Hyaluronic acid optimizes therapeutic effects of Hydrogen peroxide-induced oxidative stress on breast cancer

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Abstract

Background and Purpose: Distinguishing the multiple effects of reactive oxygen species (ROS) on cancer cells is important to understand their role in tumour biology. Conversely, elevated levels of ROS-induced oxidative stress can induce cancer cell death. However, some anti-oxidative or ROS-mediated oxidative therapies have also yielded beneficial effects.

Experimental approach: To better define the effects of oxidative stress, *in vitro* experiments were conducted on 4T1 and splenic mononuclear cells (MNCs) under hypoxic and normoxic conditions. Furthermore, H_2O_2 [10-1000μM], was used as a ROS source alone or in combination with hyaluronic acid (HA), which is frequently used as drug delivery vehicle.

Key Results: Our results indicate that treatment of cancer cells with H_2O_2+HA was significantly more effective than H_2O_2 alone. In addition, treatment with H_2O_2+HA led to increased apoptosis, decreased proliferation, and multi-phase cell cycle arrest in 4T1 cells in a dose-dependent manner under normoxic or hypoxic conditions. Also, migratory tendency and the mRNA levels of VEGF, and MMP-2,9 were significantly decreased. Of note, HA treatment combined with 100-1000μM H_2O_2+ caused more damage to MNCs as compared to treatment with lower concentrations [10-50μM]. Based on these results we propose to administer high dose H_2O_2+HA [100-1000μM] for intra-tumoral injection and low doses for systemic administration.

Conclusions & Implications: Intra-tumoral route could have toxic and inhibitory effects not only on the tumour but also on residential myeloid cells defending it, whereas systemic treatment could stimulate peripheral immune responses against the tumour. More *in vivo* research is required to confirm this hypothesis.

1. Introduction

Reactive oxygen species (ROS) including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\bullet$), have long been known as by-products of normal cellular metabolism (1). The damaging effects of high levels of ROS on lipids, proteins, and DNA, ROS can be oncogenic, promoting genomic instability and tumorigenesis (2). Furthermore, ROS play multiple roles as signalling molecules to promote cancer cell proliferation, survival, angiogenesis, adaptation to hypoxia, and metastasis (3–5). The sources of ROS include cellular components that make up the tumour microenvironment and the tumour cell itself. The proliferative and metabolic capacities of tumour cells result in ROS production by mitochondria and to a lesser extent by membrane oxidases. ROS at high levels is also involved in suppression of anti-tumour immune response. Hypoxia-inducible transcription factors (HIFs) are stabilised by ROS, leading to promotion of angiogenesis by upregulation of vascular endothelial growth factor (VEGF) in the hypoxic tumor microenvironment (6). Contributing to treatment resistance, tumour cells adapt to the harsh conditions of persistent hypoxia and consequently become more invasive and metastatic (7). Normal cells cannot typically survive under persistent hypoxic condition, whereas tumour

cells adapt and thus remain viable under these conditions due to hypoxia-mediated proteomic and genomic changes in tumour cells (8). Interestingly, a selection pressure occurs when tumour cells are subjected to hypoxia. This selection endorses the survival of more malignant subpopulations of tumour cells expressing HIF-1α, MMP-2, MMP-9, VEGF, and PDL-1(9). Hypoxia also affects other prominent cellular events such as cell cycle, proliferation, apoptosis, survival, and migratory ability (7, 10–14).

It is noteworthy that the role of ROS in cancer is double-edged. Excessive levels of ROS lead to increased oxidative stress that can induce cancer cell death (15, 16). To overcome toxic effects of ROS, cancer cells evolve to maintain a redox balance by increasing their antioxidant potential (17). Scavenging excess ROS enables cancer cells to maintain ROS at a level favouring the activation of protumourigenic signalling pathways preventing cancer cell death. Indeed, comparing with normal cells, cancer cells have a high rate of ROS production, which is counterbalanced in an altered redox environment with a high rate of ROS scavenging properties. Such a unique characteristic makes cancer cells more sensitive than normal cells to fluctuations in ROS or redox levels according to which therapeutic strategies have been designed to eliminate or produce ROS (17). Higher ROS levels correlates with higher direct killing of tumour cells(18)., Antioxidant therapy trials in cancer patients have yielded inconsistent outcomes, but mostly indicated that it worsens cancer. Preclinical studies on murine cancer models indicated that the therapy can promote tumour growth and metastasis (6, 19, 20).

Hydrogen peroxide (H_2O_2) is a physiological ROS. Medical use of H_2O_2 has produced evidence for anti-neoplastic effects of (21–24). With the aim of tumour suppression, H_2O_2 has been administered systemically or locally via intravenous or intratumoural routes, respectively (25). Nonetheless, systemic or local administration alone has not succeeded to eliminate tumours, and the mechanism by which hydrogen peroxide therapy produces anti-tumour effects is still debated. Owing to the homeostatic role of immune cells in maintaining the integrity of every tissue, these cells tend to localize at the tumour loci (26). Subsequently, immune cells adapt to the hypoxic conditions found in the tumour microenvironment and can protect and support cancerous tissues and tumour cells (27, 28).

We hypothesized that the development of tumours can be hindered and even reverted if an approach consisting of concurrent systemic and local H_2O_2 is applied. In the present study, we aimed to eliminate tumour cells and their guarding immune cells locally by intratumoural H_2O_2 therapy and simultaneously eradicate the remnants of these cancerous cells by inducing the recruitment of H_2O_2-treated systemic immune cells. In the first step, therefore, we evaluated the effect of hydrogen peroxide therapy on tumour and splenic mononuclear cells (MNCs) under normoxia, representative of systemic condition, and hypoxia, representative of the condition seen in tumour foci. In our experiments, we also examined hyaluronic acid (HA), which is commonly used as a drug delivery vehicle (29), to facilitate the production of cytotoxic ROS from hydrogen peroxide (30). Therefore, treatment using H_2O_2, as an ROS source, alone or in combination with HA was performed to further shed light on their impacts on the 4T1 breast carcinoma cell line and MNCs *in vitro*.

2. Materials & Methods

2.1 Chemicals

Fetal bovine serum and RPMI1640 medium were purchased from GIBCO/Life Technologies Inc. 3- [4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), neutral red (NR), annexin V/PI, Hyaluronic acid 8-15000 mol wt. (HA), acridine orange (AO), Propidium Iodide (PI), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA) and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) were obtained from Sigma-Aldrich. TRIzol was procured from AMERCO, US. The anti PDL-1 antibody was purchased from Abcam ab224027, UK.

2.2 Cell Culture

The mouse breast cancer cell line 4T1 was obtained from the Pasture Institute, Cell Bank of Iran (NCBI, Tehran, Iran). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% glutamine (Thermo Fisher Scientific), 100 IU/ml streptomycin and 100 IU/ml penicillin at 37 °C in a 5% CO_2 atmosphere.

2.3 Isolation of mononuclear cells (MNCs) from spleen

Balb/c mice were sacrificed and their spleens were collected under sterile condition, then transferred to a 40 mm petri dish containing the perfusion medium (5 ml of RPMI medium supplemented with 10% FBS). The harvested cell suspension was centrifuged at 400 g for 8 minutes at 4–8 °C, the supernatant was discarded, and RBC lysis buffer was added. Cells were washed three times with RPMI, and thereafter 1 ml of culture medium was added to the pelleted cells. Finally, cells were counted and applied for tests.

2.4 Induction of hypoxic or normoxic condition and treatments with H_2O_2 and H_2O_2 + HA

Normoxic (18% O_2, 5% CO_2) or hypoxic (1% O_2, 5% CO_2 and 94% N_2) condition in two different incubators (Labotec C200, Germany) in 95% humidity were established. For all tests, 4T1 cancer cells and MNCs were treated by increasing concentrations of H_2O_2 (0, 10, 20, 50, 100, 200, 500 and 1000 µM) or H_2O_2 + HA (%0.83 of 8-15000 mol wt) (0, 10, 20, 50, 100, 200, 500, 1000 µM and HA%0.83) according to normoxic or hypoxic condition and incubated for 24 hrs.

2.5 Characteristic hypoxic model by anti PDL-1

To characterize the hypoxic condition, 4T1 cells were seeded in 6-well plates (1×10^6 cells/well) in regular growth medium and incubated in normoxic (18% O2, 5% CO2) and hypoxic (1% O2, 5% CO2 and 94% N2) conditions in two distinct incubators (Labotec C200, Germany) with 95% humidity overnight. Then, cells were collected and washed with phosphate buffered saline (PBS), suspended with 100 µl PBS. Next, 5 µl Anti PDL-1 mouse antibody conjugated with FITC was added to cells and incubated for 30 minutes at room temperate. Lastly, we added 400 µl flow cytometry staining buffer and performed flow cytometery (BD Biosciences, San Diego, CA, USA). The data was analysed using Flowjo version 7.6.1.

