and the formation of apoptotic bodies (17). Understanding these ultrastructural changes is crucial, as they provide critical insights into the processes of apoptosis and the effectiveness of anticancer therapies.

The morphological changes in cancer cells can be examined through ultrastructural analysis by utilizing high-resolution imaging, including scanning electron microscopy (18). Nonetheless, research on the effects of caffeine on apoptosis, along with its connection to telomerase activity and ultrastructural changes in TNBC cells, remains insufficiently understood. Further investigation is essential to elucidate these complex interactions, which could pave the way for the development of more effective therapeutic strategies targeting apoptotic pathways and telomerase activity in TNBC.

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Methods

Materials

MDA-MB-231 cell line (Cat. No. 92020424) was purchased from the European Collection of Authenticated Cell Cultures (ECACC) with passage number 39. Caffeine (Cat. No. C0750), Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. D6429), fetal bovine serum (FBS) (Cat. No. F7942), antibiotic-antimycotic solution (Cat. No. A5955), trypsin/EDTA solution (Cat. No. T4299), phosphate-buffered saline (PBS) (Cat. No. P2272), poly-L-lysine (Cat. No. P8920) were purchased from Sigma–Aldrich (Darmstadt, Germany). Doxorubicin (Cat. No. DD0813) was purchased from Global Onkolab Farma (Jakarta, Indonesia). Counting Kit-8 (Cat. No. CK04) was obtained from Dojindo (Japan).

Annexin V-FITC/Propidium Iodide (PI) Kit (Cat. No. CV0001) was purchased from Assay Genie (Dublin, Ireland). Glutaraldehyde (Cat. No. G5882), osmium tetroxide (Cat. No. 75632), and hexamethyldisilazane (HMDS) (Cat. No. 8.04324) were purchased from Gibco (New York, USA). Blood/Cell Total RNA Mini Kit (Cat. No. RBD100) was purchased from Geneaid Biotech Ltd. (New Taipei, Taiwan). ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Cat. No. FSQ-301) and KOD SYBR® qPCR Mix (Cat. No. QKD-201) were purchased from Toyobo, Co. Ltd. (Shanghai, China).

Cell culture

MDA-MB-231 cells were cultured in high-glucose DMEM with 10% FBS and 1% antibiotic-antimycotic solution (1×) under optimal conditions in a humidified incubator at 37°C with 5% CO_2 . Before initiating the experiment, the cells were harvested at approximately 70–80% confluency to provide consistent growth rates across experimental groups. Only cells with passages 40–45 were used for data collection (19).

Viability assay

The impact of caffeine on MDA-MB-231 cell viability and proliferation was assessed using the CCK-8 assay according to the manufacturer's guidelines (20). Briefly, 10.000 cells/well were seeded and pre-incubated in DMEM for 24 hours. Subsequently, the medium was replaced with 100 µL of fresh medium containing caffeine at caffeine concentrations of 10, 15, and 20 mM for the experimental group. The control group consists of negative control (untreated), vehicle control (DMSO), and positive control (0.9 µM doxorubicin). The cells were incubated at 37°C with 5% CO_2 for 24 hours. After treatment, 10 µL of CCK-8 solution was added to each well, followed by incubation at 37°C for 3.5 hours. Absorbance was then measured at 450 nm using a BioTek 800 TS Absorbance Reader.

Apoptosis assay

To evaluate apoptosis, MDA-MB-231 cells were plated in a 6-well plate at a density of 2×10^6 cells per well and subsequently treated with caffeine. Following 24 hours of incubation, the cells were harvested by trypsinization, collected by centrifugation at 300 × g for 5 minutes, and washed twice with PBS. The cell pellet was first resuspended in 100 µL of Annexin V Binding Buffer and transferred into a 5 mL test tube. Next, 5 µL of Annexin V-FITC and 10 µL of PI solution were added. The mixture was gently vortexed and left to incubate in the dark at room temperature (25°C) for 15 minutes. After incubation, the stained cells were centrifuged at 400 × g for 5 minutes at 25°C. Finally, the cell pellets were resuspended in 400 µL of 1X Annexin V Binding Buffer and immediately analyzed using the AGILENT NovoCyte Advanteon Flow Cytometer (21, 22).

Ultrastructure Analysis

For ultrastructure analysis, glass coverslips were cleaned with ethanol and coated with poly-L-lysine for 15 minutes to promote cell attachment. The coverslips were then rinsed with PBS and air-dried. MDA-MB-231 cells were seeded onto the treated coverslips at a density of 2×10^5 cells and incubated for 24 hours to allow attachment. Next, the cells were treated with caffeine for 24 hours. After treatment, the cells were washed with PBS and fixed in 2.5% glutaraldehyde for 30 minutes at room temperature. Post-fixation was performed using 1% osmium tetroxide (OsO_4) for 15 minutes. The cells were gradually dehydrated using a series of ethanol dilutions (70%, 80%, 90%, and 100%) for 10 minutes in each concentration. To complete dehydration, hexamethyldisilazane (HMDS) was applied until the samples were fully dried. The dried samples were then sputter-coated with an Au to enhance conductivity and examined under a scanning electron microscope (SEM) with an operating voltage of 10 kV (23).

