

Sub-Lethal Hyperthermia Enhances Anticancer Activity of Doxorubicin in Chronically Hypoxic HepG2 Cells Through ROS-Dependent Mechanism

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Abstract

Background and aims:

Thermal ablation in combination with transarterial chemoembolization (TACE) has been reported to exert a more powerful anti-tumor effect than thermal ablation alone in hepatocellular carcinoma (HCC) patients. However, the underlying mechanisms remain unclear. The purpose of this study was to evaluate whether sub-lethal hyperthermia encountered in the peri-ablation zone during thermal ablation enhances the anticancer activity of doxorubicin in chronically hypoxic (encountered in the tumor area after TACE) liver cancer cells and to explore the underlying mechanisms.

Methods

HepG2 cells pre-cultured under chronic hypoxic conditions (1% oxygen) were treated in a 42 °C water bath for 15 min or 30 min, followed by incubation with doxorubicin. Assays were then performed to determine intracellular uptake of doxorubicin, cell viability, apoptosis, cell cycle, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), and total antioxidant capacity.

Results

The results confirmed that sub-lethal hyperthermia enhanced intracellular uptake of doxorubicin into hypoxic HepG2 cells. Hyperthermia combined with doxorubicin led to a greater inhibition of cell viability and increased apoptosis in hypoxic HepG2 cells compared to hyperthermia or doxorubicin alone. In addition, the combination induced apoptosis by increasing ROS and causing disruption of mitochondrial membrane potential. Pretreatment with the ROS scavenger N-acetyl cysteine (NAC) significantly inhibited the apoptotic response suggesting that cell death is ROS-dependent.

Conclusions

These findings suggest that sub-lethal hyperthermia enhanced the anti-cancer activity of doxorubicin in hypoxic HepG2 cells through ROS-dependent mechanism.

Background

Hepatocellular carcinoma is one of the most common malignancies worldwide [1, 2]. Thermal ablation (predominantly radiofrequency ablation (RFA) and microwave ablation (MWA)), has shown excellent therapeutic efficacy in hepatocellular carcinoma and is a commonly used therapy following surgery and embolotherapy (transarterial chemoembolization/transarterial embolization, TACE/TAE) [3–5].

Considerable studies have shown that RFA can achieve complete necrosis in most lesions smaller than 3 cm in diameter [6, 7]. However, for lesions more than 3 cm in diameter, especially for those over 5 cm,

complete necrosis cannot be obtained in a minority of patients, even if the procedure is repeated. Clinical studies have reported that residual viable tumor cells have been identified in up to 20% of clinical cases following ablation [8, 9].

Several studies have shown that TACE in combination with RFA increased the size of the ablation zone compared to RFA treatment alone, and efficacy has been demonstrated in the treatment of small and medium-sized HCC [10–13]. The underlying mechanisms behind the potential advantage of combined therapies remain unclear. One competitive theory is that hyperthermia during thermal ablation may increase sensitivity of tumor cells to cytotoxicity from chemotherapeutic agents, such as doxorubicin [14, 15]. However, the underlying mechanisms have not been sufficiently investigated and further studies are needed.

It is well known that during thermal ablation, the temperature inside the ablation zone decreases from center to margin where the temperature is sub-lethal and residual viable tumor cells often exist, potentially causing tumor relapse [16, 17]. In our previous study, we reported that chronic hypoxia, an important characteristic following TACE, can trigger chemoresistance to doxorubicin in HepG2 cells [18]. In the present study, we used in vitro methods to investigate whether sub-lethal hyperthermia, which is usually encountered in the peri-ablation zone during thermal ablation, promotes sensitivity of hypoxic HepG2 cells to cytotoxicity from doxorubicin.

Methods And Materials

Cell culture

Human liver cancer-derived HepG2 cells were grown in a monolayer in tissue culture flasks (Corning, NY, USA) in RPMI-1640 medium (Thermo Fisher, MA, USA) containing 10% fetal bovine serum (Thermo Fisher, MA, USA), 1% sodium pyruvate (Caisson Labs, UT, USA), 1% nonessential amino acids (Thermo Fisher, MA, USA), and 1 mM gentamicin (Thermo Fisher, MA, USA) at 37°C in a humidified incubator with 5% carbon dioxide. Cells were used in the logarithmic growth phase.

The chronic hypoxia condition was achieved as described previously. Briefly, cells were subjected repeatedly to hypoxia (1% O₂, 5% CO₂, 94% N₂) for 4 h daily for 7 consecutive days. The following treatments and assays were performed under normoxic conditions.

Heat treatments

Confluent cells in 6-well culture plates containing 2 mL of medium per well were heated for 15 min or 30 min at 42°C, relative to controls (37°C), in temperature-controlled precision water baths ($\pm 0.1^\circ\text{C}$) (Haake D8, Fisher Scientific, Montreal, QC). Cells were then cultured in fresh medium at 37°C in the presence (1 mM, working concentration) or absence of doxorubicin for various lengths of time until the following assays were performed.

Intracellular uptake of Doxorubicin

Cells were incubated in fresh culture medium in the presence or absence of doxorubicin (1 mM, working concentration) for 30 min at 37°C. After recovering in fresh medium without doxorubicin for 3 h, intracellular doxorubicin was measured by fluorescence intensity using flow cytometer (BD Biosciences, CA, USA) at 488 nm. Data were analyzed using FlowJo X software.

Cell viability assay

After culturing for 24 h, cell viability was assessed using the MTT assay (Sigma, USA) according to the manufacturer's protocol. In brief, 10 µl of MTT solution was added to 200 µL of medium per well in 96-well plates, followed by incubation for 4 hours. The medium was discarded and 150 µl of DMSO per well was added. Absorbance at 568 nm was measured using a Thermomax microplate reader (Thermo, USA).

Cell cycle analysis

After treatment, the cells were collected for detection using a cell cycle analysis kit according to the manufacturer's manual. In brief, cells were fixed in 70% ice-cold ethanol and stored at -20°C overnight. After washing with PBS, cells were incubated with 300 µl PBS, 125 µl RNase, and 25 µl PI (1 mg/ml) for 30 min at 37°C. A FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany) was used to record 10,000 ungated events for each sample with excitation at 535 nm. Analysis of the events was performed using Modfit 3.2 software.

Apoptosis

Apoptosis assay was performed using a FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's manual. In brief, cells were harvested and washed twice with cold PBS. The cells were then resuspended in 100 mL of 1 × Annexin V Binding Buffer, supplemented with 5 mL of Annexin V-FITC and 1 mL of propidium iodide, and incubated on ice in the dark for 15 min. Thereafter, 400 mL of 1 × Annexin V Binding Buffer was added and the stained cells were immediately analyzed by flow cytometer (BD Biosciences). Data were analyzed using FlowJo X software. Annexin V (+)/PI (-) cells were considered apoptotic and were analyzed as a percentage of the entire cell population.

Mitochondrial membrane potential (MMP)

MMP was detected using the TMRM mitochondrial membrane potential assay kit according to the manufacturer's manual. After treatment, cells were labeled with 10 nmol/l tetramethylrhodamine methyl ester (TMRM), in serum free MEM medium for 20 min at 37°C. Samples were analyzed by flow cytometer. Data were analyzed using FlowJo X software.

Measurement of reactive oxygen species (ROS)

Cellular ROS levels of hydrogen peroxide were detected using an ROS assay kit according the manufacturer's manual. Briefly, cells were incubated with DCFH-DA at a final concentration of 25 µM at 37°C for 30 min. To measure ROS generation, a FACSCalibur flow cytometer (BD Bioscience, Heidelberg,

Germany) was used to detect the fluorescence intensity of dichlorofluorescein (DCF) at 535 nm. For each analysis, 10,000 events were recorded.

Total antioxidant capacity assay

Cellular total antioxidant capacity (TAC) was quantified using a TAC assay kit (Biovision, Mountain View, CA), according to the manufacturer's instructions. The concentration of TAC was calculated from the standard curves and the value was expressed as nmol/ng protein.

Statistical analysis

All of the experiments were repeated independently three times. Means \pm SD of the three experiments are presented. ANOVA plus a multiple comparison test (Fisher's protected least significant difference test, PLSD) was performed to determine group differences. P-values < 0.05 were regarded as significant for all of the statistical analyses.

