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Cell Kinetic Study of Tamoxifen Treated MCF-7 and MDA-MB 468 Breast Cancer Cell Lines

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ABSTRACT

Apoptosis could be a major mechanism of antitumor effect of tamoxifen. Therefore this study is designed to characterize the kinetic behavior of tamoxifen-induced apoptosis in the estrogen receptor positive (ER^+) and negative (ER^-) cell lines, MCF-7 and MDA-MB-468. Frequency of cell death was examined by trypan blue and acridine orange staining. Annexin V-Fluorescein/PI was used in flow cytometry for distinguishing the dividing, apoptotic and necrotic cells and Hoechst 33258 staining was also applied to detect apoptotic changes in the nuclear morphology. The results showed that tamoxifen was able to induce apoptosis in both cell lines (χ^2 test, $P<0.05$). In contrast to the MCF-7 cells, which responded to the low concentrations of tamoxifen (0.5-1µM), the treated MDA-MB-468 cells were affected at 20 µM (χ^2 test, $P<0.05$). However, the kinetic data revealed that tamoxifen, at higher doses stimulates the ER^+ cell proliferation. This is the first study showing these opposite effects in the kinetics of tamoxifen treated cells. Therefore it may serve as a guideline for the precautionary evaluation of suggested high doses of tamoxifen. *Iran. Biomed. J. 7 (2): 51-56, 2003*

Keywords: Apoptosis, Flow cytometry, Annexin V/PI, Tamoxifen, Breast cancer cell lines

INTRODUCTION

The balance between proliferation and cell death, mainly apoptosis, is crucial in determining the overall growth or regression of the tumor [1]. Reduced apoptosis may lead to a shift in tissue kinetic toward the expansion of cell numbers and to the preservation of genetically aberrant cells, favoring clonal expansion and neoplastic development [2]. Breast cancer is the commonest malignancy and comprises 18% of all cancers in women [3]. Since 1990, death rates from breast cancer have decreased by over 25% and this is at least in part due to the improved use of adjuvant tamoxifen and chemotherapy [4]. Tamoxifen, a non-steroidal anti-estrogen, was primarily exploited as a drug against hormone-dependent breast cancer [5]. We previously reported that daily administration of tamoxifen (0.5 mg/kg) resulted in the regression of N-nitroso-N-methylurea (NMU)-induced rat mammary tumor [6]. It has recently been shown that tamoxifen is effective for hormone insensitive breast cancer in advanced stages [7]. It inhibits cell proliferation and induces apoptosis irrespective of the status of estrogen receptor expression [8-10]. To improve the chemotherapeutic effects of tamoxifen and to expand its application in the treatment of wide variety of cancers, it is essential to get more insight into the dose-response analysis. A quanti-tative study of cell apoptosis and proliferation in a static-cell population, such as tissue sections, provides little information on the actual rapidity of cell replication and loss. Therefore, this study was designed to characterize the *in vitro* kinetic behavior of tamoxifen-induced apoptosis in the estrogen receptor positive and negative cell lines of the breast cancer, MCF-7 and MDA-MB-468. Since these cell lines are originally derived from infiltrating carcinoma, the commonest type of breast cancer in the world, the results will most likely be resemble the molecular events involved in the tamoxifen treated breast cancer patients.

MATERIALS AND METHODS

Chemicals, culture media and related compounds were purchased from Sigma Company (Germany). Cell culture plasticwares were from Nunc Company (Denmark). Annexin V-FLOUS staining kit (Cat. No. 1 988 549) was obtained from Roche Diag-

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nostic (Germany). Tamoxifen citrate was kindly gifted by Iran Hormone pharmaceutical company (Iran).

Cell culture. MCF-7 and MDA-MB-468 breast cancer cell lines obtained from National Cell Bank of Iran (NCBI) were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 μ/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2, 95% air. Cultures were regularly examined using inverted microscope (Micros, Austria).