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Gene expression analysis

To analyze gene expression, MDA-MB-231 cells were cultured at a density of 1×10^6 cells/well in a 6-well plate. After caffeine treatment, total RNA was isolated using the Geneaid Total RNA Mini Kit (Blood/Culture Cell) for adherent cells, following the manufacturer's instructions. For cDNA synthesis, total RNA was reverse-transcribed using the ReverTra Ace cDNA Synthesis Kit [Toyobo].

Quantitative real-time PCR (qPCR) was conducted using the Rotor-Gene Q Thermal Cycler to analyze *BAX*, *BCL2*, *CASP8*, and *hTERT* expression. The primers are listed in Table 1. The qPCR conditions included an initial denaturation at 98°C for 2 minutes, followed by 40 cycles of denaturation at 98°C for 10 seconds, annealing at 61°C for 10 seconds, and extension at 68°C for 30 seconds. A final extension step was performed at 60°C for 1 minute. Each reaction was performed in triplicate. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, with *ACTB* as the reference gene, and fold changes were calculated relative to controls (24).

Table 1. Primer sequences for gene expression analysis

Gene	Orientation	Sequence (5' to 3')	References
ACTB	Forward Reverse	TGACGTGGACATCCGCAAAG CTGGAAGGTGGACAGCGAGG	(25)
BAX	Forward Reverse	GCGAGTGTCTCAAGCGCATC CCAGTTGAAGTTGCCGTCAGAA	(26)
BCL2	Forward Reverse	ATGTGTGTGGAGAGCGTCAA GAGACAGCCAGGAGAAATCAA	(27)
hTERT	Forward Reverse	CCCATTTCATCAGCAAGTTTGG CTTGGCTTTCAGGATGGAGTAG	(28)
CASP8	Forward Reverse	GTTGTGTGGGGTAATGACAATCT CCATTCCTGTCCCTAATGCTG	(29)

Data analysis

In this study, data were analyzed using one-way ANOVA, followed by Dunnett's post hoc test to assess statistical differences between the control and treatment groups. All statistical analyses were conducted using IBM SPSS Statistics 23 and GraphPad Prism 10 software, with statistical significance at $p < 0.05$. Flow cytometry data were analyzed using NovoExpress 1.6.2 software.

Results

Inhibitory effect of caffeine on cell viability across different concentrations

Our results demonstrate that caffeine reduces the MDA-MB-231 cell viability after 24 hours of incubation. As presented in Figure 1, cell viability in both the control and vehicle (DMSO) groups remained approximately 100%, indicating that the vehicle did not induce cytotoxic effects. Statistically, no notable differences were observed between these two groups. However, a substantial reduction was detected in the doxorubicin (Dox) and caffeine-treated groups compared to the control, confirming its cytotoxic properties. The concentration of Dox (0.9 μM) used in this study was chosen based on previous literature reporting effective cytotoxic activity in MDA-MB-231 cells within this dose (30). To confirm the suitability of this concentration, we performed a preliminary assay using CCK-8 across a concentration range of 0.3–1.8 μM.

The results demonstrated that 0.9 μM reduced cell viability by approximately 50%, supporting its suitability as an effective concentration for comparative analysis. Interestingly, the cytotoxic effects of caffeine at all concentrations were comparable to those of doxorubicin, a commonly used chemotherapy drug, the outcome is a viability percentage of approximately 40%. This suggests that caffeine may have potential as an adjuvant or alternative cytotoxic compound. Furthermore, treatment with caffeine at concentrations of 10, 15, and 20 mM did not result in a further decline in cell viability beyond that induced by Dox alone, as

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evidenced by the plateau observed in the viability curve. The reduction of cell viability could be associated with cell death, particularly apoptosis (31, 32). Thus, further investigation is needed to confirm whether apoptosis is the predominant mechanism underlying caffeine-induced cytotoxicity.

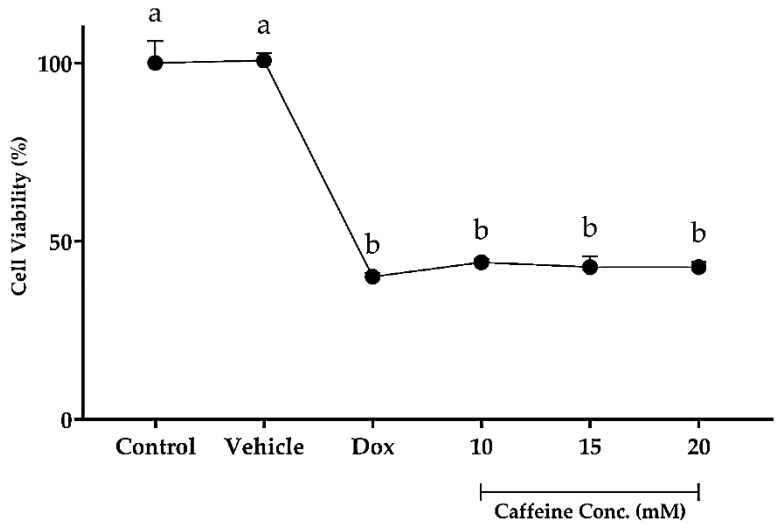


Figure 1. The inhibitory effects of caffeine on the MDA-MB-231 cell viability. Quantitative data are presented as mean ± SEM, represented by error bars; n = 3.