Results

Enhanced cytotoxicity of doxorubicin following sub-lethal hyperthermia

To evaluate the effects of sub-lethal hyperthermia alone on chronically hypoxic liver cancer cells, hypoxic HepG2 cells were cultured in a water bath at 42 °C for 15 min and 30 min and cell viability was measured using an MTT assay. The results demonstrated that cell viability significantly decreased in cells treated with sub-lethal hyperthermia at 42 °C compared to those at 37 °C. However, the difference in cell viability between cells exposed to hyperthermia for 15 min and 30 min was insignificant. For cells co-treated with hyperthermia and doxorubicin, cell viability was significantly lower than cells treated with hyperthermia or doxorubicin alone. (Fig. 1a, b)

Numerous studies have shown that hyperthermia enhanced intracellular uptake of doxorubicin in different types of tumor cells [19, 20]. In this study, we measured doxorubicin positive cells using flow cytometer. The results showed that sub-lethal hyperthermia significantly enhanced intracellular uptake of doxorubicin in HepG2 cells pre-cultured under chronically hypoxic conditions. However, there was no significant difference between doxorubicin positive cells exposed to hyperthermia for 15 min and 30 min (Fig. 1c).

Role Of Ros Generation And Redox Equilibrium

Give that ROS generation is an important early event associated with mitochondrial membrane injury and apoptosis, we then measured ROS levels in cells treated with hyperthermia and doxorubicin. After exposing chronically hypoxic HepG2 cells to hyperthermia for 15 min and 30 min, ROS levels increased

significantly compared to controls. However, the difference between cells exposed to hyperthermia for 15 min and 30 min was not statistically significant. Cells treated with a combination of hyperthermia and doxorubicin had much higher ROS levels than those treated with hyperthermia or doxorubicin alone (Fig. 4a, b). We then used a scavenger (N-acetyl cysteine) to block the function of ROS to further investigate its role in apoptosis. The results demonstrated that apoptosis decreased significantly (Fig. 4c), accompanied by an increase in mitochondrial membrane potential.

We also measured TAC levels in cells treated with hyperthermia. The results showed a slight increase in TAC level in cells exposed to hyperthermia for 15 min. However, a higher TAC level was measured in cells treated with hyperthermia for 30 min compared to cells treated for 15 min (Fig. 4d).

Discussion

Treatment of liver cancer patients with trans-arterial chemoembolization can lead to a tumor microenvironment characterized by chronic hypoxia. This may in turn trigger a more aggressive phenotype in the residual cancer cells [21–25]. We have previously reported that chronic hypoxia up-regulates gene expression of NRF2/ABCB1 and PARP-1 in HepG2 cells. The NRF2/ABCB1-mediated efflux effect may lead to decreased intracellular uptake of doxorubicin and result in chemo-resistance in hypoxic liver cancer cells. In addition, PARP-1 mediated dampening of DNA damage may also play an important role in doxorubicin resistance in chronically hypoxic HepG2 cells [18].

In this study, sub-lethal hyperthermia combined with doxorubicin exerted a synergistic anti-cancer effect *in vitro* in chronic hypoxic liver cancer cells. Our results also showed that doxorubicin positive cells increased significantly in cells pre-treated with heat at 42 °C. These results are consistent with results reported in the literature where hyperthermia ranging from 41–47 °C altered cell membrane permeability and in turn allowed uptake of chemotherapeutic agents [20, 26, 27]. However, there was no significant difference in the inhibition of cell viability between cells pre-treated with heat for 30 min and cells treated for 15 min, although the difference in doxorubicin positive cells between these two groups was statistically significant. These results indicated that the synergistic effect of co-treatment cannot be fully explained by hyperthermia-mediated intracellular uptake of doxorubicin.

Both hyperthermia and doxorubicin have been shown to cause cell cycle arrest in a broad range of cancer cells. Cell cycle analysis in this study showed that hyperthermia or doxorubicin alone or a combination of both had a slight, direct effect on cell cycle distribution in chronically hypoxic HepG2 cells. This finding is inconsistent with those reported by Fatfat et al. and Lim et al., in which either hyperthermia or doxorubicin dramatically affected cell cycle distribution *in vitro* in liver cancer cells [28, 29]. The inconsistency may be explained by the dose of hyperthermia or doxorubicin applied in this study being different from what was reported previously [30, 31]. In addition, the same dose of inducer may have a different effect on cells in various states.

Many studies have shown that the biological effect of hyperthermia is correlated with chemically reactive molecules containing oxygen, also known as reactive oxygen species [32, 33]. In the present study, results

showed that both hyperthermia and doxorubicin induced the generation of ROS, resulting in a decrease in mitochondrial membrane potential and apoptosis. These results suggest that inhibition of ROS reversed hyperthermia- and doxorubicin-induced apoptosis. However, exposure of cells to sub-lethal hyperthermia for a relatively long time (30 min) did not result in a higher ROS level. This suggests that generation of ROS following sub-lethal hyperthermia was not time-dependent. Interestingly, our results also showed that the 30 min exposure of cells to hyperthermia led to a higher level of TAC compared to cells exposed for 15 min. The high level of TAC may aid tumor cells in maintaining a high redox equilibrium for survival under heat stress, thus triggering thermo-tolerance in cancer cells [34, 35]. These results hint that a longer time period of thermal ablation may theoretically lead to increased necrosis in the lethal hyperthermia zone. Alternatively, it may also lead to thermo-tolerance in the sub-lethal hyperthermia zone through redox equilibrium. Thus, further studies are needed to investigate the potential dual effects of sub-lethal hyperthermia during thermal ablation.

The limitations of this study include that the hyperthermia condition used in this study may not fully mimic the actual status of the peri-ablation zone during thermal ablation, where a gradual decrease in temperature often occurs. In addition, our results showed that sub-lethal hyperthermia induced high levels of TAC, which may trigger thermal tolerance in hypoxic liver cancer cells. However, the underlying mechanism remains to be determined. Thus, further study is needed to investigate the effects of a wider range of sub-lethal thermal heating doses on hypoxic liver cancer cells, as well as the detailed underlying mechanisms.

Conclusions

In summary, this study showed that sub-lethal hyperthermia enhanced doxorubicin-induced cytotoxicity in chronically hypoxic HepG2 cells through increased intracellular uptake of doxorubicin, generation of ROS, and mitochondrial membrane injury. In addition, a relatively long exposure time to sub-lethal hyperthermia induced high levels of TAC, which may trigger thermo-tolerance in chronically hypoxic liver cancer cells. These findings suggest a new strategy for improving efficacy and overcoming potential thermo-tolerance in tumor cells in the peri-ablation zone during thermal ablation in HCC patients.

Abbreviations

ROS, reactive oxygen species; TACE, transarterial chemoembolization; HCC, hepatocellular carcinoma; MMP, mitochondrial membrane potential; NAC, N-acetyl cysteine; RFA, radiofrequency ablation; MWA, microwave ablation; TAE, transarterial embolization

Declarations

Ethics approval and consent to participate

This work was approved by the Ethical Board of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Consent of publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

QW, HZ, QR, and TY carried out the study. YL, SZ and GZ performed the statistical analysis. QW and HZ wrote the manuscript. XX designed this study. All authors read and approved the final manuscript.