2.6 Measurement the intracellular ROS

In order to measure the net intracellular intensities of generated Reactive oxygen species (ROS), DCFH-DA which is a non-fluorescence dye was used, in which it combines with intracellular ROS for generating fluorescent 2,7- dichlorofluorescein (DCF). Briefly, 4T1 Cells were cultured in a 6 well plate at a concentration of 1×10^6 cells/well and MNCs (1×10^6 cells/well) in 24-well plate and incubated for 24 hrs under normoxic or hypoxic condition and treated with H_2O_2 or H_2O_2 + HA for 24 hrs. The supernatant was discarded and the cells were washed twice with PBS. The cells were incubated with 10 µM DCFH-DA for 45 min. After incubation, the plates washed twice with PBS, and finally suspended in 500 µl of PBS. Subsequently, the amount of ROS estimated by DCF production and measured by flow cytometer (BD Biosciences, San Diego, CA, USA). The data analysed by using Flowjo version7.6.1.

2.7 Measurement the intracellular malondialdehyde

MDA was measured as thiobarbituric acid reactive substance (TBARS). Briefly, tumour cells were seeded in 1×10^6 per well in a 6-well plate and MNCs (1×10^6 cells/well) in 24-well plate incubation under normoxic or hypoxic condition overnight, and were treated with H_2O_2 or H_2O_2 + HA for 24 hrs. After the incubation time, the cells were collected and lysed using freeze-and-thaw and ultra-sonication. 500 µL of cell lysate was mixed with 500 µL of 20% (w/v) cold TCA to precipitate the proteins. 500 µL 0.80% (w/v) thiobarbituric acid (TBA) was added to the supernatant and heated at 95 °C for 1 hr. Also, standard curve was generated using Tetra-epoxypropane. The pink colour product (MDA–TBA complex) was determined by absorbance at 532 nm spectrophotometrically.

2.8 Evaluation cell viability/proliferation by MTT assay

MTT assay was performed to determine the viability of breast cancer cell line and normal cells. 4T1 and MNCs were seeded in 96-well plates (5×10^4 cells/well) and (1×10^6 cells/well) containing 200 µl regular growth medium under normoxic or hypoxic condition overnight, respectively. After 24hrs of incubation, in order to achieve our goal, similar procedure of Kukia et al (31) was applied. Briefly, 20 µl MTT solution (5 mg/ ml) was added to each well and incubated for 4 hrs in 5% CO_2 atmosphere at 37° C. The supernatant in all wells were removed and 100 µl of DMSO was added instead. The absorption was measured at 490 nm by microplate Reader. Data was calculated based on viability difference between control and treated cells by the following formula: Cell viability was expressed as 100% for untreated cells (control). All samples were performed in triplicates and the survival rate (%) was calculated as the following equation.

Survival rate (%) = (OD in treatment group/OD in control group) × 100

2.9 Evaluation of lysosomal vitality by Neutral Red uptake

In order to test the survival and vitality, we used Neutral Red Uptake Assay by using the ability of viable cells to incorporate and bind to NR. Neutral red uptake was measured according to Abbasi et al(32).Briefly, 4T1 and MNCs cells were seeded in 96-well plates (5×10^4 cells/well) and (1×10^6

cells/well) containing 200 µl regular growth medium under normoxic or hypoxic condition overnight, respectively. Cells were treated under normoxic or hypoxic condition and incubated at 37 °C in 5% CO_2 for 24hrs. After that, 20 µl of NR solution (3.3 mg/ ml) was added to each well and incubated for 4 hrs in 5% CO_2 atmosphere at 37^0 C. After 24hrs of incubation, the upper medium was removed and the internalized NR was solubilized with PBS by mixing 200 µl of 10% acetic acid plus 40% ethanol solution under 30-mins of incubation. The optical density was measured at 630 nm. All samples were performed in triplicates and the survival rate (%) was calculated.

Survival rate (%) = (OD in treatment group/OD in control group) × 100

2.10 Evaluation of cell apoptosis by acridine orange and propidium iodide

Apoptosis was also detected by using acridine orange (AO) and propidium iodide (PI). Cells were cultured in a 6 well plates at a concentration of 1×10^6 cells/well and MNCs (1×10^6 cells/well) in 24-well plates for 24 hrs under normoxic or hypoxic condition and treated with H_2O_2 or H_2O_2 + HA for 24hrs. The supernatant was discarded and cells were stained with 10 µl of Acridine Orange (AO, 10 µg/ml) for 15 min at room temperature in dark condition and immediately 10 µl PI (PI, 10 µg/ml)) was added and tested by fluorescence microscopy. Apoptotic modifications in cells were determined with fluorescent microscopy and the percentage of cells exhibiting apoptosis was counted and calculated.

2.11 Evaluation of cell apoptosis using annexin V/PI

4T1 Cells were seeded in six-well plates at a density of 1×10^6 per well and MNC (1×10^6 cells/well) in 24-well plate cultured overnight under normoxic or hypoxic condition for overnight incubation. Then, the cells were treated with different concentration of H_2O_2 or H_2O_2 + HA and incubated for 24 hrs again. After termination of incubation time, the cells were harvested, washed with PBS, suspended in 500 µl binding buffer, added 5 µl annexin V-FITC plus 5 µl Propidium iodide (PI), and incubated for 15 minutes at room temperature in the dark room. Cell apoptosis was measured by flow cytometer (BD Biosciences, San Diego, CA, USA) and data analysed using Flowjo version7.6.1.

2.12 Cell cycle analysis by flow cytometry

For analysing the cell cycle, DNA staining by Propidium iodide (PI) was used and measured by flow cytometry. Cells were seeded in six-well plates at a density of 1×10^6 per well and MNCs (1×10^6 cells/well) in 24-well plates and cultured overnight under normoxic or hypoxic condition and treated with H_2O_2 or H_2O_2 + HA for 24 hrs. Cells were trypsinized, harvested, and centrifuged at 300 g for 5 minute at 4 °C. Finally, the pellet was washed with PBS, fixed in ice-cold ethanol (70%) and stored at 4 °C for at least 15hrs. Before the staining cells being washed with PBS, they were centrifuged at 300 g for 5 minute in 4 °C and stained with 500 µl of staining solution containing 0.5 µg/ml RNase A and 50 µg/ml PI and

incubated in the dark at 37 °C for 30 minutes, and stored at 4 °C until used. DNA content analysis was performed by flow cytometry at an excitation and emission wavelength of 488 nm and 610 nm respectively. Flowjo 7.6.1 soft-ware was used to analyse the data.

2.13 Cell cycle analysis by DAPI staining

DNA staining for cell cycle characteristics was evaluated by DAPI stain. Intact nuclei within the cells were stained by the DAPI method. Briefly, after treatment with different concentration of $H_2O_2$and H_2O_2 + HA, cells were washed once with PBS, fixed in acetone 70% for 30 min at 4 ^{0}C, then stained by 1 µg/ml DAPI for 15 minutes. Results were expressed with complementary nuclear morphological observations gathered using fluorescence microscope. Eventually, analysed by Image J software (version 1.50i, National Institute of Health, Bethesda, MD, USA), with cell cycle tool macro.

2.14 Measurement of cell migration gene expression

Briefly, Cells were cultured in a 6 well plate at a concentration of 1×10^6 cells/well plate for 24hrs under normoxic or hypoxic condition and treated with H_2O_2 or H_2O_2 + HA for 24hrs. Total RNA was extracted from 4T1 and control cells and treated with TRIzol, according to the manufacturer's instructions of Thermo Fischer kit, USA). Concentration and purity of RNA were quantified spectrophotometrically by measuring; the ratio A260/A280, which was approximately 1.9. Complementary DNAs were synthesized with cDNA synthesis Kit (Thermo Fischer, USA) according to the manufacturer's instructions. The expression levels of MMP-2, MMP-9, VEGF and HIF-1α were determined with step one plus Real time PCR system (Applied Bio-systems). Real time PCR was set up with the set of primers (Table 1S) was used in all reactions to yield the amplification of an endogenous control gene (HPRT) and the specific target gene of interest. Following amplification, fold expression analysis was performed.

2.15 Measurement cell migration using wound healing assays

The wound-healing assay is a standard method for investigating cell migration. Briefly, the 4T1 cells (2×10^6 cells/well) were plated in 6-well plates using RPMI 1640 with 10% FBS at 37 °C for 24 hrs under normoxic or hypoxic condition up to a confluency of about 90%, then wounded by scratching with a p1000 pipette tip, followed by three times of washing in serum-free medium, after that, cells were treated under normoxic or hypoxic condition once again and incubated in regular medium for 24hrs. Afterward, they were treated with H_2O_2 or H_2O_2 + HA for 24hrs.Finally, the 4T1 cell migration was assessed by mono-layer gap closure migration assay, embedded by ImageJ software (version 1.50i, National Institute of Health, Bethesda, MD, USA), with a wound healing tool macro (MRI_Wound _Healing Tool).

2.16 Statistical analysis

Statistical significance was performed by one-way and two-way ANOVA analysis of variance using Graph Pad Prism 7.0 for Windows (Graph Pad Software, Inc., San Diego, CA, USA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ were considered as significance level for all analyses performed. Data were presented as mean ± SD in triplicates experiments.