Elevated caffeine concentrations increase apoptosis in MDA-MB-231 cells

To further investigate whether caffeine-induced cytotoxicity is mediated through apoptosis, we performed an apoptosis assay utilizing flow cytometry (Figure 2a—2f). The quantitative analysis of apoptotic cells is presented in Figure 2g. In the negative control group, the majority of cells (99.16%) remained viable, with a very low percentage undergoing early apoptosis (0.54%), late apoptosis (0.16%), and necrosis (0.15%).

This indicates that untreated MDA-MB-231 cells were in a healthy state, showing no significant signs of apoptosis. The vehicle group exhibited a high percentage of viable cells (96.51%), while only a minor fraction of cells underwent early apoptosis (2.83%), late apoptosis (0.56%), and necrosis (0.10%). This suggests that the vehicle treatment had minimal cytotoxic effects on MDA-MB-231 cells, as the viability and apoptotic rates were similar to those observed in the negative control group.

On the other hand, the significant induction of apoptosis in the doxorubicin group validates its role as a positive control. A substantial decrease in viable cells, along with a marked increase in early apoptotic cells, demonstrates its ability to trigger programmed cell death in MDA-MB-231 cells. At 10 mM caffeine, a significant decrease in cell viability was observed (82.62%), along with a notable increase in early apoptosis (16.58%). These results indicate that caffeine at this concentration effectively induces apoptosis, particularly in the early phase. The trend became more evident at 15 mM caffeine, with cell viability decreasing to 74.89% and early apoptosis rising to 21.55%. In addition, at 15 mM caffeine, necrosis slightly increased to 3.11%, suggesting that while some cells were transitioning to cell death, the majority remained in the early apoptotic phase.

At 20 mM caffeine, the effects were more pronounced, with cell viability significantly dropping to 60.60% and early apoptosis rising to 38.54%. Despite the strong induction of early apoptosis, the progression to late apoptosis (0.17%) and necrosis (0.69%) remained minimal, indicating that caffeine predominantly induces early-phase apoptosis at higher concentrations.

Caffeine affects the morphological structure of MDA-MB-231 cells

Morphological analysis of MDA-MB-231 cells was conducted using a scanning electron microscope to examine the ultrastructural changes induced by caffeine (Figure 3). The results indicated that the control and vehicle-treated cells maintained a normal spherical shape with intact membranes (Figure 3a—

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3b). Moreover, tunneling nanotubes (TNTs) were observed (Figure 3a), suggesting an active cell-to-cell communication. On the other hand, cells treated with doxorubicin exhibited characteristic apoptotic features, including the formation of hollow structures and disruption of their spherical shape (Figure 3c). These changes reflect the cytotoxic effect of doxorubicin as a positive control to trigger cell death in cancer cells (26). Caffeine treatment led to ultra-structural morphological changes consistent with apoptotic characteristics, which were observed across all concentrations. At 10mM, exposure of caffeine led to the disruption of the cell's spherical structure, accompanied by membrane blebbing. These findings further confirm the cytotoxic effects of caffeine at 10 mM concentration, leading to a clear shift toward apoptosis (Figure 3d). At 15mM of caffeine treatment, the cells showed significant structural disruptions (Figure 3e). Moving on, cells treated with 20mM caffeine demonstrated prominent apoptotic features, including the formation of membrane blebbing and hollow structures (Figure 3f).

