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Antiproliferative action of menadione and 1,25(OH)$_2$D$_3$ on breast cancer cells

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ABSTRACT

Calcitriol or 1,25(OH)$_2$D$_3$ is a negative growth regulator of MCF-7 breast cancer cells. The growth arrest is due to apoptosis activation, which involves mitochondrial disruption. This effect is blunted in vitamin D resistant cells (MCF-7^{DRes} cells). Menadione (MEN), a glutathione (GSH)-depleting compound, may potentiate antitumoral effects of anticancer drugs. The aim of this study was to investigate whether MEN enhances cellular responsiveness of MCF-7 cells to 1,25(OH)$_2$D$_3$. Cells were cultured and treated with different concentrations of 1,25(OH)$_2$D$_3$ ± MEN or vehicle for 96 h. GSH levels and the activity of antioxidant enzymes were determined by spectrophotometry and ROS production by flow cytometry. Both drugs decreased growth and enhanced ROS in MCF-7 cells, obtaining the maximal effects when 1,25(OH)$_2$D$_3$ was combined with MEN ($P < 0.01$ *vs.* Control and *vs.* each compound alone). MCF-7^{DRes} cells were not responsive to 1,25(OH)$_2$D$_3$, but the cell proliferation was slightly inhibited by the combined treatment. Calcitriol and MEN separately enhanced antioxidant enzyme activities, but when they were used in combination, the effect was more pronounced ($P < 0.05$ *vs.* Control and *vs.* each compound alone). MEN, calcitriol and the combined treatment decreased GSH levels ($P < 0.05$ *vs.* Control). The data indicate that MEN potentiates the effect of 1,25(OH)$_2$D$_3$ on growth arrest in MCF-7 cells by oxidative stress and increases the activities of antioxidant enzymes, probably as a compensatory mechanism.

1. Introduction

The secosteroid 1,25(OH)$_2$D$_3$ or calcitriol, the most active form of vitamin D$_3$, is considered a potent negative growth regulator of breast cancer cells both in vitro and in vivo [1]. The effects of 1,25(OH)$_2$D$_3$ on breast cancer cells include cell cycle arrest in G_0/G_1, induction of differentiation markers and activation of apoptosis [2]. It has been proposed that vitamin D or analogs inhibit cell growth and induce apoptosis by downregulation of surviving via TGFbeta signaling and activation of p38 MAPK pathway [3]. Sergeev [4] and McConkey and Orrenius [5] have demonstrated that apoptosis induced by 1,25(OH)$_2$D$_3$ depends on Ca^{2+} signaling. It has also been shown that 1,25(OH)$_2$D$_3$ induces apoptosis in MCF-7 cells by disruption of mitochondrial function, which was associated with Bax translocation to mitochondria, cytochrome *c* release, and generation of reactive oxygen species (ROS) [6]. It appears that nanomolar concentrations of 1,25(OH)$_2$D$_3$ mediate growth regulatory effects via mechanisms requiring the nuclear VDR, but micromolar concentrations of vitamin D compounds can exert non-VDR-mediated effects [7].

Toxicity studies have demonstrated that 1,25(OH)$_2$D$_3$ induces hypercalcaemia in animals at concentrations proved to protect against cancer formation or progression [8]. Therefore, new pharmacological approaches have emerged in order to prevent or inhibit breast tumor growth. The increasing number of vitamin D analogs, with similar effects to calcitriol on growth and differentiation but fewer side effects, indicate the considerable interest of these compounds as potential anticancer drugs.

