Original Article

The Cytotoxicity of 27-Hydroxycholesterol in MCF-7 and MDA-MB-231

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Abstract

Background: Although several roles of 27-hydroxycholesterol (27-HC), the most abundant oxysterol in blood circulation, in cancers have been elucidated, its impact on breast cancer proliferation and its pathway remain unknown.

Materials and Methods: The effect of 27-HC on breast cancer cell proliferation and its pathway was evaluated using Michigan Cancer Foundation - 7 (MCF-7) and M.D. Anderson - Metastatic Breast 231 (MDA-MB-231) cell lines. The MTT assay was applied after 24- and 48-hour incubation to distinguish cell proliferation. To determine the cause of different viability results from the MTT assay, the Annexin-FITC/PI test was used at concentrations of 0.1, 1, and 10 μM after 24- and 48-hour incubation.

Results: 27-HC in concentrations of 5, 10, and 20 μM induced cell cytotoxicity compared with control. Also, the annexin V conjugated with fluorescein isothiocyanate/propidium iodide (Annexin-FITC/PI) test revealed an increase in total apoptotic cells treated with 0.1, 1, and 10 μM of 27-HC after 48 hours (P value < 0.05). Besides, the cytotoxic effect of 27-HC was observed at 10 μM concentration in both cell lines, MCF-7 and MDA-MB-231 (P value < 0.05).

Conclusion: The identification of 27-HC's cytotoxic effects on both estrogen receptor (ER)-negative and ER-positive breast cancer cell lines is a novel discovery that may be linked to LXRβ.

Keywords: 27-Hydroxycholesterol, cell viability, estrogen receptor, liver X receptor, MCF-7, MDA-MB-231

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Introduction

Breast cancer is a prevalent type of cancer that affects many women worldwide. It is the second most common type of cancer and the leading cause of cancer-related deaths among women. In 2017, there were approximately 16,700 cases of breast cancer per 100,000 women globally.[1,2] Breast cancer is classified based on the presence or absence of certain proteins, including progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (Her2), which are identified through histopathological analysis.[3] In this regard, breast tumors categorized as PR-positive and ER-positive have a better prognosis.[4] Among environmental factors affecting breast cancer, high-cholesterol diet has an important role.[5] It was assumed that cholesterol influences breast cancer, but recent studies showed the crucial role of 27-hydroxycholesterol (27-HC), one of the cholesterol metabolites.[6]

It is shown that 27-HC is the most prevalent oxysterol in circulation, with concentrations ranging from 0.2 to 0.9 μM,

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which are associated with cholesterol levels.[7] This metabolite has essential effects on high-burden diseases such as cardiovascular diseases, malignancies, and neurodegenerative disorders. In addition, 27-HC plays roles in cell cycle checkpoints and immune system impairment.[8-11] A cytochrome P450 enzyme called CYP27A1 converts cholesterol to 27-HC, while CYP7B1 catabolizes 27-HC to bile acid. 27-HC primarily exerts its effects through the activation of two nuclear receptors, ER and Liver X receptor (LXR).[12] While 27-HC functions as an LXR agonist, it can also act as a selective ER modulator (SERM), acting as either an antagonist or agonist depending on the presence or absence of estrogen.[13]

In vitro studies have demonstrated that 27-HC can affect breast cancer progression through the activation of LXR and ER.[14] There is conflicting evidence regarding the effects of 27-HC on breast cancer progression through these receptors. While 27-HC has been shown to promote cancer progression through ER activation in the MCF-7 cell line,[15] some studies have suggested that 27-HC may act as an antiproliferative agent through the LXR receptor.[16,17]

However, clinical studies have shown a reverse correlation of 27-HC with breast cancer. A nested case–control study showed that higher serum 27-HC concentration among postmenopausal women was associated with lower breast cancer risk.[18] Another clinical study on 1472 cases compared with matched controls by DeRouen *et al*.[19] supported the fact that circulating 27-HC decreased the risk of breast cancer.

This study aimed to shed light on the effect of 27-HC on breast cancer progression or elimination by evaluating the proliferation of MCF-7 as ER-positive and MDA-MB-231 as ER-negative breast cancer cell lines exposed to 27-HC.

