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Doxorubicin-Hyaluronan Conjugated Super-Paramagnetic Iron Oxide Nanoparticles (DOX-HA-SPION) Enhanced Cytoplasmic Uptake of Doxorubicin and Modulated Apoptosis, IL-6 Release and NF-kappaB Activity in Human MDA-MB-231 Breast Cancer Cells

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Abstract

Triple negative breast cancer exhibit increased IL-6 expression compared with matched healthy breast tissue and a strong link between inflammation and cancer progression and metastasis has been reported. We investigated whether doxorubicin-hyaluronan-super-paramagnetic iron oxide nanoparticles (DOX-HA-SPION) would show greater therapeutic efficacy in human triple negative breast cancer cells (TNBC) MDA-MB-231, as was recently shown in drug-sensitive and multidrug-resistant ovarian cancer cells. Therefore, we measured cellular DOX uptake via confocal microscopy; observed morphologic changes: mitochondrial and nuclear changes with electron microscopy, and quantitated apoptosis using FACS analysis after Annexin V and PI staining in MDA-MB-231 cells treated with either DOX alone or DOX-HA-SPION. We also measured both proinflammatory and anti-inflammatory cytokines; IL-6, IL-10 respectively and also measured nitrate levels in the conditioned medium by ELISA. In addition, NF-kB activity was measured by luciferase assay. Confocal microscopy demonstrated greater cytoplasmic uptake of DOX-HA-SPION than free DOX. We also demonstrated reduction of Vimentin with DOX-HA-SPION which is significantly less than both control and DOX. DOX-HA-SPION enhanced apoptosis and significantly down regulated both pro-inflammatory mediators IL-6 and NF-kB in comparison to DOX alone. The secretion levels of anti-inflammatory mediators IL-10 and nitrate was not decreased in the DOX or DOX-HA-SPION treatment groups. Our data suggest that DOX-HA-SPION nanomedicine-based drug delivery could have promising potential in treating metastasized and chemoresistant breast cancer by enhancing the drug efficacy and minimizing off-target effects.

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Keywords

Breast Cancer; Doxorubicin; Nanoparticles; IL-6; NF-kappaB; Apoptosis

1. INTRODUCTION

Breast cancer is among the most frequently diagnosed cancers in women. It is estimated that 232,340 women and 2,240 men will be diagnosed with invasive breast cancer will be diagnosed among women in the US in 2013. Overall death rate has been estimated at 14%¹ but actual death rates vary among various breast cancers. Breast cancers are classified depending on cell receptors; triple negative breast cancer (estrogen, progesterone and Her-2 receptor, TNBC) accounts for about 15–25% of all breast cancers² and is known to have the worst prognosis.^{3–5} TNBC is more prevalent among low socioeconomic groups, especially Latino and African-American.^{6, 7}

Current treatments include surgery,⁸ hormonal therapy,^{9, 10} chemotherapy,^{11–14} radiation^{15–17} and/or immunotherapy.^{18–23} Neoadjuvant chemotherapy is the first line therapy for TNBC^{24–28} and large clinical trials have concluded that these women respond best to cytotoxic chemotherapy.^{29–31} Adriamycin (doxorubicin, DOX) has been the first line chemotherapy agent³² but it exhibits high short and long-term morbidity and mortality with severe heart failure occurring in more than 20% of patients and has a lifetime dose limit, commonly leading to under treatment of patients to reduce fatal side effects, but possibly causing more recurrence.^{33, 34} Thus the major limitations of current chemotherapy are:

- (a) serious immediate side effects including but not limited to sepsis;
- (b) long term side effects like heart failure seen in 20% of females after a few years; and
- (c) drug resistance.³⁵

The serious off-target side effects and the development of resistance by cancer cells is a serious limitation to the use of chemotherapeutic agents.^{36, 37} Targeting drugs to tumor can not only increase cytotoxicity to tumor cells but also help decrease systemic side effects by reducing the administered dose.³⁸

Tumors have a unique property to accumulate small particles like nanoparticles through enhanced permeability and retention (EPR).^{39, 40} Abnormalities of the tumor vasculature, namely hypervascularization, aberrant vascular architecture, extensive production of vascular permeability factors that stimulate extravasation within tumor tissues, and lack of lymphatic drainage can be exploited for selective drug targeting for solid tumors.⁴⁰ Multiple investigators have shown role of nanoparticle in breast cancer.⁴¹ Indeed, this selective high local concentration of nano-sized anticancer drugs in tumor tissues has proven superior in therapeutic effect with minimal side effects in both preclinical and clinical settings.

Recently, we synthesized doxorubicin coated nanoparticle platform. This nanoparticle consists of an iron oxide core to which a low molecular weight hyaluronan (HA) polymer has been conjugated. HA polymer can simultaneously bind to multiple copies of CD44

leading to avid binding.⁴² CD44 is a multistructural cell adhesion molecule located on chromosome 11 in humans and chromosome 2 in mice and has both protumorigenic and antitumorigenic properties.⁴³ The attachment of DOX to NP enhanced its cytotoxicity not only in drug sensitive cancer cell lines but also in cancer cells displaying drug resistance phenotype.⁴⁴ DOX delivered by the HA-nanoparticle is much more potent than free DOX against ovarian cancer *in vitro*⁴⁴ and *in vivo*.^{45–47} As CD44 is overexpressed in ER-/PR-/Her-2- or Triple Negative Breast Cancer (TNBC) cell lines.⁴⁸ We became interested in investigating whether HA coated nanoparticles can be utilized to enhance the drug efficacy against CD44-expressing TNBC cancer cells such as MDA-MB-231. There are several other doxorubicin-based nanoparticles have been documented and being getting investigated.^{49–72}

The human breast tumors exhibit increased IL-6 expression compared with matched healthy breast tissue⁷³ and a strong link between inflammation and cancer progression and metastasis has been reported.⁷⁴ Vyas et al. have extensively dissected up-regulation of inflammatory modulators in cancer cells with multiple common chemotherapies.^{75, 76} HA is known to modulate inflammation and we exploited this property of HA for high inflammatory milieu in TNBC to further enhance the potency of DOX. HA is known for dual pro-inflammatory and anti-inflammatory properties in addition to effect of nitrate and we studied IL-6 changes with DOX-HA-SPION nanoparticles. Elevated IL-6 levels indicate poor prognosis in patients with recurrent breast cancer. Indeed, serum IL-6 levels are an important prognostic indicator of poor outcome in metastatic breast cancer patients in terms of size of tumor, lymph node positive status, and the number of metastatic sites.⁷⁷ However, the level of IL-6 does not correlate with hormonal receptors status, tumor grade, menopausal status or site of metastasis.⁷⁷ Serum IL-6 levels also correlate with poor survival in patients with hormone-refractory metastatic breast cancer.⁷⁸ The production and regulation of IL-6 in MDA-MB-231 is complex and is regulated through the JAK/STAT3 as well as the NF- κ B pathways.⁷⁹ Our unpublished data with MDA-MB-231 breast cancer cell line suggest that a combination therapy including agents that reduce IL-6 levels could be a new strategy for aggressively treating recurrent breast cancer. Therefore, we hypothesize that DOX-HA-SPION could modulate inflammatory cytokines in the tumor milieu, thus dampening the effect of the IL-6/NF kappa B signaling pathway. We also hypothesized DOX-HA-SPION could modulate metastasis proteins in TNBC like Vimentin and E-Cadherin.