3. Results

3.1 Evaluating hypoxia induced PDL-1 expression

To evaluate the effect of hypoxic or normoxic conditions on 4T1 cells, we used PD-L1 as a marker of malignancy and resistance to immunotherapy. PD-L1 helps tumours escape from adaptive immunity (33). Flow cytometry analysis of 4T1 cells under normoxic or hypoxic condition revealed that PD-L1 surface expression under hypoxic condition was significantly increased by almost four times compared to 4T1 cells under normoxic condition as demonstrated in Fig. 1.

3.2. ROS responses in tumour cells under hypoxic conditions

In this study we evaluated the intracellular responses of cells treated with H_2O_2 or H_2O_2 + HA

3.2.1 Measurement of ROS level in 4T1 cell treated with H_2O_2 or H_2O_2 + HA

We evaluated ROS production provoked following treatment with H_2O_2 or H_2O_2 + HA under normoxic or hypoxic condition in 4T1 and MN cells using a flow cytometry assay. This was performed because of the crucial role ROS plays in cell proliferation (34) and tumorigenesis (35). We first estimated the effect of H_2O_2 or H_2O_2 + HA on 4T1 cells on ROS generation. Figure S1a&b and Fig. 2(A,B,E&F) demonstrate that treatment with either of H_2O_2 or H_2O_2 + HA led to a significant increase in ROS level under normoxic or hypoxic condition. However, the trend of increase under normoxic condition was milder in the cells treated by H_2O_2 + HA rather than H_2O_2. Noteworthy, once the concentration of 1000 μM H_2O_2 alone or H_2O_2 + HA were used, ROS level were more than 24- and 20-fold, respectively. Under hypoxic condition, when 1000 μM H_2O_2 alone or H_2O_2 + HA were applied, ROS level increased more than 7- and 6-fold, respectively. These results demonstrate that either of H_2O_2 or H_2O_2 + HA could stimulate ROS production in a dose-dependent manner. MN cells were also evaluated for ROS levels induced by either H_2O_2 or H_2O_2 + HA treatment. Under normoxic conditions, the ROS level was significantly decreased at10μM H_2O_2 but remained unchanged at 20 and 50 μM. Treatment with H_2O_2 concentrations > 100 μM provoked more significant ROS production. However, treatment with 10–50 μM H_2O_2 + HA resulted in decreased ROS

levels relative to H_2O_2 alone and remained unchanged at concentrations of H_2O_2 more than 100 μM. Treatment of MN cells under hypoxic conditions with 10–50 mM H_2O_2 did not affect ROS levels and gradually increased after treatment with 100–1000 μM H_2O_2. MN cells treated with H_2O_2 + HA under hypoxic condition showed a significant decrease in ROS level at 10 μM H_2O_2 + HA, remained unchanged at 20–200 μM H_2O_2 + HA and significantly increased in 500 and 1000 mM H_2O_2 + HA compared to control. The results suggested 10–200 μM H_2O_2 + HA as a suitable range which does not affect ROS levels.

3.2.2 Malondialdehyde (MDA) levels in 4T1 cell treated with H_2O_2 and H_2O_2 + HA

Malondialdehyde (MDA) is an end product of polyunsaturated fatty acid (PUFA) peroxidation in cells. Free radicals cause overproduction of MDA, which is a marker of oxidative stress and the antioxidant status in cancer patients (36). This test was additionally performed as a complementary test for ROS generation. Similar to ROS results, treatment with either of H_2O_2 or H_2O_2 + HA led to a significant increase in MDA level under normoxic or hypoxic condition in a dose-dependent manner (Fig. 2C,D,G&H). MN cells were also evaluated for MDA production by either H_2O_2 or H_2O_2 + HA treatment. Treatment with either of H_2O_2 or H_2O_2 + HA led to increased MDA levels under normoxic or hypoxic conditions. Nevertheless, MDA production in MN cells was not as much as in cancer cells even at the higher concentrations of H_2O_2 or H_2O_2 + HA treatment.

3.3 Biological changes in tumour cell treated with H_2O_2 or H_2O_2 + HA

To assess 4T1 cell line and MNC cell responses to normoxic or hypoxic conditions after treatment with doses 10-1000 μM H_2O_2 alone or along with HA, we examined proliferation, viability, vitality and apoptosis.

3.3.1 Measurement of cell proliferative capacity by assessing metabolic activity

MTT assay is a colorimetric assay for assessing cell metabolic activity. Our data presented in Fig. 3(A,B,E&F) shows the viability of 4T1 and MNC cells treated with 10-1000 μM H_2O_2 alone or with HA under normoxic or hypoxic condition and morphological change in 4T1 and MNc cells after treatment (Figure S2a& b). While 10–50 μM H_2O_2 under normoxic conditions did not affect cells viability, the viable cell number at 1001000 μM H_2O_2 was significantly decreased in a concentration-dependent manner comparing to the control. However, MTT assay indicated that 4T1 and MNC cells viability decreased at any concentration of H_2O_2 under normoxic condition comparing with the untreated control group. On the other hand, 10100 μM H_2O_2 + HA under normoxic condition did not affect 4T1 and MNC cells viability

compared to the control. Furthermore, concentrations between 200–1000 μM H_2O_2 + HA under normoxic condition significantly decreased 4T1 and MNC cell viability in comparison with the untreated control cells.

3.3.2 Measurement of cell vitality using lysosomal activity

Neutral Red (NR) is a weak cationic dye that penetrates into the cellular membranes and accumulates in lysosomes. Microscopic view of 4T1 stained with NR is shown in Figure S3. The data indicates vitality of 4T1 cells and MNCs treated with 10-1000 μM H_2O_2 alone or along with HA under normoxic or hypoxic condition. While 10–50 μM H_2O_2 under normoxic condition did not affect 4T1 cell vitality, the viable cell number at 1001000 μM H_2O_2 was significantly decreased in a concentration-dependent manner comparing to the control. However, 4T1 cell vitality decreased at any concentration of H_2O_2 under normoxic condition compared to the untreated control group. On the other hand, 10100μMH_2O_2 + HA under normoxic condition did not affect 4T1cell vitality compared to the control as measured by NR assay. Furthermore, concentrations between 200–1000 μM H_2O_2 + HA under normoxic condition significantly decreased 4T1 cell vitality in comparison with the untreated control 4T1 cells. However, as Fig. 3(C,D,G&H) shows, NR assay indicated that under normoxic condition 4T1 cells vitality significantly decreased at any concentration of H_2O_2 alone or along with HA compared to control, though in a biphasic manner. Indeed, based on NR assay the rate of 4T1 cells vitality after treatment with concentrations of H_2O_2 + HA decreased compared to viable control cells.

To compare treatment-mediated damage to normal cells, the same experiments were performed on MNC cells as normal cells. Figure 3 show cell viability and vitality based on MTT and NR assay, on MN cells treated with 10-1000 μM H_2O_2 alone or along with HA under normoxic condition. While 10–50 μM H_2O_2 under normoxic condition did not affect MN cell viability, the viable cell number at 1001000 μM H_2O_2 significantly decreased comparing to the control group as measured by NR assay. However, MTT assay indicated that MN cell viability decreased at any concentration of H_2O_2 under normoxic condition compared to the control. On the other hand, treatment with 1050 μM H2O2 + HA under normoxic condition did not significantly decrease MN cell viability compared to the control as measured by MTT assay. Similarly, and based on NR assay, concentrations between 10–100 μM H_2O_2 + HA under normoxic condition did not decrease MN cell vitality in comparison with the control untreated MN cells. Alternatively, treatment of MN cells with either of H_2O_2 or H_2O_2 + HA under hypoxic condition significantly deceased MN cell viability compared to the control as estimated by MTT assay. The results were confirmed by NR assay in a dose-dependent manner. However, treatment of MN cells with 10 μM H_2O_2 + HA conserved cell vitality as evaluated with NR assay.