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Figures

a

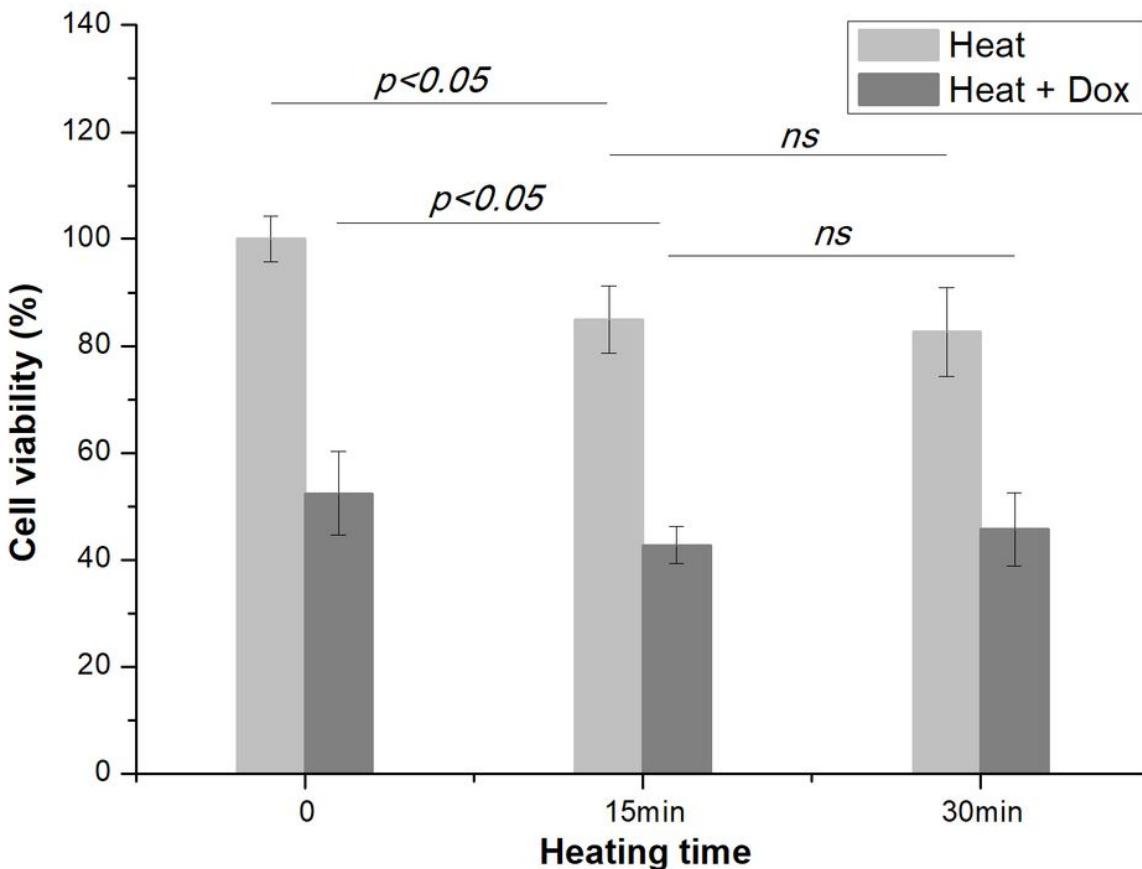


Figure 1

Cell viability and intracellular uptake of doxorubicin. (a) Co-treatment of sub-lethal hyperthermia and doxorubicin synergically inhibit cell viability in chronically hypoxic HepG2 cells.

b

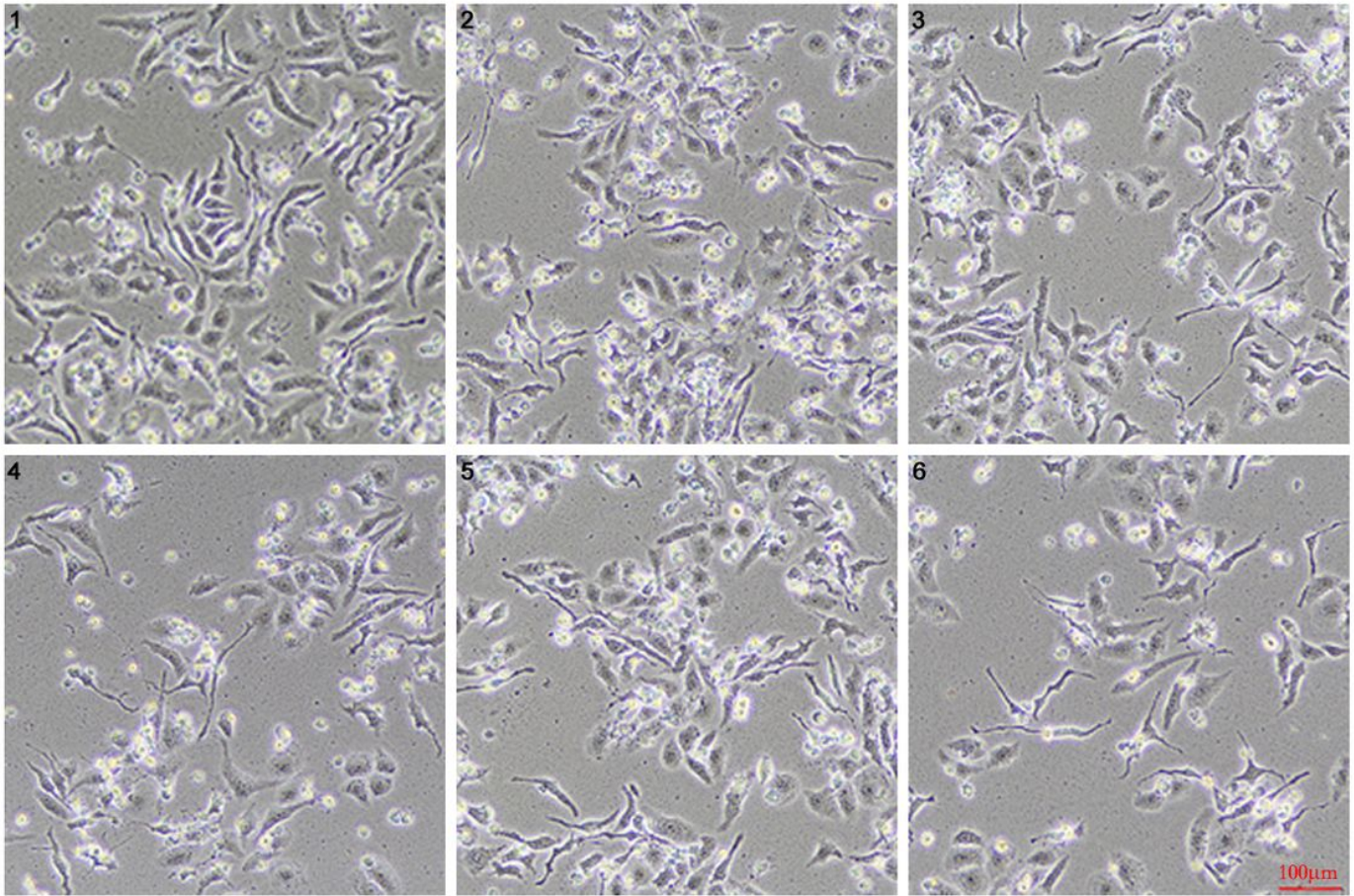


Figure 2

Cell viability and intracellular uptake of doxorubicin. (b) Cell growth of representative experiment in each group. 1, Heat (-), Dox (-); 2, Heat (+) 15min, Dox (-); 3, Heat (+) 30min, Dox (-); 4, Heat (-), Dox (+); 5, Heat (+) 15min, Dox (+); 6, Heat (+) 30min, Dox (+).