Since glutathione (GSH)-depleting drugs potentiate the effect of some anticancer drugs [9], it is reasonable to think that the calcitriol antiproliferative effect could be enhanced by simultaneous menadione (MEN) treatment. MEN is a quinone that can be toxic by interacting with thiol groups on essential molecules such as proteins and GSH and by oxidative stress resulting from its metabolic redox cycle via one electron reduction to the semiquinone radical [10,11]. It has been shown to produce DNA single-strand breaks and to modify the DNA bases in S30 breast cancer cells [12]. MEN genotoxicity has been attributed to its ability to damage DNA via ROS generation [13]. MCF-7 cell growth is estrogen-dependent [14]. MEN impairs the ability of these cells to bind endogenous estrogen receptor to DNA [15] and directly binds to estrogen receptor inhibiting the transcriptional activity of 17β-estradiol [16]. Therefore, it is possible that the antiproliferative effect of calcitriol could

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be increased by combined treatment with MEN, which would cause higher ROS production and alteration of GSH content and of the antioxidant defences.

It is known that the sensitivity of MCF-7 cells to $1,25(OH)_2D_3$ is different depending on the MCF-7 clones. Continuous exposure of MCF-7 cells to $1,25(OH)_2D_3$ produces apoptosis of those cells unable to differentiate, generating a vitamin D resistant cell population named MCF-7^{DRes} cells [17]. Towsend et al. [18] have done transcriptomic analyses to determine responsive and resistant gene signatures in MCF-7 and MCF-7^{DRes} cells, respectively. The proapoptotic gene BAX was one of the most significantly upregulated genes (3.25-fold) by $1,25(OH)_2D_3$ in MCF-7 cells. In contrast, no clear gene targets associated with the induction of apoptosis and/or cell cycle arrest were upregulated in MCF-7^{DRes} cells by the hormone. However, co-treatment of MCF-7^{DRes} cells with TGF-β_2 plus $1,25(OH)_2D_3$ increased antiproliferative and vitamin D receptor transcriptional effects.

The aim of the present study was to address the following questions: (1) Does combined treatment of $1,25(OH)_2D_3$ and MEN increase the antiproliferative action of vitamin D hormone on MCF-7 and MCF-7^{DRes} cells? (2) To what extension, ROS production is involved in the combination treatment? (3) Is there any compensatory effect of the antioxidant defences in the $1,25(OH)_2D_3$ responsive cells?

2. Material and methods

2.1. Chemicals

Tissue culture media (Dulbeccoĭs Modified Eagle Medium), phosphate buffered saline (PBS) and trypsin–EDTA (0.05%) were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Fetal bovine serum (FBS) was from Natocor (Argentina). $1,25(OH)_2D_3$ was a generous gift from LEO Pharma (Ballerup, Denmark). Menadione (2-methyl-1,4-naphtoquinone sodium bisulfite, purity: 95%) was obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Cell culture and treatments

MCF-7 and MCF-7^{DRes} [6] cells were cultured in DMEM supplemented with 5% FBS, penicillin and streptomycin at 37 °C under 95% humidity. Cells were subcultured twice weekly and 24 h before the initiation of an experiment. Cells were plated onto 10 cm plates and allowed to grow in 10 ml of complete media for 24 h (until 70–80% confluency). Cultures were treated with $1,25(OH)_2D_3$ with or without MEN or vehicle for the indicated times and concentrations, and then harvested for enzymatic assays. Vehicle ethanol was added to control cultures in a concentration below 0.1%.

2.3. Cell growth assay and morphology

Cell density was quantified by crystal violet staining. Briefly, cells were seeded at 1000 cells/well into 24-well plates and treated with $1,25(OH)_2D_3 \pm$ MEN or vehicle at the indicated times. Cells were fixed with 1% glutaraldehyde for 15 min, incubated with 0.1% crystal violet for 30 min, destained with H_2O and finally solubilized with Triton X-100 (0.2%). Absorbance was measured at 562 nm (minus background at 630 nm). The morphology of the cells was assessed by phase contrast using an Olympus inverted microscope equipped with a Spot RT digital camera.

