

Low ozone concentrations do not exert cytoprotective effects on tamoxifen-treated breast cancer cells *in vitro*

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ABSTRACT

Medical treatment with low ozone concentrations proved to exert therapeutic effects in various diseases by inducing a cytoprotective antioxidant response through the nuclear factor erythroid derived-like 2 (Nrf2) transcription factor pathway. Low ozone doses are increasingly administered to oncological patients as a complementary treatment to mitigate some adverse side-effects of antitumor treatments. However, a widespread concern exists about the possibility that the cytoprotective effect of Nrf2 activation may confer drug resistance to cancer cells or at least reduce the efficacy of antitumor agents. In this study, the effect of low ozone concentrations on tamoxifen-treated MCF7 human breast cancer cells has been investigated *in vitro* by histochemical and molecular techniques. Results demonstrated that cell viability, proliferation and migration were generally similar in tamoxifen-treated cells as in cells concomitantly treated with tamoxifen and ozone. Notably, low ozone concentrations were unable to overstimulate the antioxidant response through the Nrf2 pathway, thus excluding a possible ozone-driven cytoprotective effect that would lead to increased tumor cell survival during the anti-neoplastic treatment. These findings, though obtained in an *in vitro* model, support the hypothesis that low ozone concentrations do not interfere with the tamoxifen-induced effects on breast cancer cells.

Key words: MCF7 cells; oxidative stress; Nrf2; cell death; cell migration.

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Introduction

Tamoxifen (TAM) is a selective estrogen receptor modulator that is widely used for the treatment or prevention of estrogen receptor-positive breast cancer, as well as for chemoprevention in women at high risk of developing breast cancer.¹ Although the antitumoral mechanisms of TAM are not completely understood, it has been demonstrated that it induces an increase of reactive oxygen species (ROS) which plays a primary role in inhibiting cell proliferation while inducing cell death in breast cancer cells.^{2,3}

Medical treatment with low ozone (O₃) concentrations (generally ranging from 10 to 50 µg O₃/mL O₂) proved to exert therapeutic effects in various pathological conditions by promoting antioxidant and anti-inflammatory responses, wound healing, angiogenesis and tissue protection from injury (*e.g.*,⁴⁻¹⁰). Most of these beneficial effects rely on O₃-driven antioxidant activity: low O₃ concentrations are able to induce an oxidative “eustress”¹¹ thus activating the nuclear factor erythroid derived-like 2 (Nrf2) transcription factor that, after detaching from its cytoplasmic inhibitor Kelch-like ECH-associated protein1 (Keap1), migrates into the nucleus where it promotes the expression of several genes under the control of antioxidant response element (ARE) enhancers. As a result, many antioxidant factors are synthesized that, through a cascade of molecular events, give rise to the cytoprotective response.^{4,12,13}

In the medical practice, low O₃ doses are increasingly administered to oncological patients as a complementary treatment due to the observed efficacy of O₃ in mitigating some adverse side-effects of antitumor therapy.¹⁴⁻¹⁶ However, widespread concern exists about the possibility that the cytoprotective effects of Nrf2 activation may confer drug resistance to cancer cells or at least reduce the efficacy of antitumor drugs.¹⁷ On the other hand, some studies demonstrated the ability of O₃ to cause direct injury to different tumor cells or to enhance the efficacy of the antitumor treatments.^{14,18-20} Thus, the suitability of medical O₃ administration to oncologic patients still remains an open issue.

The present investigation aims at exploring the effect of low O₃ concentrations on TAM-treated MCF7 human breast cancer cells, a cellular model widely used for *in vitro* studies as these cells retain several peculiar features of the mammary epithelium and are a well-recognized target for TAM since they express estrogen receptors.²¹ This simple *in vitro* model allowed the histochemical and molecular analysis of cell viability, proliferation and antioxidant response in TAM-treated cells exposed to low O₃ concentrations under strictly controlled experimental conditions.

Materials and Methods

Cell culture and treatments

Human breast cancer MCF7 cells (ECACC 86012803) were grown in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (all reagents were purchased from Gibco, Waltham, MA, USA) at 37°C in a 5% CO₂ humidified atmosphere in T75 flasks. When sub-confluent (80%), the cells were detached using 0.25% trypsin/EDTA (Gibco) and seeded for specific experiments.

