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ORIGINAL ARTICLE

Calcitriol enhances the effect of photodynamic therapy in human breast cancer

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Summary

Purpose: *To investigate the killing effect of photodynamic therapy (PDT) mediated by hematoporphyrin derivative (HPD) on human breast cancer MCF7 and MDA-MB-231 cells in vitro.*

Methods: *MCF7 and MDA-MB-231 breast cancer cells cultured in vitro were incubated with calcitriol (concentration of 10^{-8}M, 10^{-10} M, 10^{-12} M, 10^{-14} M, 10^{-16} M, 0 M) to determine a proper concentration. The cells were divided into experimental group (calcitriol, HPD group and laser), HPD group (HPD and laser), calcitriol group (calcitriol and laser), blank laser group (laser alone) and blank group (no drugs and laser). Then the cells were preconditioned with calcitriol for 48 hrs and incubated with HPD for 6 hrs. After light exposure with 630 nm laser, the cells' viability and the reactive oxygen species (ROS) were assessed. After 8 hrs, flow cytometry was applied to detect the rate of cell apoptosis. The fluorescence intensity in cells was detected. Furthermore, the expression of porphyrin synthetic enzymes in pretreated breast cancer cells was analyzed.*

Results: *MTT assay showed that the viability of cells in the experimental group was lowest ($p<0.05$). The ROS intensity of the experimental group was higher ($p<0.01$). The rate of cell apoptosis was higher in the experimental group ($p<0.05$), and the fluorescence of the experimental group was higher ($p<0.01$). Furthermore, mechanistic studies documented that the expression of the porphyrin synthesis enzyme coproporphyrinogen oxidase (CPOX) was increased by calcitriol at the mRNA level.*

Conclusion: *This research revealed a simple, non-toxic and highly effective preconditioning regimen to selectively enhance protoporphyrin IX (PpIX) fluorescence and the response of HPD-PDT in breast cancer search. This finding suggests that the combined treatment of breast cancer cells with calcitriol plus HPD may provide an effective and selective therapeutic modality to enhance HPD-induced PpIX fluorescent quality for improving discrimination of tumor tissue and PDT efficacy.*

Key words: *breast cancer, HPD, MCF7, MDA-MB-231, photodynamic therapy*

Introduction

Breast cancer is one of the most common cancers in women worldwide [1]. Nowadays, patients tend to care more about the appearance, function and psychology than treating their disease alone. PDT plays an increasingly important role in the treatment of cancer because of its advantages such as less trauma, less side effects and so on [2]. Calcitriol, namely 1, 25(OH)2 vitamin D3, can inhibit cancer cell proliferation and can be combined with calcitriol receptor to adjust the proliferation and differentiation of target cells [3]. To understand the effect of calcitriol combined with HPD-PDT on breast cancer cells and its mechanism, MCF7 and MDA-MB-231 breast cancer cell lines were chosen to conduct this research which may provide theoretical proof for the clinical application of calcitriol combined with HPD PDT.

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Methods

Source of reagents

1,25(OH) 2 vitamin D3 (Calcitriol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPD (5mg/ml) was purchased from ChongQing HuaDing Pharmaceutical Company (Chong Qing, China) and then stored at -20 ℃. Chloroform dimethylsulfoxide (DMSO) and MTT were purchased from YiKe Biology Company (Guangzhou, China).

Cell culture

The human breast cancer cell lines MCF7 and MDA-MB-231 from the Cancer Center of Southern Medical University were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin and incubated at 37℃ in a humidified atmosphere with 5% CO_2.

Detection of calcitriol concentration

Breast cancer cells were cultured and seeded into 96-well plates at a density of $3x10^3$ per well and incubated with 10^{-8} M, 10^{-10} M, 10^{-12} M, 10^{-14} M, and 10^{-16} M calcitriol for 48 hrs. Then the viability was assessed by MTT assay. In comparison with the blank group without calcitriol, proper concentration of calcitriol was chosen.

Photodynamic treatment

The cells were grouped into 5 subgroups, namely experimental group (calcitriol+HPD+laser), HPD group (HPD+laser), calcitriol group (calcitriol+laser), laser group (laser alone) and blank group. Breast cancer cells were seeded into 96-well plates at a density of $3x10^3$ per well and incubated with calcitriol for 48 hrs. Cells were then washed with PBS and serum-free RPMI 1640 medium containing HPD (10μg/ml) for cells' incubation for an additional 24 hrs. Cells were then irradiated with PDT machine at a power density of 7.5J/cm^2 (XD-635AB; Xingda, Guilin, China). Immediately following irradiation, the medium was replaced with RPMI 1640 medium supplemented with 10% FBS and cells were incubated for 24 hrs.