In the current study, we investigated the interactions of DOX-HA-SPION nanoparticles with human MDA-MB-231 breast cancer cells. We observed that DOX-HA-SPION increased cellular uptake of DOX, enhanced apoptosis, and reduced secretion of the pro-inflammatory IL-6 and transcriptional activity of NF- κ B without significant changes in IL10 or nitrate secretion. Our data suggest that DOX-HA-SPION nanoparticles have desirable effects on pro-survival and inflammatory cytokines and transcription factors without decreasing anti-inflammatory pathway and it may be more beneficial in reducing angiogenesis, invasion and metastasis than DOX alone in the treatment of breast cancer.

2. MATERIALS AND METHODS

2.1. Materials

The doxorubicin (DOX) and nanoparticle conjugated DOX (DOX-HA-SPION) were synthesized as previously described.⁴⁴ IL-6 and IL-10 ELISA kits for and the Annexin V Apoptosis detection kit (FACS) were obtained from BD Biosciences (San Diego, CA). The Signal Lentiviral Particles for NF- κ B assay was from Qiagen (Frederick, MD) and the Steady-Glo Luciferase Assay system was from Promega Corporation. The nitrate fluorometric assay was purchased from Cayman chemical company (Ann Arbor, MI). All reagent grade chemicals were obtained from Sigma. MDA-MB-231 cells were obtained from the laboratory of Dr. Susan Conrad, Michigan State University.

2.2. Confocal Laser Microscopy

MDA-MB-231 cells (1×10^5 cells/well) in FBS containing-DMEM medium were inoculated in a 4-chambered plate and incubated overnight at 37 °C with 5% CO₂. After removing the supernatant and washing the cells, free DOX (2 μ M) and DOX-HA-SPION with equivalent amount of DOX were added in serum-free DMEM. The cells were incubated for 5 h. The cytotoxic agents were removed, and the cells were washed with PBS twice and fixed with 10% formalin (0.5 mL/well) for 10 min. After washing the cells with PBS, PBS (200 μ L) and DAPI (300 nM, 300 μ L) were added for 5 min. The cells were washed with PBS and water, and the plate was covered with aluminum foil until the time of analysis. Images were taken on an Olympus FluoView 1000 LSM confocal microscope. For Vimentin and E-Cadherin detection, cancer cells were grown in DMEM containing 10% FBS on glass slides coated with poly-D-lysine (BD Biosciences). The cells were allowed to attach overnight. Antigen detection with specific 1° antibodies was accomplished with immunofluorescence (IF) detection kits (Zyagen, San Diego, CA). Confocal images will be acquired using the Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Olympus America, Inc., Center Valley, PA).

2.3. FACS (Fluorescence Activated Cell Sorting) Analysis for Apoptosis

MDA-MB-231 cells (1×10^5 cells/well) in FBS containing-DMEM medium were inoculated in a T-25 flask and incubated overnight at 37 °C and 5% CO₂. After removing the supernatant and washing the cells, free DOX (2 μ M) and the equivalent amount of DOX-HA-SPION were added in complete medium. The cells were incubated for 24 hours and agents were removed. Cells were incubated for an additional 24 hours in complete medium without DOX or DOX-HA-SPION. Next day, the cells were trypsinized and analyzed for apoptosis analysis using FACS, as described by the manufacturer. Briefly, cells were washed four times with serum-containing DMEM, resuspended in the growth medium, and transferred to FACS tubes. 5 μ L each of Propidium Iodide and FITC Annexin V were added and incubated for 15 min at room temperature in the dark. 400 μ L of binding buffer was added and fluorescence was assessed using a BD Vantage SE Flow Cytometer. FACS experiments were done in multiple sets with 24-hour incubation period with DOX, DOX-HA-SPION followed by 24 hour incubation period in the medium to study early and late apoptosis

2.4. IL-6 and IL-10 Cytokine Assay

MDA-MB-231 cells (1×10^4 cells/well) were plated in 96-well plates in FBS containing-DMEM medium overnight at 37 °C and 5% CO₂. Next day, cells were exposed to DOX (2 µM) and the equivalent amount of DOX-HA-SPION for 24 hours in complete medium. At the end of experimental period, the conditioned media were collected and centrifuged for 5 minutes at 2000 rpm to remove cell debris. Enzyme-linked immunosorbent assay (ELISA) for the human IL-6 and IL-10 were performed as described previously.^{80–82} Briefly, 96 well BD Falcon Elisa plates were coated with 100 µL diluted capture antibodies to each well and incubated overnight at 4 °C. The wells were aspirated and washed three times with 1× wash buffer. The plates were then blocked with assay diluents (200 µL/well) and incubated at room temperature for 1 hour. The plate was then aspirated and washed thrice with wash buffer. Standard and sample dilutions were prepared in assay diluents as per manufacturer's recommendation. 100 µL/well of standard or samples were added and the plates were sealed and incubated at room temperature for 2 hours. After aspirating and washing the plates for 5 times with wash buffer, 100 µL of working detector was added to each well and incubated at room temperature for 1 hour. The plates were aspirated and washed with wash buffer 6 times with 30 sec to 1 min soaks and 100 µL/well of substrate solution was added and incubated at room temperature for 30 min in dark. After adding 50 µL of stop solution to each well, the plates were read at 450 nm within 30 min with λ correction 570 nm.⁸³

2.5. NF Kappa-B Transcriptional Activity Assay

MDA-MB-231 cells (1×10^4 cells/well) were plated in 96-well plates in FBS containing-DMEM medium for overnight at 37 °C and 5% CO₂. Cells were transduced after 24 hours with Cignal Lentiviral NF- κ B reporter gene per the recommendation by manufacturer (Qiagen, Frederick, MD). Briefly, 20 µL of Cignal Lentiviral particle was added to each cell after removing the medium and growth medium was added without antibiotics to make a total volume of 50 µL in each cell to achieve an MOI (Multiplicity of Infection) of 10.⁸⁴ Sure ENTRY Transduction Reagent was then added to make a final concentration of 8 µg/ml in each well and the plates were incubated at 37 °C in humidified 5% CO₂ for 24 hours. The following day, the growth medium containing Lentiviral particles was removed and cells were exposed to DOX (2 µM) and the equivalent amount of DOX-HA-SPION for 24 hours in complete medium. The NF-Kappa B promoter activity was measured using Steady-Glo luciferase reagent assay (Promega Inc.) per manufacturer's instructions. The luminescence was measured with a VERITAS microplate Luminometer.