Materials and Methods

Culture of cancer cell lines

The MCF-7 and MDA-MB-231 cells (Institute Pasteur of Iran, Tehran, Iran) were cultured in RPMI-1640 media and Dulbecco's Modified Eagle Medium (DMEM) High-Glucose H-21 media, respectively, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin or streptomycin (100 IU/mL and 100 mg/ml, respectively) (Gibco BRL, Rockville, MD, USA). The cells were cultured in a humidified incubator at 37°C with 5% CO2 until they reached 80% confluency. At this point, they were subcultured using trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco BRL, Rockville, MD, USA) for a maximum of two passages before being transferred to a new medium.

Preparation of 27-HC solution

To prepare a master stock, 27-HC (Tocris Bioscience, Bristol, United Kingdom) was dissolved in 96% ethanol (Gibco BRL, Rockville, MD, USA) with a concentration of 10 mM and stored at -20°C. The working solution was immediately prepared by adding a complete culture medium. The operational solutions' ethanol concentration was equal to the toxic dose for human cancer cell lines (2.5% (v/v)).[20]

Viability of cancer cell lines exposed to 27-HC

The cells were added to a 96-well plate at a density of 4×10^4 cells per well. After 24 hours of incubation, the remaining culture medium was removed once the cells had adhered to the plate. After confirmation of cells' adhesions and 80% confluency, the cells were incubated with the medium alone (control) or 0.1, 1, 5, 10, or 20 μM concentrations of 27-HC for 24 and 48 hours. Notably, each well's volume was equal to 160 μL.

Following the incubation period, 16 μL of MTT solution was added to each well and mixed. MTT solution was prepared by dissolving 25 mg thiazolyl blue tetrazolium bromide (Gibco BRL, Rockville, MD, USA) in 3 mL phosphate-buffered saline (PBS) (Gibco BRL, Rockville, MD, USA) after three hours of incubation, the supernatants were removed, and 100 μL dimethyl sulfoxide (DMSO) (Gibco BRL, Rockville, MD, USA) was added to each well and shaken. An enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek, Winooski, VA, USA) was used to estimate cell viability by measuring absorbance at 570 nm. The viability percentage was determined by calculating the absorbance ratio of wells incubated with 27-HC using formula 1. The MTT assay was repeated three times with the same materials and method.

$$Cell\,Viability\,Percent = \frac{sample\,OD - blank\,OD}{control\,OD - blank\,OD} \times 100 \qquad (1)$$

Apoptosis analysis of cancer cells after exposure to 27-HC

To determine the cause of different viability found in cytotoxicity assay, Annexin-FITC and propidium iodide (PI) double staining was utilized by detecting the phosphatidylserine exposed apoptotic cells via flow cytometry. The protocol was acquired from Lakshmanan's recommendation.[21] The flow cytometry was performed by the ZE5 Cell Analyzer (Bio-Rad, Hercules, CA, USA) three times (n = 3). Before flow cytometry, cells were incubated with 27-HC at 0.1, 1, and 10 μM for 24 and 48 hours. Cells that tested negative for both PI and Annexin V were classified as viable. Cells that tested negative for PI and positive for Annexin V were classified as early apoptotic, while cells that tested positive for both PI and Annexin V were classified as late apoptotic. Cells that tested positive for PI and negative for Annexin V were classified as necrotic. The dot plot graphs displayed the results, showcasing the distribution of viable, early-phase apoptotic, late-phase apoptotic, and dead cells in the lower left, lower right, upper right, and upper left quadrants, respectively. We used the summation of early-phase apoptotic and late-phase apoptotic cells as total apoptotic cells for analysis.

Statistical analysis

Data were collected from three repetitions of experiments. The data presented in the study included both qualitative

and quantitative data, which were expressed as percentages and mean ± standard error of the mean (mean ± SEM). The statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) Statistics for Windows, version 26. The Mann–Whitney U-test was utilized, and a *P* value of 0.05 or less was considered statistically significant. Graphs were made using Microsoft Excel 2019 software.

Results

Effect of 27-HC on cell viability of MCF-7 cell line

The impact of 27-HC on the viability of the MCF-7 cell line was assessed using the MTT assay. MCF-7 cells were exposed to varying concentrations of 27-HC (0.1, 1, 5, 10, and 20 μM) for 24 and 48 hours. The results showed the variable effect of 27-HC. In concentrations less than 5 μM, there were few changes in cell viability, but analyses found a significant difference between control and 5, 10, and 20 μM of 27-HC regarding cell viability (IC50 = 2.19 μM) [Figure 1].