Cell viability assay

Cell viability was examined by the MTT assay. Breast cancer cells were incubated with medium containing MTT for 4 hrs in the dark at 37°C. One hundred microliters DMSO were added into each well after removing the medium and incubation for 20 min followed. Ultraviolet (UV) absorption was measured at 492 nm using a 96-well plate reader. Three independent experiments were conducted. The inhibition rate was calculated by the following formula:

Inhibition rate= (Optical density/OD of blank group/ OD of target group)/ OD of blank group x100.

Cell apoptosis analysis

The apoptosis ratio was analyzed using Annexin V FITC Apoptosis Detection Kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Apoptotic cells were analyzed and quantified using FACScan flow cytometry (Becton Dickinson, USA). Tests were carried out in triplicate.

Reactive oxygen species detection

The ROS value was detected by Multiscan Spectrum(Spectra Max M5, Molecular Devices, USA). The excitation wavelength was set at 480 nm, and emission was recorded at 510nm . The test was performed in the dark because light could affect the results of ROS detection.

Spectrofluorometric analysis

The PpIX content of cells was assessed by Multiscan Spectrum (Spectra Max M5, Molecular Devices, USA). The excitation wavelength was set at 410 nm and emission was recorded at 633 nm. An emission spectrum was recorded at the range of 600 – 750 nm. This fluorescence analysis was performed in the dark to minimize PpIX loss due to photobleaching.

Table 1. RT-PCR primers to quantitatively measure the mRNA levels of the PBGD, UROS, CPOX, and FECH genes

Gene	*F/R*	*Primer sequence*
PBGD	Forward	TTCGCTGCATCGCTGAAAGG
	Reverse	GGGCAGGGACATGGATGGTA
UROS	Forward	TTCTCATCCTGAAGATTACGGG
	Reverse	CACAGGGAAATAGAAGAGGCAGT
CPOX	Forward	TGAATCAAGAAGACGCTG
	Reverse	CACAGGGAAATAGAAGAGGCAGT
FECH	Forward	CACACAGTATCCACAGTACAG
	Reverse	GCAGAAAACAGAATGACCACC

Real-time PCR

Total RNA of breast cancer cells was extracted with TRIzol (Invitrogen, Calif, USA) reagent according to the manufacturer's instructions. First-strand cDNA was synthesized by using the high-capacity cDNA reverse transcription kit (Takara, Dalian, China) with random primers. To examine changes in the level of mRNA for PBGD, UROS, CPOX 0 and FECH, RT-PCR was performed on a ABI-7500 using SYBR Green PCR Master Mix (Invitrogen, Shanghai, China). The primer pairs of these genes are listed in Table 1. Parallel reactions were performed using primers to β-actin as internal control.

Statistics

Statistical analyses were performed using the SPSS19.0 software (SPSS Inc., Chicago, IL, USA). Least-significant difference (LSD) t-test was applied when appropriate. A p value < 0.05 was considered statistically significant. All values were expressed as mean±standard error of the mean (SEM) from three different experiments.

Results

Determination of calcitriol concentration

Figure 1 shows that the cytotoxic effect of a 48-hr treatment with calcitriol was dose-dependent, and calcitriol at 10^{-12}M was non-cytotoxic. Therefore, 10^{-12} M calcitriol was considered optimal for use in breast cancer cells.

Inhibition rate of cells after PDT

The MCF7 cells' mean inhibition rate of the experimental group, HPD group, calcitriol group and laser group was 89.03±0.79, 77.76±2.27, 20.54±2.32 and 18.06±3.60, respectively. The MDA-MB-231 cells' mean inhibition rate of the experimental group, HPD group, calcitriol group, laser group was 75.82±1.43, 46.38±0.64, 16.98±0.28 and 13.49±2.04, respectively. The experimental group was highest in both kind of cells among the 4 groups (p<0.01). There was no significant difference between the calcitriol group and laser group (p>0.05) (Figure 2).