2.6. Nitrate Assay

MDA-MB-231 cells (1×10^4 cells/well) were plated in 96-well plates in FBS containing-DMEM medium for overnight at 37 °C and 5% CO₂. Next day, cells were exposed to DOX (2 µM) and the equivalent amount of DOX-HA-SPION for 24 hours in complete medium. At the end of the experimental period, the conditioned medium was collected and centrifuged for 5 minutes at 2000 rpm to remove cell debris. The secretion levels of nitrate were measured according to the manufacturer's recommended protocol (Cayman chemical company Ann Arbor, MI).

2.7. Scanning Electron Microscopy

MDA-MB-231 cells were grown as described before. The cells were trypsinized and fixed with 2.5% glutaraldehyde +2.5% paraformaldehyde in 0.1 M cacodylate buffer. Cells were stored in 4 °C till examination under scanning electron microscopy.

2.8. Statistical Analysis

All studies were done independently at least three times unless indicated otherwise. Data are expressed as means $\pm$ standard error (SE) and analyzed using Student's *t*-tests with Bonferroni corrections as appropriate, seeking 95% confidence.

3. RESULTS

3.1. DOX-HA-SPION Intra-Cytoplasmic Uptake in MDA-MB-231 Cells is Greater Than That of DOX with Equal Total Intracellular Uptake

MDA-MB-231 cells were incubated with free DOX or DOX-HA-SPION as described above. Confocal images are shown in Figure 1. We observed significantly increased cytoplasmic fluorescence (Fig. 1(B); 2201 ± 275 vs. 1514 ± 95 in DOX alone, $n = 5$, $p < 0.05$) as well as modestly but significantly decreased nuclear uptake of DOX-HA-SPION in comparison to DOX alone (Fig. 1(B); 1754 ± 348 vs. 2274 ± 245 , $n = 5$, $p < 0.05$).

3.2. DOX and DOX-HA-SPION Induce Morphological Changes in MDA-MB-231 Cells

We also assessed whether treatment resulted in morphological changes in our cells. Figure 2 images are representative of typical cellular, mitochondrial and nuclear changes after initial treatment with free DOX, DOX-HA-SPION when compared to untreated MDA-MB-231 cells. Electron microscopy showed decreased cellular size, absence of cellular pseudopodia (sign of invasion), mitochondrial matrix disruption, and nuclear clumping in both DOX and DOX-HA-SPION treated cells as compared to untreated control cells.

3.3. DOX-HA-SPION Enhances Apoptosis in MDA-MB-231 Cells

We then investigated whether the enhanced cellular accumulation and morphological changes caused apoptosis. Typical FACS histograms are shown in Figures 3(A) and (B). Significantly enhanced early and late apoptosis is evident in DOX-HA-SPION treated cells in comparison to DOX alone (Fig. 3(C) 8.1 ± 1.2 vs. 3.4 ± 0.6 , $n = 5$, $p < 0.01$); 4.1 ± 0.8 vs. 1.2 ± 0.6 , $n = 5$, $p < 0.02$). Early apoptotic cells are positive with FITC Annexin V and late apoptotic cells are positive with both Propidium Iodide and FITC Annexin V. Total apoptosis was increased by approximately two to three folds ($12\% \pm 0.6$) in comparison to free DOX ($4.6\% \pm 0.6$).

3.4. DOX-HA-SPION Inhibits Secretion of IL-6 without Changing IL-10 or Nitrate in MDA-MB-231 Cells

The pro-inflammatory milieu of TNBC is thought to contribute to its invasiveness.⁸⁵ To determine whether DOX-HA-SPION modulates MDA-MB-231 cell function, in part, by altering cytokine secretion, we determined secretion levels of two cytokines, IL-6 and IL-10 expressed in pg/ml. We observed that cells exposed to DOX-HA-SPION secreted

significantly less IL-6 in comparison to both untreated control as well as DOX alone (Fig. 4(A), 367 ± 4 vs. 516 ± 30 , 646 ± 32 , $n = 3$, $p < 0.05$). DOX significantly increased the production of IL-6 in comparison to untreated control (Fig. 4(A), 646 ± 32 vs. 516 ± 30 , $n = 3$, $p < 0.05$). We also measured the secretion level of IL-10 and did not observe any significant difference in untreated; DOX or DOX-HA-SPION treated groups (Fig. 4(B), 18 ± 4 vs. 19 ± 2 , 15 ± 3 , $n = 3$, $p > 0.05$). Similarly, nitrate levels were unchanged in various treatment groups. Figure 5 shows nitrate release levels in control, DOX and DOX-HA-SPION treatment groups to be 15 ± 0.4 nM, 19 ± 2.3 nM, and 16 ± 0.5 nM respectively. The results are not statistically significant.

3.5. DOX-HA-SPION Suppresses NF- κ B Transcriptional Activity in MDA-MB-231 Cells

NF- κ B activity was measured by transducing a retrovirus reporter gene with NF- κ B promoter or the empty vector and NF- κ B activity expressed as Relative Luminescence Unit (RLU). Activity was enhanced by approximately 2.5 fold in cells exposed to free DOX in comparison to untreated control cells (Fig. 6, 22821 ± 2394 vs. 12282 ± 177 , $n = 3$, $p < 0.05$) but cells incubated with DOX-HA-SPION showed significantly reduced NF- κ B activity (40%) (Fig. 6, 10776 ± 880 vs. 22821 ± 2394 , $n = 3$, $p < 0.05$).

3.6. DOX-HA-SPION Suppresses Vimentin in MDA-MB-231 Cells

Vimentin expression was compared to baseline untreated cells on incubation with free DOX, or DOX-HA-SPION as described above. We observed significantly decrease cytoplasmic fluorescence (332 ± 31 vs. 192 ± 14 in basal vs. DOX alone, $n = 8$, $p < 0.05$) and this decrease was potentiated with DOX-HA-SPION (148.9 ± 8.4) with both statistically significant from baseline and DOX alone.

4. DISCUSSION

In the current investigation, we are the first to demonstrate enhanced cytoplasmic uptake, more apoptosis, and down-regulation of both IL-6, a pro-inflammatory cytokine, and NF- κ B activity with DOX-HA-SPION nanoparticle treatment in comparison to treatment with free DOX in the human MDA-MB-231 invasive breast cancer cells. The higher level of IL-6 and increased activity of NF- κ B have been reported to be higher in TNBC than in other breast cancer cell lines or normal breast tissue.^{86,87} The low levels of pro-inflammatory cytokines are known to reduce proliferation, metastasis and angiogenesis suggesting that reduction of IL-6 and down-regulation of NF- κ B activity could be beneficial in breast cancer therapy.