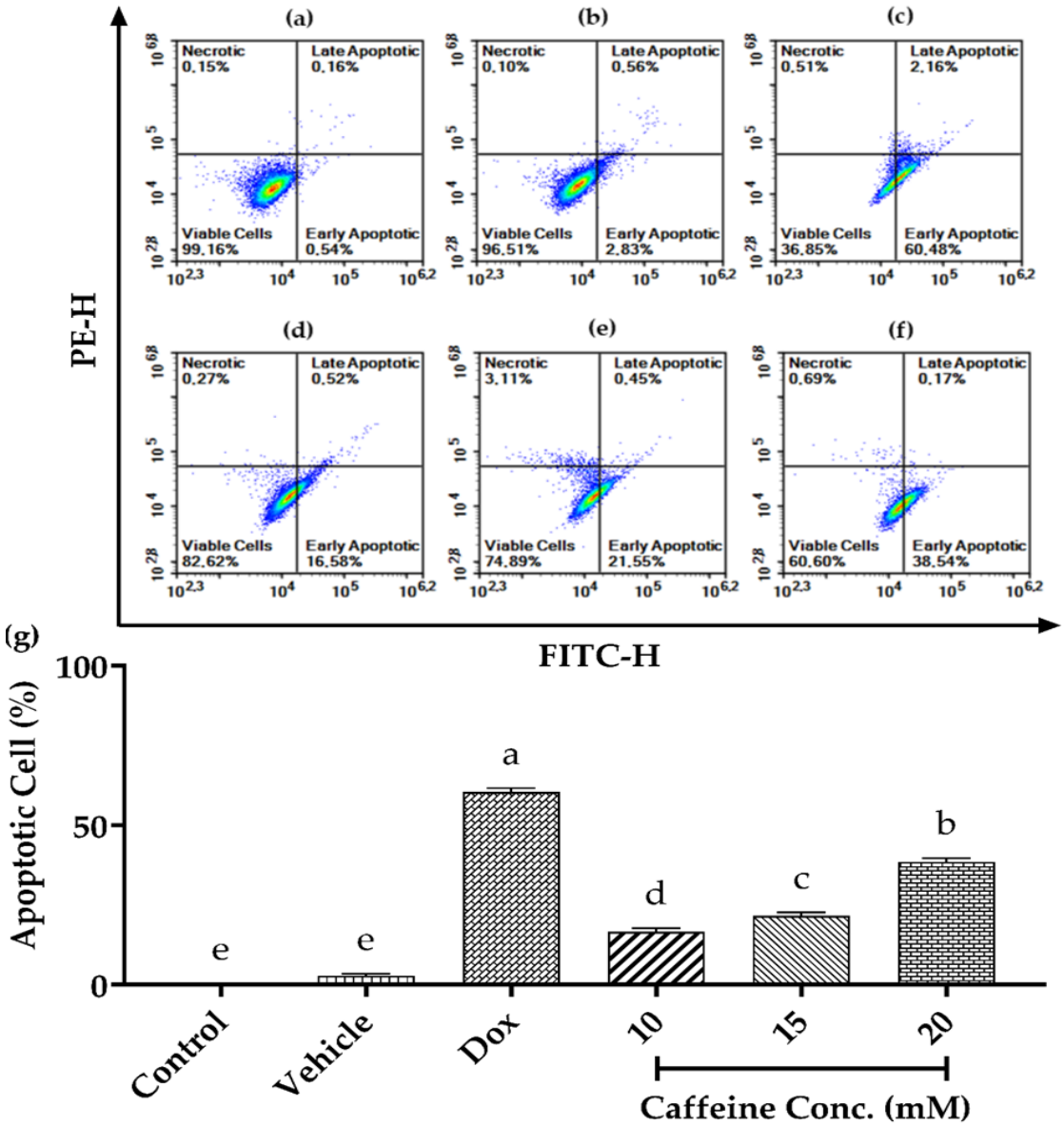


Figure 2. Caffeine-induced apoptosis in MDA-MB-231 cells after 24 hours of treatment. (a) Control; (b) Vehicle; (c) Doxorubicin; (d) 10 mM caffeine; (e) 15 mM caffeine; (f) 20 mM caffeine; (g) Quantitative analysis of apoptotic cell (% of total cells) at various caffeine concentrations, n = 3. The data emphasize the dose-dependent pro-apoptotic effect of caffeine treatment in MDA-MB-231 cells.

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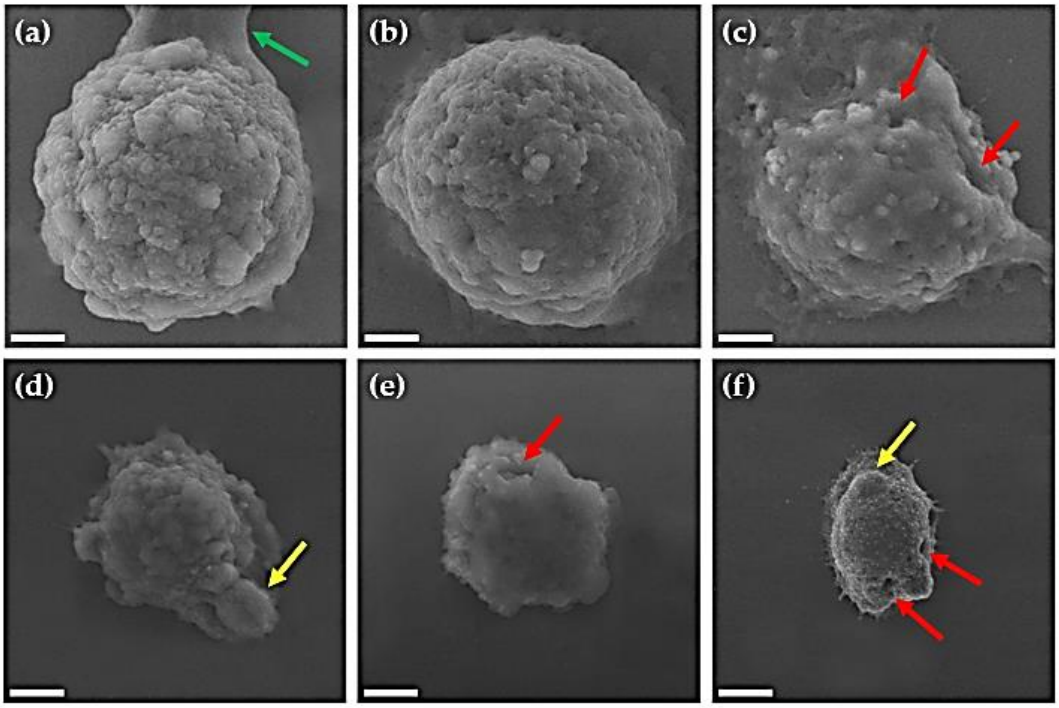


Figure 3. Scanning electron microscopy (SEM) analysis showing the ultrastructural changes in MDA-MB-231 cells. (a) untreated cells with the presence of tunneling nanotubes (TNTs) (green arrow); (b) vehicle-treated cells; (c) doxorubicin-treated cells; (d) 10 mM caffeine highlight a membrane blebbing (yellow arrow); (e) 15 mM caffeine displayed a significant hollow cell (red arrow); (f) 20 mM caffeine exhibited a hollow cell, membrane blebbing, and loss of TNTs. Magnification 5000x. Bars: 3 µm.

