의학박사 학위논문

Low dose doxorubicin increases the invasiveness of osteosarcoma through ROS/MMP-9 pathway in vitro

저용량 doxorubicin 투여로 활성화된 ROS/MMP-9 경로를 통한 골육종의 침습성 증가에 대한 실험실적 연구

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by
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ABSTRACT

Introduction: Doxorubicin (DOX or adriamycin) is a major chemotherapeutic agent for osteosarcoma (OS). It expectedly eliminates micro-metastasis, but suffers from cardiotoxicity that can be lethal. Trials have sought to reduce the cardiotoxicity by reducing the peak plasma levels of DOX, but results have been controversial. Besides, these attempts involving low dose DOX may produce still other adverse effects. The purpose of this study was to evaluate the effect of low concentrations of DOX on the invasiveness of OS, and the involvement of the reactive oxygen species/matrix metalloproteinase-9 (ROS/MMP-9) pathway.

Methods: The human OS cell line U2OS was used for the experiments. Hydrogen peroxide (H_2O_2) and the antioxidant compound N-acetylcysteine (NAC) were used to investigate the involvement of ROS signaling. The effects of DOX, H_2O_2, and NAC on U2OS proliferation was evaluated by the MTT assay. Invasiveness of U2OS was evaluated by Matrigel^{TM} invasion assay and gelatin zymography in the presence of various doses of DOX or H_2O_2, with or without NAC pre-treatment. In each experimental condition, MMP-9 expression was measured using quantitative real time polymerase chain reaction (qPCR).

Results: In proliferation assays, DOX (up to 500 nM), H_2O_2 (up to 50 μM),

and NAC (up to 10 mM) did not affect U2OS proliferation significantly for up to 24 h. Matrigel^{TM} invasion assay and gelatin zymography revealed increased invasiveness of U2OS at around 100 nM of DOX, which is a subclinical concentration. In qPCR, MMP-9 expression was increased in the presence of DOX doses that increased the invasiveness of U2OS. H_2O_2, a representative source of ROS, also increased the invasiveness of U2OS. No DOX- or H_2O_2-mediated increased invasiveness or MMP-9 expression was evident when pre-treated with NAC.

Conclusions: Low-dose DOX increased U2OS invasiveness through ROS activation and MMP-9 induction in vitro. DOX may have oncologic adverse effects at low doses, which should be further investigated. The ROS/MMP-9 pathway could be a novel target for future research and targeted therapies for OS.

Keywords: osteosarcoma, invasiveness, doxorubicin, reactive oxygen species, MMP-9

Student number: 2011-30555

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LIST OF ABBREVIATIONS

DOX: doxorubicin

FBS: fetal bovine serum

MEM: Modified Eagle's Medium

MMP-9: matrix metalloproteinase-9

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC: N-acetylcysteine

OS: osteosarcoma

qPCR: quantitative real time polymerase chain reaction

ROS: reactive oxygen species

INTRODUCTION

Osteosarcoma (OS) is the most common primary bone malignancy. Although surgical removal is essential for cure, more than 80% of OS patients treated with surgery alone develop metastatic disease [1]. This is presumably due to the subclinical metastases present at diagnosis in the majority of OS patients [2]. Chemotherapy is expected to eradicate these deposits, and greatly improves the survival rate of OS patients, from 20-30% to 60-70%. Although various new methods including target agents have been tried, the mainstream OS chemotherapy involves traditional anticancer drugs, among which doxorubicin (DOX) is a crucial member [3].

DOX intercalates between DNA bases and inhibits the action of topoisomerase II, resulting in the cessation of cell replication. However, DOX has other mechanisms of action that have not yet been clearly identified including one that is associated with reactive oxygen species (ROS). DOX induces ROS, which additionally damage cancer cells. However, ROS are also one of the major causes of DOX toxicity, which dose-dependently damages the heart and limits the therapeutic use of doxorubicin [4].

The response of cancer cells to ROS depends on various factors including the amount of ROS. High levels of ROS induce apoptosis or death of cancer cells, while lower levels can stimulate cell survival signals or activate oncogenic signals [5]. Low plasma peak regimens and modified drug formulations have been explored to minimize adverse events and maximize

therapeutic benefits in cancer chemotherapy [6, 7]. However, these attempts may produce still other adverse effects. As the intracellular mechanism of such adverse effects including that for the DOX-induced ROS have not yet been clearly identified, new regimens should be investigated for possible unknown effects.

The purposes of this study were to evaluate the effect of low concentrations of DOX on the invasiveness of OS and to explore the involvement of the ROS/matrix metalloproteinse-9 (MMP-9) pathway.

MATERIALS AND METHODS

Experimental scheme

All the experiments in this study were performed in vitro. In our pilot study, the U2OS human OS cell line showed more invasiveness than the MG63 or SaOS2 OS cell lines (data not shown). As the literature was consistent with these findings [8], we chose U2OS for this study, which focused on the invasiveness of OS. Before the experiments for invasiveness, we sought to determine the time duration and drug concentrations in which the viability of U2OS cells was not significantly affected. To evaluate the influence of DOX on the invasiveness of U2OS, a Matrigel^{TM} invasion assay and gelatin zymography were performed using various concentrations of DOX. We also investigated the cellular expression level of MMP-9, which we previously suggested as a major proteolytic enzyme for OS invasiveness [9]. We hypothesized that the influence of DOX to invasiveness is mediated by ROS, and performed the same experiments for invasiveness with H_2O_2, a representative source of ROS, instead of DOX. Finally, to confirm that the DOX-induced change of invasiveness was mediated by ROS, we investigated whether the DOX-induced change of invasiveness could be abrogated by N-acetylcysteine (NAC), a potent antioxidant.

Cell cultures

The U2OS cell line was kindly provided by Prof. Dohyun Nam (Samsung Medical Center, Seoul, Korea). The cells were maintained in Modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and1% Antibiotic-Antimycotic solution (Gibco, Gaithersburg, MD).