3.3.3 Measurement the cell Apoptosis using Acridine orange

We also evaluated cytotoxic effect of H_2O_2 on 4T1 and MN cells using acridine orange, a dsDNA intercalating dye Live nucleated cells fluoresce green and apoptotic cells fluoresce red. The rate of apoptosis was evaluated under normoxic or hypoxic condition after the cells were exposed to H_2O_2 or H_2O_2 + HA for 24 hrs. The concentration of H_2O_2 alone or with HA to which the cells were exposed was in the range of 10-1000 μM, as shown in Fig. 4(A,B,E&F). Microscopic view of 4T1 and MN cells stained with Acridine and PI is shown in Figure. s4a&b. We found out that under normoxic condition, H_2O_2 had a strong cytotoxic effect on 4T1 cells compared to the untreated control cells. Under hypoxic condition, exposure of 4T1 cells to either of H_2O_2 or H_2O_2 + HA led to a significant increase in the rate of apoptosis. Noteworthy, hypoxia itself had increasing effect on the apoptosis rate as apoptosis in control cells under hypoxic condition was markedly more than that of control cells under normoxic condition. Accordingly, the rate of apoptosis in each concentration of H_2O_2 or H_2O_2 + HA under hypoxic condition was more than that of the corresponding concentration under normoxic condition. In general, an increasing trend in the apoptosis rate of 4T1 cells was observed under normoxic or hypoxic condition when exposed to either of H_2O_2 or H_2O_2 + HA. We also evaluated apoptosis rate of MN cells at the same condition that 4T1 cells were exposed. Under normoxic condition, the apoptosis rate unchanged at 10 and 20 μM H_2O_2 and significantly increased at 50-1000 μM compared to the control group. Inversely, H_2O_2 + HA decreased the rate of apoptosis in MN cells at concentration range of 10–500 μM and had no effect at 1000 μM compared with the untreated control group. Notably, HA conferred resistance to apoptosis in MN cells, as exposure of MN cells to concentrations of 10–500 μM H_2O_2 + HA under normoxic condition led to a significant decrease in the rate of apoptosis. Under hypoxic condition, MN cells were resistant to apoptosis in a broader range of 10-1000 μM H_2O_2 compared to the untreated control group. The apoptosis rate in MN cells treated with 10–100 μM H_2O_2 + HA was significantly lower than that of control cells and showed no difference with the control in concentration range of 200–1000 μM H_2O_2 + HA. H_2O_2 under hypoxic condition had less apoptotic effect than that of normoxic condition. This phenomenon was inversed for H_2O_2 + HA.

3.3.4 Measurement of cell Apoptosis using AnnexinV-FITC/PI

We also used Annexin V-FITC/PI to evaluate the quantity of apoptosis rate after treatment with H_2O_2 or H_2O_2 + HA. Our data indicates that under normoxic condition, 4T1 treated with 10-1000 μM H_2O_2 showed a significant increase in apoptosis rate. However, under hypoxic condition, 4T1 cells treated with H_2O_2 + HA showed a significant decrease in apoptosis rate compared to 4T1 cells treated with H_2O_2. MN cells treated with H_2O_2 + HA showed a significant decrease in apoptosis rate compared to MN cells treated with H_2O_2 under normoxic or hypoxic condition (Fig. 4 (C,D,G&H) and Figure S5 a&b).

3.4 Estimation of malignancy in 4T1 cells treated with H_2O_2 or H_2O_2 + HA

Assessment of malignancy after treatment of 4T1 cell line with H_2O_2or H_2O_2 + HA was based on five indices including HIF-1α, MMP-2, MMP-9, and VEGF-A.

3.4.1 Measurement of 4T1 cell response to hypoxia represented by HIF-1α level

Hypoxia activates HIF-1α transcription factor to help the cell adapt hypoxic condition (37). We estimated HIF-1α expression in 4T1 cells following exposure to H_2O_2 or H_2O_2 + HA under normoxic or hypoxic condition. Under normoxia, treatment of 4T1 cells with 20–100 μM H_2O_2 significantly decreased the HIF-1α level in a dose-dependent manner while treatment with 10 μM H_2O_2 did not change the HIF-1α level. Alternatively, treatment of 4T1 cells with 50–100 μM H_2O_2 + HA significantly decreased the HIF-1α level in a dose-dependent manner while treatment with 10 or 20 μM H_2O_2 + HA did not change the HIF-1α level under normoxic condition. Under hypoxic condition, treatment of 4T1 cells with H_2O_2 or H_2O_2 + HA significantly decreased the HIF-1α level compared to control cells Fig. 5(A&C).

3.4.2 Assessment of 4T1 angiogenic activity

We assessed VEGF level in 4T1 cells after treatment with H_2O_2 or H_2O_2 + HA. VEGF, which is a marker of angiogenic potential (38), was significantly decreased following treatment with either of H_2O_2 alone or H_2O_2 + HA under normoxic or hypoxic condition (Fig. 5(B&D)).

3.4.3 Evaluation of tumour metastatic potential

MMP-2 and 9 are regarded as markers of metastatic potential in malignancies (39). The level of these two markers were evaluated in 4T1 cells following treatment with H_2O_2 or H_2O_2 + HA as oxygen source. As Fig. 6(A,B,D&E) demonstrates, both of MMP-2 and − 9 were significantly decreased following treatment with either of H_2O_2 alone or H_2O_2 + HA under normoxic or hypoxic condition.

3.3.4 Evaluation of 4T1 cell line migratory tendency after treatment with H_2O_2 or H_2O_2 + HA

Considering the importance of tumour cell migration and metastasis, we further investigated migratory potential of 4T1 cell line after treatment with H_2O_2 or H_2O_2 + HA under normoxic or hypoxic condition. We observed that treatment with 10-1000 μM H_2O_2or 50-1000 μM H_2O_2 + HA under normoxic condition led to a marked decrease in cell migration potency. As shown in Figure S6 and Fig. 6 (C&F), treatment with 10 and 20 μM H_2O_2 + HA did not affect cell migration potency and was effective at concentration 50-1000 μM H_2O_2 + HA in a dose-dependent manner under normoxic condition. Treatment with H_2O_2 or H_2O_2 + HA under hypoxic condition was able to significantly decrease cell migratory ability at any concentration applied in the range of 10-1000 mM H_2O_2 or H_2O_2 + HA compared to the control in a dose-dependent manner. Treatment with 1000 μM H_2O_2 under normoxic or hypoxic condition led to a sudden

decrease by less than 2.5-fold in migration rate, while treatment with H_2O_2 + HA did not have such an effect.

3.4.5 Evaluation of cell cycle status in 4T1 and MN cells treated with H_2O_2 or H_2O_2 + HA

Flow cytometry was utilized to analyse 4T1 cell cycle after treatment with various concentration of H_2O_2 or H_2O_2 + HA under normoxic or hypoxic condition. As shown in Figure S6a&b and Fig. 7, treatment of 4T1 cells with either H_2O_2 or H_2O_2 + HA under normoxic or hypoxic conditions significantly decreased G1 phase and increased G2 phase comparing with the corresponding control group. The results were confirmed by the DAPI method as illustrated in Fig. 7 and Figure S7a,b&c. The results suggest that proliferation inhibition could be attributed to 4T1 cell cycle arrest at G1 and G2 phase under normoxic or hypoxic condition.

We also examined the effect of H_2O_2 or H_2O_2 + HA treatment on MN cells under normoxic or hypoxic condition. The results indicated that treatment with either H_2O_2 or H_2O_2 + HA under normoxic condition significantly increased S phase, which was not mainly affected in 4T1 cells, comparing with the corresponding control. G1 phase was unaffected at concentration 10 and 20 μM H_2O_2 and changed in a biphasic manner at concentration range 50-1000 μM H_2O_2 and 10-1000 μM H_2O_2 + HA. G2 phase was not affected in the range of 10–50 μM of H_2O_2 and increased in the range of 100–1000 μM H_2O_2. Treatment with H_2O_2 + HA significantly decreased G2 phase at concentration range of 10–500 μM and remained unchanged at 1000 μM compared to the control. Under hypoxic condition, treatment with either of H_2O_2 or H_2O_2 + HA significantly decreased M phase compared to the control. G2 phase was significantly decreased only at concentration range of 10–100 μM H_2O_2 and remained unchanged at concentration range of 200–1000 μM H_2O_2 compared to the control. Furthermore, treatment with H_2O_2 + HA made a significant decrease in G2 phase compared to the control. The results suggest that MN cells are mainly at G1 and S phase under normoxic and G1 under hypoxic condition.

4. Discussion

Medical application of H_2O_2 has been suggested before but it is still debatable whether hydrogen peroxide therapy can combat hypoxia by increasing the cellular levels of oxygen by decomposing into its resultant products (water and oxygen) or inducing oxidative stress by producing OH radical mainly depend on the microenvironment (40, 41).

Given the tumour as a systemic disease with local manifestations (42), we thus propose that an approach consisting of simultaneous systemic and local H_2O_2 therapy could provide more efficient results in eradicating tumour cells by recasting the sound microenvironment of solid tumours (43, 44). Here, we chose to use H_2O_2alone or H_2O_2 + HA and initially attempted to assess the *in vitro* effects of H_2O_2 therapy on both 4T1 tumour and splenic mononuclear (MN) cells as normal cells. In our

experiments, tumour cells under normoxia represent circulating tumour cells with the ability to metastasise to other healthy tissues and normal cells under hypoxia are equivalent to tumour-resident immune cells defending the tumour. Moreover, the application of hyaluronic acid in the experiments was based upon several grounds including: 1) hyaluronic acid is naturally found in the form of hyaluronan in the extracellular matrix (45), 2) by affecting H_2O_2 decomposition, it promotes oxygen release from H_2O_2, which can significantly dwindle the side effects of H_2O_2, 3) it holds clinical significance and its medical application extends to various conditions (46). For instance, hyaluronic acid, in conjunction with H_2O_2, is illustrated to boost tumour radio sensitivity, which is strongly oxygen-dependent, and simultaneously lessen H_2O_2 side effects (45).