Caffeine tends to increase intrinsic apoptosis via *BAX/BCL2* modulation in MDA-MB-231 cells

To further investigate the molecular mechanisms driving caffeine-induced apoptosis, we analyzed the expression levels of key apoptotic markers. Figure 4a illustrates a notable increase in the BAX/BCL2 mRNA expression ratio following caffeine treatment. The control and vehicle groups exhibited relatively low expression levels with no significant difference. Dox treatment resulted in a marked increase in *BAX/BCL2* expression (~3.3-fold change), confirming its pro-apoptotic effects. The increasing *BAX/BCL2* ratio observed at caffeine concentrations of 10 mM and above indicates activation of the intrinsic apoptosis pathway.

A high *BAX/BCL2* ratio reflects an upregulation of the pro-apoptotic protein *BAX* alongside a reduction in the anti-apoptotic protein *BCL2*, shifting the balance toward cell death.At 10 mM caffeine, this pro-apoptotic effect becomes highly significant, with the *BAX/BCL2* ratio approaching levels observed in doxorubicin-treated cells (~2.7-fold change), suggesting that caffeine at this concentration may mimic doxorubicin's cytotoxic effects through apoptosis induction. A higher caffeine concentration (15 mM) resulted in a significant increase (~4.2-fold change), while the highest concentration (20 mM) exhibited the most pronounced effect (~5.2-fold change), indicating an increased activation of the intrinsic apoptosis pathway.

Statistical analysis showed distinct groupings demonstrate an enhanced apoptotic response at higher caffeine concentrations. Interestingly, while the intrinsic pathway is strongly activated, the expression of *CASP8*, a key marker of the extrinsic apoptosis pathway, did not change significantly in comparison with untreated cells. As shown in Figure 4b, the control and vehicle groups showed similar *CASP8* expression, while dox treatment slightly increased *CASP8* expression (~1.5-fold change). Similarly, caffeine treatment at 10 mM maintained *CASP8* expression at a level comparable to doxorubicin (~1.5-fold change). However, at higher caffeine concentrations (15 mM and 20 mM), *CASP8* expression decreased (~0.9- and ~0.4-fold change, respectively). This suggests that the intrinsic pathway becomes more dominant as caffeine concentration increases, while the extrinsic pathway remains relatively unaffected.

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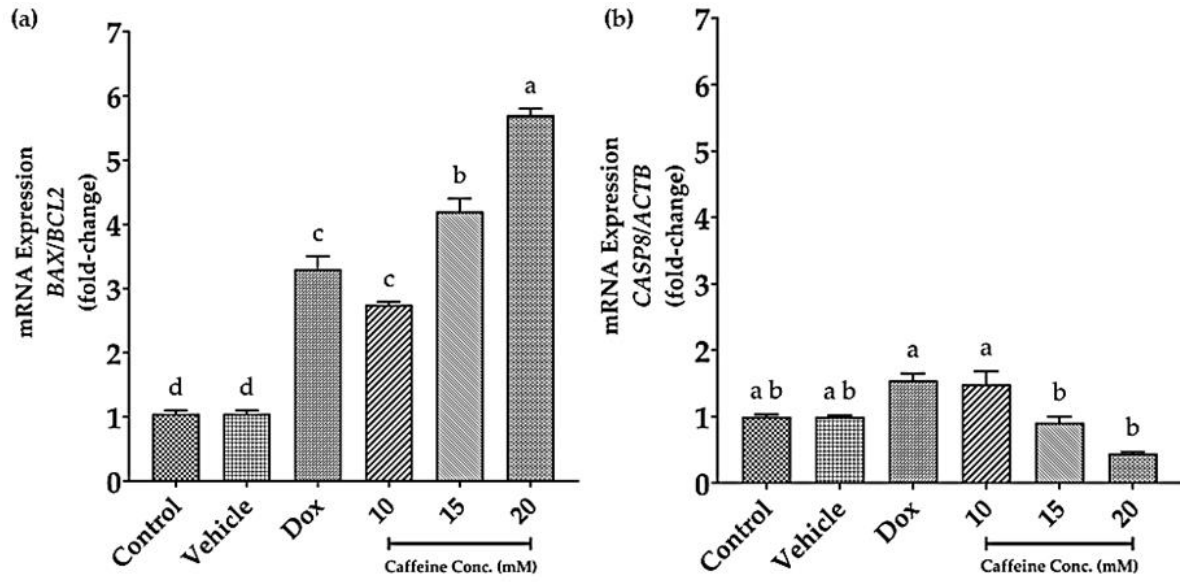


Figure 4. Apoptotic genes expression analysis in MDA-MB-231 cells after caffeine treatment for 24 hours. (a) Increased mRNA expression of BAX/BCL2 ratio in a dose-dependent manner. (b) Exposure to caffeine did not significantly affect CASP8 mRNA expression. Quantitative data are presented as mean ± SEM, represented by error bars; n = 3. Different letters denote significant differences between groups at $p < 0.05$.

Caffeine shows upregulation effects on *hTERT* mRNA expression

Furthermore, our results showed that caffeine not only affected the regulation of apoptotic-related genes but likewise influenced the human telomerase reverse transcriptase *(hTERT)* gene in telomerase activity, which is a key marker of cell immortality. The mRNA expression of *hTERT* is presented in Figure 5. In the negative control (untreated cells), positive control (doxorubicin treated cells), and vehicle (DMSO treated cells), *hTERT* expression was relatively low (~1.0-fold change).

