

THE EFFECTS OF NAVELBINE APPLICATION AS A SINGLE AGENT AND WITH TAMOXIFEN, EPIRUBICIN OR CARBOPLATIN ON GROWTH KINETICS OF FM3A CELLS

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Abstract

In this study, after investigation of cytotoxic effects of Navelbine on tumour derived cells, the most efficient drug or drug combinations were explored using Navelbine+Tamoxifen, Navelbine+Epirubicin and Navelbine+Carboplatin. FM3A cells derived from C3H mouse mammary carcinoma were used in this investigation. Growth rate, mitotic index, labelling index and as being cell kinetics parameters were evaluated in the experiments. Although Navelbine application on FM3A cell cultures was successful, Tamoxifen application showed better experimental results. Therefore Tamoxifen was indicated as decreasing cell kinetics parameters. If drugs were considered according to their effects on cultures, Epirubicin and Navelbine were also efficient respectively compared Tamoxifen application. Carboplatin application did not change growth kinetics of FM3A cells. When drug combinations were applied on FM3A cell cultures, Navelbine+Epirubicin combination on cell kinetics parameters was more effective than the others. Navelbine+Tamoxifen was indicated as a second effective combination.

Introduction

Breast cancer risk factors include duration of exposure to female hormones (early menarche and late menopause); reproductive factors (nulliparity, late age of first pregnancy); dietary and low physical activity factors (obesity; high-fat diet); ionizing radiation during breast development; chronic use of hormone replacement therapy; and genetic inheritance (family history) of breast cancer such as BRCA1, BRCA2, or p53 germline mutations (Ruddon, 2007).

The tubulin-binding agents are the most widely used chemotherapeutic drugs in cancer treatment (Pasquier and Kavallaris, 2007). Molecular target of the activity of Navelbine is tubulin / microtubule dynamic stability. Navelbine shows its activity by inhibiting tubulin polymerization and especially affecting the mitotic microtubule. Navelbine (vinorelbine) leads to cell death in interphase or next mitosis by inhibiting mitosis in G₂ + M phase (Dumontet and Sikic, 1999).

The first “classic anti- estrogen” is Tamoxifen (Bai and Gus, 2009). For more than 35 years, tamoxifen has been the gold standard for the endocrine treatment of all stages of estrogen-receptor-positive breast cancer (Jordan, 2003). Tamoxifen create an antiestrogen impact by blocking estrogen receptors in target cells. The using rate of tamoxifen as adjuvant treatment is increasing day by day in the treatment of breast cancer and metastases containing estrogen receptors. Tamoxifen enters into specific interactions with other cell structures other than receptors in mammary cells (Gorlich and Jandrig, 1997).

Anthracyclines which have very important role in tumor chemotherapy exhibit their cytostatic effects by causing maximum cell death during S and G₂ phases of the cell cycle. Effect mechanism of epirubicin which is an antibiotic from the class of anthracycline and has antineoplastic activity is due to the ability of binding DNA. Cell culture studies showed that Epirubicin passes to cell quickly, localized in the nucleus inhibit nucleic acid synthesis and mitosis (Di Marco, 1984; Yeung et al., 1988; Young and Weenen, 1984).

Cisplatin is one of the most potent chemotherapy drugs widely used for cancer treatment (Florea and Büsselberg, 2011). These drugs exert their antiproliferative effects by creating intrastrand and interstrand DNA cross-links, which block DNA replication (Wagner and Karnitz, 2009).

In this study, we aimed to investigate Navelbine widely used in the chemotherapy of tumor show how impact on mouse mammary carcinoma and whether this effect vary during combination with Tamoxifen, Epirubicin and Carboplatin. Also detection of the most effective drugs and drug combinations have been tried by applying Tamoxifen, Epirubicin and Carboplatin alone to FM3A cell cultures. To achieve the objective of the study, Mouse FM3A after administration of drugs to FM3A mouse mammary carcinoma cells, cell kinetic parameters including growth rate, mitotic index and labelling index were determined. As well as this, it is aimed to contribute to research of tumor with this preliminary study demonstrating the effects of antineoplastic drugs such as Navelbine, Tamoxifen, Epirubicinve Carboplatin and their combined use.

Methods

Cell line and Cell Culture

FM3A cell line originated from C3H mouse mammary carcinoma used in this study was obtained from European animal cell culture collection and grown in our cell and tissue laboratory since 1995. These cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 µg/ml streptomycin (Streptomycin sulphate, I. E. Ulugay), 100 IU/ml penicilin (Pronapen, Pfizer), amphotericin B (Sigma, USA) and 2 mM glutamine at 37 °C in humidified atmosphere of 5% CO₂ in air. The pH of the medium was adjusted to 7.2 with NaHCO₃.

Cell Seeding

For experiments in 24-well plates, 15 000 cells are seeded per well in 1 ml of cell growth medium. For 24 hours incubation was continued at 37 °C in a mixture of 95% air and 5% CO₂ and the cells were ready for experiments.

Determination of Estrogen receptor

ER levels were studied by the methods of (Lippman and Huff, 1976) with minor modifications. ER activity as demonstrated by the dextran-coated charcoal technique is closely correlated with the clinical ability of arimidex to inhibit tumour growth. Cell suspension was mixed with buffer. This mixture was homogenized with a Teflon-glass homogenizer for 5-6 seconds 800 rev/min. The homogenate was centrifuged at 100.000 g for 60 min and then 0.1 ml cytosol sample was incubated 1 nM [³H] estradiol with 60 nM diethylstilbestrol and without diethylstilbestrol at +4 °C for one night. After incubation, non-bound estradiol was destroyed by

treatment with dextran-coated charcoal and the radioactivity of the supernatant was counted in a liquid scintillation counter. Specific binding was determined from tritium composition differences between incubated samples and diethylstilbestrol. Data were analyzed according to Scatchard (Klotz, 1982) and estrogen content was found as 12.1 fmol/mg protein. Such a level is a characteristic of estrogen responsive tumors (Mc Guire et al., 1990).

Preparing drug concentrations

Drug concentrations that were used in the this study were determined based on previous *in vitro* and clinical studies (Topçul et al., 2002). 10 mg / ml stock solution in sterile injectable Navelbine (Navelbine[®], Pierre Fabre Medicament Boulogne, France), 10 mg Tamoxifen drage (Nolvadex[®], Zeneca Pharmaceuticals, A.B.D.), 10 mg Epirubicin sterile lyophilized powder in admixture (Farmarubicin[®], Farmitalia Carlo ErbaS.p.A., Italy) and 15 mg/150 mL stock solution of sterile injectable Carboplatin (Carbo Whether[®], Pharmachemi BV Netherlands) were dissolved in sterile suitable medias. The required final concentrations are 0.02 µg/ml for Navelbine (dissolved in RPMI-1640), 0.01 µg/ml for Tamoxifen (dissolved in Balanced Salt Solution (BSS), Gibcolab.), 0.01 µg/ml for Epirubicin (dissolved in RPMI-1640) and 0.01 µg/ml for Carboplatin.