To acquire an initial estimation, tumour cells were treated with H_2O_2alone or H_2O_2 + HA under normoxic or hypoxic condition. Meanwhile, normal MN cells were exposed to the same treatments and conditions as tumour cells to evaluate the potential side effects of these agents on normal cells under normoxic or hypoxic condition. Our results demonstrated that when H_2O_2 was used in conjunction with hyaluronic acid, it could exert robust and increased antitumour effects, which was in accordance with the previous reports on the beneficial role of hyaluronic acid as a drug delivery agent (47). Indeed, H_2O_2 therapy using H_2O_2 + HA encompasses two vital aspects including the immune system and the tumour itself.

With regard to cancer-immune cells (the immune system), our results endorse the use of H_2O_2 + HA at high concentrations [100–1000μM] for intratumoural injections as high doses of H_2O_2 + HA have toxic and inhibitory effects not only on the tumour but also on its residential immune cells defending it. On the other hand, low doses of H_2O_2 + HA [10–50μM] is recommended for systemic administration since our results have shown that these concentrations are far more toxic for tumour cells than normal cells. Based on the results obtained from this study and one previous report, dual administration of H_2O_2 + HA (i.e. systemic and local) could be considered as a suitable candidate for depleting local immune cells in favour of the tumour and further replacing them with newly-oxygenated systemic immune cells (21). In fact, the substitution of local immune cells guarding the tumour could be of benefit as it could alter the tumour microenvironment and thus provide a golden opportunity for the eradication of tumour cells. Tumour resident cells mostly include regulatory T cells (48), M2 macrophages(49), N2 neutrophils (50), myeloid-derived suppressor cell (MDSCs) (51) and other immunosuppressive cells (52). Accordingly, H_2O_2 therapy in the form of systemic H_2O_2 therapy could effectively bend the local immune responses towards a stimulatory and inflammatory phenotype against the tumour (52).

With regard to tumour cells, various cellular features were investigated including cell cycle arrest, oxidative stress, and gene expression. Our data demonstrated that H_2O_2 therapy with H_2O_2 + HA could potently induce multi-phase cell cycle arrest. Presumably, H_2O_2 + HA affects the cell cycle through targeting signalling pathways related to cell death (53). Furthermore, the observed cell cycle arrest in our experiments was consistent with previous reports performed on mouse fibroblasts and NIH3T3 cell line (54). Another crucial element in the survival of solid tumours is the regulation of oxidative stress (17, 55). Indeed, it is observed that high levels of ROS may impose a severe obstacle on the development of the

tumour (56). Similarly, we provided scientific evidence that H_2O_2 + HA could considerably hinder the development of solid tumours by intensifying ROS levels in 4T1 tumour cells in a dose-dependent manner. Also, treating 4T1 cells with H_2O_2 + HA significantly decreased hypoxia along with parameters indicative of angiogenesis, malignancy, and metastasis.

5. Conclusion

These data suggest that hypoxic conditions within the tumour microenvironment lead to two major consequences: 1) deviation of the immune system towards immune responses favouring the development of the tumour and thus opposing anti-tumour effects and 2) adaptation of tumour cells to hypoxia, which ultimately transforms these cells into a more resistant and invasive form. These showed by the invivo underway experiments The immune response in the tumour microenvironment might be reprogrammed by combining systemic and local H_2O_2 therapy, which Our advantage that the side effect is lowered and the speed of response was high, these supported by our invivo studies underway. Therefore, H_2O_2 + HA merits further research as a therapeutic strategy.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author statement

Ardeshir Abbasi: Writing- Original draft preparation, Software, Validation, Investigation, Formal analysis, Software

Nafiseh Pakravan: Methodology, Investigation, Writing- Reviewing and Editing, Visualization

Zuhair Mohammad Hassan: investigation, Visualization, Writing- Reviewing and Editing, Resources, Visualization

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Ethics Approval and consent to participate

All of experimental procedures were established and performed in accordance to the guidelines Ethics Committee of our University (code Number: 708/TDT/10).

Consent for Publication

Not applicable

Availability of Data and Materials

The authors agree to share the results of this study

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Figures

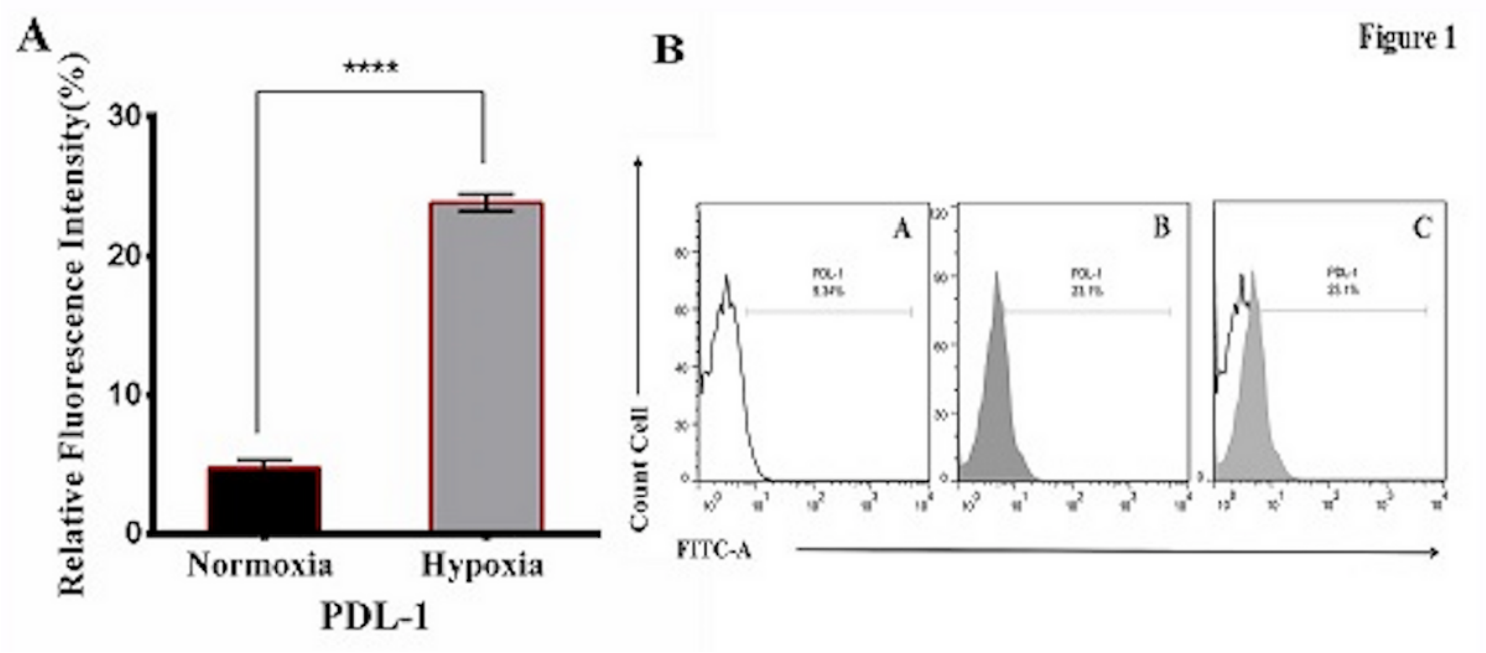

Figure 1

Characterization of hypoxia model; investigation the rate of surface expression PDL-1 on 4T1 cancer cells under normoxic or hypoxic condition after 24hrs of incubation (a). The amount of difference rate of Surface Expression PDL-1 on 4T1 cancer cells under normoxic or hypoxic condition after 24hrs of incubation (b). A: Normoxia, B: Hypoxia, and C: merge of A & B.