Proliferation assay

Monolayer culture proliferation was measured using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. For each assay, 5×10^4 cells were seeded in wells of a 24-well culture plate. The confluent monolayer cultures were trypsinized and replated in triplicate. Cell survival was determined 24 h after each experimental treatment involving the addition of DOX, H_2O_2, or NAC at different concentrations. MTT (200 μg/mL) was added to each well and the plate was incubated at 37 °C for 1 h. The MTT solution was removed and 200 mL of dimethyl sulfoxide was added to dissolve the formazan crystals that had formed. The optical density at 595 nm was read using a spectrophotometer. The viability was determined by the ratio of the optical density value for the cells with each experimental treatment relative to that for the control cells.

Invasion assay

Ten microliters of diluted Matrigel™ (10.5 mg/mL; BD Biosciences, San Jose, CA) in cold serum-free MEM was added into the upper chamber of a 24-well plate with Transwell® Permeable Supports (Corning Incorporated, Corning, NY) and incubated at 37°C for 1 h to form an even and thin layer. The U2OS

cells (1×10^5) were seeded with 200 μL of serum-free MEM in the upper compartment of the chamber, and 500 μL of MEM containing 0.1% FBS was added in the lower compartment of the chamber. DOX or H_2O_2 was added in the lower compartment at different concentrations. To evaluate the effect of antioxidant pre-treatment, NAC (2-10 mM) was added in the upper compartment 1 h before each experimental treatment. The plate was incubated at 37 °C for 6 h. After the incubation, non-invading cells that remained on the upper surface of the Matrigel™ membrane were completely removed by wiping with a cotton swab. The invading cells on the bottom of the Transwell® Permeable Support were fixed with 100% methanol for 1 min and stained with Hematoxylin and Eosin. Digital photomicrographs were taken from five fixed locations (at the center, and at the perimeters of the Transwell® Permeable Support in the 3, 6, 9 and 12 o'clock positions) at a magnification of ×200. The number of invaded cells was counted in these locations.

RNA isolation and quantitative polymerase chain reaction

After each experimental treatment (incubation of the cells with of DOX or H_2O_2 at different concentrations for 18 h, with or without NAC pre-treatment for 1 h), total cellular RNA was extracted with a RNeasy® Mini kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. For quantitative real time polymerase chain reaction (qPCR), cDNA was synthesized from 1 μg of total RNA using a SuperScript® III reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. qPCR was conducted

with 0.5 μL of cDNA template in triplicate in 384 well plate, using 7900HT Fast Real Time PCR System (Applied Biosytems, Foster City, CA) and AccuPower® 2× GreenStar™ qPCR Master Mix (Bioneer, Daejeon, Korea). Quantification was carried out using the efficiency-corrected ΔΔCq method. The specific oligonucleotide sequences for target genes were as follows: MMP-9, 50-CACTGTCCACCCCTCAGAGC-30 (sense) and 50-GCCACTTGTCGGCGATAAGG-30 (antisense); GAPDH, 50-TGATGACATCAAGAAGGTGAAG-30 (sense) and 50-TCCTTGGAGGCCATGTGGGCCAT-30 (antisense). All assays were performed in triplicates.

Gelatin zymography

After each experimental treatment (incubation of the cells with of DOX or H_2O_2 at different concentrations for 18 h, with or without NAC pre-treatment for 1 h), the medium was harvested and prepared with standard sodium dodecyl sulfate (SDS)-gel-loading buffer containing 0.01% SDS without β-mercaptoethanol and were not boiled before loading. The prepared samples were then subjected to electrophoresis in 8% SDS polyacrylamide gels containing 0.1% gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 and subsequently incubated at 37 °C for 24–48 h in a development buffer containing 10 mM $CaCl_2$, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5). The gel was stained with 0.2% Coomassie brilliant blue and destained with methanol-acetic acid-water (100/100/800, v/v/v) and

photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Statistical analysis

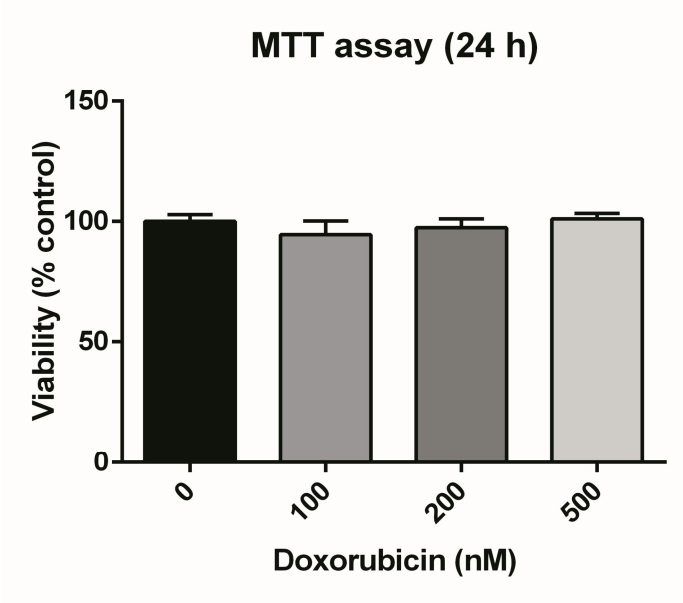
Statistical analysis and graphical representations were performed using the GraphPad Prism for Windows software version 6.00 (GraphPad Software, La Jolla, CA). Mann-Whitney test was used to determine the differences between the experimental results, with the significance level set at $p < 0.05$. Statistically significant differences are marked by asterisks in figures.