Cell survival/cytotoxicity assay. A 1.0 mM stock solution of tamoxifen citrate was prepared in absolute ethanol and stored at -20°C. Cells were plated at density of 5×10^5 cells/ml in a 24-well plate. Next day, cells were treated with various concentrations of tamoxifen (0.1, 0.5, 1, 2.5, 5, 10, 15 and 20 μM) and after 6, 12, 24, 36 and 48 h, cell viability was assessed by trypan blue (0.4% w/v) and acridine orange (1μg/ml) exclusion assay. "Dead cells" were defined as those stained with trypan blue or those whose nucleus emitted brilliant green fluorescence with acridine orange. In all experiments, control sister cultures were treated with an equivalent volume of the vehicle ethanol (maximum 0.1%).

Flow cytometry. Annexin V-FLUOS staining kit was used as manufacturer's recommendation. Approximately 10^5 cells/well were plated in a 24-well plate and allowed to adhere after 48 h. The medium was replaced with fresh culture medium containing tamoxifen at final concentrations of 0.1, 0.5, 1, 2.5, 5, 10, 15 and 20 μM. Non-treated wells received an equivalent volume of ethanol (<0.1%). To determine optimal time course for the induction of apoptosis, cells were harvested after 12, 24, 36 and 48 h. Cells were trypsinized, washed in PBS and centrifuged at 200 ×g for 5 minutes. The pellets were resuspended in 100 μl of staining solution (containing annexin V–fluorescein and propidium iodide in Hepes buffer), and were mixed gently and incubated for 15 min at room temperature (15-25ºC) in the dark. The samples were read in a FACS calibur flow cytometer (USA) using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection, and a filter >600 nm for Propidium iodide detection. Analyses were performed by the software supplied in the instrument.

Analysis of nuclear morphology. Cells were plated in 8-well chamber slides and allowed to adhere. Tamoxifen treated cells were fixed with methanol–acetic acid 3:1 (v/v) for 10 min and then stained with Hoechst 33258 (1 mg/ ml) at 37°C in the dark (10 min). Slides were washed in distilled water and mounted in a mounting medium (containing citric acid and disodium orthophosphate dissolved in distilled water and glycerol (1:1)) and examined by an epifluorescence microscope (Micros, Austria). Apoptotic cells were defined on the basis of characteristic changes in the nuclear morphology.

RESULTS AND DISCUSSION

To evaluate cytotoxicity of tamoxifen, viability tests were applied using trypan blue and acridine orange dyes. Both dyes showed similar results, although, acridine orange test was more precise than trypan blue method.

The flow cytometer has recently become the instrument of choice for analysis of cell kinetics and offers the possibility of rapid and accurate analysis of a large population of individual cell [11]. Therefore, fluorescein-conjugated annexin V (FL1-H) and PI (FL2-H) staining detected by flow cytometry were used as criteria for distinguishing dividing, apoptotic and necrotic cells. In this study, the treatment of ER^- breast cancer MDA-MB-468 cells with 20 μM tamoxifen resulted in a significant (χ^2 test, $P<0.05$) increase in the cell death, apoptosis and growth inhibition in a time-dependent manner and reached the peak value at 36 h. However, treatment with lower doses (0.1, 0.5, 1, 2.5, 5, 10 and 15 μM) was unable to increase the number of dead cells significantly (χ^2 test, $P>0.5$) (Figs.1A, 2A and 3A). Hashimoto *et al.* [12] using different ER^- cell line (GT1-7 cells) have observed the same dose-response (20 μM) within 24 h. Other investigators have also reported a consistent occurrence of apoptosis at higher doses of tamoxifen in ER^- non-breast cancer cell lines [13, 14].

In the estrogen receptor positive breast cancer MCF-7 cells, tamoxifen at 0.1 μM concentration exert only a cytostatic effect (Fig. 2B). At 1 μM concentration, it resulted in a significant (χ^2 test, $P<0.05$) increase in cell death, early and late apoptosis, as well as growth inhibition, in a time-dependent manner and reached the peak value at 24 and 36 h, respectively (Figs. 1B & 3B). Similar effects, although less potent were also observed

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when 0.5 μM tamoxifen was used (χ^2 test, P<0.1). These findings are consistent with the previous studies [15,16] and reveal that estrogen receptor positive cells are more susceptible to the apoptotic effect of tamoxifen than estrogen receptor negative ones. However, it is important to note that not only tamoxifen at higher doses (i.e. > 2.5μM) is unable to exert apoptotic or growth inhibitory effect, but rather has a stimulatory action on the proliferation of MCF-7 cells (Fig. 2B). This kinetic study revealed that tamoxifen is a potent antagonist of estrogen and induces apoptosis in ER^+ cells, however, at concentrations higher than 2.5 μM, it shows an estrogenic behavior and acts as an agonist of estrogen.