Preparing ³H-thymidine

9 ml deionized water was added to a vial containing 1 mCi/ml ³H-thymidine (TRA-120, Amersham, England) and stock solution was prepared. Then 100µCi/ml solution was diluted to 1 µCi/ml with cell culture medium. The cells will be labelled with this solution.

Application of Drug and ³H-Thymidine

The drug concentrations applied to FM3A cells were determined according to previous *in vitro* and clinical studies. Cells were exposed to experimental doses of drugs 0-64 h for all the cell kinetic parameters. At the end of these periods, to investigate the labeling index parameter cells were incubated in medium containing 1 µCi/ml ³H-thymidine for 20 min. and cells were labelled. Then fixation was carried out.

Autoradiography

After labelling, the cells were fixed with Carnoys fixative [ethanol: glacial acetic acid (3: 1)] and remaining radioactive materials were washed twice with 2% perchloric acid at 4°C for 30 mins. After preparing slides, they were coated with K.2 gel emulsion (Ilford, England) prepared with distilled water at 40°C to determine the thymidine labelling index. After 3 days of exposure at 4°C, autoradiograms were washed with D- 19 b developer (Kodak) and fixed with Fixaj B (Kodak) (Bassleer et al., 1968). The slides were evaluated after being stained with Giemsa for 3 minutes.

Cell Kinetic Parameters

Hemocytometer assay

The growth rate of control and experimental groups of FM3A cells was determined with in treatment period. After these periods, the cells were selected in each well with 0.25% trypsin. Both control and treatment groups were counted on hemocytometer with trypan blue under light microscope. Then growth rate was evaluated by counting viable and dead cells from each well.

Mitotic Index (MI)

Mitotic index was determined by Feulgen method. Feulgen stain is a staining technique used to identify chromosomal material or DNA in cell specimens. It depends on acid hydrolysis of DNA, therefore fixating agents using strong acids should be avoided (Bedi and Goldstein, 1976; Kjellstrand, 1980). Before the cells were treated with Feulgen, they were treated with 1 N HCl at room temperature for 1 min and then hydrolyzed with 1 N HCl for 10.5 min at 60°C. After slides were treated with Feulgen, they were rinsed for a few minutes in distilled water and stained with 10% Giemsa stain solution (pH 6.8) for 3 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water. And then the slides were air dried. At last mitotic index was calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control. At least 3.000 cells were examined from each slide for MI.

Labelling index (LI)

Autoradiograms were stained with Giemsa stain at 16 °C for 3 min. For each drug concentration and time period of each application 3000 cells were counted under light microscope and percentage of cells labelling was calculated.

Statistics analysis

Arithmetic mean and standard deviation of the data of growth rate, mitotic index and labeling index obtained from the control and experimental groups were calculated. The significance between control and experimental groups was determined by DUNNETT's test and the significance between experimental groups was determined by Student's t-test.

Results

Growth Rate

After administration of Navelbine, Tamoxifen, Epirubicin, Carboplatin alone; Navelbine+Tamoxifen, Navelbine + Epirubicin and Navelbine + Carboplatin combinations to FM3A cell line for 0, 2, 4, 8, 16, 32 and 64 h, growth rate values of the cells were shown in Figure 1, 2, 3. The differences between the control and all experimental groups were significant ($p < 0.01$).

According to data obtained from the growth rate, Tamoxifen administered to FM3A alone suppressed the cell proliferation with the maximum degree. After Tamoxifen, Epirubicin and Navelbine came respectively. There was no inhibitory effect on growth rate of FM3A cell proliferation. Consequently amongst the investigational drugs of Tamoxifen appears to be the best medication for treatment. Then Epirubicin and Navelbine comes.

As a result of application of Navelbine+Tamoxifen, Navelbine+Epirubicin and Navelbine+Carboplatin, it was seen that Navelbine combination with Epirubicin was the most effective drug combination than the others. It is considered that the effect of Navelbine+Carboplatin combination on growth rate of FM3A cells was based on Navelbine.

Mitotic Index (MI)

After administration of Navelbine, Tamoxifen, Epirubicin, Carboplatin alone; Navelbine+Tamoxifen, Navelbine+Epirubicin and Navelbine+Carboplatin combinations to FM3A cell line for 0, 2, 4, 8, 16, 32 and 64 h, mitotic index values of the cells were shown in Figure 4, 5, 6. The differences between the control and all experimental groups were significant ($p < 0.01$).

Although values that are obtained by applying Navelbine alone to the cell, it could not reach conclusions tamoxifen alone implementation. Hence Tamoxifen was determined as the most effective drug in terms of reducing mitotic index values in FM3A cell cultures. Epirubicin and Navelbine came respectively after Tamoxifen according to their effects. Carboplatin did not make any changes in mitotic index values of FM3A cells.

As a result of application of Navelbine+Tamoxifen, Navelbine+Epirubicin and Navelbine+Carboplatin combinations, the best answer is taken from Navelbine + Epirubicin combination. These two drugs make interact in a synergistic manner with each and they make a positive effect in reducing mitotic index. It was determined that Navelbine and Tamoxifen increase the effects of each other when applying Navelbine+Tamoxifen combination, but they are not as effective as Navelbine + Epirubicin. Navelbine+Carboplatin combination was effective on FM3A cells, but it was seen that this effect was based on Navelbine.

Labelling index (LI)

After administration of Navelbine, Tamoxifen, Epirubicin, Carboplatin alone; Navelbine+Tamoxifen, Navelbine+Epirubicin and Navelbine+Carboplatin combinations to FM3A cell line for 0, 2, 4, 8, 16, 32 and 64 h, labelling index values of the cells were shown in Figure 7, 8, 9. The differences between the control and all experimental groups were significant ($p < 0.01$).

As a result of the analysis in terms of labeling index, considering the application of drugs alone, it was seen that Tamoxifen is the most successful drug. After Tamoxifen, Epirubicin and Navelbine come. There is no effect of Carboplatin on FM3A cell culture was seen.

When Navelbine+Tamoxifen, Navelbine+Epirubicin, Navelbine+Carboplatin combinations were examined, Navelbine+Epirubicin combination with synergistic effect was reached the most successful results. Navelbine+Tamoxifen combination followed Navelbine+Epirubicin combination. In Navelbine and Tamoxifen combination, both of the drugs increased the effects of each other.

Discussion

The aim of this study was determination of cytotoxic effect Navelbine which is a chemotherapeutic agent on FM3A cell line, evaluating Navelbine+Tamoxifen, Navelbine+Epirubicin and Navelbine+Carboplatin drug combinations and as well as determining drug or drug combinations showing the most cytotoxic action *in vitro*. In our study, FM3A cells derived from C3H mouse mammary carcinoma were used. For this purpose various cell kinetic parameters including growth rate, mitotic index and labelling index were used.