RESULTS

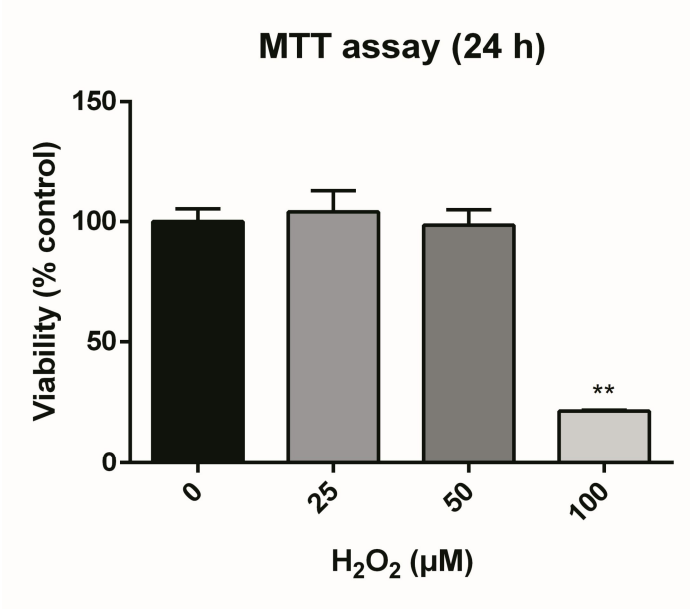
Effect of DOX, H_2O_2, and NAC on proliferation

For the invasion analysis, cell numbers should be adjusted to be equal among the experimental groups. Since doxorubicin or H_2O_2 has known to affect proliferation of cells, we had to find the proper range of dose where the growth of U2OS cells was not significantly hampered. Thus, we performed MTT assays to analyze the growth rate of U2O2 in different doses of DOX, H_2O_2, and NAC. DOX (up to 500 nM), H_2O_2 (up to 50 μM), and NAC (up to 10 mM) did not significantly affect U2OS cell proliferation for up to 24 h (Figure 1). Subsequent experiments were performed within 24 h using DOX, H_2O_2, and NAC at concentrations keeping the U2OS viability unaffected.

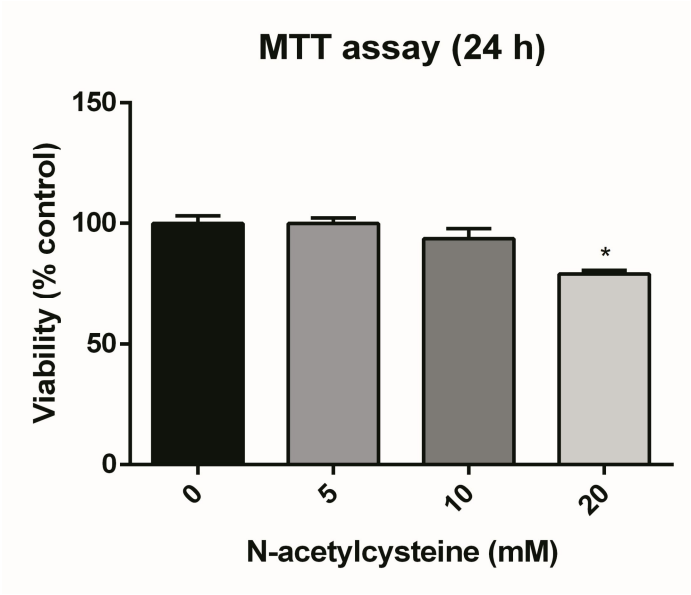
Figure 1. Effects of DOX, H_2O_2, and NAC on U2OS cell proliferation.



(A) Until 24 h, DOX did not affect the proliferation of U2OS significantly, up to a concentration of 500 nM.



(B) Until 24 h, H_2O_2, did not affect the proliferation of U2OS significantly, up to a concentration of 50 μM.

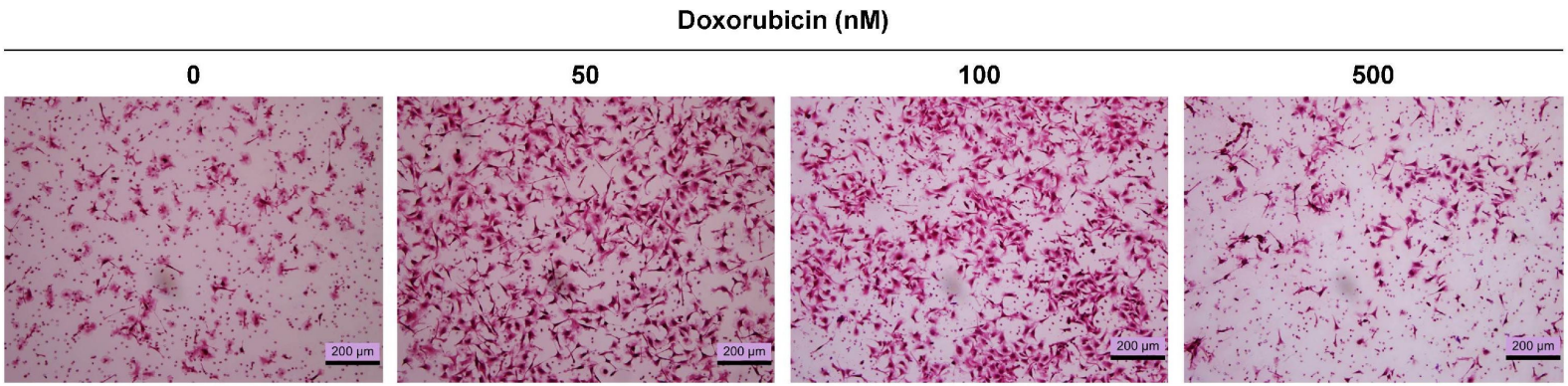


(C) Until 24 h, NAC did not affect the proliferation of U2OS significantly, up to a concentration of 10 mM.