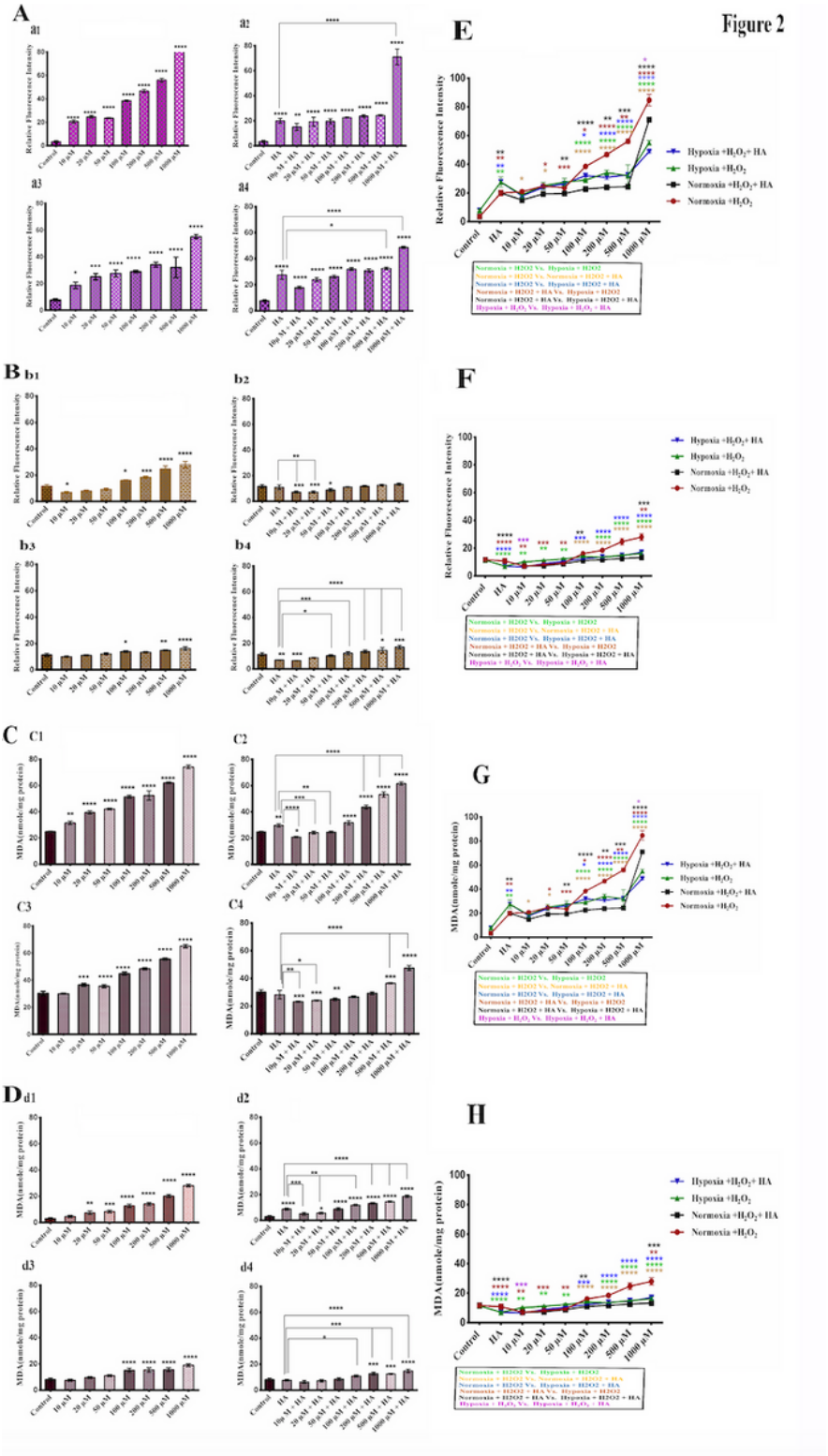

Figure 2

H2O2 or H2O2 + HA -induced modulation of oxidative damage (A B,E,F) and intracellular determined with malondialdehyde (MDA) production(C,D,G,H) in different levels of 4T1 cancer cells (A&C) and MNCs (B&D) treated with different concentration of H2O2 or H2O2 +HA under normoxia or hypoxia for 24hrs. (a1,b1,c1&d1): Cells under normoxia and treated with H2O2. (a2,b2,c2&d2): Cells under normoxic condition and treated with H2O2 +HA. (a3,b3,c3&d3): Cells under hypoxia and treated with H2O2.

(a4,b4,c4&d4): Cells under hypoxia and treated with H2O2 +HA.ROS activity and MDA were assessed. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. respective control). Comparison of 4T1 cancer cells (E&G) and MNCs (F&H) reactive oxygen species generation in treatments with different concentrations under normoxia or hypoxia for 24hrs determined by DCF-DA flow cytometry and . MDA production. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. similar concentrations in different conditions and each index is represented with assigned colour in the legend).

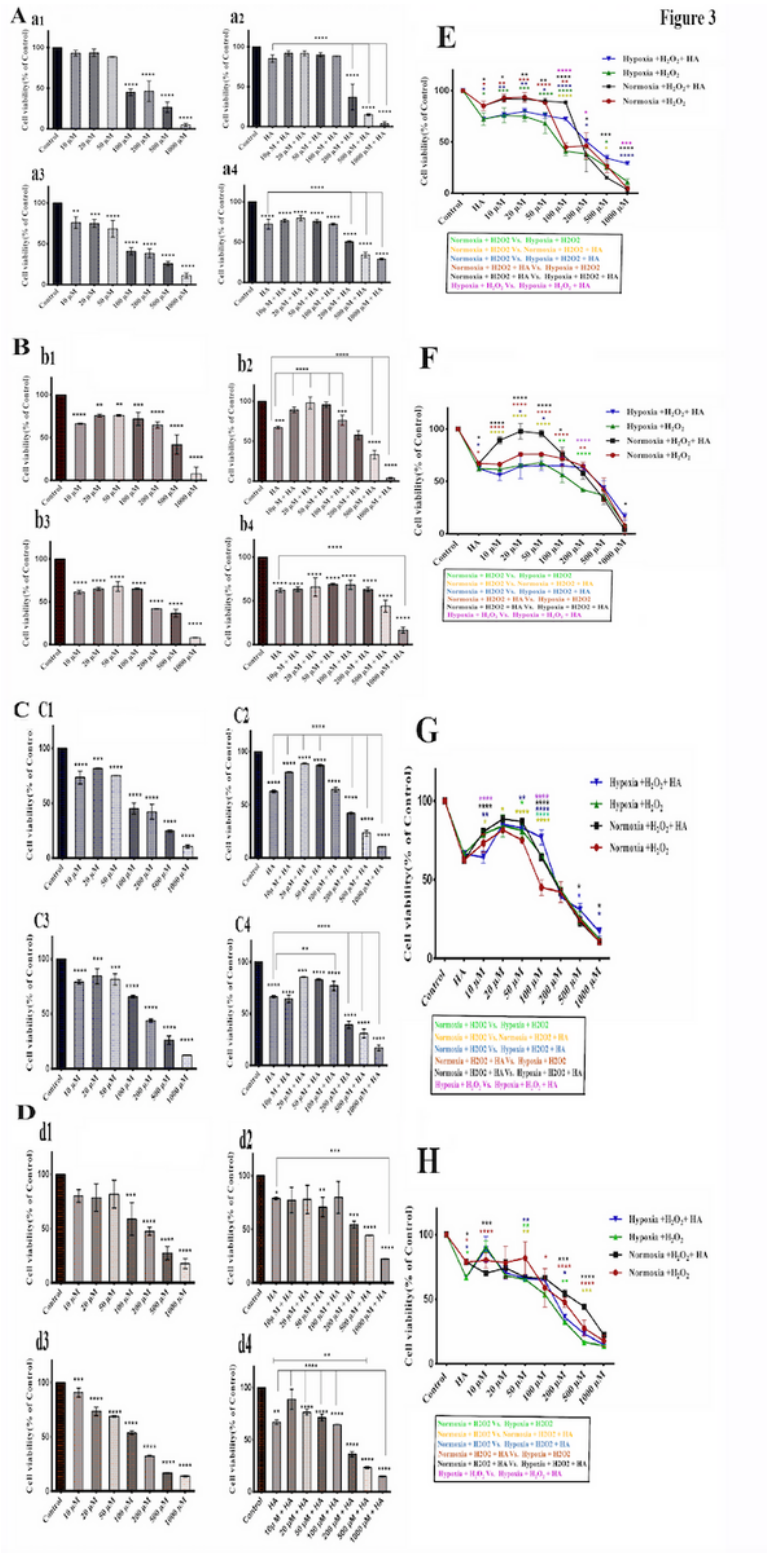

Figure 3

H2O2 or H2O2 + HA -induced modulation of Viability (A B,E,F) and vitality(C,D,G,H) in different levels of 4T1 cancer cells (A&C) and MNCs (B&D) treated with different concentration of H2O2 or H2O2 +HA under normoxia or hypoxia for 24hrs. (a1,b1,c1&d1): Cells under normoxia and treated with H2O2. (a2,b2,c2&d2): Cells under normoxic condition and treated with H2O2 +HA. (a3,b3,c3&d3): Cells under hypoxia and treated with H2O2. (a4,b4,c4&d4): Cells under hypoxia and treated with H2O2 +HA. Viability and vitality activity were assessed. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. respective control). Comparison of 4T1 cancer cells (E&G) and MNCs (F&H) viability and vitality in treatments with different concentrations under normoxia or hypoxia for 24hrs determined by MTT and NR assay. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. similar concentrations in different conditions and each index is represented with assigned colour in the legend).