Using confocal laser microscopy, we demonstrated that DOX-HA-SPION mainly accumulates in the cytoplasm and it might be slowly released into the nucleus. In comparison according to known fact, free DOX diffuses directly and concentrates in the nucleus. The difference in cellular distribution of these two compounds can be explained based on their different uptake mechanisms. El-Dakdouki and colleagues have shown increased total cellular uptake with DOX-HA-SPION in comparison to free DOX in ovarian cancer cells.^{44, 88} Moreover, they reported that (we did not show DOX is taken up by passive diffusion) DOX-HA-SPION is taken up by endocytosis via CD44 followed by escape from endosomes.⁴⁴ The exact cytotoxic mechanism of DOX is only partially

understood but the major cellular target for DOX is the DNA inside the nucleus.⁸⁹ Another possible target for DOX has been reported to be mitochondria, where DOX can induce collapse of the mitochondrial transmembrane potential, leading to oxidative stress and apoptotic signals.⁹⁰

The enhanced cytotoxicity of DOX-HA-SPION after the initial time period can be attributed to the significant accumulation of DOX in mitochondria delivered by NPs in addition to that in the nucleus.⁴⁴ This hypothesis can be substantiated with the help of our SEM pictures that suggest cellular, nuclear as well as mitochondrial changes. Our particle is different from other nanoparticle studied in TNBC,⁹¹ as it not only increases apoptosis, but also has effects on a pro-inflammatory pathway.

Our results also indicate that nanoparticle-induced changes in inflammatory cytokines could potentially lead to a positive effect in slowing progression and metastasis of breast cancer. We also observed increased IL-6 levels in MDA-MB-231 cancer cells. Furthermore, we observed down-regulation of secretion of IL-6 with DOX-HA-SPION whereas free DOX accentuated secretion.

Tumor progression in the highly invasive MDA-MB-231 breast cancer cell line follows distinct steps. These include tumor growth, angiogenesis, tumor cell detachment, epithelial-mesenchymal transition (EMT), and growth of the macro metastasis.⁹²

IL-10 is an anti-inflammatory cytokine involved in the regulation of other cytokines and immune response. Increased levels of IL-10 have been associated with the inability to mount an effective immune response to Hodgkins lymphoma.⁹³ Increased levels of IL-10 have been reported in patients with breast cancer and this was directly correlated to suppression of the immune response.⁹⁴ We saw no changes in IL-10 levels, suggesting that the nanoparticle effect is specific to IL-6. Our study demonstrates DOX-HA-SPION did not stimulate secretion of nitrate as compared control. Nitric oxide (NO) is a pleiotropic regulator, critical to numerous biological processes, including vasodilation, neurotransmission, and macrophage-mediated immunity.⁹⁵ Increased NO generation in cancer cells may contribute to tumor angiogenesis by up-regulating vascular endothelial growth factor (VEGF), and thus may increase the tumors' metastatic ability.⁹⁶ Our study showed DOX-HA-SPION did not increase the levels of nitrate indicating that nanoparticle does not contribute to negative pro-inflammatory mechanism.

It is well documented that NF- κ B is activated in human breast cancer tissues and cell lines.^{86, 87, 97} In this study, we showed that DOX-HA-SPION inhibits activation of NF- κ B while free DOX stimulates it. Down-regulation of NF- κ B suggests that DOX-HA-SPION promotes apoptosis much more than DOX alone. Moreover, activation of NF- κ B has also been shown to enhance production of cytokines (IL-6), inhibit apoptosis as well as contribute to drug resistance.⁹⁸

Vimentin is a known epithelial-mesenchymal transition (EMT) marker. EMT is an epithelial cellular pathway to lose cell polarity and cell-cell adhesion, eventually leading to migration and gaining invasive properties. In breast cancer Vimentin expression causes high tumor invasiveness and chemoresistance.⁹⁹ We demonstrated that in addition to lowering

inflammation, DOX-HA-SPION lowered the Vimentin expression. We also performed E-Cadherin but it did not show a similar trend.

In conclusion, we demonstrate a promising role for DOX-HA-SPION in exploiting the application of HA targeting CD44, thus leading to increase DOX uptake. In future work, we will use an *in-vivo* model and MRI to show localization of nanoparticle. We also will need long-term studies to determine whether selective targeting to the tumor reduces cardiac toxicity. Nonetheless, our findings that DOX-HA-SPION increases apoptosis and reduces proinflammatory but not anti-inflammatory cytokine release indicates that this treatment could be beneficial in reducing angiogenesis and metastasis in TNBC.

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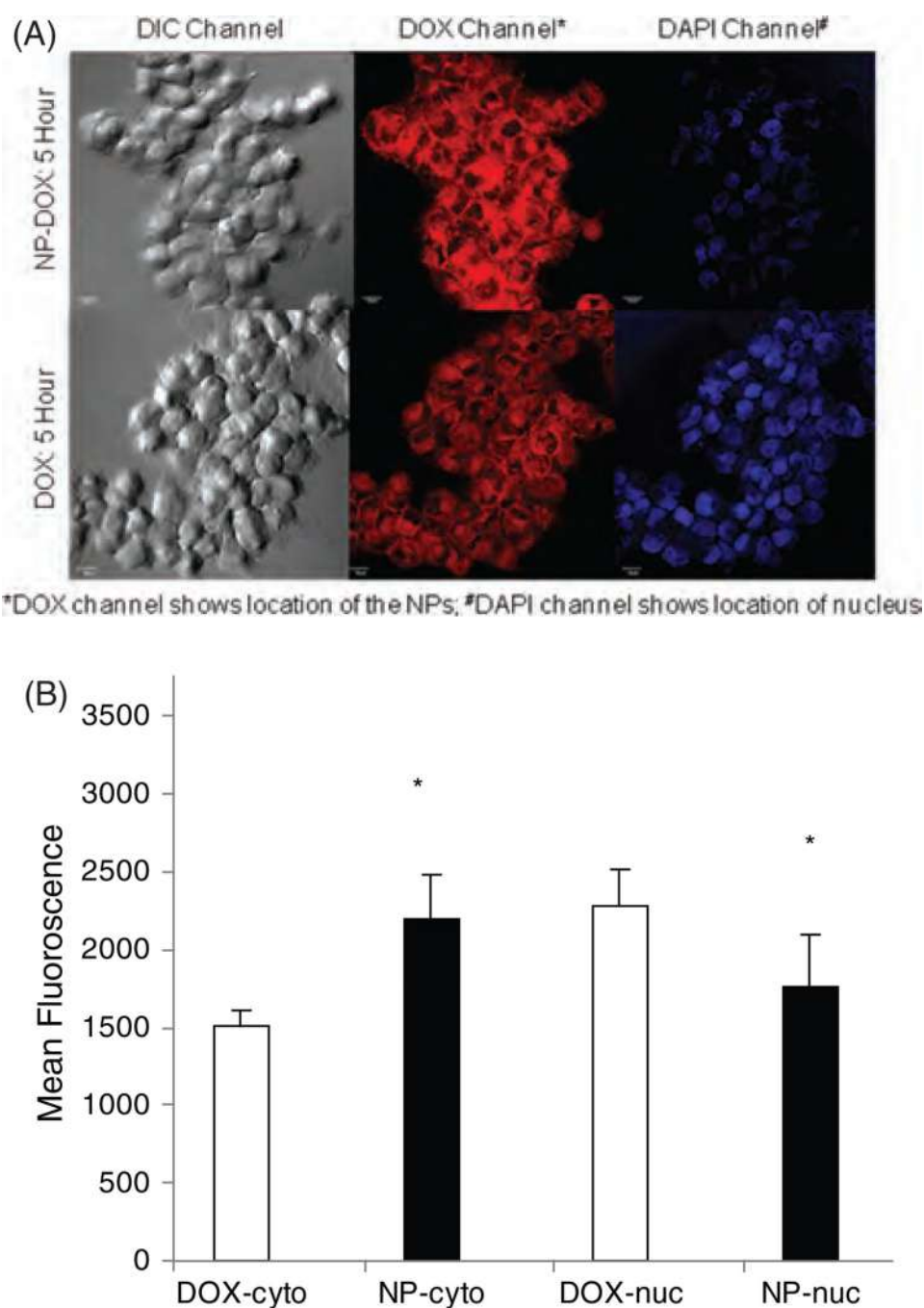