Detection of reactive oxygen species

Thirty min after exposure, cells were collected to detect ROS. The MCF7 cells' ROS mean value of the experimental group, HPD group, calcitriol group, laser group and blank group was 3.903±0.098, 2.713±0.218, 2.239±0.945, 1.835±0.127, 0.561±0.004, respectively. The MDA-MB-231 cells' ROS mean value of the experimental group, HPD group, calcitriol group, laser group and blank group was 3.628±0.045, 2.269±0.175, 1.649±0.296, 1.472±0.098 and 0.573±0.096, respectively. The experimental group was highest in both kind of cells among the 5 subgroups (p<0.01). There was no significant difference between the calcitriol group and laser group (p>0.05) (Figure 3).

Detection of cell apoptosis

Cells were collected 8 hrs after light exposure. The MCF7 cells' mean rates of apoptosis of the experimental group, HPD group, calcitriol group, laser group and blank group were 0.4407±0.01255,

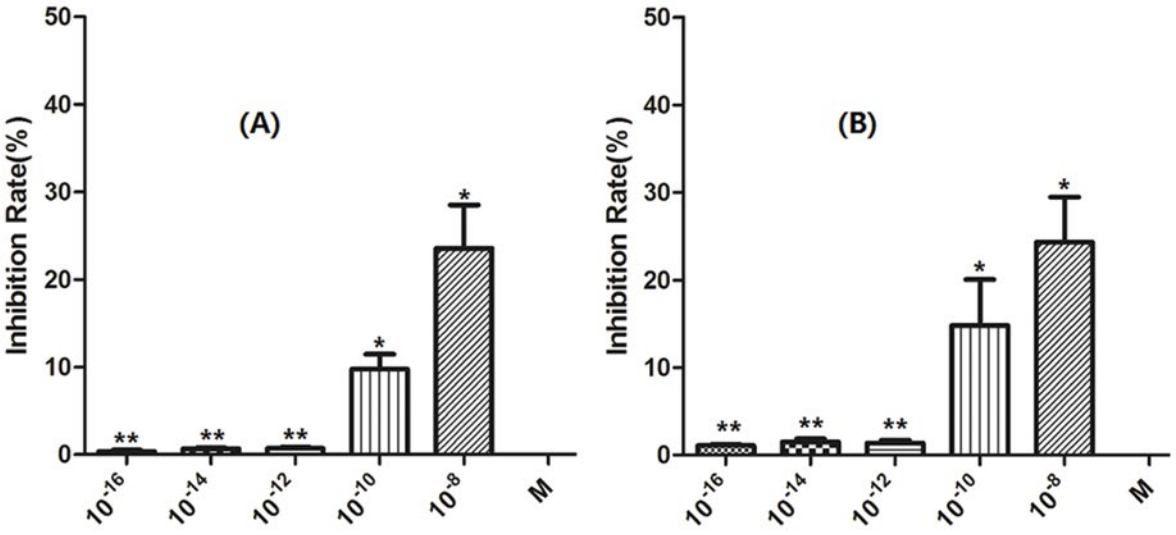

Figure 1. Effects of calcitriol on viability of breast cancer cells. The figure shows the inhibition of MCF7 cells **(A)** and MDA-MB-231 cells **(B)**. In comparison with blank group, when the concentration was 10-12M or below, there was no statistical significance in the inhibition rate between them (*p<0.05, ** p>0.05).