Cytotoxic potential of Navelbine used in our study as main drug was investigated against human tumor cell lines and it is indicated that it showed activity against a variety of tumor types such as lung, breast, leukemia, colon, urinary bladder, central nervous system and melanoma (Cros and Wright, 1989).

Creating microtubule damage and microtubule binding capacity of Navelbine compared with other vinca alkaloids, it was shown that microtubule damage rate of Navelbine is 20:1 and minimum concentration is sufficient for creating spindle fiber depolymerization (Meninger et al., 1990).

As a result of applying of 0.02 $\mu\text{g/ml}$ Navelbine alone to FM3A cells for 0, 2, 4, 8, 16, 32 and 64 hours, when we examine cell kinetic parameters including growth rate, mitotic index, labeling index, it is considered that Navelbine inhibits the tumor growth depending on reducing the percentage of cells in mitosis and the synthesis phase.

Tamoxifen treatment is considered as the main selection for the patients for patients with hormone-dependent and advanced breast tumors. Typically, this patient group consist of postmenopausal people who have estrogen receptor positive malignant tumor and in these patients there is a long period between the first appearance of tumor and recurrence. There are literatures which enounce that even in people who do not carry these properties are made hormonal treatment and this is a good approach to alleviate this disease (palliation) (Cannobio, et al., 1989; Adams and Knick, 1995; Clean et al., 1991; Maral et al., 1984; Maral et al., 1981). Tamoxifen was shown to be more effective in postmenopausal women than premenopausal women and is used as initial therapy in postmenopausal women nowadays (Fracchi et al., 1968).

Random trials in women with breast cancer to determine the effectiveness of the administration of the hormonal therapy alone or combination with cytotoxic drugs was evaluated by Mouridsen (1990) and it was shown that in postmenopausal women, administration of the hormonal therapy combination with cytotoxic drugs lead to a reduction in premorbid reversion rate ($26\pm 5\%$) and the risk of death ($10\pm 7\%$) (Mouridsen, 1990).

In studies by Kiang et al. (1985) and an Australian-New Zealand research group (1986) it was found that the median survival of patients increased with co-administration of hormone therapy with chemotherapy and The time taken to disease progression was prolonged (Kiang, 1985; Australian and New Zealand Breast Cancer Trial Group, 1986). Numerous studies were performed stating that because of its high cytotoxic activity, the use of Navelbine+Tamoxifen therapy offers an important opportunity (Van Praagh et al., 1995; Chollet et al., 1995; Adenis et al. 1995; Namer et al., 1995).

In our study by examining cell kinetic parameters it was shown that the effect of Tamoxifen alone on FM3A cell culture was more successful than the effect of Navelbine alone. The cytotoxic effect obtained by combined application Navelbine+Tamoxifen was more successful than the administration of these drugs alone. These data indicated that these two drugs enhance the effects of each other. The results of our study seem to be concordant with the above mentioned studies.

Suppression of cell proliferation, reduction of mitotic index and labelling index values by combined administration of Navelbine+Epirubicin support each other and are parallel with the research in literature.

Initially Navelbine+Carboplatin combination created the idea that these two agents may be practical advantage because of their different side effect profiles. In the literature survey that was made with this idea, it was seen that studies about Navelbine+Carboplatin combination was very limited and it was detected that existing studies were often about lung cancer (Bunn, 1989; Calvert, 1989; Calvert et al., 1989).

In a phase II trial by Kakolyris et al., Navelbine, Carboplatin Mitoxantrone used concomitantly and applied to the patients with metastatic breast cancer as $30 \text{ mg/m}^2/\text{day}$ Navelbine, $250 \text{ mg/m}^2/2$ days Carboplatin and $12 \text{ mg/m}^2/\text{day}$ Mitoxantrone at 21-days period. As a result of these applications, it was explained that Carboplatinis treatment method that can be tolerated for breast cancer, but has more side effects (Kakolyris et al., 1999).

The evaluation of the data obtained in our experiments showed that Tamoxifen is the most effective drug when administered alone on FM3A mammary carcinoma cell line and the most effective drug combination is Navelbine+Epirubicin. This situation is concordant with the above mentioned researches. As well as in this study which extensively investigated application of Navelbine alone, with anthracycline, without anthracycline and combination with hormone therapy for the first time, administered drugs and drug combinations to FM3A cells show different effects depending on, especially cell type in literature is based on properties such as metastatic growth of the tumour, estrogen receptor status, tumour size, and histological grading.

Knowledge of the course of the cancer disease, determining whether the tumour has aggressive potential, initial determination of parameters that could indicate the presence of resistance to treatment and then

making an oncobiogram on a small amount of a cell population taken from the tumor may reduce the costs of treatment. As a result of such application patients will treat with the most effective drug or drug combinations for the tumour unlike standard treatment and random treatment of the patients will be prevented. When considering in this aspect, the results obtained from this study will be a significant source in terms of regulation of treatment regimens applied in clinic after testing on larger group of cells *in vitro* and *in vivo*.