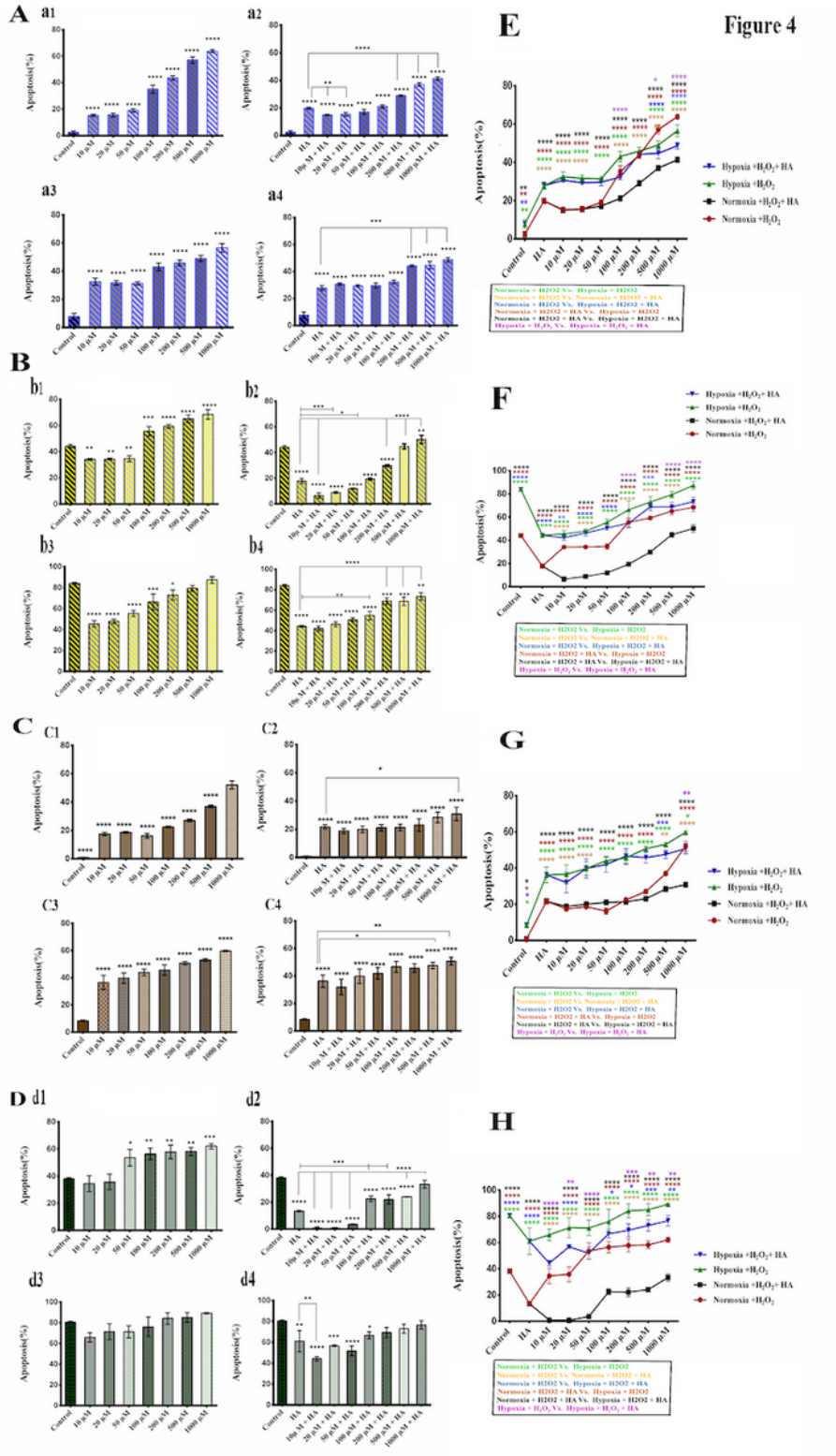

Figure 4

Apoptosis activity was determined by Acridin orange/ propidium iodide (A B,E,F) and AnexinV/PI (C,D,G,H) for 4T1cancer cells(A&C) and MNCs(B&D) treated with different concentration of H2O2 or H2O2+HA under normoxic or hypoxic condition for 24hrs. (a1,b1,c1&d1): Cells under normoxia and treated with H2O2. (a2,b2,c2&d2): Cells under normoxic condition and treated with H2O2 +HA. (a3,b3,c3&d3): Cells under hypoxia and treated with H2O2. (a4,b4,c4&d4): Cells under hypoxia and treated with H2O2 +HA.

Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and****p<0.0001 vs. respective control). Comparison of 4T1 cancer cells (E&G) and MNCs (F&H) cancer cells apoptosis activity in treatments with different concentrations under normoxic or hypoxic condition for 24hrs determined by Acridin orange/ propidium iodide and AnexinV/PI. Apoptosis activity was assessed. Data shown are means ± SD (n=3) (*p <0.05, **p<0.01, ***p <0.001 and ****p<0.0001 vs. similar concentrations in different conditions and each index is represented with the assigned colour the in the legend).

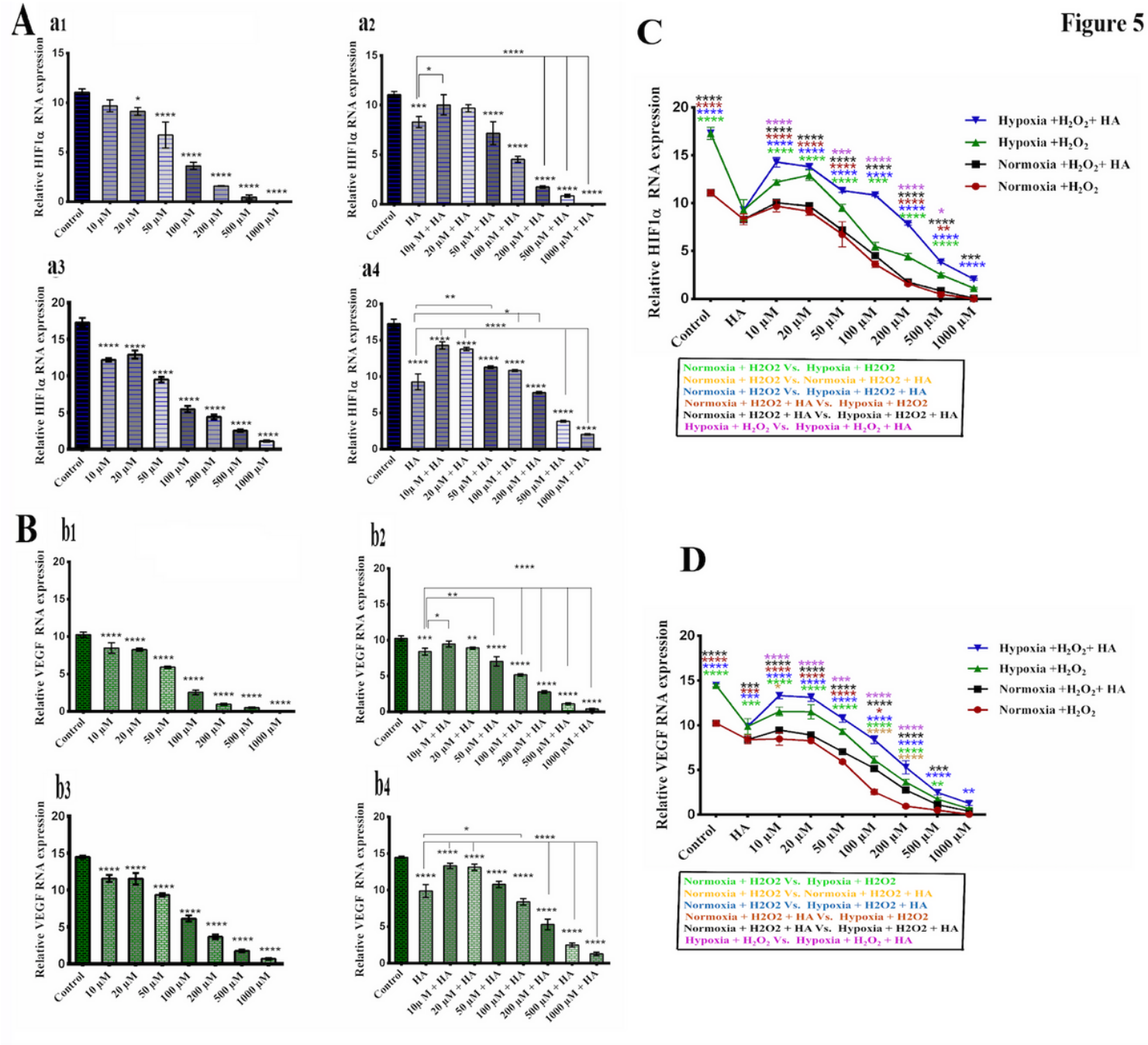

Figure 5

HIF-1α (A&C) and VEGF(B&D) mRNA expression levels of 4T1 cancer cells treatment with different concentration of H2O2 or H2O2 +HA under normoxia or hypoxia for 24 hrs . HPRT was used as an endogenous control. (a1&b1): Cells under normoxic condition and treated with H2O2. (a2&b2): Cells

under normoxic condition and treated withH2O2 +HA. (a3&b3): Cells under hypoxic condition and treated with H2O2. (a4&b4): Cells under hypoxic condition and treated with H2O2 +HA. Data were expressed as the mean ± SD from three independent experiments (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. respective control). Comparison of 4T1 cancer cells HIF-1α(C) and VEGF(D) mRNA expression levels in treatments with different concentrations under normoxic or hypoxic condition for 24hrs determined by Real Time PCR. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. similar concentrations in different conditions and each index is represented with the assigned colour in the legend).