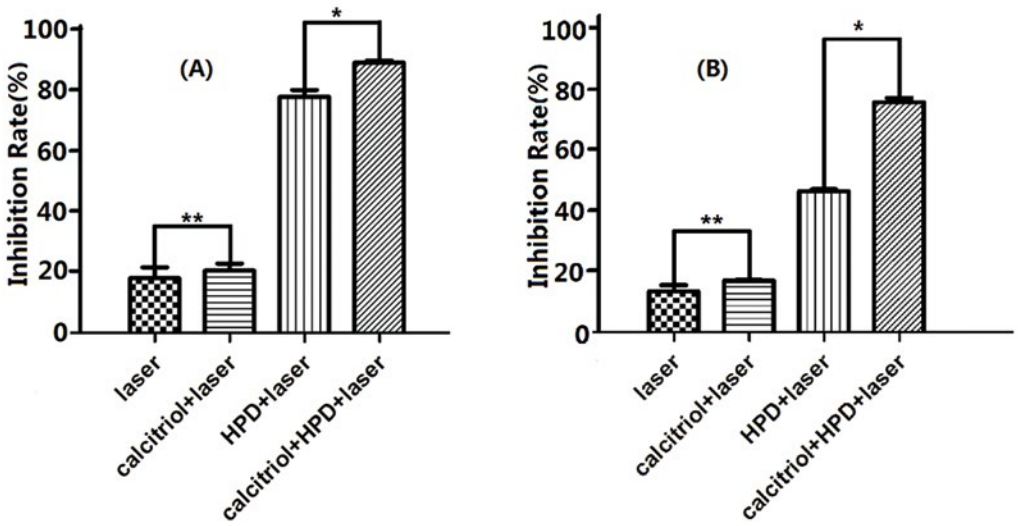

Figure 2. The viability of the cells after PDT. The Figure shows the inhibition rate of MCF7 cells **(A)** and MDA-MB-231 cells **(B)** after PDT. The inhibition rate in the experimental group was higher than in the HPD group, while there was no statistical significance between laser group and calcitriol group. The Figure shows that calcitriol can enhance the effect of HPD-PDT, while the calcitriol alone cannot strengthen the killing effect (*p<0.05, **p>0.05).