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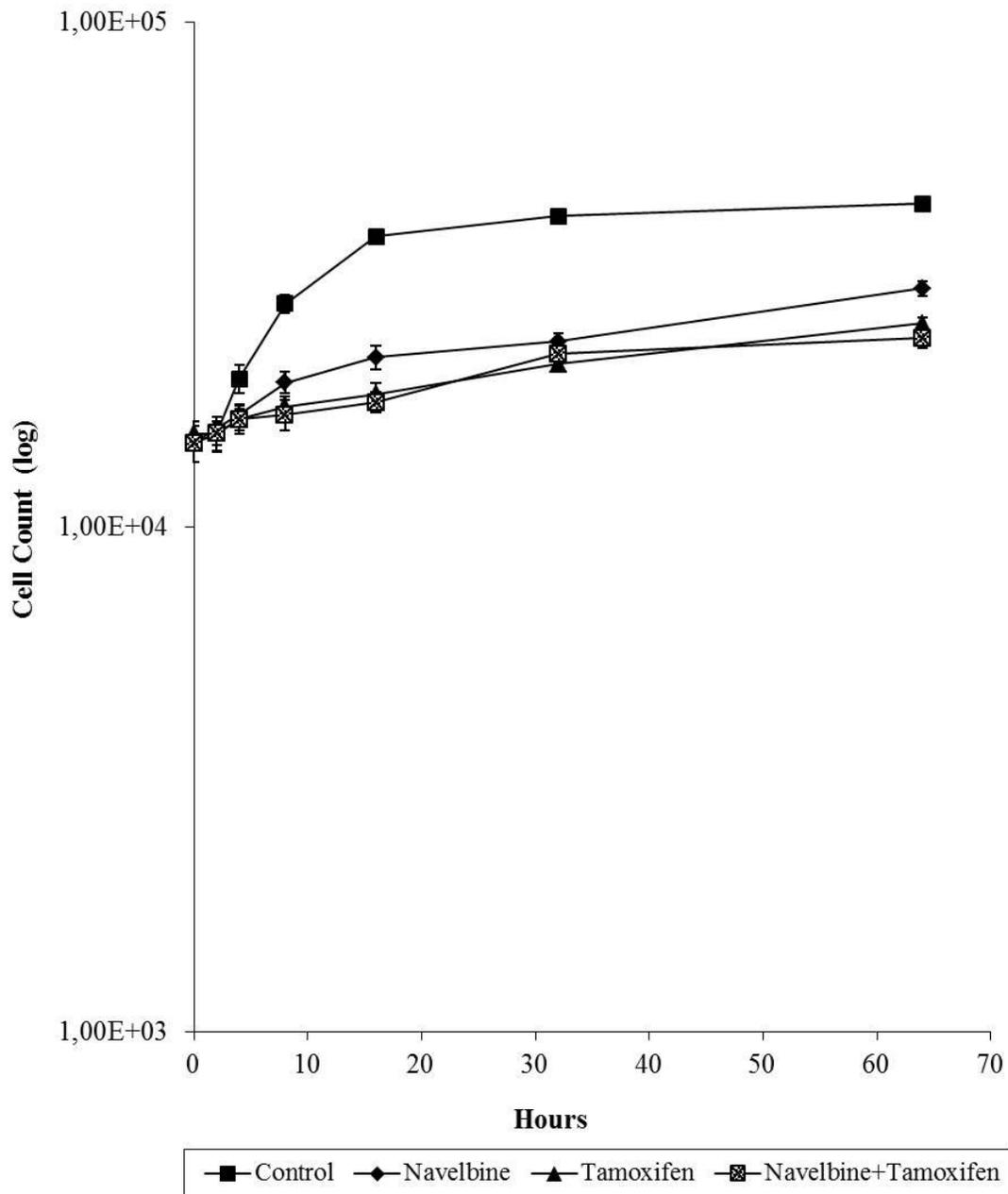


Figure 1: Growth rate values of FM3A cells treated with Navelbine, Tamoxifen alone and Navelbine combination with Tamoxifen ($p < 0.01$).

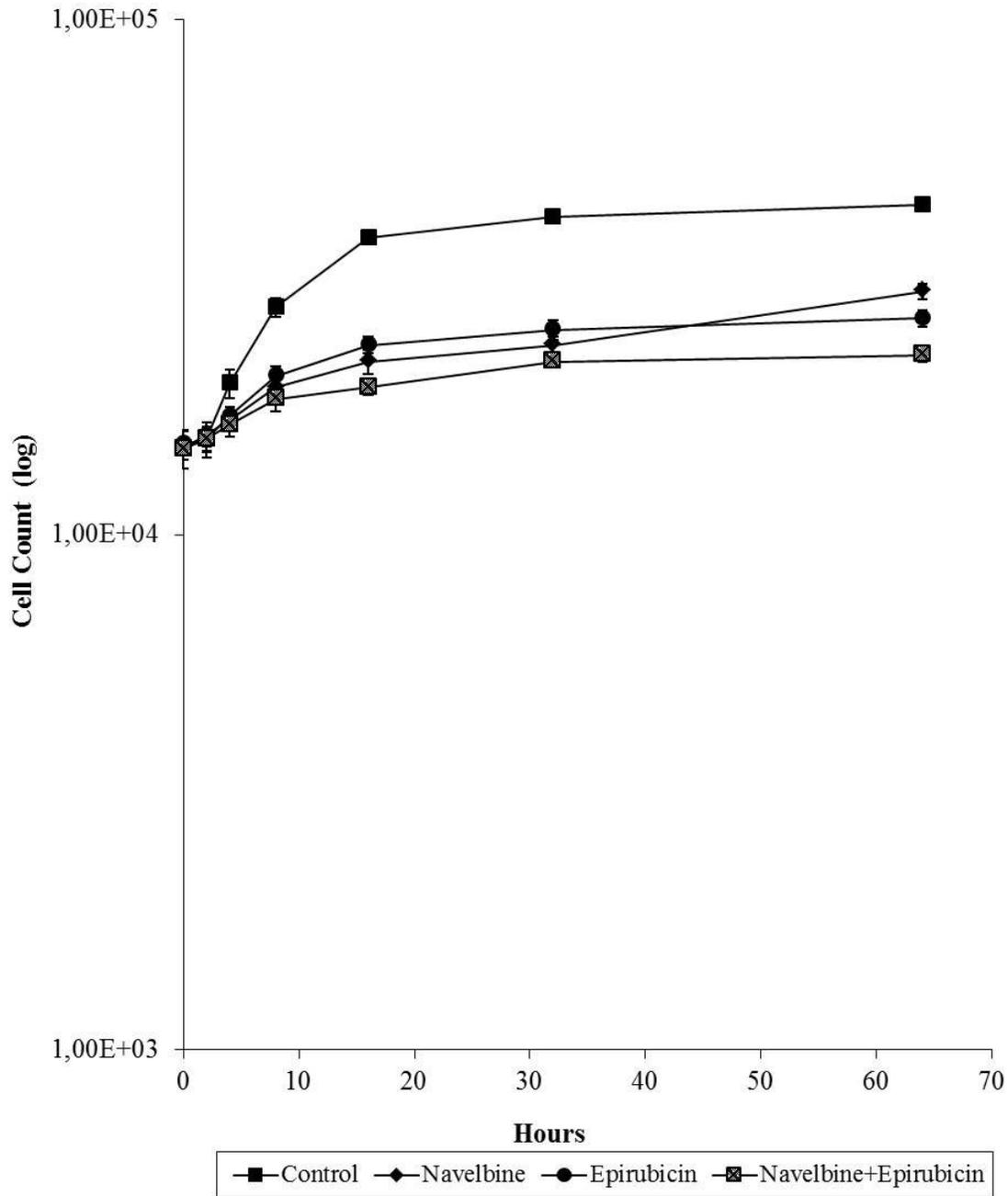


Figure 2: Growth rate values of FM3A cells treated with Navelbine, Epirubicin alone and Navelbine combination with Epirubicin ($p < 0.01$).