C

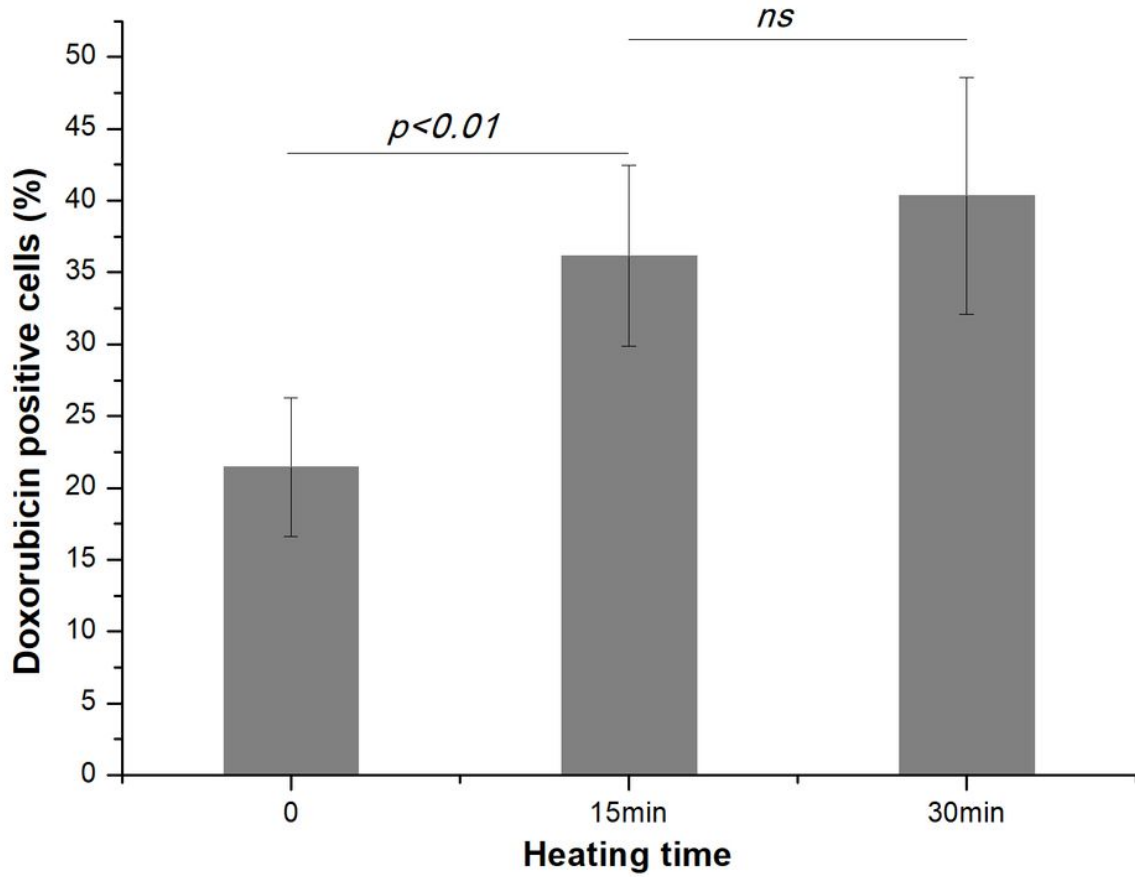


Figure 3

Cell viability and intracellular uptake of doxorubicin. (c) Percentage of doxorubicin positive cells increases significantly following sub-lethal hyperthermia treatment. ns, no significance (similarly hereinafter).

a

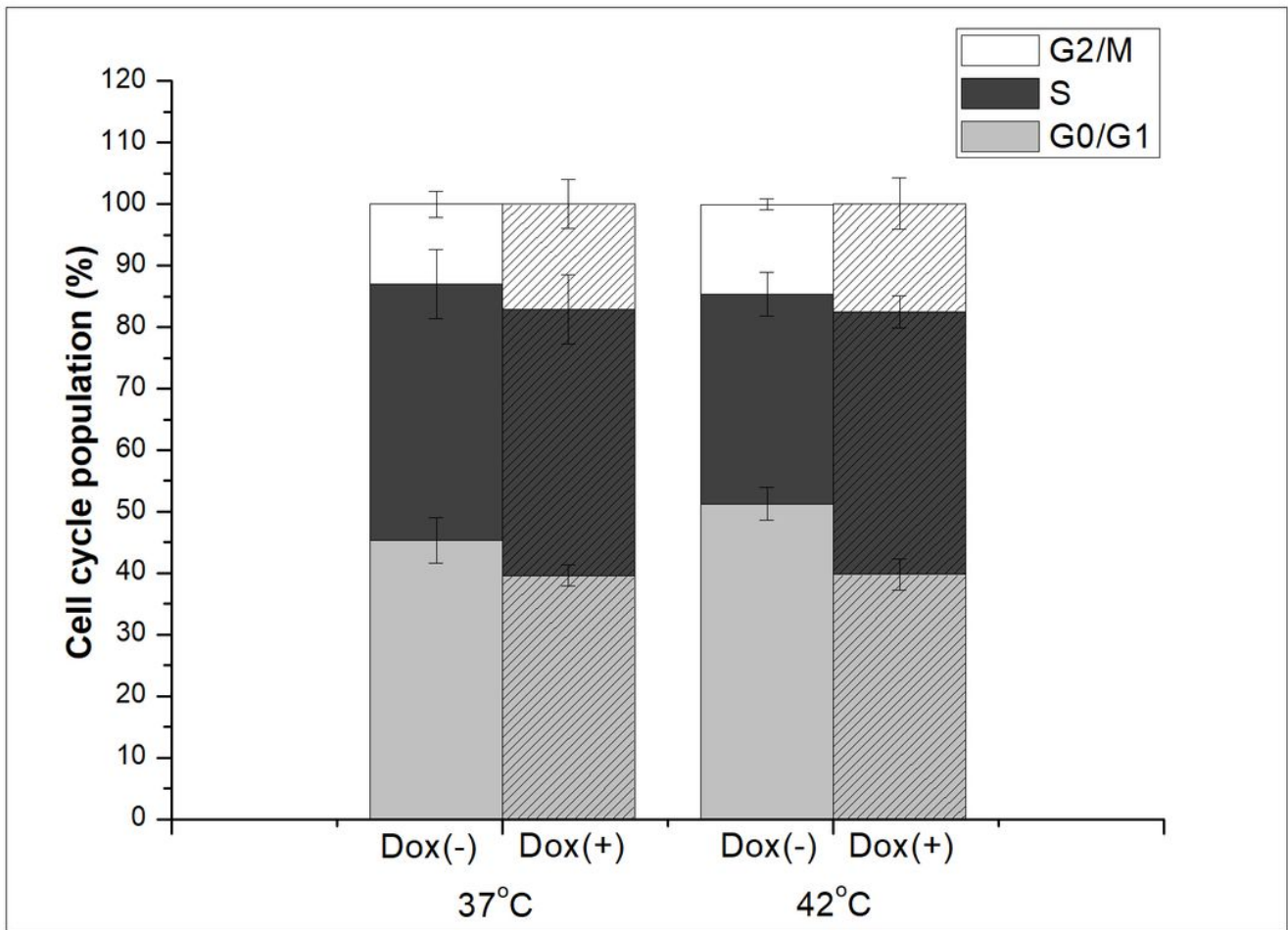


Figure 4

Sub-lethal hyperthermia or doxorubicin alone produces slight influence on cell cycle in chronic hypoxic HepG2 cells. (a) Treatment with sub-lethal hyperthermia for 15 min or doxorubicin alone produces slight influence on cell cycle, with G2/M population increases from $13 \pm 2.11\%$ (control) to $14.56 \pm 0.9\%$ (hyperthermia) and $17.07 \pm 3.94\%$ (doxorubicin), but the differences are insignificant. Hyperthermia in combination with doxorubicin slightly enhanced the G2/M cell cycle arrest to $17.63 \pm 4.23\%$ with statistical insignificance.