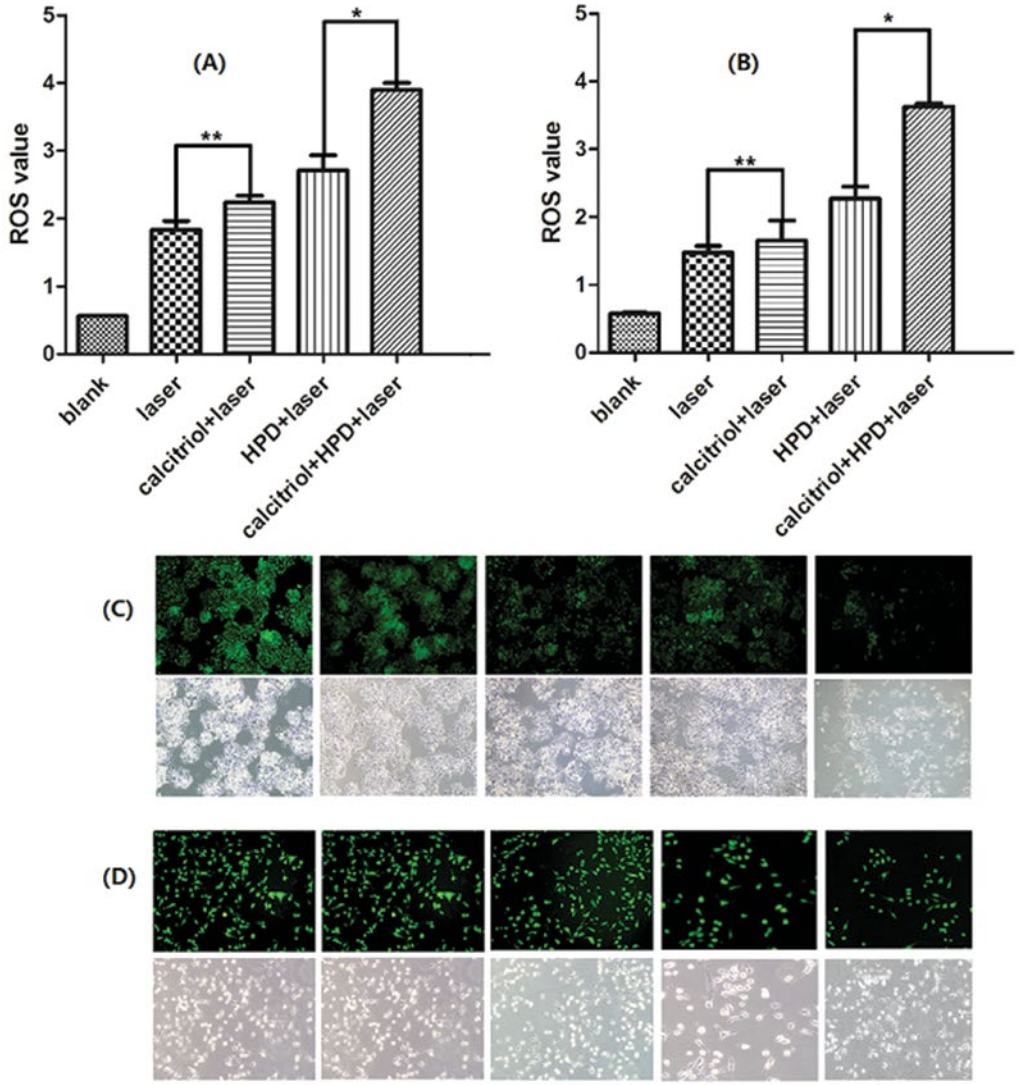

Figure 3. ROS of the cells after PDT. The figure shows the ROS value and fluorescence image of MCF7 cells (A,C) and MDA-MB-231 cells (B,D) after PDT. The ROS value in the experimental group was higher than in the HPD group, while there was no statistical significance between laser group and calcitriol group (*p<0.05, **p>0.05).