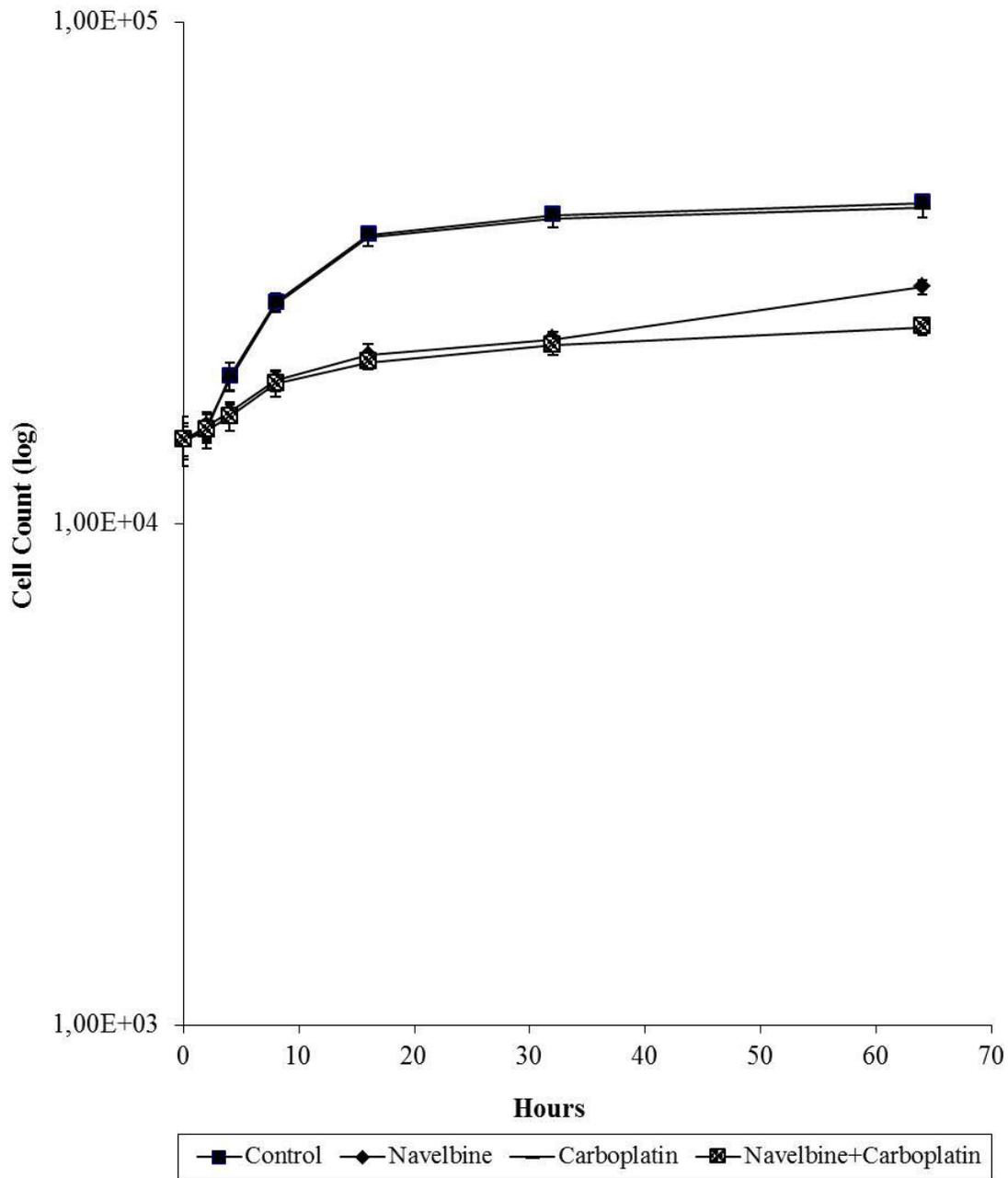


Figure 3: Growth rate values of FM3A cells treated with Navelbine, Carboplatin alone and Navelbine combination with Carboplatin ($p < 0.01$).

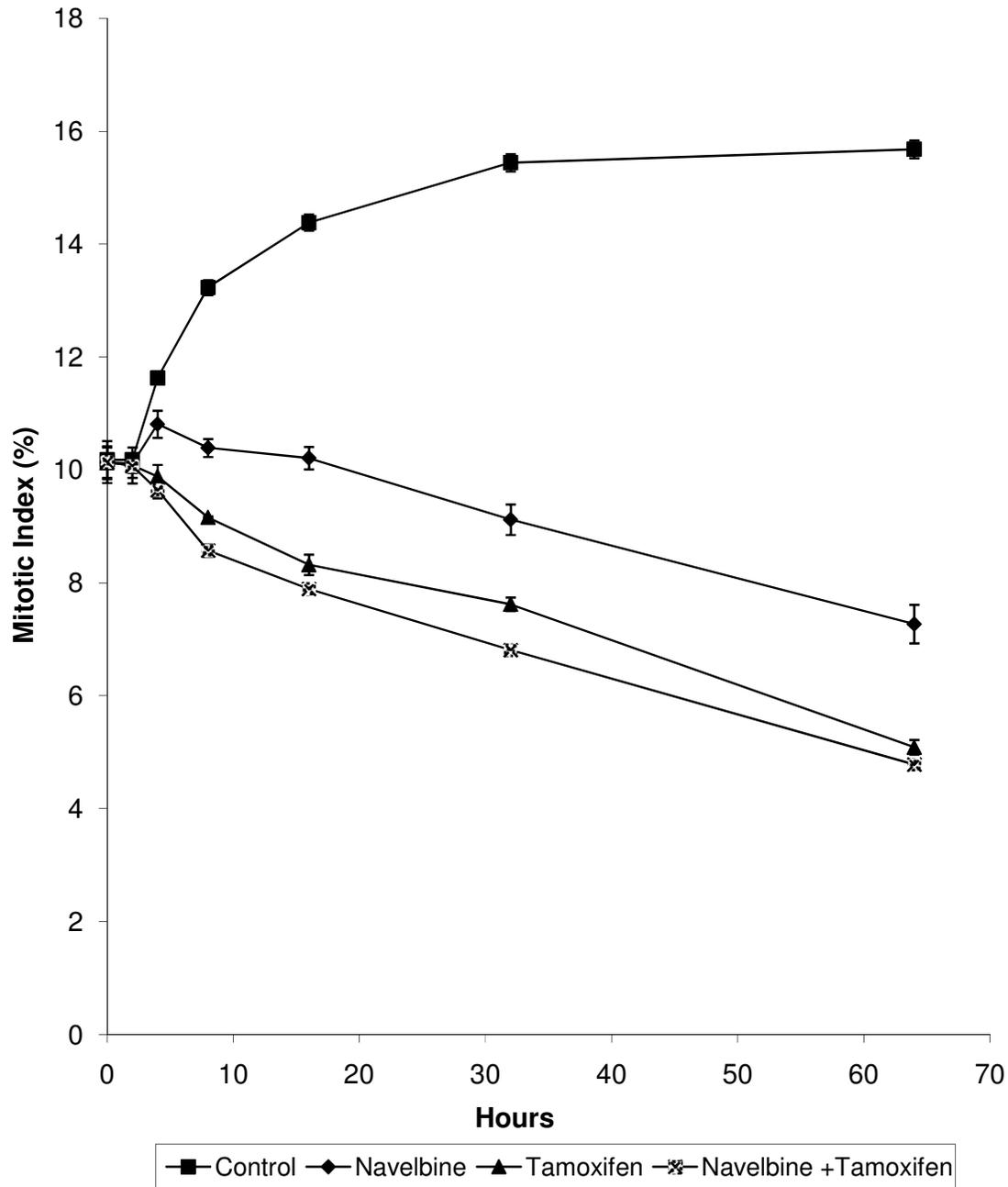


Figure 4: Mitotic index values of FM3A cells treated with Navelbine, Tamoxifen alone and Navelbine combination with Tamoxifen ($p < 0.01$).