2.4. Flow cytometry

Cells were harvested by trypsinization and pelleted by centrifugation. ROS were analyzed on an Epics XL Flow Cytometer in cell suspensions (1×10^6 cells) incubated with 4 μM hydroethidine (HE, Molecular Probes, Invitrogen, Grand Island, NY, USA) in PBS for 15 min at 37 °C. Conversion of HE to ethidium by superoxide anion was analyzed by flow cytometry on FL3 using a 620-nm band pass filter. Data were analyzed using MULTIPLUS AV analysis software.

2.5. Spectrophotometric procedures

After incubation, cells were washed twice with PBS and resuspended in 1 ml PBS buffer containing 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF, 1% Triton X-100. Cells were harvested and finally homogenized manually at 4 °C. The crude homogenate was cleared by centrifugation ($15{,}000 \times g$) for 10 min at 4 °C, and the resulting supernatants were used for the enzyme assays. Superoxide dismutase (SOD) activity was determined in 1 μM EDTA, 50 mM potassium phosphate buffer, pH 7.8, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 40 μM riboflavine. SOD activity is defined in terms of its ability of inhibiting superoxide anion (O_2^-)-dependent reaction due to the competition between SOD and NBT [19]. Catalase (CAT) activity was assayed in 50 mM potassium phosphate buffer pH 7.4 and 0.3 M H_2O_2. The H_2O_2 decomposition rate is directly proportional to enzyme activity [20]. GSH-peroxidase (GPx) assay was performed as described by Cheng et al. [21] with 400 μM tert-butylhydroperoxide as an artificial substrate. Total GSH content was assayed by the glutathione disulfide reductase-5,5′-dithiobis(2-nitrobenzoate) recycling procedure [22]. Protein concentrations were determined by the Bradford assay [23] using purified serum albumin as standard.

2.6. Statistics

Results were evaluated by one-way analysis of variance (ANOVA) and Bonferroni test was used as a post hoc test. Differences were considered significant at $P < 0.05$.

3. Results

The morphology of MCF-7 cells under MEN, $1,25(OH)_2D_3$ or the combined treatment is depicted in Fig. 1. The cells demonstrated a typical epithelial morphology upon exposure to vehicle or MEN. However, when the cells were treated with 100 nM $1,25(OH)_2D_3$ alone or in combination with 5 μM MEN, displayed morphological features of apoptosis. Cells were shrunken and laid above the plane of adherent monolayer; these features were more pronounced after the combination treatment. No effects of any compound alone were observed in MCF-7^{DRes} cells, but the combined treatment induced some morphological characteristics of apoptosis (data not shown).

Growth assays indicate proliferation inhibition of MCF-7 cells treated with $1,25(OH)_2D_3$ alone and/or in combination with MEN. As shown in Fig. 2 (Panels A and B), cell growth of MCF-7 cells was not inhibited by 1 or 10 nM calcitriol alone or in combination with 1 or 2.5 μM MEN. Only a slight reduction on MCF-7 proliferation was observed when 5 μM MEN was added alone to the cultured medium (Fig. 2, Panels A–C). The combination of 1 or 10 nM calcitriol with 5 μM MEN caused a small reduction in the proliferation of MCF-7 cells (Fig. 2, Panels A and B). However, MCF-7 cells treated with 100 nM calcitriol alone or in combination with different doses of MEN showed a sharp inhibition of cell growth (Fig. 2, Panel C), being maximal at 5 μM MEN. Instead, MCF-7^{DRes} cells were not responsive to 1–10 nM calcitriol or in combination with MEN at the concentrations tested, but they were sensitive to the combined action of 100 nM $1,25(OH)_2D_3$ plus 5 μM MEN (Fig. 2, Panel D). An acute high dose of 50 μM MEN for 1 h significantly reduced growth of MCF-7 cells by 30% (data not shown). When cells were treated with 100 nM calcitriol for 96 h previously to the addition of the acute dose of