Effect of 27-HC on apoptosis of MCF-7 cells

To investigate whether 27-HC induces apoptosis or reduces cell proliferation in the MCF-7 cell line, we utilized flow cytometry with Annexin V and PI staining. MCF-7 cells were exposed to 27-HC at concentrations of 0.1, 1, and 10 μM for 24 and 48 hours. Results showed an increase in total apoptotic cells for all treatment groups after 48 hours (*P* value <0.05) [Figure 2].

Evaluation of 27-HC effect on both ER-positive and ER-negative cell lines

Cell viability was measured using MTT assay on MCF-7 and MDA-MB-231 cell lines to evaluate the role of ER on the observed cytotoxic effect of 27-HC. Both cell lines showed a cytotoxic effect of 27-HC at 10 μM concentration (*P* value <0.05) [Figure 3].

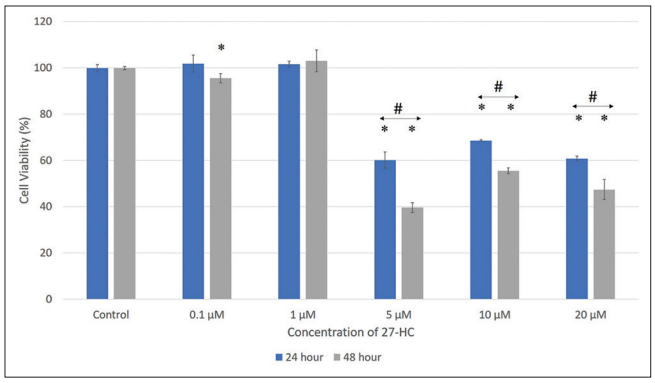

Figure 1: Cell viability of MCF-7 cell line at different concentrations of 27-HC for 24- and 48-hour treatment by MTT assay. Cell viability was decreased significantly in 5, 10, and 20 μM compared with control in both treatment times. * Shows Mann–Whitney test significance compared with control, and # shows Mann–Whitney test significance with different treatment times (*P* value <0.05). Data are presented as mean ± SEM

Discussion

This study assessed the effect of 27-HC on cell viability in MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) breast cancer cell lines. Cytotoxic effects were observed in the MCF-7 cell line at 27-HC concentrations of 5, 10, and 20 μM in 24- and 48-hour incubations, resulting from apoptosis induction by 27-HC. In addition, a comparison of different concentrations of 27-HC (0.1, 1, and 10 μM) on MCF-7 and MDA-MB-231 found toxicity in both cell lines, suggesting the cytotoxic effects of 27-HC may be independent of ER.

Studies showed that the physiologic serum concentration of 27-HC does not reach more than 1 μM. However, two studies support that the concentration of 27-HC in the breast cancer environment is higher than the serum level. Wu *et al*.[22] showed increased tumor 27-HC content due to diminished expression of CYP7B1, 27-HC catabolizing enzyme. A study by Warns *et al*.[23] reported no toxic effect of 27-HC up to 300 μM, which is in line with our flow cytometry result, showing that the cytotoxic effect of 27-HC is not due to toxic concentration.

Numerous studies have investigated the effect of 27-HC on the proliferation of breast cancer cell lines. However, there are controversial findings regarding the impact of different concentrations. In a study on MCF-7 and Ishikawa, endometrial cancer cell lines, an increase in proliferation was observed at 0.01, 0.1, and 1 μM concentrations via ER through RET pathway.[22] Mashat *et al*.[24] also observed an increase in proliferation in MCF-7 in an ER-α-dependent manner. Another study by Raza *et al*.[25] revealed a significant proliferative effect of 27-HC in concentrations of 0,1 and 1 μM, similar to the estradiol effect. However, they did not observe the proliferative impact of 27-HC in the MDA-MB-231. In contrast, Avena *et al*.[26] reported a proliferative effect of 27-HC on MDA-MB-231 cells at concentrations of 0.001, 0.01, 0, and 1 μM through the G protein-coupled estrogen receptor, which activates ERK1 or ERK2 and NFκB. Cruz *et al*. investigated the proliferative effect of 27-HC in MCF-7 and MCF-10, non-tumorigenic breast cell lines, at 0.1, 0.5, 1, and 10 μM concentrations. Despite the 27-HC-induced proliferation in the MCF-7 cell line at 1 μM in an ER-dependent manner, they showed decreased proliferation at 1 and 10 μM after 96 hours in the MCF-10.[27]