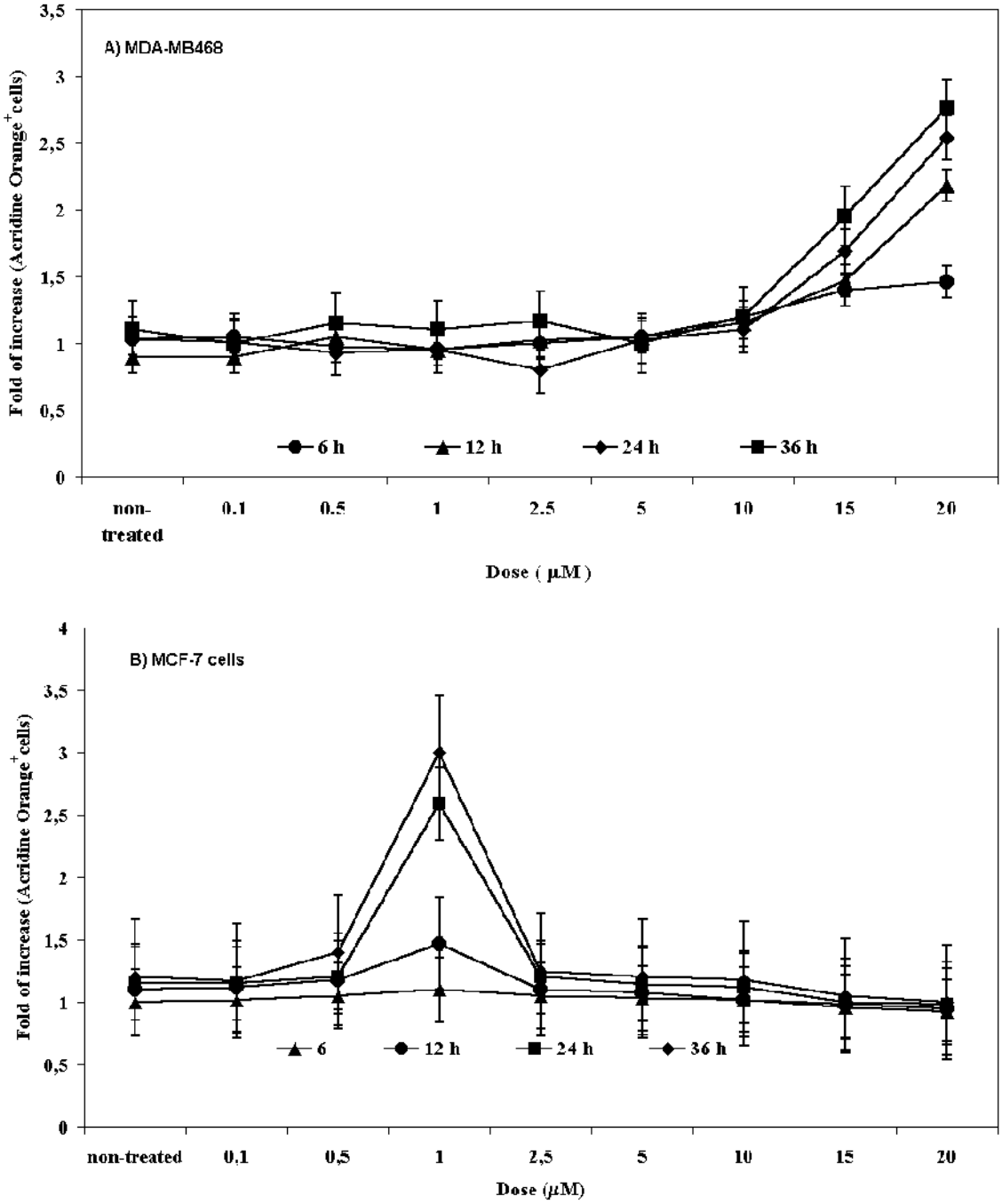

Fig. 1. Acridine orange-based dye exclusion cell viability/cytotoxicity test. The results showed that in the MDA-MB468 **(A)**, tamoxifen at 20 μM induces a significant increase in the cell death (χ^2 test, P<0.05). In