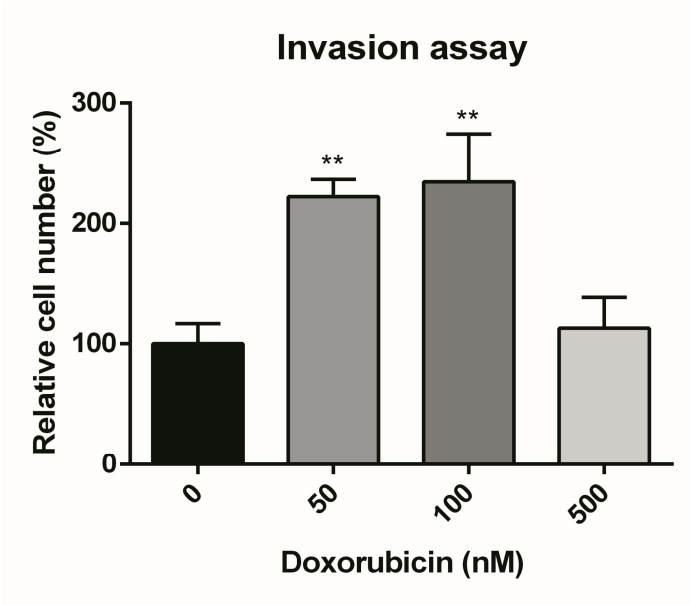
DOX increases U2OS invasiveness

To evaluate the influence of DOX on the invasiveness of U2OS cells, we performed Matrigel^{TM} invasion assay, gelatin zymography, and MMP-9 qPCR, with variable concentrations of DOX. In the Matrigel^{TM} invasion assay, after 6 h of incubation, the number of invading U2OS cells was significantly increased in the presence of 50 nM and 100 nM DOX, compared to the absence of DOX or 500 nM DOX, in which the invasion was not significantly different from the control (Figures 2A and 2B). qPCR revealed maximum MMP-9 expression at 100 nM DOX; the pattern was similar to that of the invasion assay. The enzymatic activity of MMP-9 also showed a similar pattern with respect to the DOX concentration in gelatin zymography, with peak activity observed at 100 nM DOX (Figures 2C and 2D).

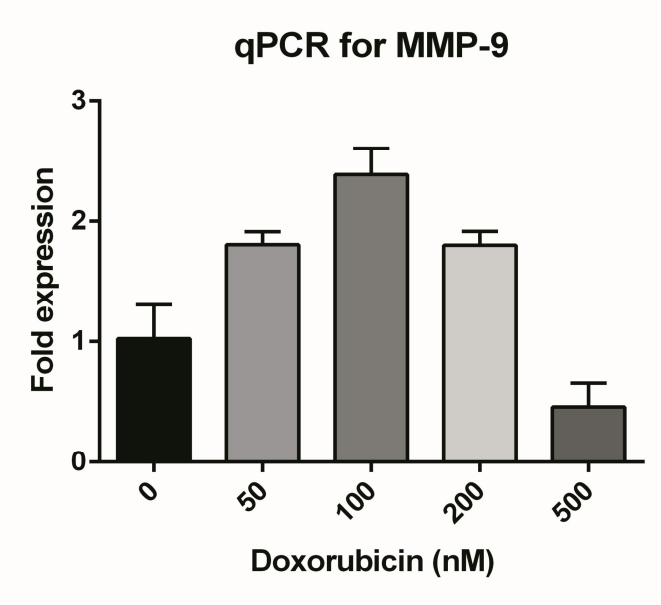
Figure 2. DOX increases the invasiveness of U2OS cells.



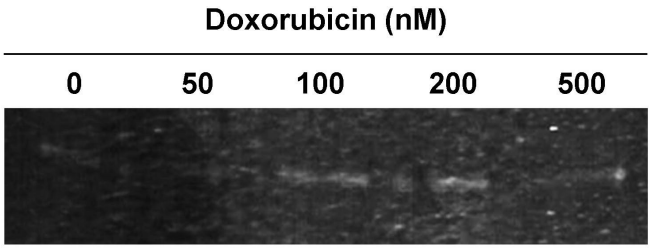
(A) The Matrigel™ invasion assay revealed an increased number of invading U2OS cells in 50 nM and 100 nM of DOX, as compared to control or 500 nM DOX.



(B) Graphical representation of the invasion assay.



(C) qPCR revealed maximal MMP-9 expression level at 100 nM DOX, similar to the result of the invasion assay.

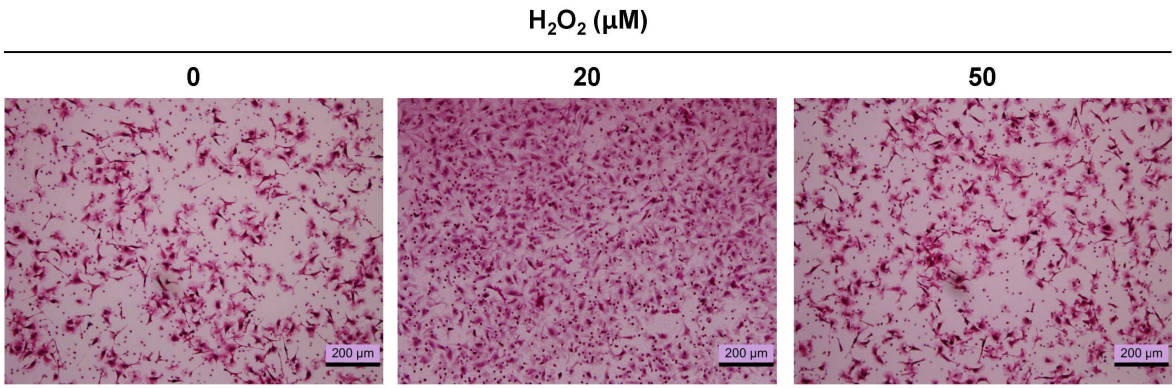


(D) Gelatin zymography also showed a similar pattern, with peak activity of MMP-9 observed at 100 nM DOX.