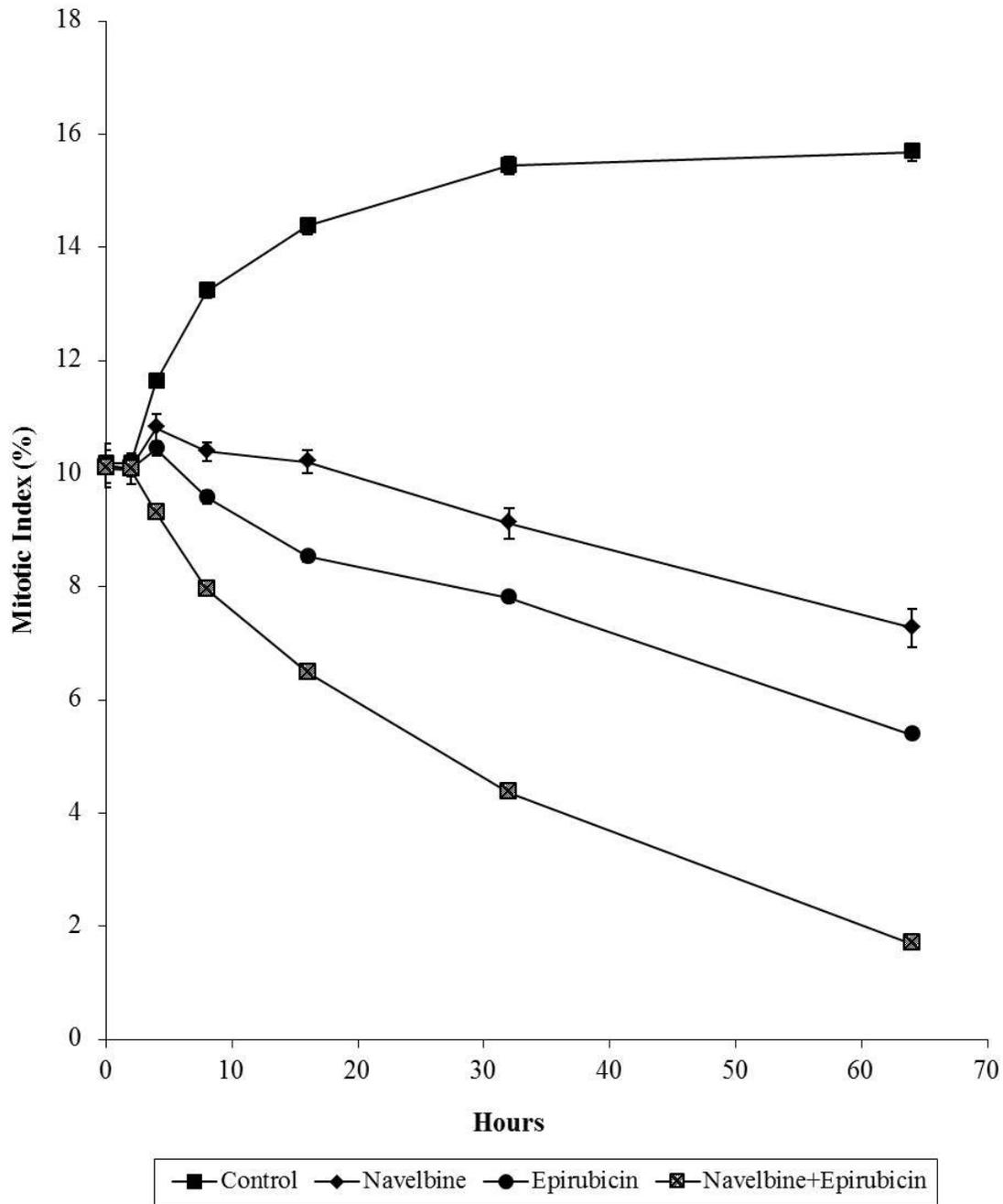


Figure 5: Mitotic index values of FM3A cells treated with Navelbine, Epirubicin alone and Navelbine combination with Epirubicin ($p < 0.01$).