the MCF-7 **(B)**, a significant increase in the cell death was occurred at 1 μM concentration (χ^2 test, P<0.05). Each point represents mean ± SD (n = 6).

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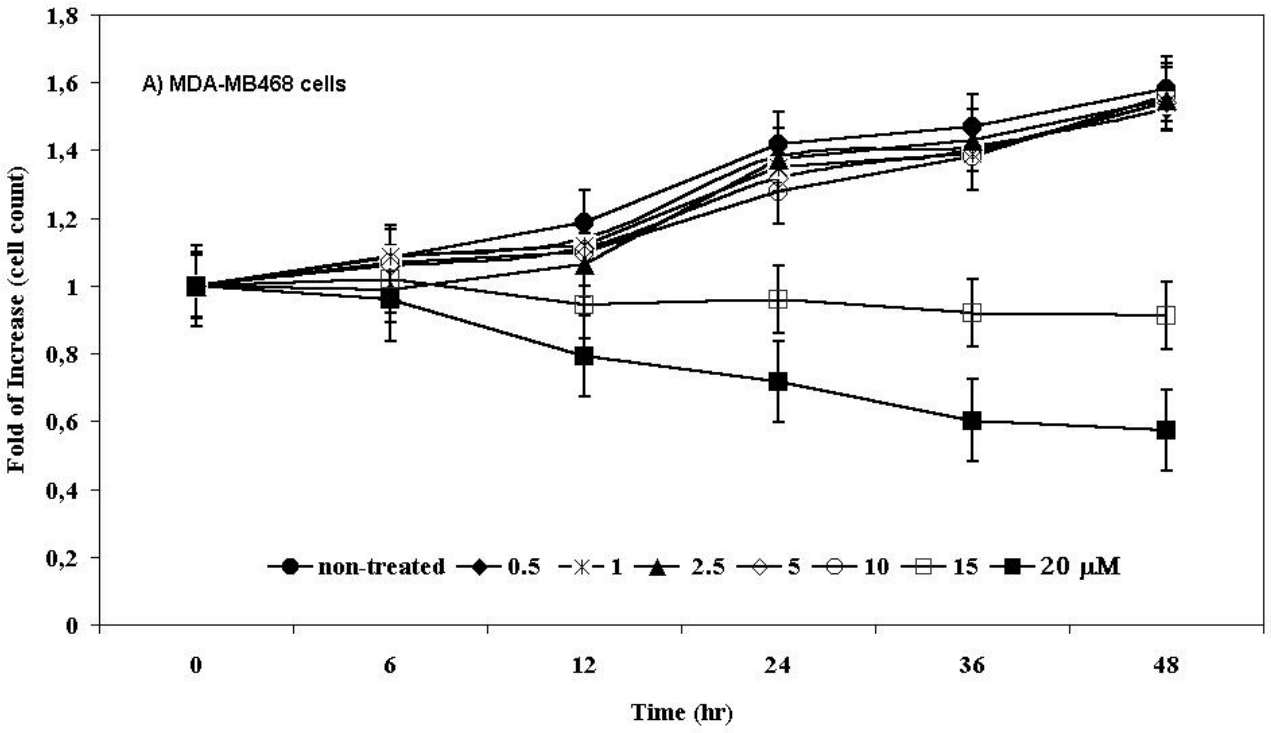