The effect of 27-HC on cell lines varies from proliferation to cytotoxicity in different cancers. Proliferation was observed in an ER-positive lung cancer cell line, H23, at 0.1 and 1 μM.[28] Besides, Hiramitsu *et al*.[29] showed that the proliferation in H1395, another lung cancer cell line, depended on ERβ through the PI3K-Akt signaling pathway. Moreover, Rossin *et al*.[30] observed the proliferative effect of 27-HC at 5 μM in Caco2 cells, a colon cancer cell line, through the AKT pathway. Conversely, Warns *et al*. showed that 27-HC reduced proliferation of this cell line at concentrations more than 10 μM, not due to cytotoxicity or apoptosis. They found that

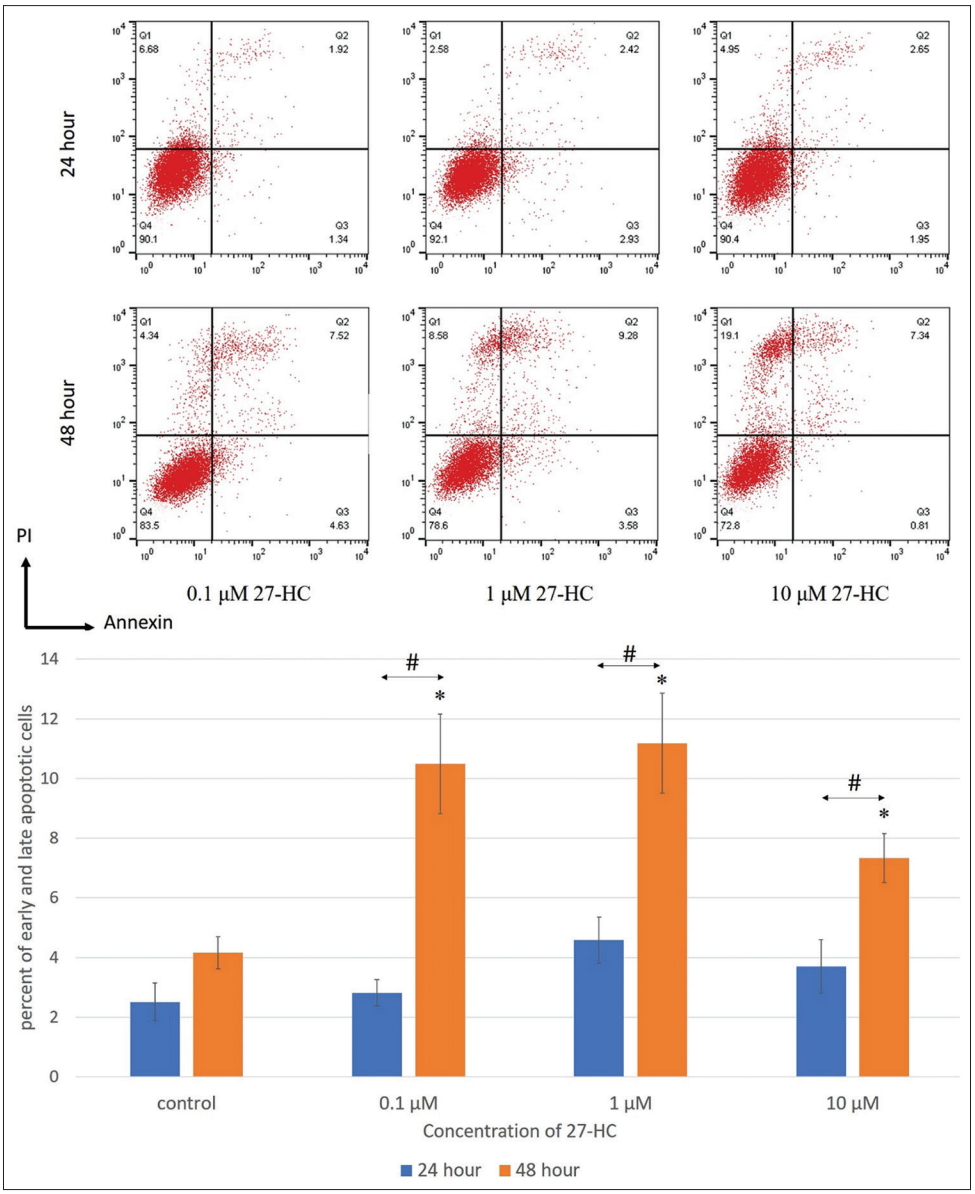

Figure 2: A flow cytometry assay using Annexin V and PI staining of MCF-7 cells treated with 0.1, 1, and 10 μM concentrations of 27-HC for 24 and 48 hours. Viable, early-phase apoptotic, late-phase apoptotic, and dead cells are shown in the lower left, lower right, upper right, and upper left quadrants, respectively. Total apoptotic cell percent (summation of early-phase apoptotic and late-phase apoptotic cells) increases after 48 hours; there is a statistically significant difference between groups and control (*P* value <0.05). * Shows Mann–Whitney test significance compared with control, and # shows Mann–Whitney test significance with different treatment times

this effect was independent of LXR and ER and may result from decreased AKT activation.[23]