Figure 1. Doxorubicin-nanoparticle (DOX-HA-SPION) enhances the *Interacellular uptake* in MDA-MB-231 breast cancer cells. MBA-MB-231 cells were grown to 80% confluency and treated with DOX or DOX-HA-SPION for 5 hours and fluorescence was measured as described in “Materials and Methods section.” (A) Typical confocal images treated with DOX or DOX-HA-SPION. (B) Mean fluorescence in the Cytoplasm and nucleus. Each treatment was repeated three times; the average values are shown with standard error (SE). The asterisk

indicates the statistically significant difference between DOX-treated and the DOX-HA-SPION treated cells with $P < 0.05$ determined by Student's t -test.

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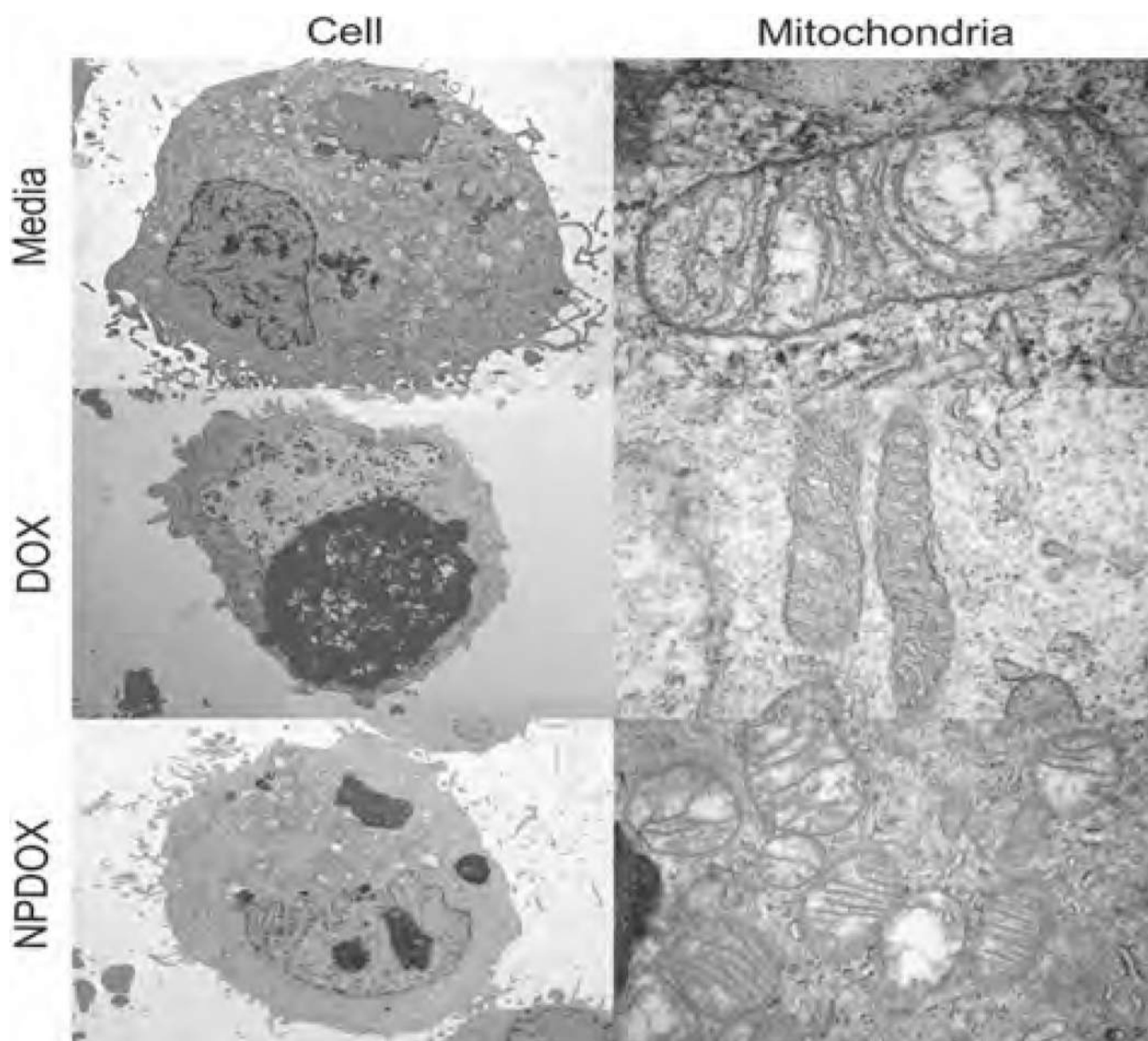


Figure 2.

Effect of DOX and DOX-HA-SPION on cellular morphology by electron microscopy in MAA-MB-231 breast cancer cells. MBA-MB-231 cells were grown to 80% confluency and treated with DOX or DOX-HA-SPION for 24 hours and morphology was examined under scanning electron microscopy as described in “*Materials and Methods section.*” Typical morphological changes shown with DOX or DOX-HA-SPION treated cells. Each treatment was repeated three times.