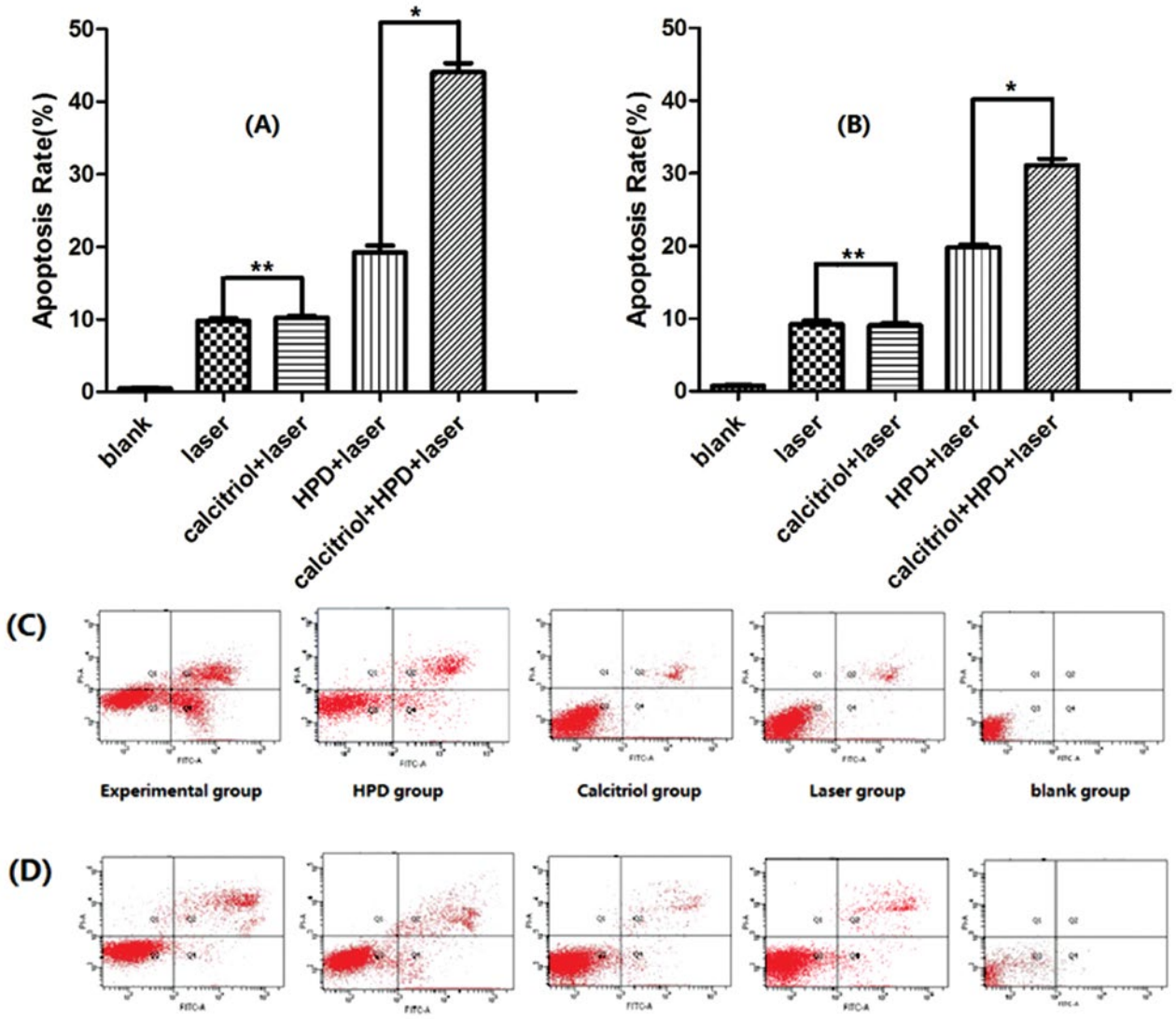

Figure 4. T The apoptosis rate of the cells after PDT. The figure shows the apoptosis rate of MCF7 cells **(A,C)** and MDA-MB-231 cells **(B,D)** after PDT. The apoptosis rate in the experimental group was higher than in the HPD group. No statistical significance existed between the laser group and the calcitriol group (*p<0.05, **p>0.05).

0.19±0.0094, 0.1023±0.0023, 0.0680±0.02703 and 0.0047±0.0009, respectively. The MDA-MB-231 cells' mean rates of apoptosis of the experimental group, HPD group, calcitriol group, laser group and blank group were 0.3110±0.0087, 0.1980±0.0040, 0.0903±0.0030, 0.0623±0.02630 and 0.0073±0.0009, respectively. The apoptosis rate of the experimental group in both kinds of cells was highest among the 5 subgroups (p<0.01). There was no significant difference between the calcitriol group and laser group (p>0.05) (Figure 4).

Determination of fluorescence intensity

The MCF7 cells' mean value of fluorescence intensity of the experimental group, HPD group, calcitriol group, laser group and blank group was 386.056±13.540, 285.871±13.292, 2.702±0.199, 2.750±0.239 and 2.904±0.142, respectively, while the mean value of MDA-MB-231 cells was 313.254±6.084, 250.048±3.156, 2.798±0.181 and 2.683±1.400, respectively. The fluorescence intensity of the experimental group was highest (p<0.01). The results of fluorescence are shown in Figure 5.

Real-time PCR experiments

The porphyrin biosynthetic enzymes (PBGD, UROS, CPOX and FECH) were used to investigate the mechanism by which calcitriol could enhance the effect of PDT. The results showed that only the expression level of CPOX was upregulated after calcitriol pre-treatment, while PBGD, UROS and FECH showed no changes in the mRNA level (Figure 6).