To describe the cytotoxic effect of 27-HC, the role of LXR should be considered. El Roz *et al*.[31] found that LXR agonists inhibited proliferation and induced apoptosis in MCF-7. Also, Gibson *et al*.[16] suggested that while 27-HC may promote proliferation through ER activation, it can also reduce cell proliferation in endometrial cancer cell lines by activating LXR. In HGC-27 cells, a gastric cancer cell line, 27-HC suppressed proliferation through LXRβ.[17] Wang *et al*. supported the hypothesis that proliferation reduction was mediated through LXRβ by suppressing Wnt signaling.[32] Another study on small-cell lung cancer cells showed activation of LXRβ-induced ATP Binding Cassette Subfamily A Member 1 (ABCA1) expression, efflux of cholesterol, and inhibition of proliferation.[33] We hypothesized that LXRβ was responsible for the observed cytotoxic effect of 27-HC in MCF-7 and MDA-MB-231. For future studies, we recommend the evaluation of LXR with specific LXR subtype antagonists and downstream cascade.

Conclusion

In conclusion, our results showed a novel finding of 27-HC cytotoxicity on both ER-negative and ER-positive

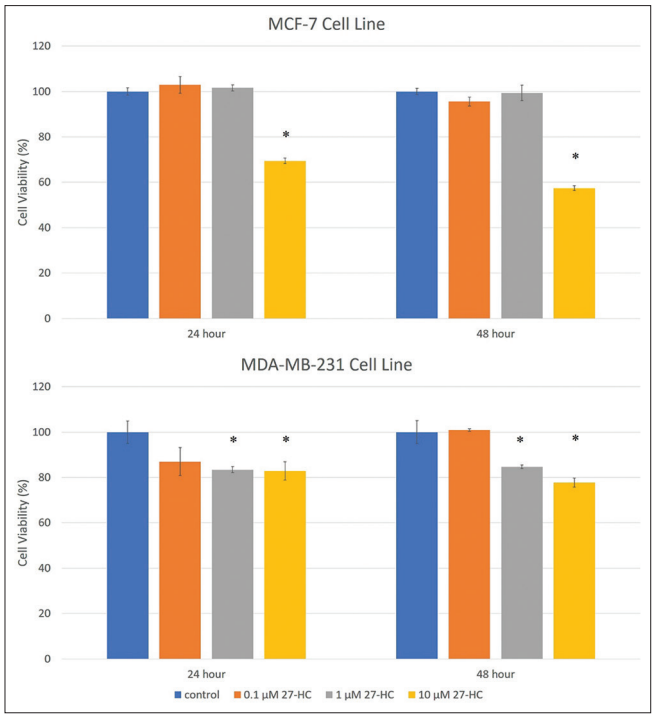

Figure 3: Cell viability of MCF-7 as ER-positive cell line (a) and MDA-MB-231 as ER-negative cell line (b) treated with 27-HC for 24- and 48-hour treatment. Cell viability was calculated after the MTT assay. * Shows Mann–Whitney test significantly compared with control (*P* value < 0.05). Data are presented as mean ± SEM

breast cancer cell lines. We hypothesized that LXRβ was responsible for the observed cytotoxic effect of 27-HC.

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Conflicts of interest

There are no conflicts of interest.

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