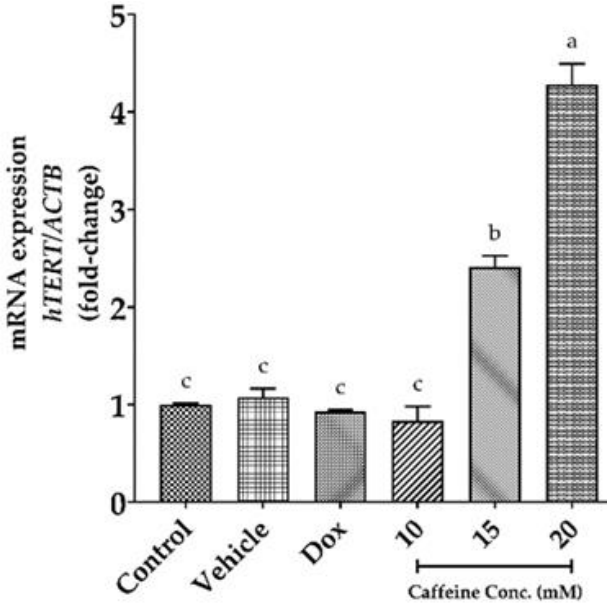


Figure 5. hTERT gene expression showed increasing effects in MDA-MB-231 cells after various concentrations of caffeine treatment for 24 hours. Quantitative data are presented as mean ± SEM, represented by error bars; n = 3. Different letters denote significant differences between groups at $p < 0.05$; a, b < 0.0001.

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Similarly, at 10 mM caffeine, there are no significant changes observed in *hTERT* expression relative to the control group. In contrast, higher concentrations of caffeine treatment induced upregulation effects on *hTERT* expression. Treatment with 15 mM caffeine resulted in approximately a ~2.4-fold upregulation, while 20 mM caffeine further elevated *hTERT* expression to approximately ~4.2-fold, significantly higher than in all other treatment groups. These findings indicate that caffeine at higher concentrations may promote telomerase activation through *hTERT* expression.

Discussion

Our study investigates the effects of various concentrations of caffeine in MDA-MB-231 cells. According to the viability results, all concentrations of caffeine treatment have a low viability, approximately 40%. This finding aligns with a previous study which reported that 10 mM concentrations of caffeine may decrease cell viability (33). Another study reported that caffeine treatment increases the percentage of apoptotic cells in glioblastoma cell lines (34). Based on our results, the cytotoxicity of caffeine-treated cells is statistically significant compared to the untreated group. However, there are no significant differences between all caffeine concentrations.

These stable viability results could be attributed to the activation of compensatory survival mechanisms, which may limit further reductions in viability at higher concentrations (34).Furthermore, we utilize the flow cytometry assay to explore the apoptotic effect of MDA-MB-231 cells following caffeine treatment. The apoptotic pathway can be characterized based on 4 categories: viable, early apoptosis, late apoptosis, and necrosis. Our findings indicated that caffeine exerts an apoptotic effect on MDA-MB-231 cells in a dose-dependent manner, with higher concentrations associated with increased early apoptosis and reduced cell viability. These findings align with earlier studies indicating the anticancer effects of caffeine in breast and liver cancers, where apoptosis induction and inhibition of tumor cell proliferation were identified as the primary mechanisms (35).

The observed dose-dependent increase in apoptosis also supports evidence that caffeine reduces cell proliferation by upregulating *p21* expression, a critical regulator of cell cycle arrest and apoptosis (36). As quantification of apoptotic processes is essential for evaluating potential anticancer therapies, our findings highlight caffeine's potential as a pro-apoptotic agent (10).

It is important to highlight that the caffeine treatment in this study has no necrosis effect, suggesting that caffeine induces cell death mediated through the apoptosis pathway. Previous studies have highlighted that anticancer drugs should ideally avoid inducing necrosis, as it leads to unregulated cell lysis that may be toxic to surrounding cells (37). Unlike apoptosis, which is a controlled process that prevents damage to neighboring tissues, necrosis has been linked with tumor progression by enhancing the angiogenesis of cancer cells. Thus, apoptosis-mediated cell death is considered a more controlled and desirable pathway for anticancer therapy (38).

To visualize the apoptotic effects of caffeine in MDA-MB-231 cells, we performed the ultrastructure observation using a scanning electron microscope. Our results are consistent with the flow cytometry results which indicate higher apoptotic effects on higher caffeine concentrations. On the untreated cells, the cell surface still has an active cell-to-cell communications, as indicated by the tunneling nanotubes (TNTs) (39). At 10 mM, the cell membrane starts to blebbing. The membrane blebbing is defined as protrusions on the cell membrane caused by actomyosin contraction in the cortical layer of the cell. This contraction triggers the rupture of the actin cortex, allowing cytosolic hydrostatic pressure to push outward, leading to the formation of blebs (40). At higher concentrations (15 mM), the cells start to shrink as the shrinkage of cells at this concentration may be caused by dehydration or the loss of cytoplasmic components, possibly resulting in the formation of hollow regions within the tumor (41). Additionally, the cell surface appeared flattened, likely due to caffeine-induced disruption of the cytoskeleton (32).