b

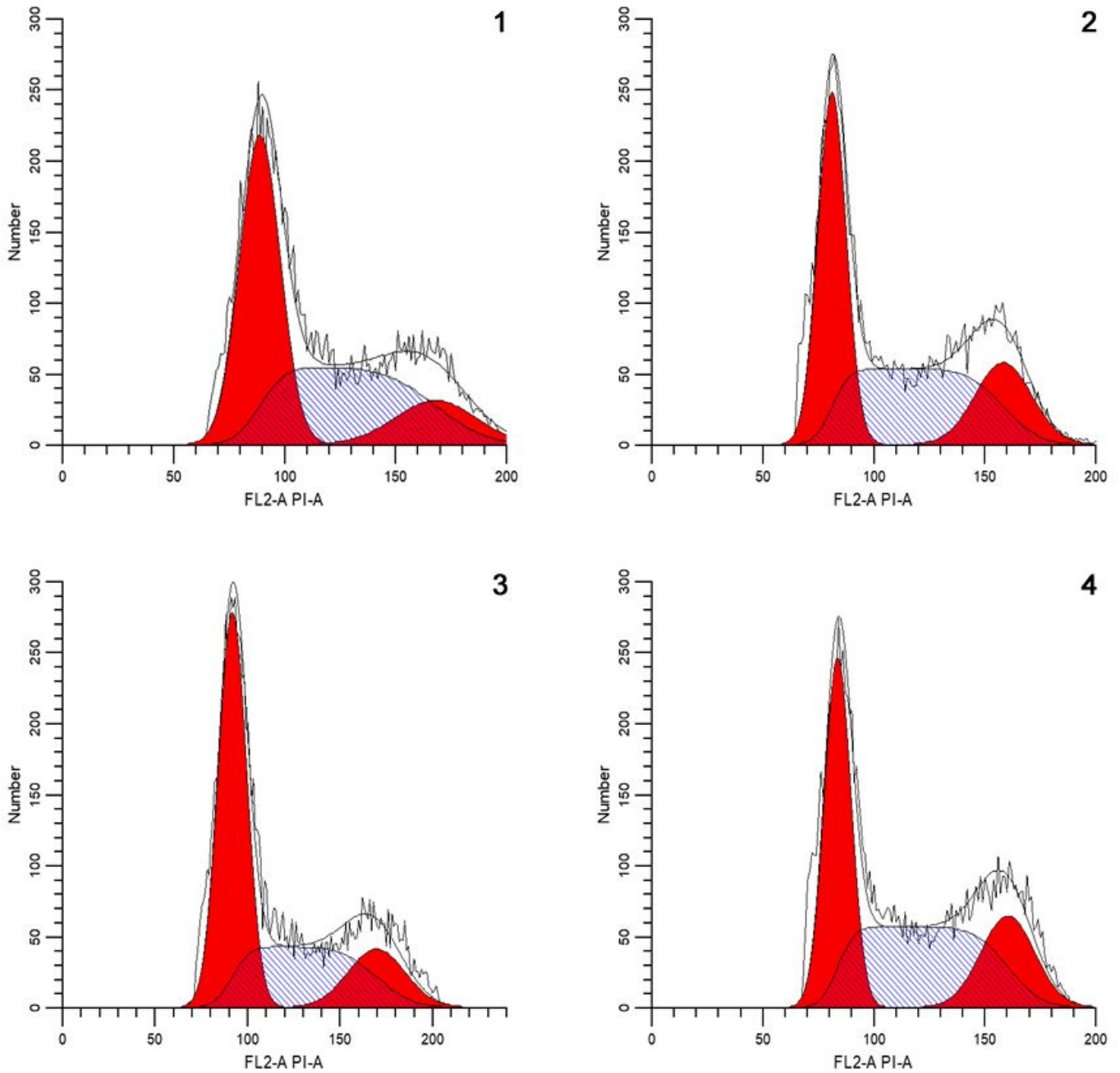


Figure 5

Sub-lethal hyperthermia or doxorubicin alone produces slight influence on cell cycle in chronic hypoxic HepG2 cells. (b) Cell cycle analysis by flow cytometer of representative experiment in each group. 1, Heat (-), Dox (-); 2, Heat (-), Dox (+); 3, Heat (+) 15min, Dox (-); 4, Heat (+) 15min, Dox (+).

a

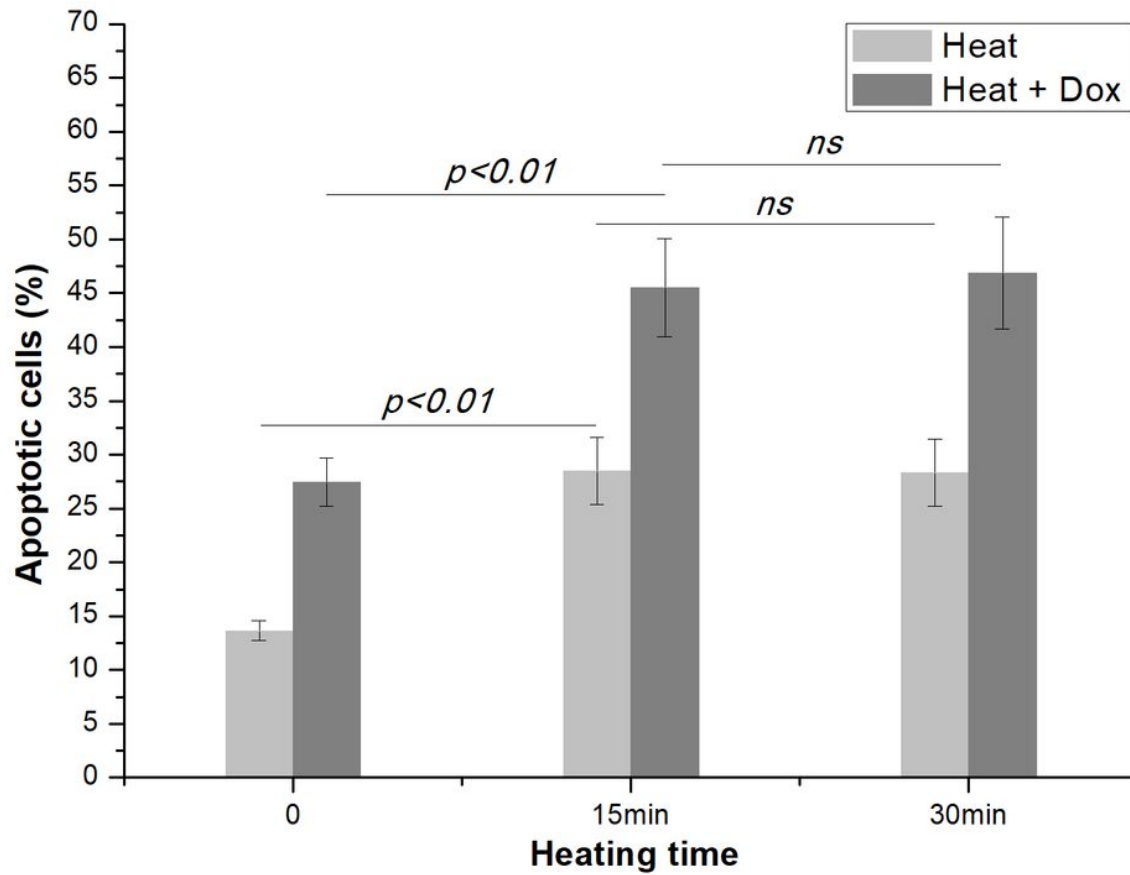


Figure 6

Co-treatment of sub-lethal hyperthermia and doxorubicin decreases MMP and increases cell apoptosis. (a) Heat and doxorubicin significantly increase the percentage of apoptotic cells.