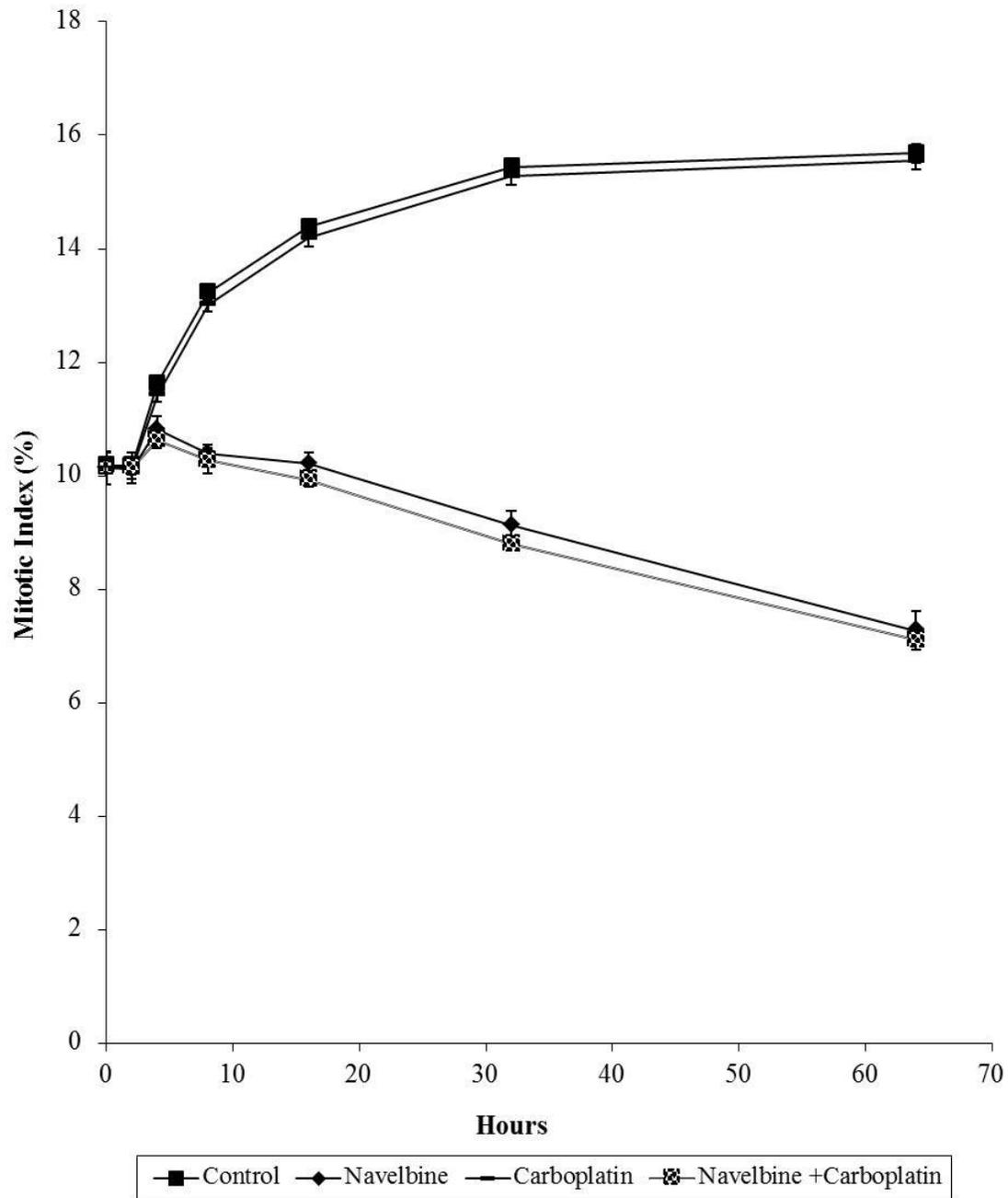


Figure 6: Mitotic index values of FM3A cells treated with Navelbine, Carboplatin alone and Navelbine combination with Carboplatin ($p < 0.01$).

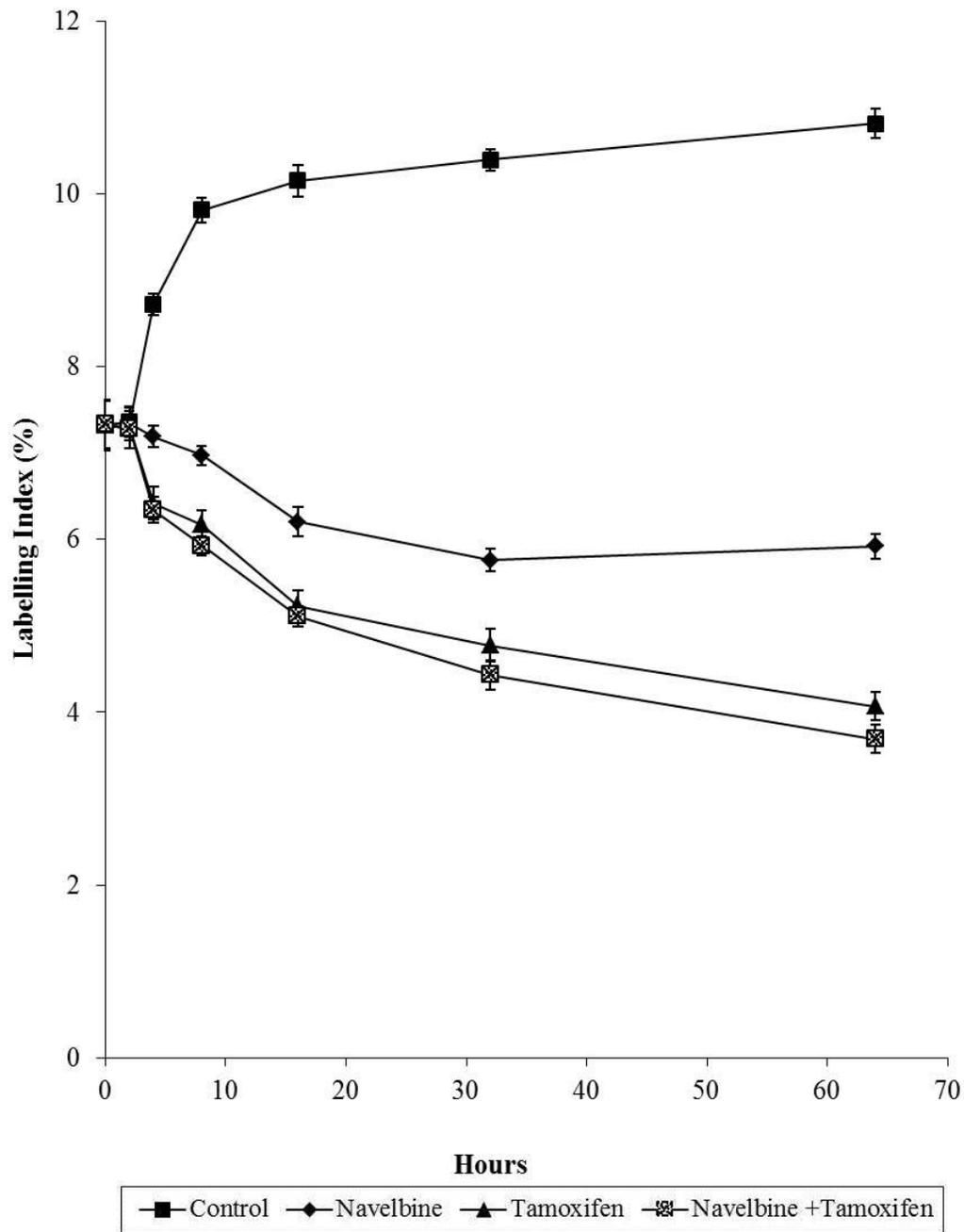


Figure 7: Labelling index values of FM3A cells treated with Navelbine, Tamoxifen alone and Navelbine combination with Tamoxifen ($p < 0.01$).