At higher concentrations (15 and 20 mM), the cells start to form a hollow structure. This condition, known as cavitation, may occur when cancer cells die and release their contents (42). Besides, the absence of TNTs at 20 mM caffeine was further observed, indicating disrupted intercellular communication. TNTs, which typically connect adjacent cells, play a crucial role in facilitating cross-talk and promoting cellular activity (43). This observation aligns with the flow cytometry results, which showed a significant

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increase in apoptotic cells at 20 mM caffeine. To gain deeper insights, we further examined the molecular interactions by analyzing gene expression profiles of key apoptotic genes, particularly those regulating the intrinsic and extrinsic pathways.

Analyzing apoptotic markers is crucial to determine whether apoptosis remains the primary mode of cell death at higher caffeine concentrations. Apoptosis, or programmed cell death, occurs via two primary pathways: the intrinsic (mitochondrial) and the extrinsic (death receptor) pathway (18). The intrinsic apoptotic pathway is regulated by the interplay between pro-apoptotic and anti-apoptotic proteins, for instance, *BAX* and *BCL2*. *BAX* promotes mitochondrial membrane permeabilization, resulting in the release of cytochrome c and activation of caspase-9. Caspase-9 subsequently activates executioner caspases, including caspase-3, triggering the degradation of cellular proteins and ultimately leading to cell death. In contrast, *BCL2* maintains mitochondrial integrity by inhibiting *BAX* activity and preventing cytochrome c release (40, 44).

Thus, an increased *BAX/BCL2* ratio, as observed with caffeine treatment, suggests that caffeine downregulates *BCL2* expression while upregulating *BAX*, disrupting mitochondrial homeostasis and inducing the release of pro-apoptotic protein via intrinsic pathway. As for the *CASP8* gene, different concentrations of caffeine had no impact on *CASP8* expression in MDA-MB-231 cells. The low *CASP8* expression suggests that apoptosis induced by caffeine in these cells is unlikely to occur through the extrinsic pathway (45). These findings align with multiple studies reporting that elevated concentrations of caffeine primarily induce apoptosis in various cell lines predominantly through the intrinsic pathway. Jafari et al. (46) demonstrated that caffeine triggers intrinsic apoptosis by suppressing the PI3K-Akt-mTOR survival signaling pathway while simultaneously activating cell death pathways. This mechanism results in increased *BAX* expression and *BCL2* phosphorylation, initiating apoptotic events. Similarly, Censi et al. (47) observed that caffeine promotes *BAX* translocation from the cytosol to the mitochondria in human lung adenocarcinoma cells. Additionally, caffeine-induced apoptosis in human glioblastoma cells has been linked to elevated caspase-3 activity and *BAX* protein expression, accompanied by a reduction in *BCL2* protein expression (45).

Taken together, these findings imply that the intrinsic pathway, rather than the extrinsic pathway, plays a key role in caffeine-induced apoptosis across different cell types, including MDA-MB-231.Our study not only investigates the apoptotic pathway in caffeine-treated cells but it additionally examines the effects of caffeine treatment on telomerase activity. Telomerase is a protein complex that elongates the ends of chromosomes by adding telomeric repeats, playing a crucial role in maintaining telomere integrity. Its activity is closely linked to cellular immortalization and tumor development. This function is primarily regulated by the expression of *hTERT*, which is frequently upregulated in cancer cells to support uncontrolled proliferation (48, 49). Our results showed that a higher concentration of caffeine treatment significantly increased *hTERT* expression. These results are consistent with previous studies demonstrating that caffeine enhances hTERT expression, thereby extending telomere length in MCF-7, HepG2, and HeLa cells (13).

In contrast, treatment with doxorubicin did not significantly affect *hTERT* expression, suggesting that doxorubicin had a limited impact on telomerase activity (28). The increase in *hTERT* expression at higher caffeine concentrations was further examined in relation to apoptosis markers, including the *BAX/BCL2* ratio and *CASP8* expression. At concentrations of 10 mM and above, a significant shift in the *BAX/BCL2* ratio was detected, indicating an increase in mitochondrial stress and activation of the intrinsic apoptosis pathway.

This shift aligns with the low *hTERT* expression at 10 mM, as low *hTERT* is typically associated with reduced cell survival and immortality. However, the observed rise in *hTERT* at higher caffeine concentrations (15 and 20 mM) may represent a cellular compensatory mechanism to counteract apoptosis, as *hTERT* has been shown to protect mitochondrial function and delay apoptotic processes (15). Another study shows that cells with elevated telomerase activity demonstrate increased cell death resistance following drug treatment exposure (17). Additionally, increased *hTERT* levels were sufficient in suppressing caspase-dependent apoptosis by decreasing the activation of caspases 3, 8, and 9. This condition could allow *hTERT* to protect the cells against mitochondrial dysfunction induced by the pro-apoptotic shift in the *BAX/BCL2* ratio (12). However,

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caffeine's effects on telomerase activity remain controversial. While in vitro studies have shown caffeine-mediated *hTERT* upregulation in cancer cells, other evidence indicates that caffeine consumption may accelerate telomere shortening.