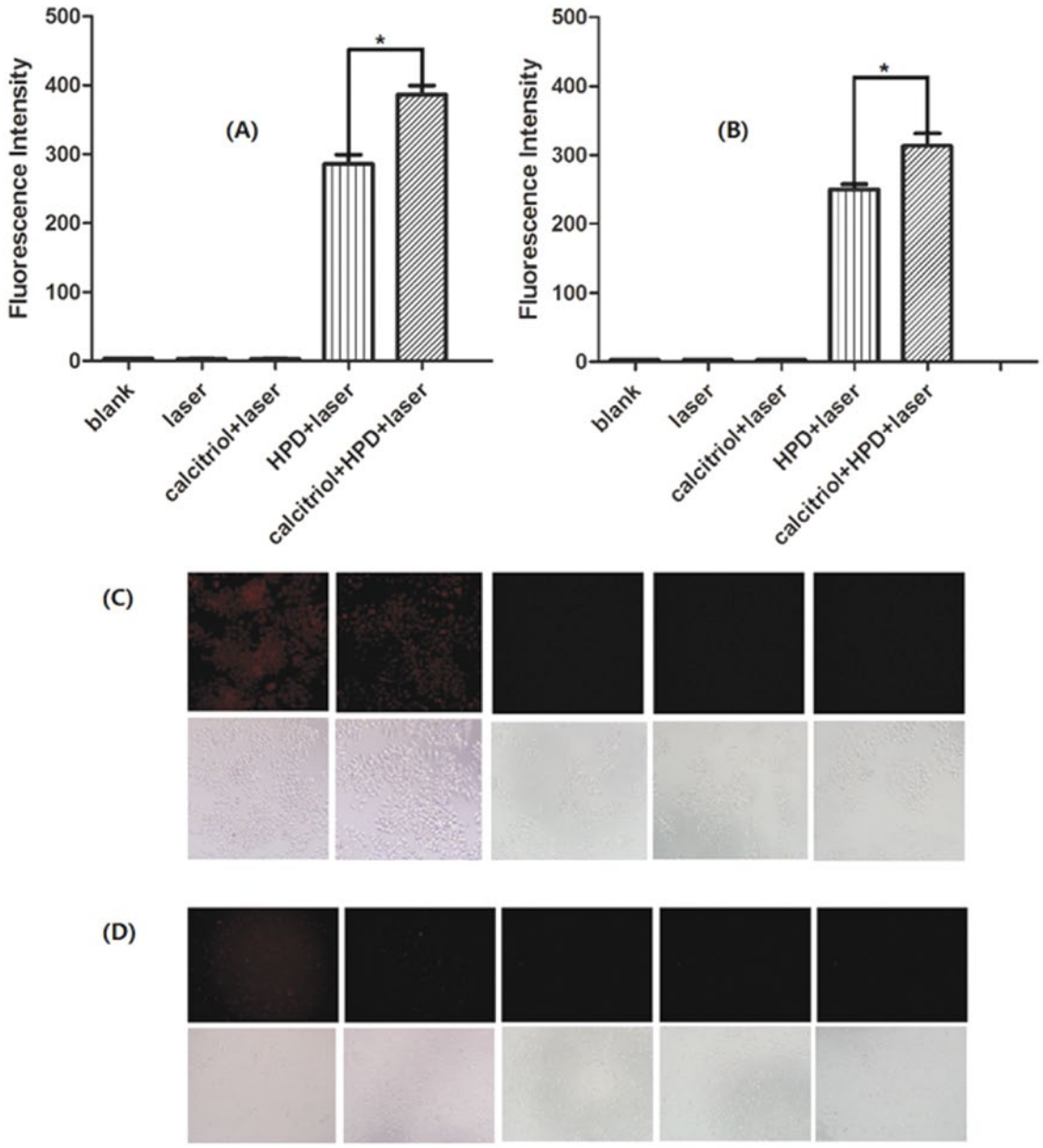

Figure 5. The fluorescence intensity of the cells. After pretreatment with calcitriol and HPD in the experimental group, only with HPD in the HPD group, none in the calcitriol group, laser group and blank group, the value of fluorescence intensity was detected. The Figure shows the value of fluorescence intensity of MCF7 cells **(A,C)** and MDA-MB-231 cells **(B,D)** after pretreatment. The value of fluorescence intensity in the experimental group was higher than in the HPD group (*p<0.05). There was no statistical significance between the laser group, calcitriol group and blank group (p>0.05).

Discussion

PDT can be applied in laryngeal cancer, esophageal cancer, lung cancer, breast cancer, and skin cancer. HPD is the first generation of photosensitizers and proved to be effective after many years' experimental researches and clinical trials [4]. PDT works by stimulating the photosensitizer accumulated in the cancer cells with laser of 630nm wavelength, producing singlet oxygen, oxygen free radical and thromboxane, causing death of cancer cells and tumor disintegration due to the embolism of microvessels [5]. Compared with traditional therapies like surgery, chemotherapy and radiotherapy, PDT has many advantages: less trauma, less toxic, more selective, more adaptive, good repeatability, applied as palliative treatment, eliminating dormant neoplastic foci, protecting face and preserving the function of organs. In the process of PDT, the mechanisms of ROS production are divided into type I (redox reaction) and type II (energy transfer reaction). There are many species of type, just like O_2^-, H_2O_2 and OH^-, which are produced by electron transfer after the inter-