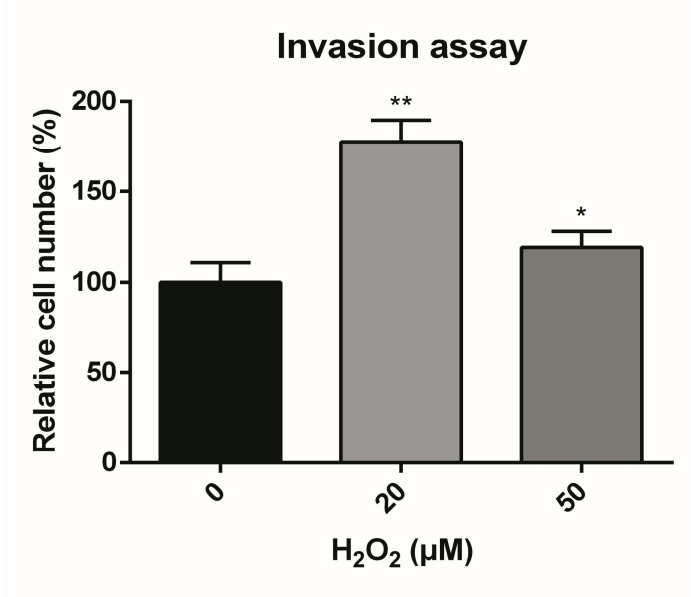
H_2O_2 mimics DOX-induced increase of U2OS invasiveness

We hypothesized that the DOX-induced increase of invasiveness is mediated by ROS, and performed the same experiments for invasiveness with H_2O_2, a representative source of ROS, instead of DOX. In the Matrigel™ invasion assay, after 6 h of incubation, the number of invading U2OS cells was significantly increased in 20 μM H_2O_2, and the increase was much reduced in 50 μM H_2O_2 (Figures 3A and 3B). Similarly, qPCR revealed increased MMP-9 expression in 20 μM H_2O_2, and the expression was similar to the control in 50 μM H_2O_2 (Figure 3C). In gelatin zymography, the enzymatic activity of MMP-9 was definitely increased in 25 μM H_2O_2, and the increase was much smaller in 50 μM H_2O_2 (Figure 3D). The invasiveness of U2OS in different concentrations of H_2O_2 showed a pattern similar to the invasion assays with DOX.

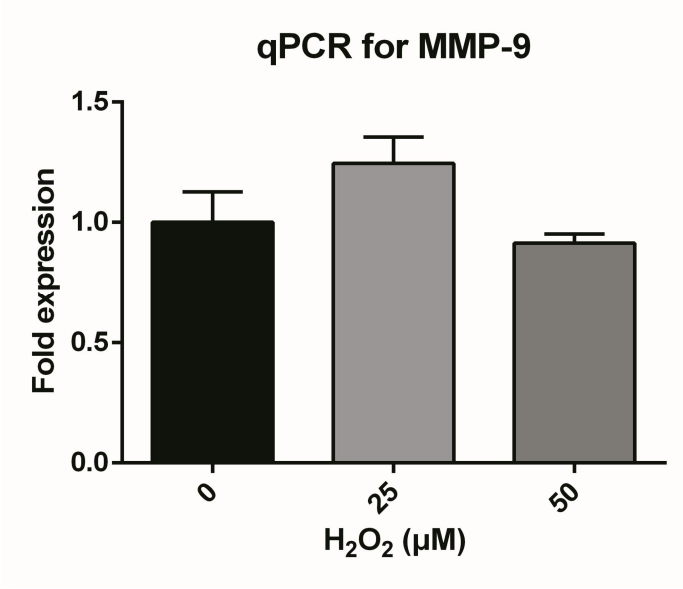
Figure 3. H_2O_2 mimics the DOX-induced increase of U2OS cell invasiveness.



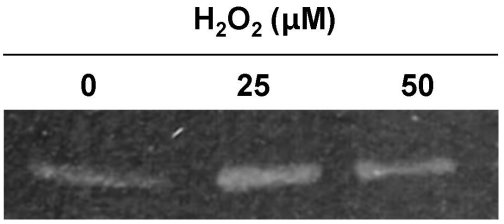
(A) The Matrigel^{TM} invasion assay revealed an increased the number of invading U2OS cells at 20 μM H_2O_2, as compared to control or 50 μM H_2O_2.



(B) Graphical representation of the invasion assay.



(C) qPCR revealed maximal MMP-9 expression at 20 μM H_2O_2, similar to the result of invasion assay.