In human observational studies, higher caffeine intake (e.g., 100mg/day) was associated with shorter telomere length (50). This outcome may be linked to impaired DNA repair and increased chromosomal instability, both of which are features of cellular aging (51). Given the limited and conflicting data on *hTERT* regulation in cancer, future studies are needed to clarify the dualistic nature of caffeine's effects. Specifically, investigations using diverse cancer models, including brain cancers where caffeine is more frequently linked to neuroprotective and aging-related pathways, may provide insight into its broader therapeutic potential.Our findings suggest that at 10–20 mM caffeine concentrations, caffeine may induce mitochondrial-associated oxidative stress, as suggested by the elevated *BAX/BCL2* ratio observed in this study, alongside previously reported downregulation of redox-related genes (*SOD2* and *GLO1*) in the same cell model, contributing to the activation of the intrinsic apoptotic pathway (52).

Although the caffeine concentrations used in this study (5–20 mM) exceed typical physiological plasma levels in humans (generally <412 µM), such concentrations are commonly applied in in vitro models to uncover mechanistic insights and evaluate compound potency (53). The elevated doses are necessary to compensate for the absence of metabolic clearance mechanisms found in vivo, such as hepatic metabolism and renal excretion, and to overcome reduced cellular uptake in vitro. Moreover, higher concentrations allow the detection of dose-dependent responses and help to identify molecular thresholds at which caffeine shifts from non-apoptotic to pro-apoptotic behavior, as observed through apoptotic markers and gene expression patterns (e.g., *BAX*, *BCL2*, *CASP8*, and *hTERT*) (9). While these concentrations may not directly translate to therapeutic dosing in clinical contexts, these are scientifically justified for mechanistic exploration, particularly in resistant and aggressive cancer models such as MDA-MB-231 triple-negative breast cancer cells. The significant increase in *hTERT* expression at these concentrations indicates an adaptive response to preserve mitochondrial function and delay cell death. Based on our findings, the potential caffeine pathway in regulating apoptosis is illustrated in Figure 6.

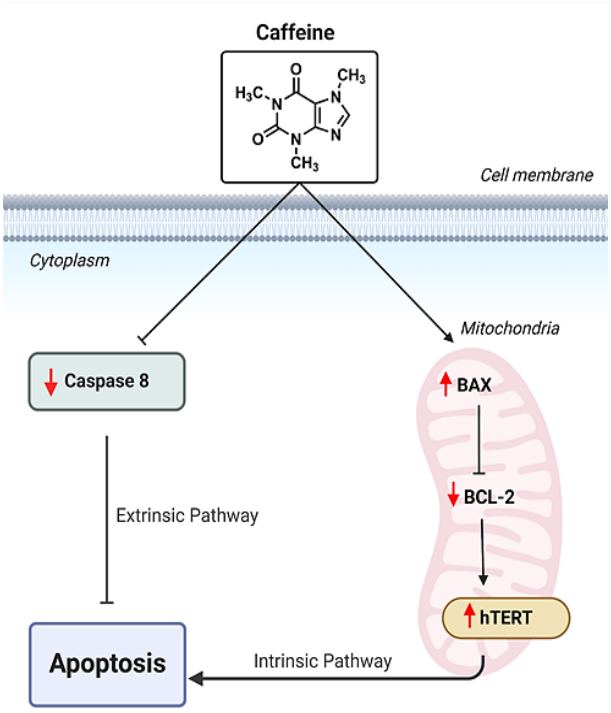


Figure 6. Illustration of the potential caffeine-mediated pathway in regulating apoptosis in MDA-MB-231 cells. Caffeine activates the intrinsic apoptosis pathway, as indicated by the elevated BAX/BCL2 ratio, while simultaneously upregulating hTERT expression, which suggests an adaptive survival mechanism to counteract cell death. Additionally, caffeine did not influence CASP8, implying a suppression of the extrinsic apoptosis pathway.

The contrasting effects of caffeine on apoptosis markers (*BAX/BCL2*, *CASP8*) and *hTERT* expression highlight a complex interaction, where caffeine appears to promote apoptotic pathways and trigger telomerase-related survival mechanisms simultaneously (15, 54). While this study provides valuable insights into the cellular and molecular effects of caffeine on TNBC cells, future research could incorporate additional TNBC cell lines or other cancer models to provide a more comprehensive representation of tumor heterogeneity. Confirming transcriptomic changes at the protein level would further clarify mechanistic insights. Moreover, investigating caffeine's effects at physiologically relevant concentrations could enhance the translational relevance of these findings and support its potential role in cancer therapy. In

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conclusion, this study demonstrates that caffeine treatment in MDA-MB-231 triple-negative breast cancer cells impacts multiple cellular processes.

For instance, caffeine significantly reduces cell viability, exhibiting a plateau effect across different concentrations, underscoring its cytotoxic potential. Additionally, caffeine induces apoptosis, as evidenced by ultrastructural changes and marked activation of the intrinsic apoptosis pathway, reflected by alterations in the *BAX/BCL2* ratio. Furthermore, caffeine upregulates *hTERT* mRNA expression, suggesting the activation of cellular compensatory mechanisms related to telomerase activity. These findings emphasize the complex of caffeine as a modulator of cellular death and telomerase activity that may offer promising therapeutic avenues for TNBC treatment. However, this study is limited by the reliance on a single cell line, the absence of protein-level confirmation, and non-physiological caffeine concentrations. Future research should incorporate multiple cell models, protein expression analysis, and physiologically relevant dosing to assess caffeine's effectiveness in therapeutic potential.

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