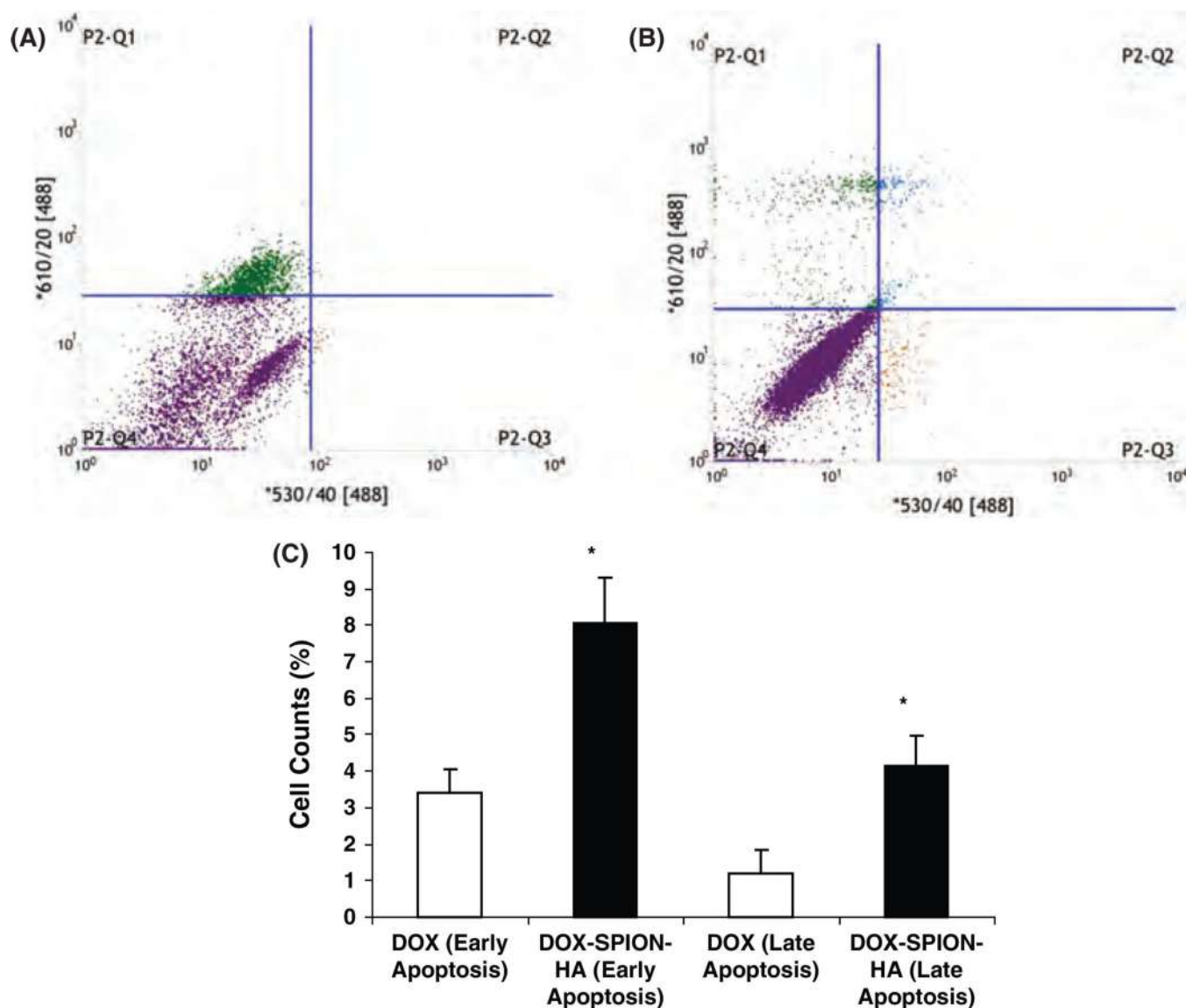


Figure 3.

Doxorubicin-nanoparticle (DOX-HA-SPION) increases apoptosis measured by FACS in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were grown to 80% confluency and treated with DOX or DOX-HA-SPION for 24 hours and followed by 24 hour incubation period in the medium to study early apoptosis and late apoptosis as described in “Materials and Methods section.” (A) Shows typical histograms treated with DOX (Q2 represents cell counts in late apoptosis and Q3 represents cell counts in early apoptosis); (B) Shows typical histograms treated with DOX-HA-SPION (Q2 represents cell counts in late apoptosis and Q3 represents cell counts in early apoptosis); and (C) shows an average values with standard error (SE). Each treatment was repeated at least three times. The asterisk indicates the statistically significant difference between DOX-treated and the DOX-HA-SPION treated cells with $P < 0.05$ determined by Student’s *t*-test.

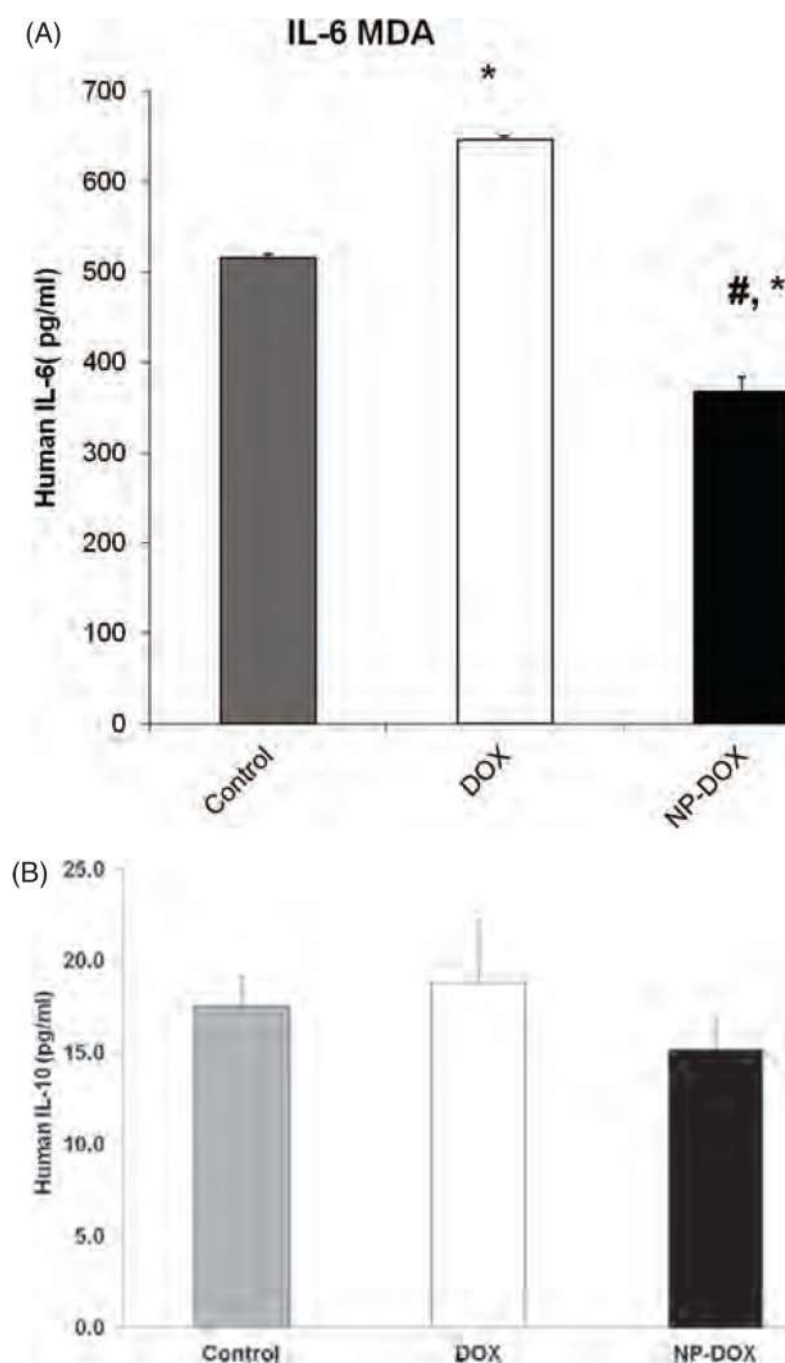


Figure 4. Doxorubicin-nanoparticle (DOX-HA-SPION) inhibits secretion of proinflammatory IL-6 without changing anti-inflammatory IL-10 cytokine level in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were incubated with DOX or DOX-HA-SPION as described in “Materials and Methods section” and conditioned medium was used to determine the secretion levels of IL-6 (A) and IL-10 (B). Each treatment was repeated three times; the average values are shown with standard error (SE). The asterisk (*) indicates the statistically significant difference between untreated control, DOX-treated and the DOX-HA-SPION

treated cells with $P < 0.05$ determined by Student's t -test. The asterisk (#) indicates the statistically significant difference between DOX-treated and the DOX-HA-SPION treated cells with $P < 0.05$ determined by Student's t -test. The IL-10 secretion levels were not statistically significant different between untreated control, DOX-treated and the DOX-HA-SPION treated cells with $P < 0.05$ determined by Student's t -test.