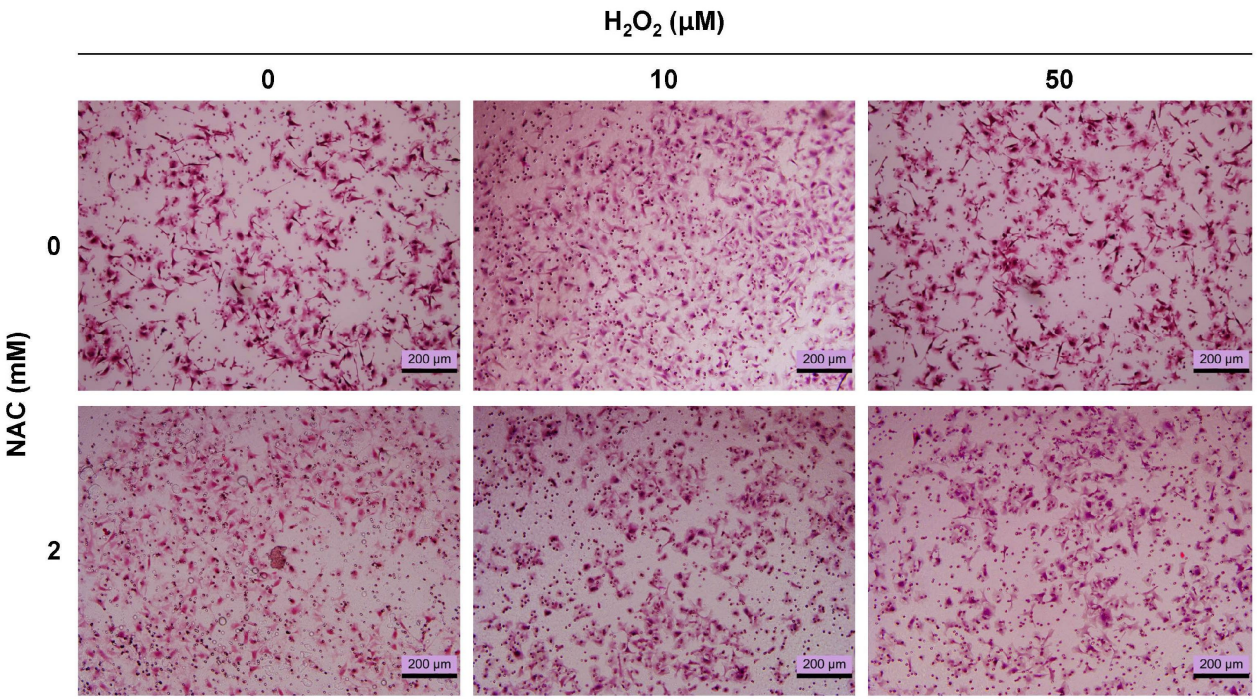


(D) Gelatin zymography also showed a similar pattern, with peak activity of MMP-9 observed at 25 μM H_2O_2.

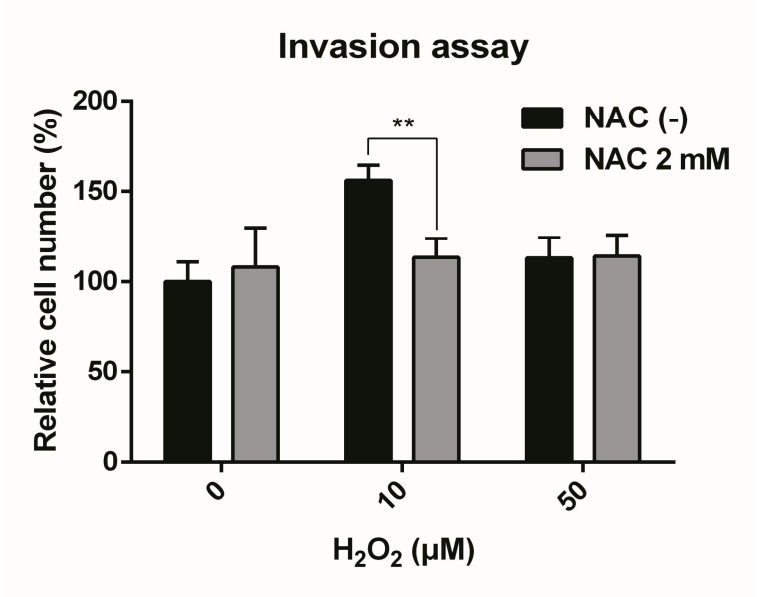
Antioxidant NAC abrogates the H_2O_2- or DOX-induced increase of invasiveness

Finally, to confirm that the DOX-induced increase of invasiveness was mediated by ROS, we investigated whether the DOX-induced increase of invasiveness could be abrogated by NAC, a potent antioxidant. When pre-treated with 2 mM of NAC for 1 h, the number of invading cells in the Matrigel^{TM} invasion assay was not increased in 10 μM H_2O_2 (Figures 4A and 4B). Similarly, when pre-treated with 10 mM NAC for 1 h, the number of invading cells in the Matrigel^{TM} invasion assay was not increased in 100 nM DOX, and was decreased with increasing concentrations of DOX (Figures 4C and 4D). In qPCR, the MMP-9 expression level, which was increased in 100 nM DOX, was not increased when samples were pre-treated with 10 mM NAC, and was decreased with increasing concentrations of DOX, showing a similar pattern shown in the invasion assays with NAC pre-treatment and DOX (Figure 4E).

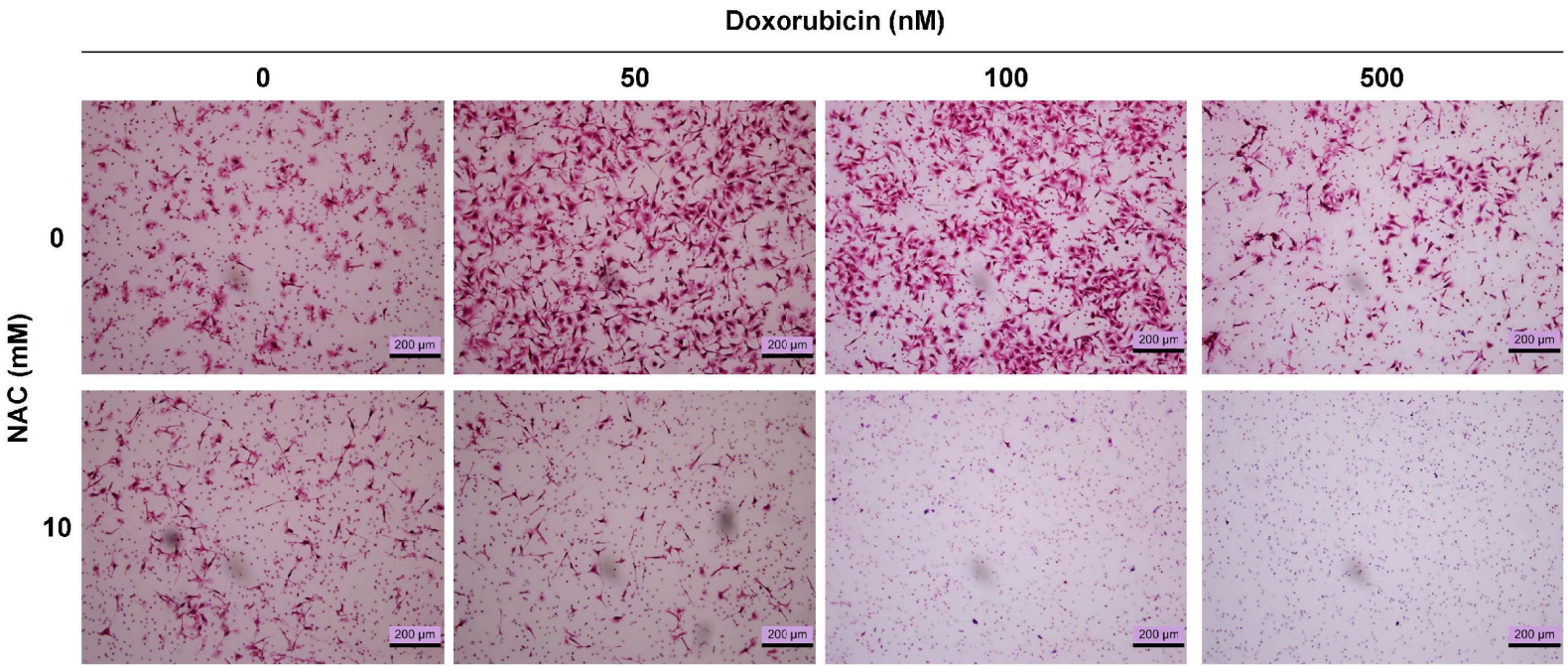
Figure 4. NAC abrogates the H_2O_2- and DOX-induced increase of U2OS cell invasiveness.