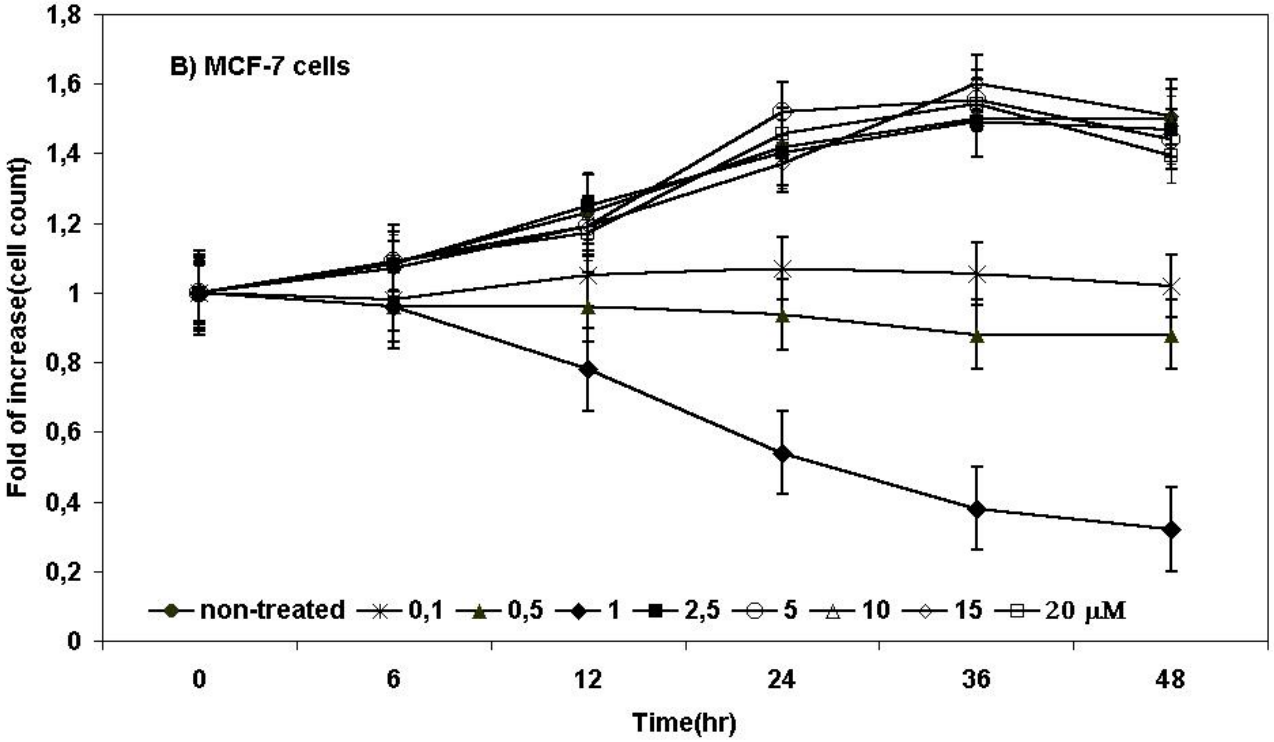

Fig. 2. Cell growth curve. In the MDA-MB468 **(A)**, tamoxifen at lower doses, i.e. 0.5,1, 2.5, 5, 10 and 15 µM, was unable to exert a growth inhibitory effect. However, at 20 µM resulted in a significant growth inhibition (χ^2 test, P<0.05). In the MCF-7 cells **(B)**, tamoxifen exerted a cytostatic effect at 0.1 µM and cytotoxic effect at 0.5 µM. A significant growth inhibition was achieved at 1 µM concentration (χ^2 test, P<0.05). Each point represents mean ± SD (n = 6).