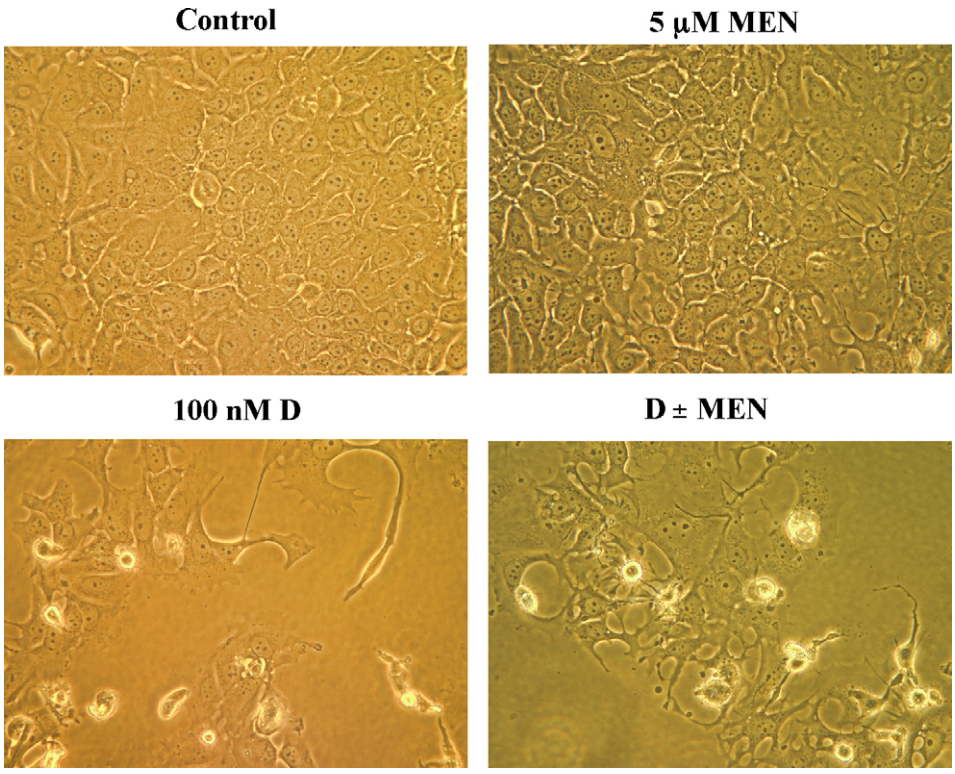

Fig. 1. Effects of 1,25(OH)$_2$D$_3$ (D) and/or MEN on the morphology of breast cancer cells. Phase contrast images (magnification, 200×) of the MCF-7cells after treatment with 100 nM D, 5 μM MEN or both for 96 h.

Table 1
Effects of 1,25 (OH)$_2$D$_3$ (D) and MEN on the activity of antioxidant enzymes in MCF-7 cells.

Treatment	SOD (U/mg prot)	GPx (mU/mg prot)	CAT (U/mg prot)
Control	25.55 ± 1.14	124.2 ± 14.70	1.31 ± 0.27
100 nM D	50.99 ± 2.69*	488.2 ± 77.88*	6.56 ± 0.89*
5 μM MEN	39.58 ± 1.66*	379.8 ± 59.90*	3.07 ± 0.73*
100 nM D + 5 μM MEN	61.95 ± 8.60*,¶	976.6 ± 74.10*,¶	14.80 ± 4.43*,¶

Cells were incubated for 96 h with 100 nM D and/or 5 μM MEN and enzyme activities were measured as indicated under Section 2.

* $P < 0.05$ *vs.* Control.

¶ $P < 0.05$ *vs.* MEN or D alone.

MEN, additive effects were observed even in MCF-7^{DRes} cells (data not shown).

To know whether the oxidative stress was triggered by calcitriol and/or MEN on MCF-7 cells, measurements of ROS and total GSH content were performed. As can be seen in Fig. 3, ROS production was slightly increased by 1,25(OH)$_2$D$_3$ alone in MCF-7 cells, but an increase greater than 20% in the ROS generation was obtained after the combined treatment of calcitriol plus MEN. Total GSH content was decreased either by calcitriol or MEN, but the combined treatment did not further enhance the effect (Fig. 4).