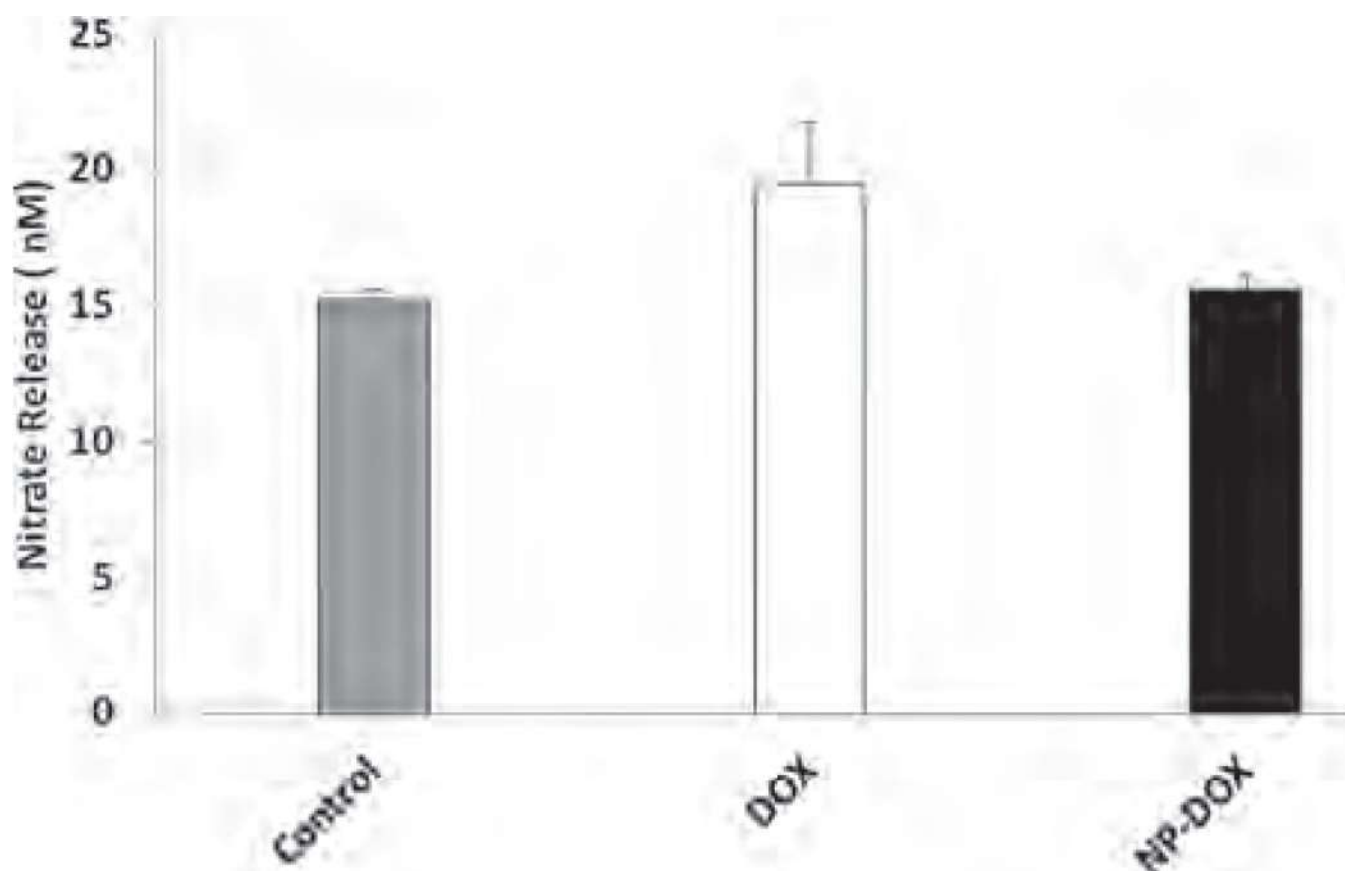


Figure 5.

Doxorubicin-nanoparticle (DOX-HA-SPION) does not change secretion of nitrate in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were incubated with DOX or DOX-HA-SPION as described in “*Materials and Methods section*” and conditioned medium was used to determine the secretion levels of Nitrate. Each treatment was repeated three times; the average values are shown with standard error (SE). The nitrate secretion levels were not statistically significant different between untreated control, DOX-treated and the DOX-HA-SPION treated cells with $P > 0.05$ determined by Student’s t -test.