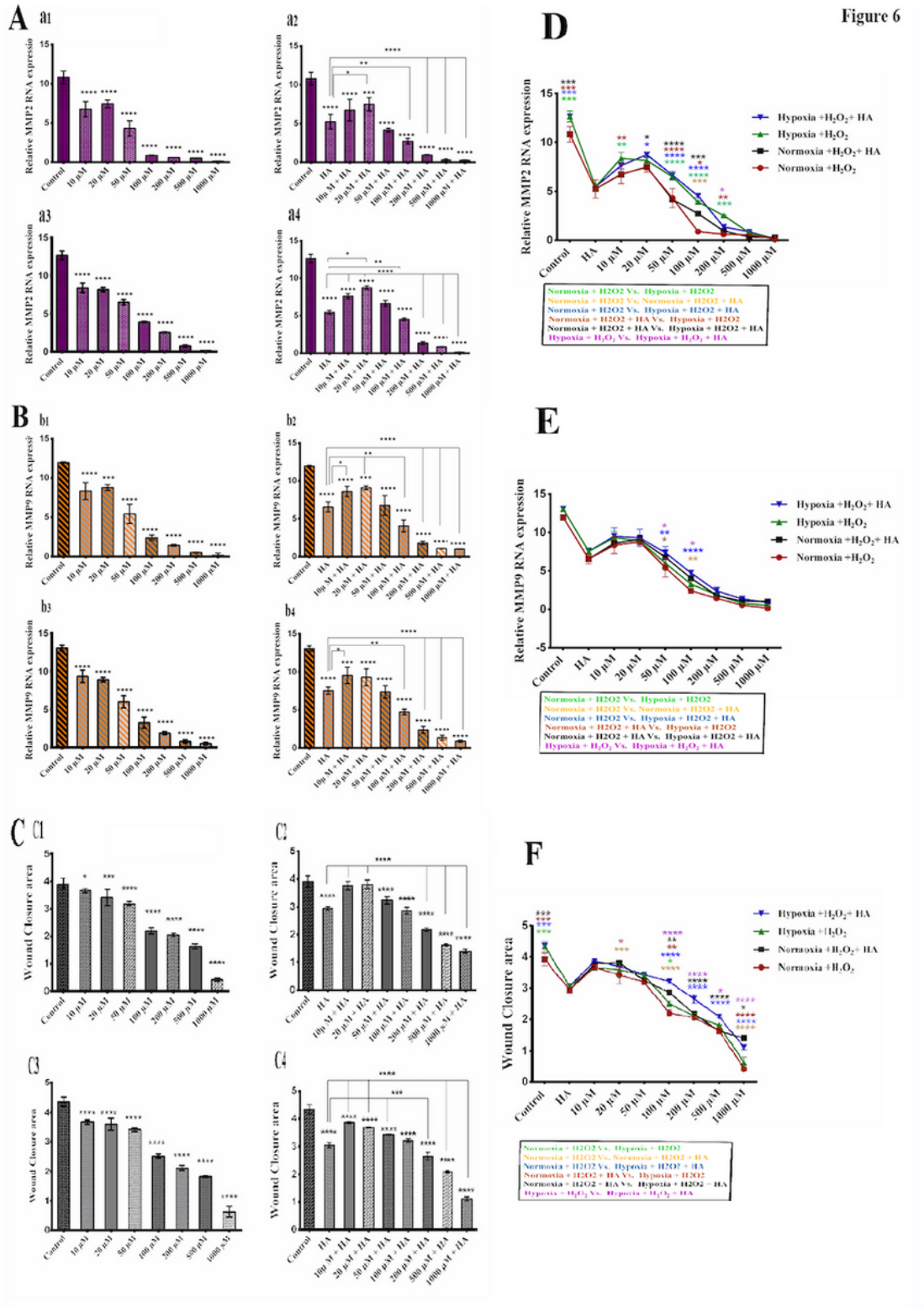

Figure 6

MMP2 (A&D) and MMP9 (B&E) mRNA expression levels and metastatic properties via targeting cell migration(C&F) of 4T1 cancer cells stimulated by different concentration of H2O2 or H2O2 +HA under normoxia or hypoxia for24hrs. HPRT was used as an endogenous control. (a1,b1&c1): Cells under normoxia and treated with H2O2. (a2,b2&c2): Cells under normoxia and treated withH2O2 +HA. (a3,b3&c3): Cells under hypoxia and treated with H2O2. (a4,b4,c4): Cells under hypoxia and treated

withH2O2 +HA. Data were expressed as the mean ± SD from three independent experiments (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. respective control). Comparison of 4T1 cancer cells MMP2 (C) , MMP2 (D) mRNA expression levels and metastatic properties via targeting cell migration(F) in treatments with different concentrations under normoxia or hypoxia for 24hrs determined by real- time PCR and wound healing assay. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. similar concentrations in different conditions and each index is represented with the assigned colour in the legend).

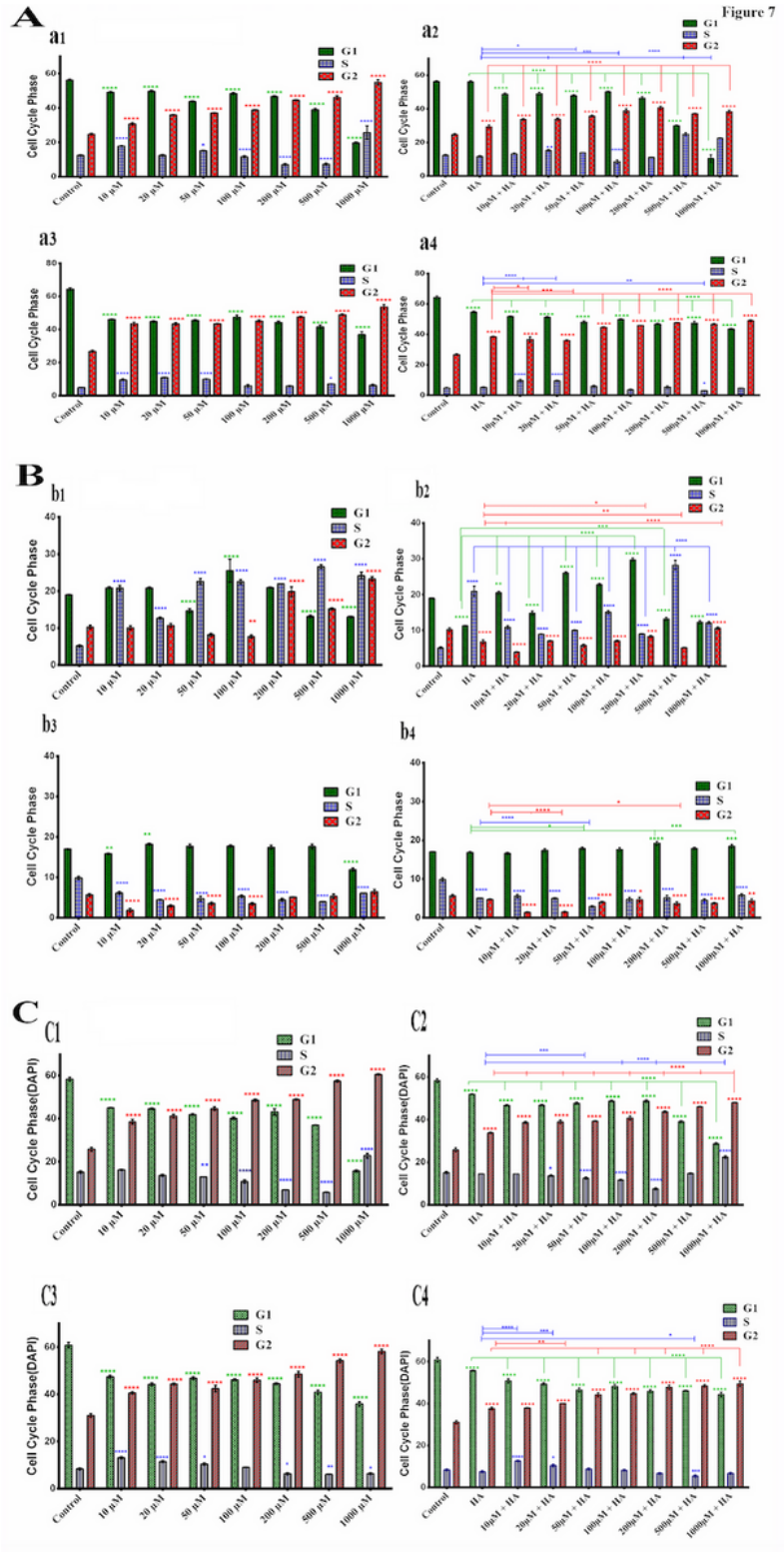

Figure 7

Effect of H2O2 or H2O2 + HA on cell cycle progression in different levels of 4T1 cancer cells (A&C) and MNCs (B) treated under normoxic or hypoxic condition for 24hrs. Cell cycle was quantified using flow cytometry (A&B) and DAPI (C). (a1, b1, c1): Cells under normoxia and treated with H2O2. (a2, b2, c2): Cells under normoxia and treated with H2O2 +HA. (a3, b3, c3): Cells under hypoxia and treated with H2O2. (a4, b4, c4): Cells under hypoxic condition and treated withH2O2 +HA. Data shown are means ± SD (n=3) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. respective control).

Supplementary Files

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- supplementry.docx