The modulation of antioxidant enzyme activities by 1,25(OH)$_2$D$_3$ and MEN is shown in Table 1. MCF-7 cells were exposed to 100 nM calcitriol and/or 5 μM MEN for 96 h and their effects on SOD, CAT and GPx activities were assayed. Both compounds significantly enhanced the antioxidant enzyme activities over non-treated controls. Moreover, if they were used in the combined form, they produced a more pronounced increment.

4. Discussion

This study shows evidence that MEN modulates MCF-7 cell sensitivity to 1,25(OH)$_2$D$_3$-mediated apoptosis. As previously shown, morphological assessments demonstrate that MCF-7 cells treated with 100 nM 1,25(OH)$_2$D$_3$ exhibit characteristic apoptotic features, leading to 50% of dead cells after 96 h of treatment. This antiproliferative action is increased by MEN, an effect that is dose dependent. The concentrations of MEN used in this study are quite similar to those employed by other authors who have explored antitumor activity of this quinone on human or mice tumor cells [24,25]. MEN concentrations used in our study may be relevant for in vivo and clinical studies, far away from the doses capable to produce toxicity [26]. The combined treatment potentiates the oxidative stress as judged by the significant increment in the ROS production. Narvaez and Welsh [6] have demonstrated that cytochrome *c* release from mitochondria and ROS production are early events in 1,25(OH)$_2$D$_3$-mediated apoptosis of MCF-7 cells, both events occurring before appearance of any morphological marker of apoptosis. MEN metabolism also produces superoxide anions and free hydroxyl radicals through its redox cycle [27]. An important signal of free hydroxyl groups has been also demonstrated in chick enterocytes after MEN administration, leading to a decrease in intestinal Ca^{2+} absorption [28].

Since the main site generating ROS is the mitochondria, the data indicate that the combined treatment might cause mitochondrial dysfunction. It is known that both drugs dissipate the mitochondrial membrane potential and alter the mitochondrial permeability with release of proapoptotic compounds [6,29]. In addition, MEN diminishes the activity of oxidoreductases from the intestinal Krebs cycle, which depresses the electron respiratory chain and the oxidative phosphorylation [29]. The ATP depletion could explain, at least in part, the antiproliferative effect of MEN on the breast cancer cells, known to have a higher requirement of this nucleotide for surviving.

Another effect of 1,25(OH)$_2$D$_3$ or MEN on MCF-7 cells is the reduction of GSH content, which contributes to the oxidative stress. Both drugs cause depletion of this thiol in MCF-7 cells, but the co-treatment does not enhance the reduction on the GSH content. This finding might indicate that there is a critical GSH level that cannot