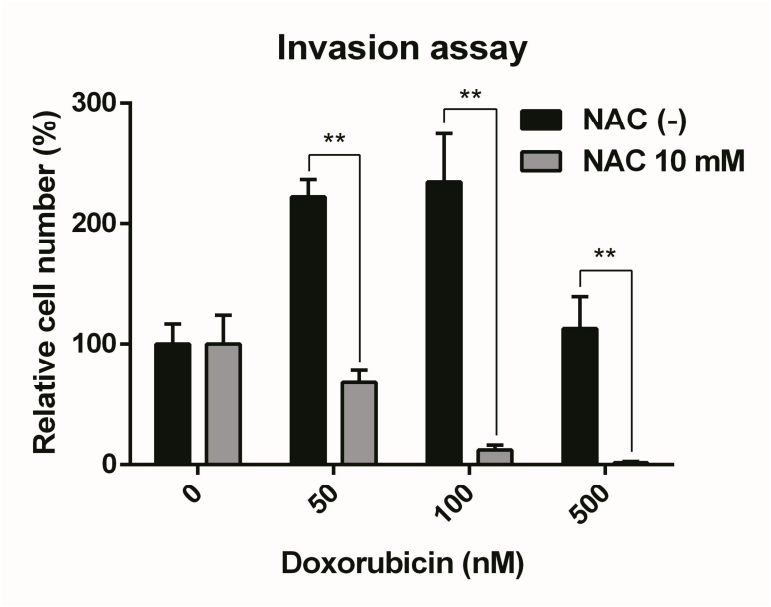
(A) NAC pre-treatment abrogated the H_2O_2-induced increased invasiveness of U2OS cells in the Matrigel^{TM} invasion assay.



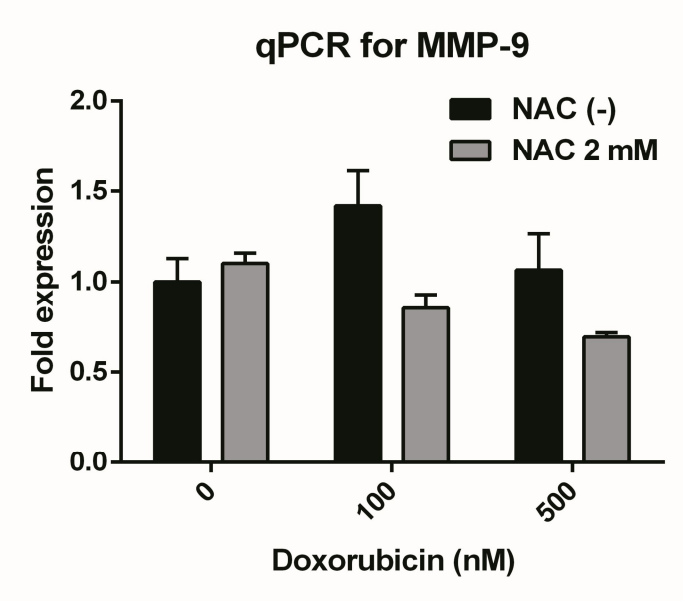
(B) Graphical representation of the invasion assay result.



(C) NAC pre-treatment abrogated the DOX-induced increased invasiveness in the Matrigel^{TM} invasion assay. Invasiveness decreased with increasing concentrations of DOX.



(D) Graphical representation of the invasion assay result.



(E) NAC pre-treatment abrogated the DOX-induced increase of MMP-9 expression level. Expression decreased with increasing concentrations of DOX, similar to the invasion assay.

DISCUSSION

High concentrations of DOX helps in eliminating cancer cells from the body [10]. However, in practice, elevated levels of DOX damage normal tissues, of which the cardiac muscle is the most vulnerable and problematic [11]. Several trials have reported encouraging results in reducing cardiac toxicity of DOX by reducing the peak plasma levels [6, 12]. However, other studies failed to show reduced cardiotoxicity [13, 14]. A recent study reported that cardiomyocyte functions may be adversely affected even by subclinical concentrations of doxorubicin (50-100 nM) [15]. Considering both the anti-tumor effect and the cardiotoxicity are associated with the peak level and cumulative dose of DOX [14, 16, 17], it should be investigated whether low-dose regimen at least has no unwanted harmful effects.