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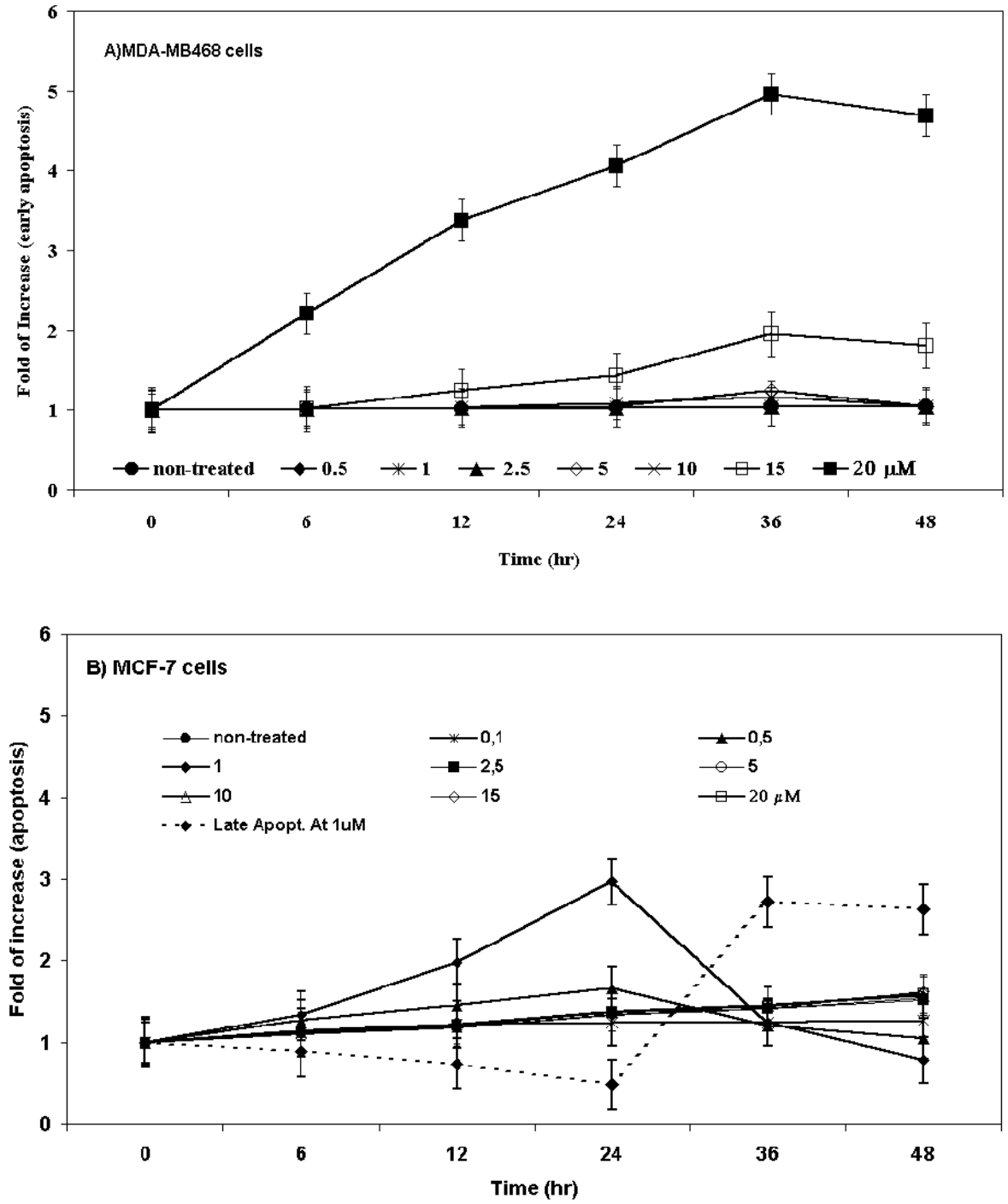

Fig. 3. Flow cytometric evaluation of apoptosis. In the MDA-MB468 **(A)**, tamoxifen at 0.5, 1, 2.5, 5, 10 and 15 µM was unable to induce apoptosis. However, at 20 µM concentration, it showed a significant increase in the early apoptosis. In the MCF-7 cells **(B)**, a significant induction of early and late (dotted line) apoptosis were achieved at 1µM concentration. Each point represents mean ± SD (n = 6).

Although, tamoxifen exhibits anti-estrogenic or estrogenic behavior, we showed here the turning point of these opposed effects in the kinetics of tamoxifen treated cells. Therefore, our findings are new and may serve as a guideline for precautionary evaluation of suggested high doses of tamoxifen for the treatment of non-breast or estrogen receptor negative cancers [13, 17-19].

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