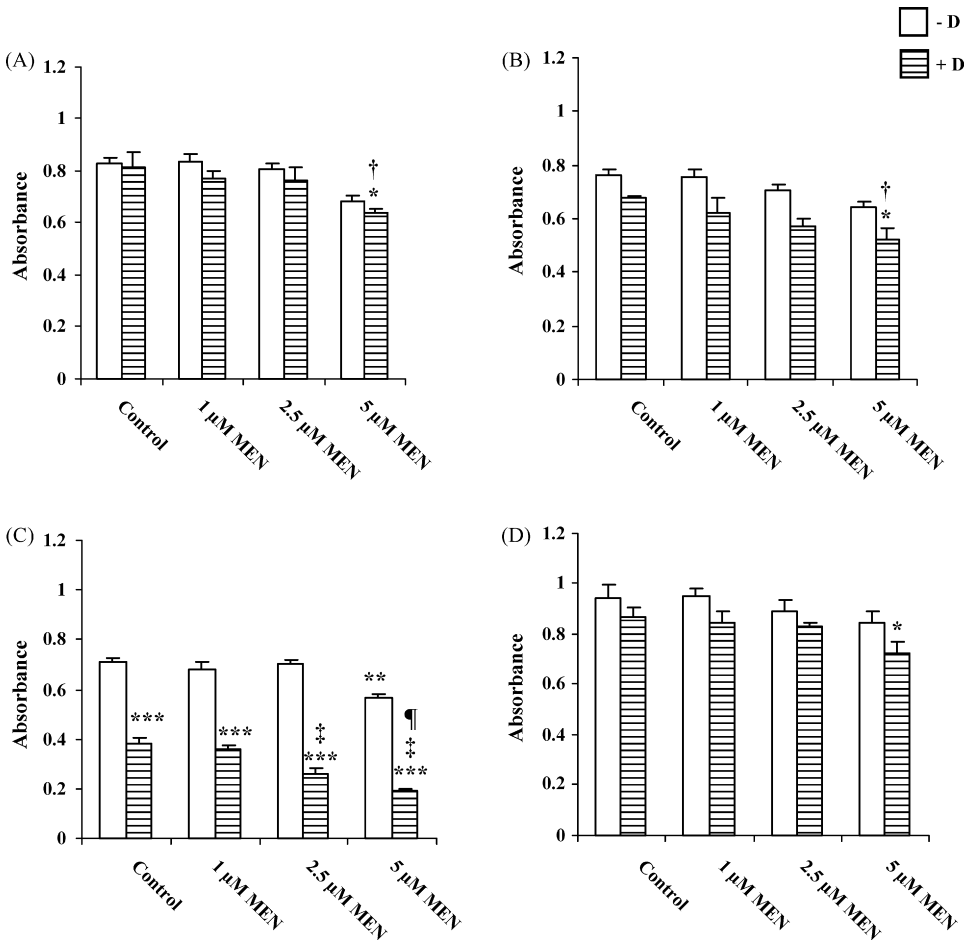

Fig. 2. Dose–response profile of 1,25(OH)$_2$D$_3$ (D) and MEN effects on cell growth of MCF-7 and MCF-7^{DRes} breast cancer cells. Cells were treated with D alone or in combination with 1–5 μM MEN for 96 h as indicated under Section 2. Panels: (A) MCF-7 cells treated with 1 nM D; (B) MCF-7 cells treated with 10 nM D; (C) MCF-7 cells treated with 100 nM D; (D) MCF-7^{DRes} cells treated with 100 nM D. Values represent means ± S.E. $^{*}P < 0.05$ *vs.* Control (−D); $^{\dagger}P < 0.05$ *vs.* Control (+D); $^{\P}P < 0.001$ *vs.* 5 μM MEN (−D); $^{\ddagger}P < 0.001$ *vs.* Control (+D); 1 μM MEN (+D); $^{**}P < 0.05$ *vs.* Control (−D); 1 μM MEN (−D) and 2.5 μM MEN (−D); $^{***}P < 0.01$ *vs.* Control (−D).

be decreased, maybe because it might compromise the elimination of ROS or the cell survival. A link between GSH depletion and suppression of cell proliferative activity has been described [30]. However, Koren et al. [31] did not find alterations in the GSH content and in CAT and GPx activities of MCF-7 cells after 1,25(OH)$_2$D$_3$ treatment. This discrepancy could be due, at least in part, to the shorter period of hormone treatment on MCF-7 cells used by those authors. Noda et al. [32] have reported that GSH depletion *per se* has no effect on cell proliferation, but a compromised intracellular GSH status renders cells more vulnerable to the suppressive effects of redox imbalance on cell proliferative activity.

The activities of SOD, GPx and CAT in MCF-7 cells are increased by treatment with 1,25(OH)$_2$D$_3$ or MEN alone. Similarly, Banakar et al. [33] have demonstrated that 1,25(OH)$_2$D$_3$ elevates SOD activity in rat hepatocarcinogenesis induced by diethylnitrosamine. Khanal et al. [34] have reported that 1,25(OH)$_2$D$_3$ increases the specific activity of SOD and its protein level in intestinal epithelial and kidney cells from chicken. In contrast, Ravid et al. [35] found a decrease in the SOD activity by calcitriol treatment of MCF-7 cells. Another difference with our data and those of other studies [36,37] is that they report much higher SOD activity. The reasons for these differences are not clear. With respect to the MEN effect on SOD activity, Kong and Fanburg [38] have also found that the quinone increases the cellular level in bovine pulmonary artery endothelial cells, which would be in agreement with our data.