b

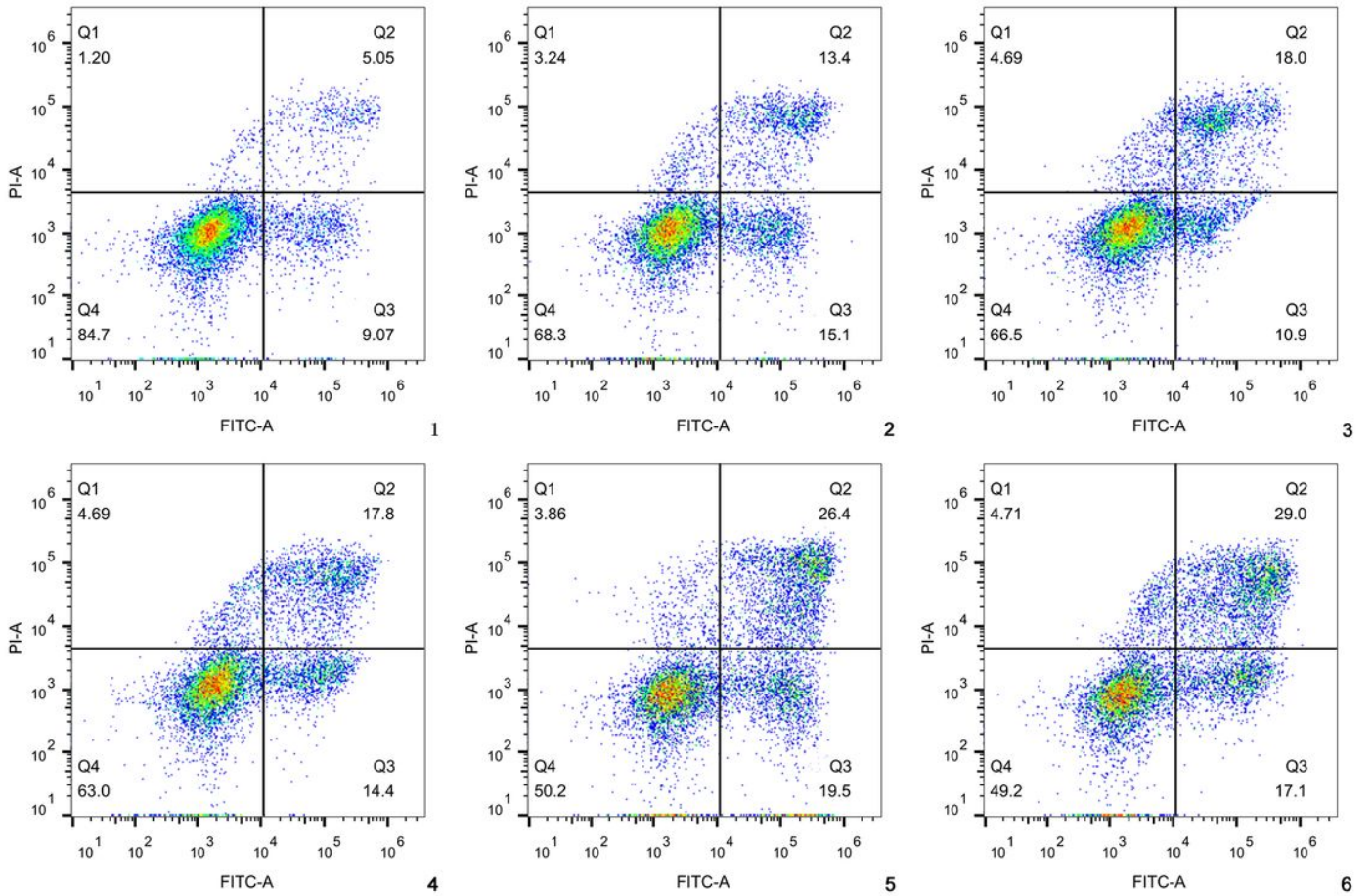


Figure 7

Co-treatment of sub-lethal hyperthermia and doxorubicin decreases MMP and increases cell apoptosis.
(b) Representative experiment of apoptosis analysis in each group;

c

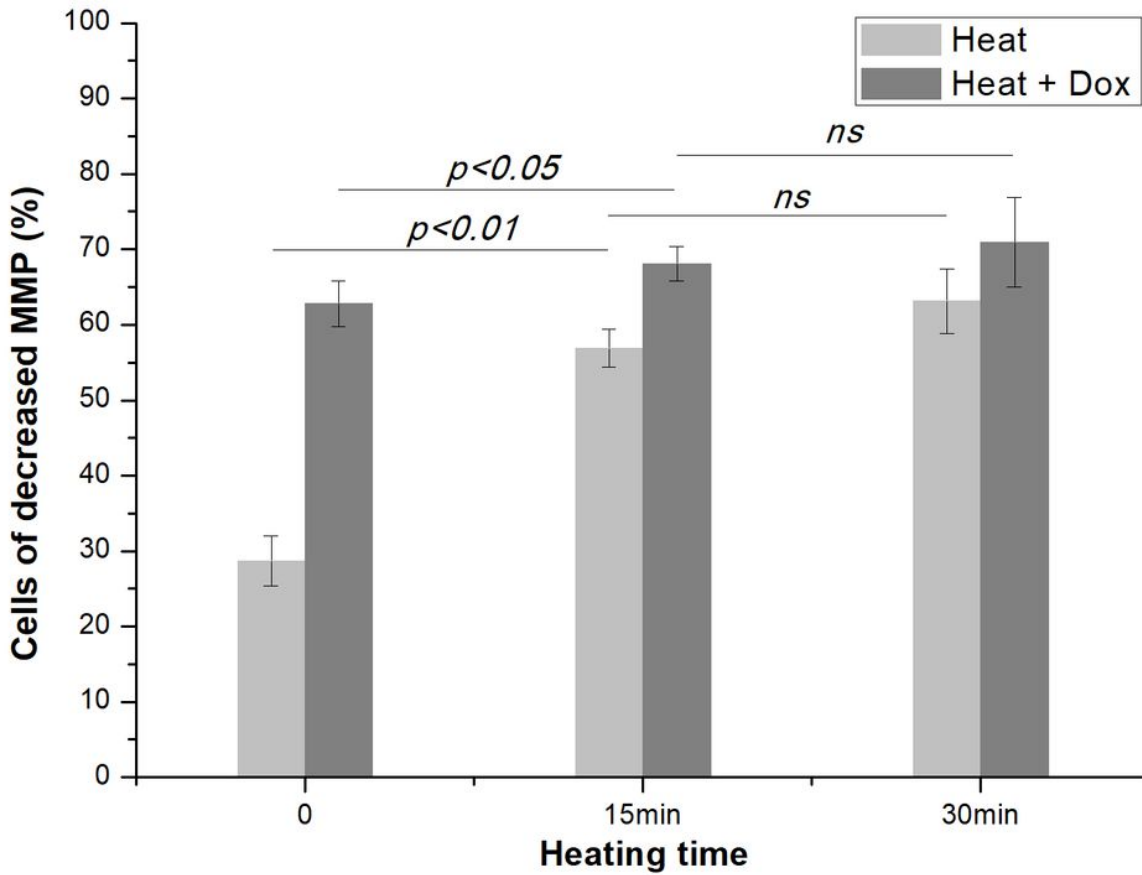


Figure 8

Co-treatment of sub-lethal hyperthermia and doxorubicin decreases MMP and increases cell apoptosis. (c) Heat and doxorubicin significantly increase the percentage of cells with decreased MMP.