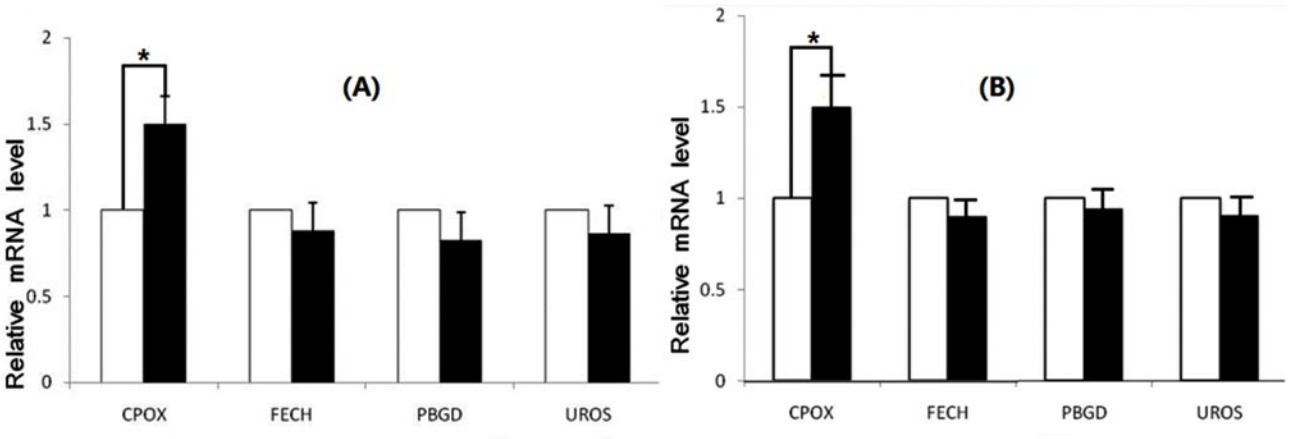

Figure 6. mRNA expression of key enzymes in the porphryrin synthetic pathway, with or without calcitriol pretreatment. Breast cancer cells were pretreated with calcitriol for 48 hrs, and mRNA expression of PBGD, UROS, CPOX and FECH was measured by real time PCR. Data are presented as mean±standard error of the mean for three separate experiments performed (*p<0.05). **A** shows that in MCF7 cells, only the expression of CPOX was upregulated (*p<0.05), while the expression of FECH, PBGD and UROS did not show significant change. In **B**, only the expression of CPOX of MDA-MB-231 was upregulated (*p<0.05), while the others did not change significantly.

action of photosensitizer and the cellular content. The O_2 from type II is the product of reaction of photosensitizer and ground state oxygen [6-8]. ROS is an important reason to induce cells' death and researches have shown that PDT could cause apoptosis and death of cancer cells [9].

However, some patients will recur after PDT with an unfavorable prognosis [10]. One of the reasons is that the accumulation of the photosensitizer in cancer cells is limited [11].

In the current study, we demonstrated that 10^{-12}M is a proper concentration of calcitriol via the MTT assay, which ensures that it will not affect the growth of breast cancer cells. After pretreatment of calcitriol, PDT was performed. Next, the viability of both kinds of cells was tested via MTT assay and showed that their viability in the experimental group was lowest (p<0.01). The HPD group took the second place (p<0.05). Statistical analysis showed no significant difference between calcitriol group and laser group. It was shown that calcitriol could enhance the effect of HPD-PDT on breast cancer cells, while it could not increase the death rate of cells by itself alone.

Concerning ROS the results showed that the value of experimental group was the highest (p<0.01), meaning that in the experimental group, the production of ROS was highest, causing more death of breast cancer cells. No difference between the calcitriol group and laser group was noted (p<0.05). Concerning cell apoptosis the results indicated that the rate of apoptosis of both kinds of cells was highest in the experimental group (p<0.01). Testing the fluorescence intensity of the 5 subgroups, it was shown that the value of the experimental group was highest (p<0.05). In order to investigate the mechanism of how calcitriol could enhance the effect of PDT, RT-PCR followed to test the the expression levels of the porphyrin biosynthetic enzymes (PBGD, UROS, CPOX and FECH). Only the expression level of CPOX was increased after calcitriol pre-treatment, while PBGD, UROS and FECH showed no changes in the mRNA level. We believe that calcitriol treatment can significantly increase CPOX expression at the mRNA level and therefore calcitriol may upregulate the CPOX expression to enhance HPD-induced PpIX level and fluorescence in breast cancer cells. CPOX is located at the mitochondrial outer membrane and plays a role in catalyzing coproporphyrinogen III to produce protoporphyrinogen [12,13]. A previous study has demonstrated that calcitriol or methotrexate increase the expression of CPOX, which results in a significant increase in the intracellular accumulation of ALA-induced PpIX in epithelial cancer cells and prostate cancer cells [14]. We claim that the induction of CPOX gene expression may play a role in the HPD-based fluorescence of tumor cells and the effect of PDT.

In summary, we have demonstrated for the first time that calcitriol can enhance the quality of HPD fluorescence imaging and can improve the efficacy of HPD-PDT by increasing the PpIX levels in cells. Furthermore, we demonstrated that calcitriol not only can increase the level of HPD-induced PpIX, but it may also increase the photosensitizer's levels by elevating CPOX mRNA expression. Future studies are needed to investigate whether calcitriol can be effectively

combined with HPD-induced PDT *in vivo* and in the clinical setting.

Conflict of interests

The authors declare no confict of interests.

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