The enhancement in the activity of all antioxidant enzymes by the combined treatment is remarkable. SOD is a tumor suppressor protein that increases the dismutation rate of superoxide anion to hydrogen peroxide and inhibits cancer cell growth in vitro as well as in xenografts growing in nude mice [39]. The higher levels of hydrogen peroxide would induce damage of macromolecules, causing alteration in the ability of the cell to undergo mitosis and, hence, inhibition of tumor cell growth. Furthermore, SOD has been proposed as a good candidate for gene therapy treatment of human primary breast cancer [39]. However, calcitriol or MEN and the combined treatment have also increased catalase and GPx activities, which might partially dampen the hydrogen peroxide induced-damage due to the degradation of hydrogen peroxide produced by these two enzymes. One possible explanation of these data is that the increment in the enzyme activity of the antioxidant system might be a compensatory response in order to protect the cells from the deleterious effects of the oxidative stress. Nevertheless, the oxidation and antioxidation balance is shifted towards oxidation leading finally to the tumor cell killing. Another explanation could be that the dismutation rate of superoxide anion by SOD ($2 \times 10^9\ M^{-1}\ s^{-1}$) is slower than the reaction rate of superoxide formation ($1.9 \times 10^{10}\ M^{-1}\ s^{-1}$); in other words, the removal of superoxide anions by SOD may be less efficient than required to eliminate the superoxide production caused by MEN treatment [40]. The data clearly support the idea that any challenge or imbalance to the existing redox status in the malignant breast cancer cells could have catastrophic results on cell viability [39].

In conclusion, the antiproliferative effect of 1,25(OH)$_2$D$_3$ on MCF-7 cells is enhanced by MEN, which is mediated by triggering