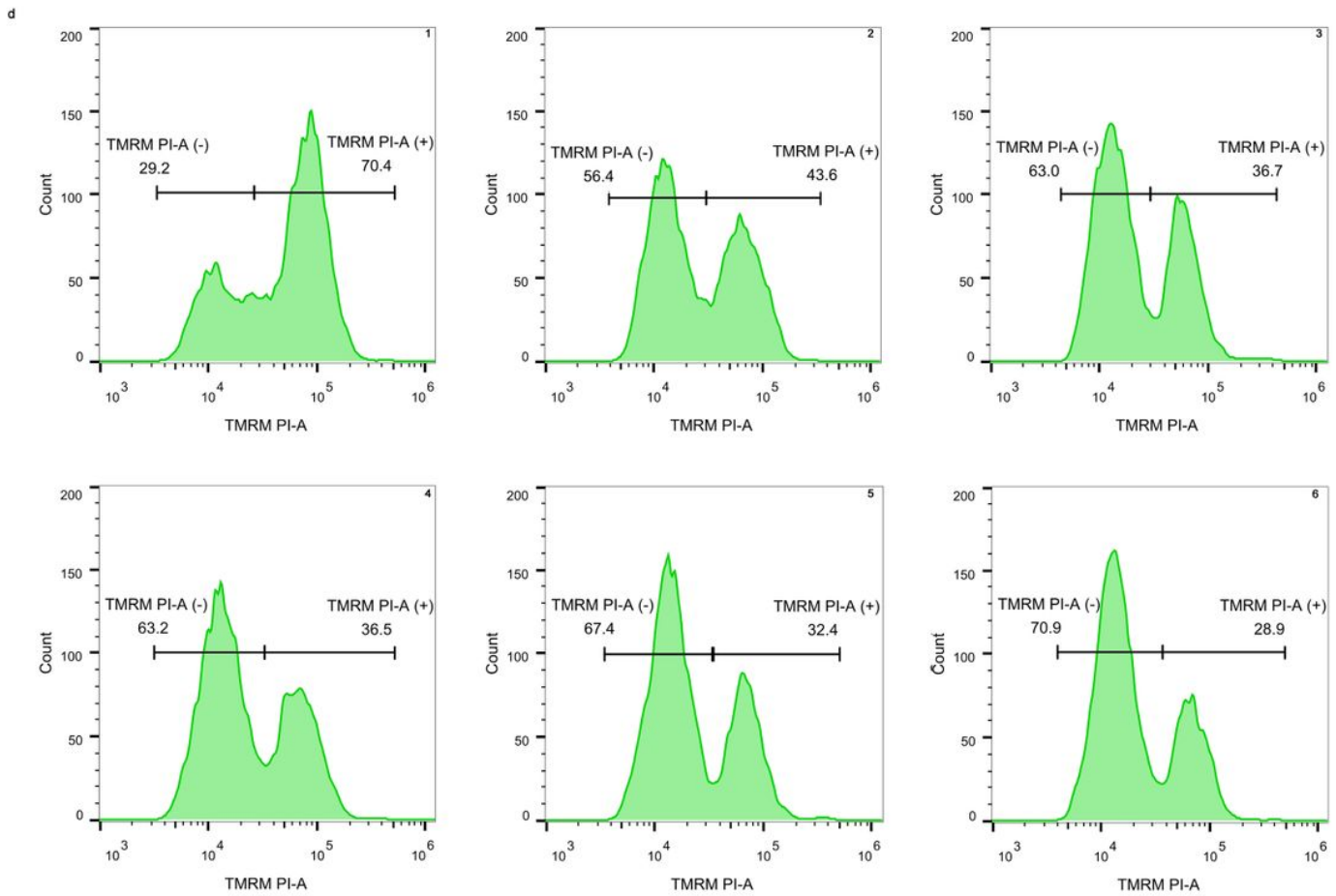


Figure 9

Co-treatment of sub-lethal hyperthermia and doxorubicin decreases MMP and increases cell apoptosis. (d) Representative experiments of MMP analysis. 1, Heat (-), Dox (-); 2, Heat (+) 15min, Dox (-); 3, Heat (+) 30min, Dox (-); 4, Heat (-), Dox (+); 5, Heat (+) 15min, Dox (+); 6, Heat (+) 30min, Dox (+)

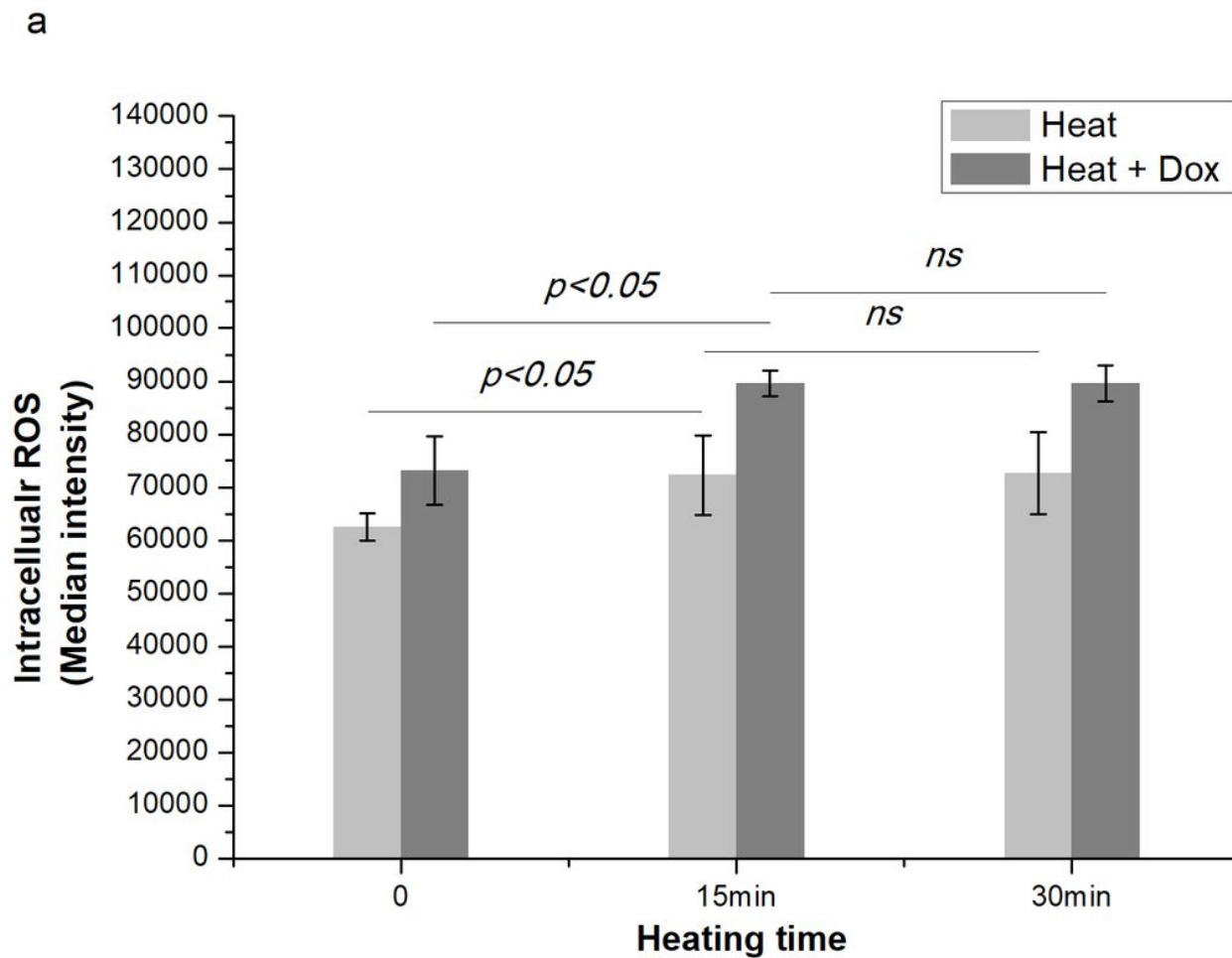


Figure 10

Redox equivalent were changed by heat and doxorubicin. (a) ROS increases significantly in cells treated with heat, doxorubicin or both.