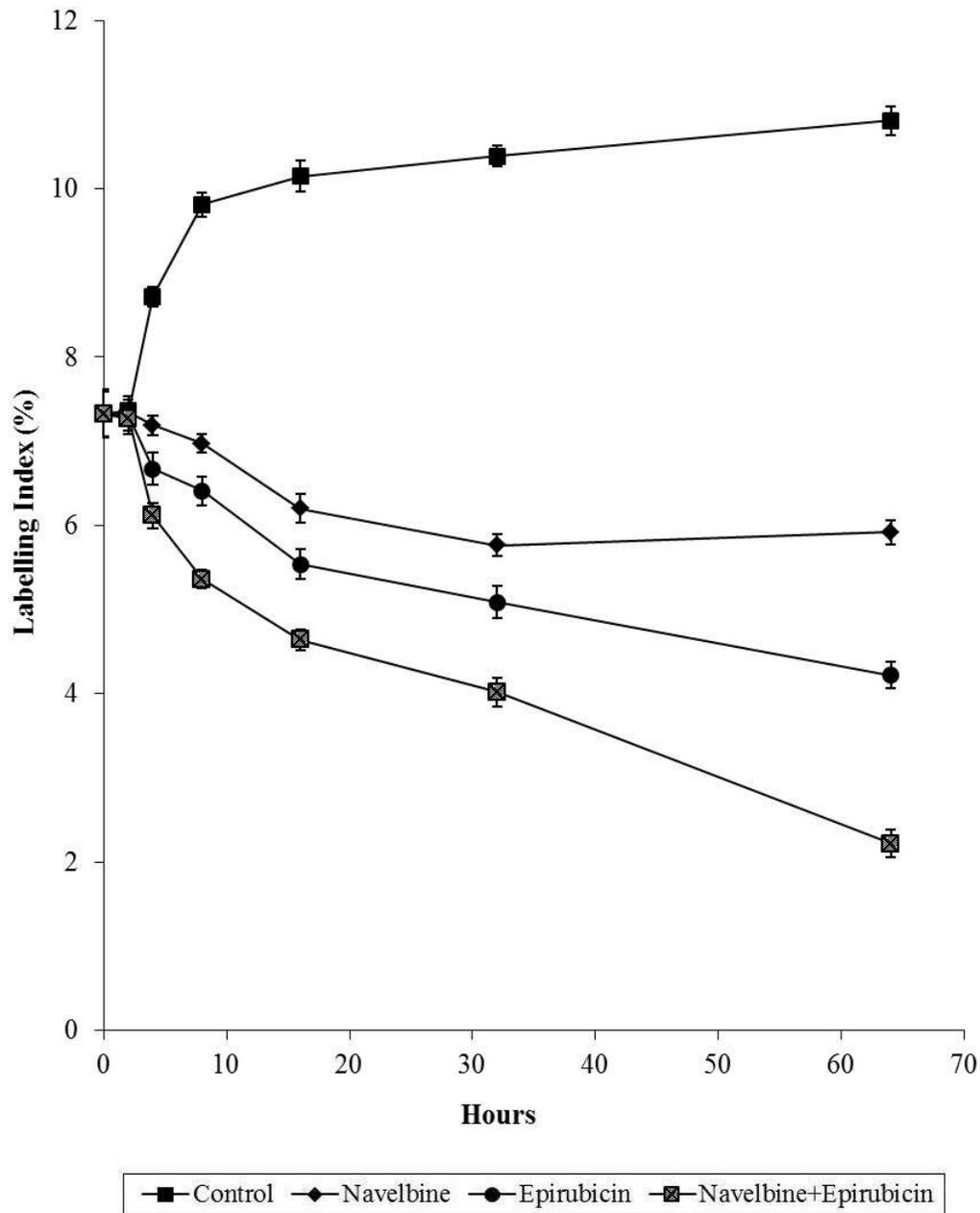


Figure 8: Labelling index values of FM3A cells treated with Navelbine, Epirubicin alone and Navelbine combination with Epirubicin ($p < 0.01$).

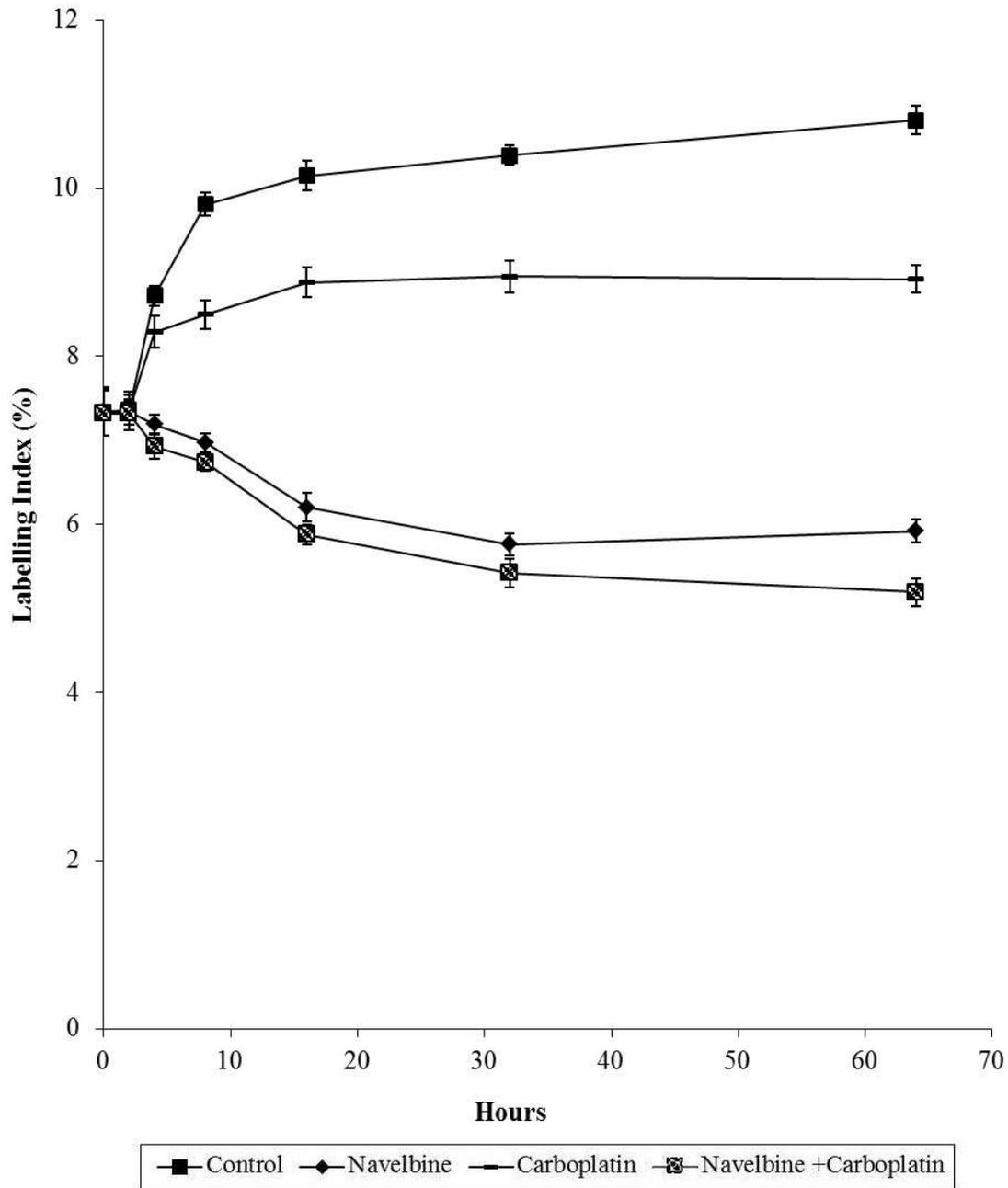


Figure 9: Labelling index values of FM3A cells treated with Navelbine, Carboplatin alone and Navelbine combination with Carboplatin ($p < 0.01$).