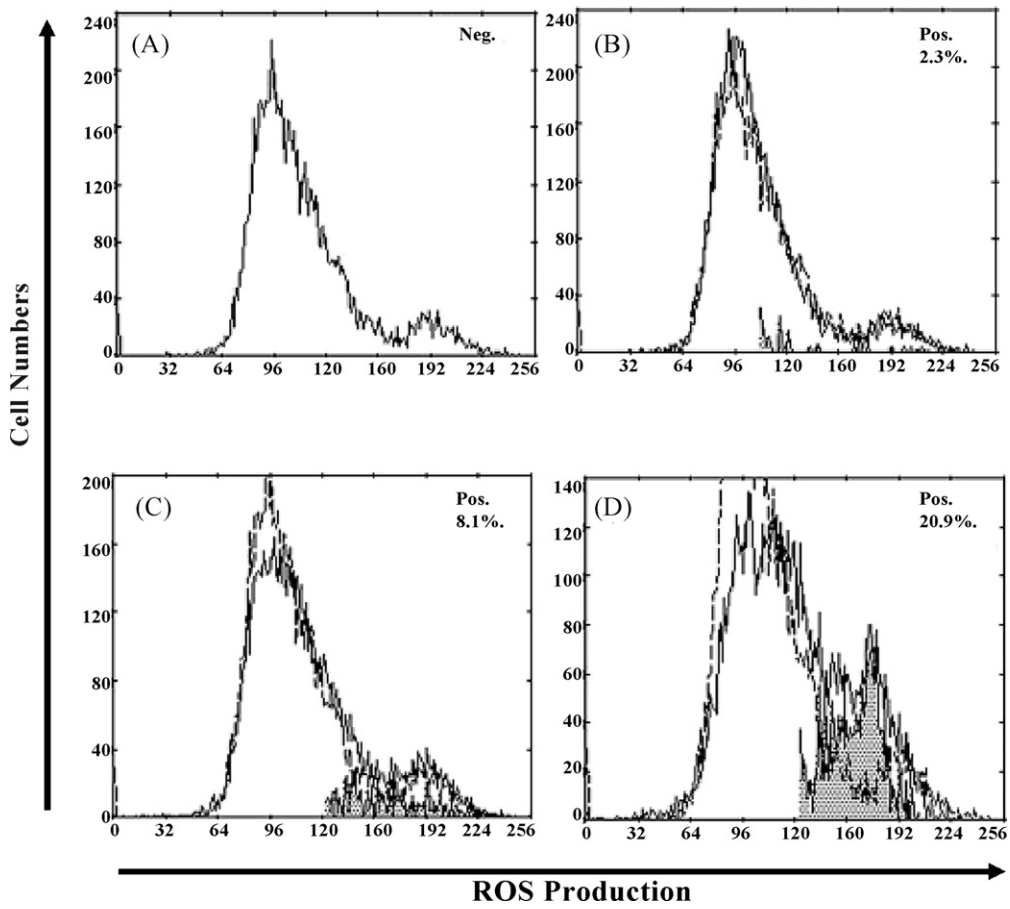

Fig. 3. Effects of 1,25$(OH)_2D_3$ (D), MEN or both on ROS production. Cells treated with 100 nM D and/or 5 μM MEN for 96 h were analyzed by flow cytometry as described under Section 2. Numbers in upper right corner of each panel indicate the percentage of positive cells after negative subtraction of data obtained with vehicle treated cells. Panels: (A) cells treated with vehicle, (B) cells treated with MEN, (C) cells treated with D, and (D) cells treated with D and MEN.

oxidative stress as suggested by an increase in the ROS production, leading finally to morphological features characteristic of apoptosis. MEN also increases the activities of antioxidant enzymes, probably as a compensatory mechanism, without overcoming the deleterious effects of the oxidative stress. Therefore, it should be possible to increase the efficacy of anticancer effect of calcitriol by simultaneously rendering the breast cancer cells more susceptible to apoptosis by changing the redox state of the cells. Although in vivo and clinical studies are needed to evaluate the antitumor effects of the calcitriol + MEN, the present data open up a new avenue for the treatment of patients with breast cancer.

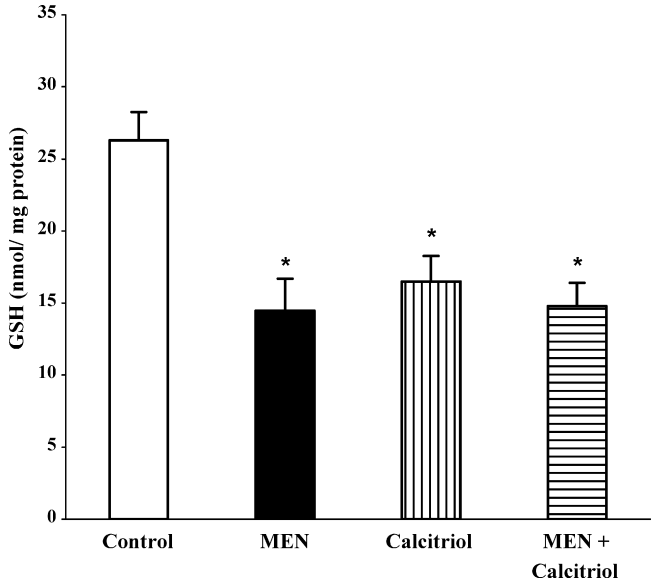

Fig. 4. Variation in GSH content of MCF-7 cells in response to 1,25$(OH)_2D_3$ (D) and MEN. Cells were incubated for 96 h with 100 nM D and/or 5 μM MEN. Total GSH content was measured as indicated under Section 2.*$P < 0.05$ *vs.* Control.

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