In standard regimens for OS, DOX is administered intravenously at 15-90 mg/m^2 body surface area a day [15]. When 75 mg/m^2 is administered, plasma concentration of DOX rapidly declines from 5 μM to 100 nM within 1 hr [18]. In steady state, plasma concentration of DOX is far below the peak concentration (5 μM), and ranges 25-250 nM after the administration of 15-90 mg/m^2 for chemotherapy [15]. Presently, doxorubicin ranged up to 500 nM and at around 100 nM, which is considered a subclinical concentration [15], it increased the invasiveness of the examined OS cell line. Although this is only an in vitro finding and should be further investigated in vivo, it gives an important message to therapeutic regimens because the invasiveness is a

crucial factor for tumor growth and metastasis [19]. Lower plasma concentrations of DOX could be less cardiotoxic, but may have adverse effects as well, which should be investigated more.

In our study, we investigated if ROS play an important role for this DOX-induced invasiveness. ROS mediate doxorubicin induced apoptosis and are the major cause DOX toxicity [20, 21]. A recent animal study directly demonstrated the dose-dependent ROS generation by cardiomyocytes exposed to DOX [22]. In the study, the antioxidant compound NAC compensated for the DOX-induced decrease of cardiomyocyte viability. ROS are reported to have other influences including the invasiveness of cancer cells [23-25]. However, little is known about the influence of DOX-induced ROS in OS. Presently, H_2O_2 as a source of ROS, mimicked the DOX-induced increase of cell invasiveness, which was inhibited by an antioxidant. These observations indicated that DOX-induced invasiveness of OS is mediated by ROS. In addition, our results revealed that such a phenomenon is mediated by MMP-9, which we previously suggested as the major proteolytic enzyme for OS invasiveness [9].

ROS has different effects depending on dose or length of exposure. While high levels of ROS can induce the apoptosis (or necrosis) of cancer cells, chronic exposure to lower concentrations may promote cancer cell survival and tumor growth [5]. Presently, H_2O_2 promoted the invasion of an OS cell line in some doses (10-20 μM) but not at higher doses. This suggests that the effect of ROS on invasiveness also depends on dose or exposure length, although this is another subject of further investigation. Notably, NAC

pre-treatment neutralized and further suppressed cell invasion when combined with high-dose DOX. As ROS is a mediator of DOX-induced cell death, antioxidants may negatively affect the anticancer effect of DOX. In this context, the use of antioxidants mostly was targeted to reduce DOX toxicity [26], and to our knowledge, no studies have addressed the anticancer effect of DOX-antioxidant combination and the mechanism of it. The present results suggest that the combination of DOX and antioxidants could be synergistic, at least for curbing the invasiveness of OS. This indicates the potential value of antioxidant supplementation for OS chemotherapy, although other biologic effects and mechanisms of DOX-antioxidant combination still require clarification.

In conclusion, low dose DOX increases the invasiveness of OSA through ROS activation and MMP-9 induction in vitro. Low dose DOX may also have other adverse effects. The ROS/MMP-9 pathway could be a novel target for future research and targeted therapies for OS, to intensify the therapeutic effects and minimize toxicity.

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국문 초록

서론: Doxorubicin(DOX, 또는 adriamycin)은 골육종에 대한 주요 항암치료약물 중 하나이다. 이는 종양의 미세전이를 억제하고자 사용되나, 그 심독성(cardiotoxicity)은 치명적일 수도 있다. DOX 의 최고 혈장 농도를 낮추어 이러한 심독성을 낮추려는 연구들이 있었으나, 결과는 논란의 여지가 있었다. 더욱이, 저용량 DOX 을 수반하는 이러한 시도들은 또 다른 부작용을 낳을 가능성도 있다. 본 연구의 목적은 저용량의 DOX 이 골육종의 침습성에 미치는 영향을 평가하고, 활성산소종/matrix metalloproteinase-9 (reactive oxygen species(ROS)/MMP-9) 경로가 이에 관여하는지 밝히는 것이었다.

방법: 실험에는 인간 골육종 세포주 U2OS 가 사용되었다. ROS 신호전달체계의 관련성을 평가하기 위하여는 과산화수소(H_2O_2) 와 항산화제 N-acetylcysteine(NAC)이 사용되었다. DOX, H_2O_2, 그리고 NAC 이 U2OS 의 증식에 미치는 영향을 MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) 실험을 이용하여 분석하였다. U2OS 의 침습성을 다양한 농도의 DOX 및 H_2O_2 조건에서 Matrigel^{TM} 침습 시험과 gelatin zymography 를 시행하여 분석하였고, 각각에서 NAC 전처치의 효과를 살펴보았다. 각 실험 조건에서 MMP-9 의

발현 정도를 정량적 실시간 중합효소연쇄반응(quantitative real time polymerase chain reaction, qPCR)을 이용하여 측정하였다.

결과: 24 시간까지의 증식능 분석에서 DOX 는 500 nM 까지, H_2O_2 는 50 μM 까지, 그리고 NAC 은 10 mM 까지 U2OS 의 증식능에 유의한 영향이 없었다. Matrigel^{TM} 침습 시험과 gelatin zymography 결과 DOX 은 임상에서보다 낮은 100 nM 부근의 농도에서 U2OS 의 침습성을 증가시켰다. qPCR 결과 침습성을 증가시킨 DOX 농도에서 MMP-9 의 발현 증가가 관찰되었다. 대표적인 ROS 발생원인 H_2O_2 또한 U2OS 의 침습성을 증가시켰다. NAC 전처치 시에는 DOX 나 H_2O_2 에 의한 침습성 증가나 MMP-9 발현 증가가 관찰되지 않았다.

결론: 저용량의 DOX 은 실험실 조건에서 ROS 활성화 및 MMP-9 유도를 통해 U2OS 의 침습성을 증가시켰다. 이는 저용량 DOX 이 종양학적 부작용이 있을 수 있음을 시사하며, 이는 향후 심도 있게 연구되어야 할 것이다. ROS/MMP-9 경로는 골육종에 대한 향후 연구와 표적치료의 새로운 대상이 될 수 있을 것으로 기대된다.

주요어 : 골육종, 침습성, doxorubicin, 활성산소종, MMP-9

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