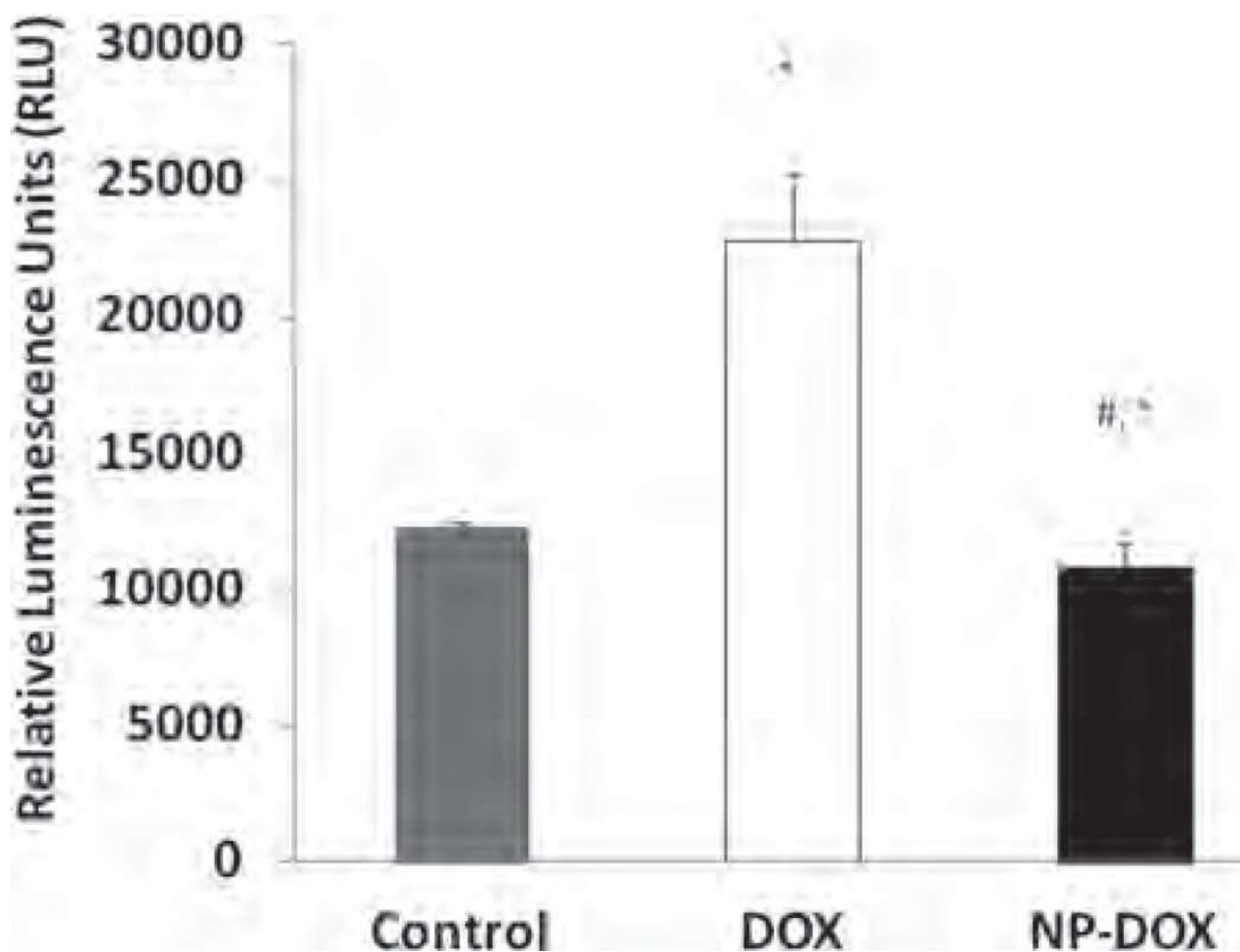


Figure 6.

Doxorubicin-nanoparticle (DOX-HA-SPION) reduces NF κ B transcriptional activity in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were grown to 80% confluency and transduced with NF κ B luciferase reporter gene. Twenty-four hours later, cells were treated with DOX or DOX-HA-SPION or not treated with either one for 24 hours and NF- κ B activity was measured as described in “Materials and Methods section.” The NF- κ B activity expressed as Relative Luminescence Unit (RLU). Each treatment was repeated three times; the average values are shown with standard error (SE). The asterisk (*) indicates the statistically significant difference between untreated control versus DOX-treated and untreated control versus DOX-HA-SPION treated cells with $P < 0.05$ determined by Student’s t -test. The asterisk (#) indicates the statistically significant difference between DOX-treated and the DOX-HA-SPION treated cells with $P < 0.05$ determined by Student’s t -test.

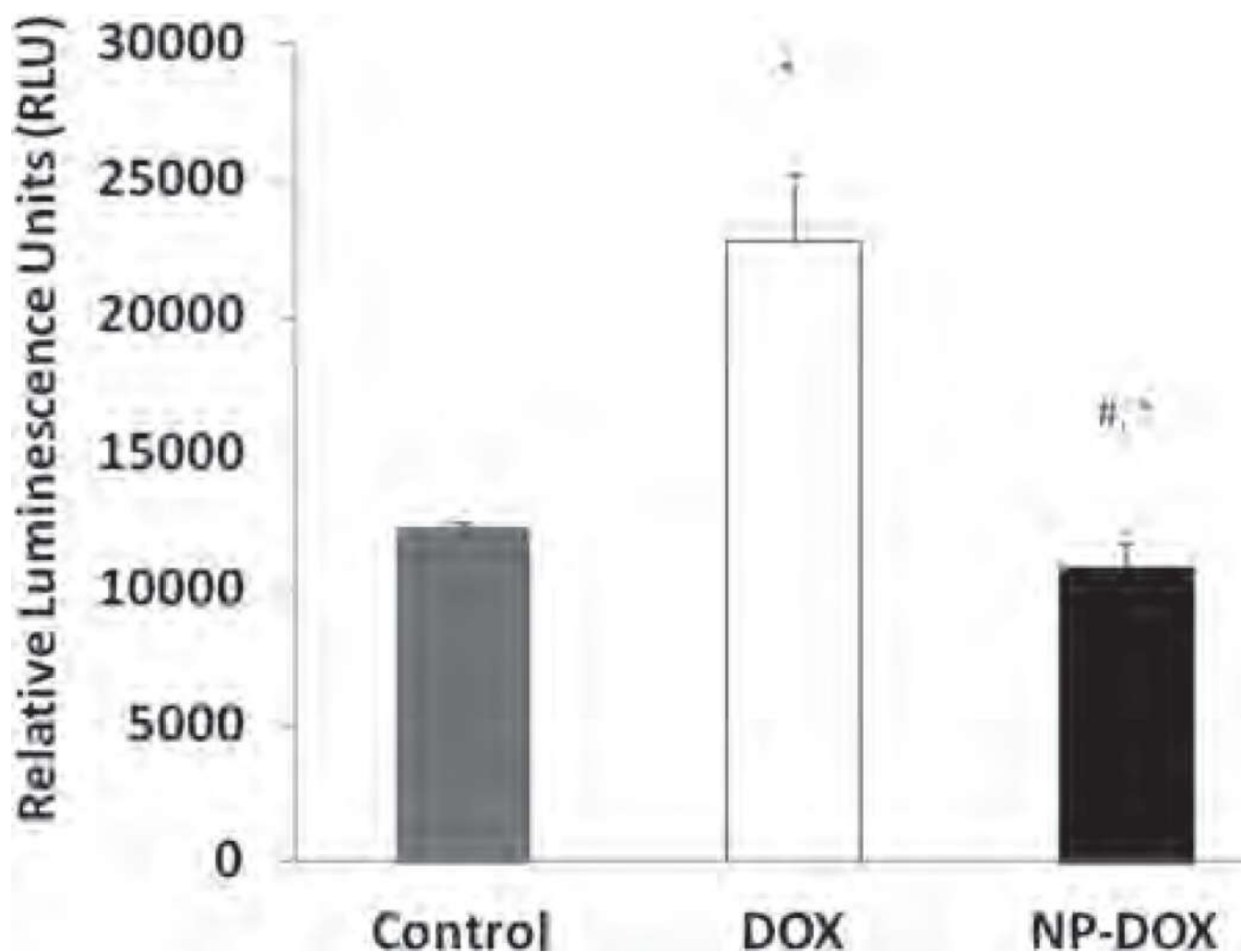


Figure 7. Proposed signal pathway by which Doxorubicin-nanoparticle (DOX-HA-SPION) may modulate cellular uptake, inflammation and apoptosis in MDA-MB-231 breast cancer cells. The hypothesis is based on the results from previous reported studies and our results from the present study.