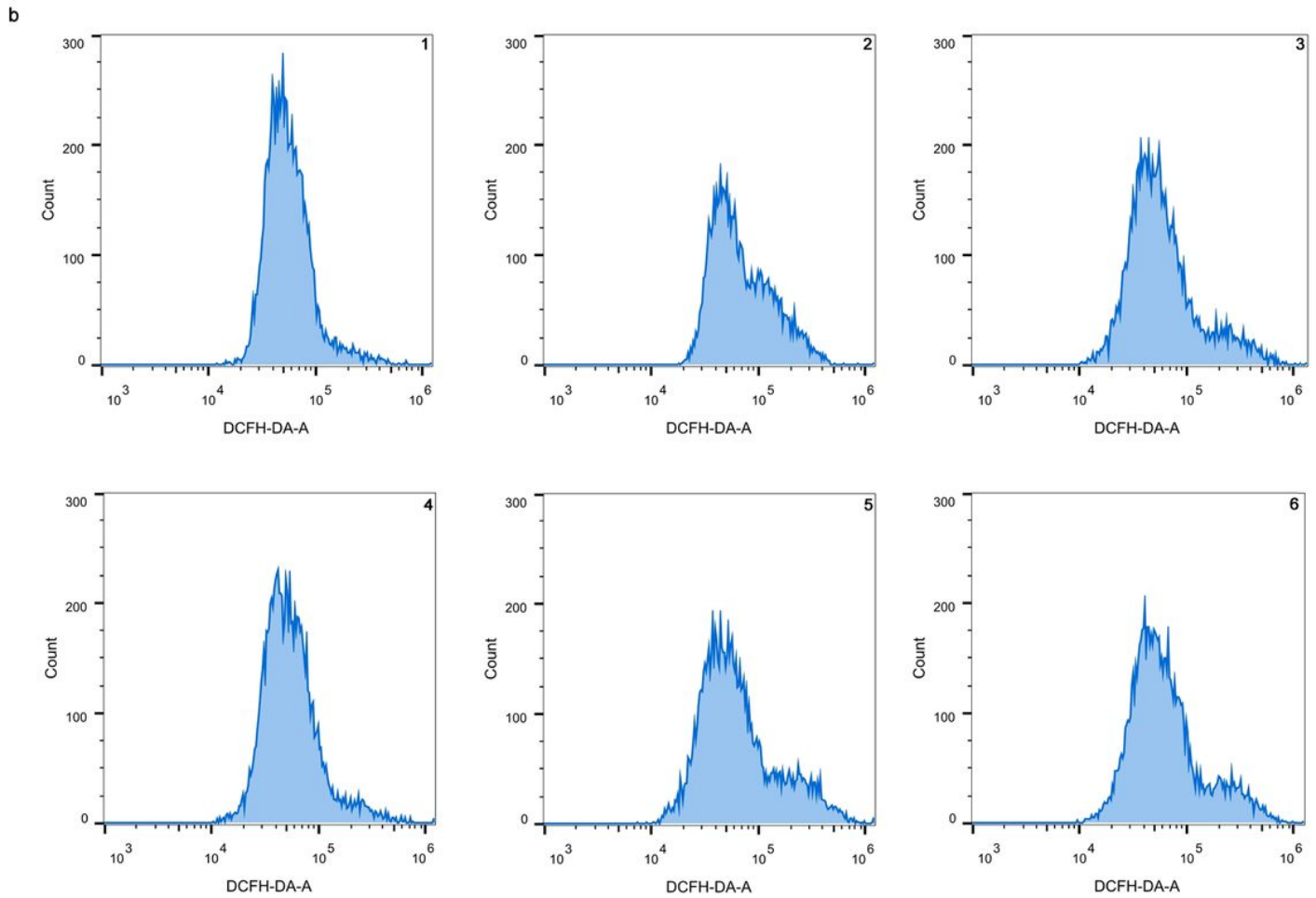


Figure 11

Redox equivalent were changed by heat and doxorubicin. (b) Representative experiment of ROS analysis by flow cytometer in each group; 1, Heat (-), Dox (-); 2, Heat (+) 15min, Dox (-); 3, Heat (+) 30min, Dox (-); 4, Heat (-), Dox (+); 5, Heat (+) 15min, Dox (+); 6, Heat (+) 30min, Dox (+).

C

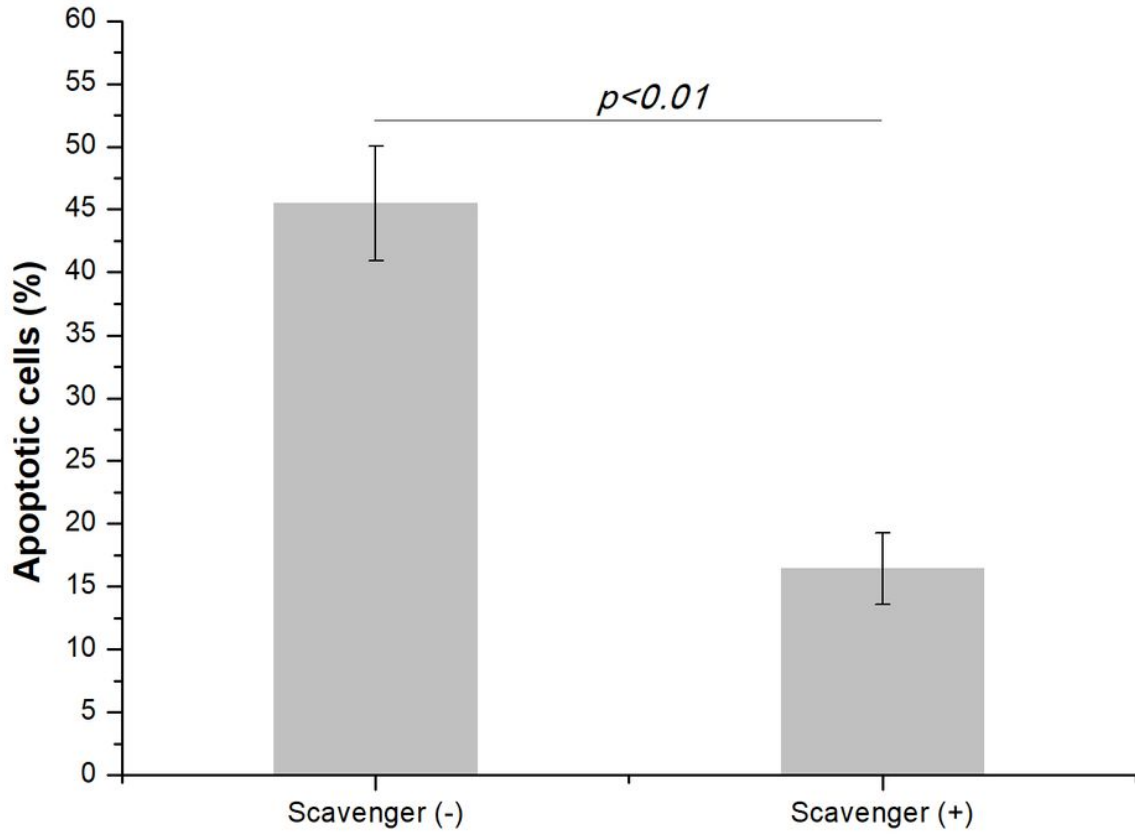


Figure 12

Redox equivalent were changed by heat and doxorubicin. (c) Cell apoptosis decreases after blocking ROS with N-acetyl cysteine.

d

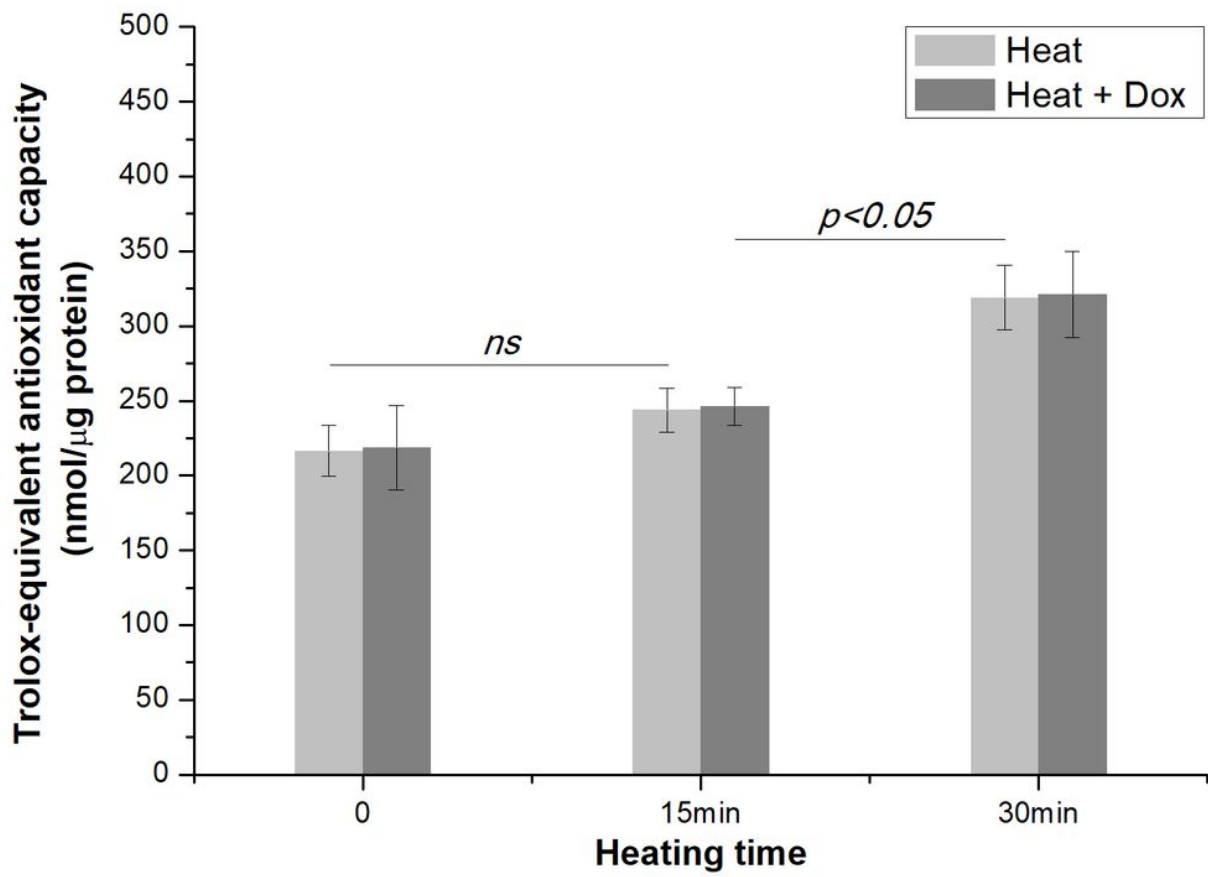


Figure 13

Redox equivalent were changed by heat and doxorubicin. (d) Treatment with hyperthermia for a longer time results in a higher TAC levels than a shorter time.