MCF7 cells were treated with 25 µM TAM (Sigma T9262, Milan, Italy) or gaseous O₂-O₃ mixtures or both. This TAM concentration was chosen because it proved to reduce cell viability by about 40% in comparison with the control (untreated) cells at 24 h post-treatment, thus reducing cell viability while maintaining an appropriate number of living cells to carry out the analyses.

TAM was dissolved in dimethyl sulfoxide (DMSO, Sigma), administered to the cells at increasing concentrations and incubated for 24 h at 37°C in a 5% CO₂ atmosphere. To evaluate the combined effects of TAM and O₂-O₃ mixture, cell samples were first treated with TAM for 24 h and then exposed to O₃ (as described below) before the analyses.

O₃ was produced from medical grade O₂ by an OZO2 FUTURA apparatus (Alnitec, Cremosano, CR, Italy); the apparatus allows the real-time photometric control of the O₃ concentration and gas flow rate. O₃ was used at the concentrations of 10 or 20 µg O₃/mL O₂, which are currently administered in the clinical practice and already proved to be non-toxic for various cultured cells and tissues.²²⁻²⁷ For Western blot, samples of 4 x 10⁶ cells were suspended in 10 mL medium into a 20 mL polypropylene syringe and an equal volume of gas (10 mL) was drawn into the syringe using a sterile filter (Alnitec); then, the cell suspension was gently shaken for 10 min,²⁸ incubated for 30 min at 37°C (Nrf2 requires a short time to move from cytoplasm to the nucleus after O₃ treatment, as demonstrated in¹³), centrifuged at 1500 rpm for 5 min and finally stored at -80°C until analysis. For MTT assay, cells were treated with gas as described above and then seeded in 96-multi-well plates. For S-phase assessment and the wound healing assays, the cells were seeded on glass coverslips and then treated with gas as described in²⁹. Some cell samples underwent the same procedure followed for the gas treatment but they were exposed to air, in order to evaluate possible handling effects, and used as control.

MTT assay

MTT assay was performed after treatment with TAM or O₂-O₃ mixtures or both. MCF7 cells were seeded in flat-bottom 96 multi-well plates at the density of 5 x 10³ cells/well. Five wells for each condition were seeded and the assay was performed on samples derived from three independent experiments.

MTT assay was performed after 24 h and 48 h from any treatment. Briefly, the medium was replaced with 100 µL of 0.5 mg/mL MTT (Sigma) in culture medium and incubated for 4 h at 37°C in an incubator. Then, MTT solution was removed, formazan crystals were dissolved in 100 µL of DMSO and the absorbance was measured at 570 nm with a spectrophotometer microplate reader (ChroMate; RayBiotech, Peachtree Corners, GA, USA). The results were reported as percentages with respect to the control value (set as 100%).

S-phase evaluation

For S-phase evaluation, samples of 8 x 10⁴ cells were seeded on 24 mm x 24 mm glass coverslips an allowed to adhere for 24 h; then they were treated with TAM, TAM+air- or TAM+O₃ as previously described (untreated samples were used as controls). 24 h post-treatment, the cells were pulse-labelled with 20 µM BrdU (Sigma) at 37°C for 30 min and fixed with cold 70% ethanol.

To partially denature DNA, the cells on coverslips were incubated with 2 N HCl for 20 min at room temperature, then neutralized for 3 min with 0.1 M sodium tetraborate (pH 8.2) (Sigma). Samples were washed with phosphate-buffered saline (PBS) and permeabilized with PBS containing 0.1% bovine serum albumin and 0.05% Tween-20 (Sigma) for 15 min, then incubated for 1 h with a mouse monoclonal antibody recognizing BrdU (BD Diagnostics, Franklin Lakes, NJ, USA) diluted 1:20 in PBS. Following two washes in PBS, the cells were incubated for 1 h with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Molecular Probes, Invitrogen, Milan, Italy) diluted 1:200, washed twice in PBS, and stained for DNA with 0.1 µg/mL Hoechst 33342 for 10 min (Abcam, Cambridge, UK) in PBS. Samples were placed on glass slides and finally coverslipped with PBS/glycerol 1:1 solution.

The percentage of BrdU-positive cells was assessed in 30 randomly selected fields (40x) for each experimental condition. Observation was performed using an Olympus BX51 microscope (Olympus Italia S.r.l., Segrate, MI, Italy) equipped with a 100W mercury lamp, under the following spectral conditions: 450-480 nm excitation filter (excf), 500 nm dichroic mirror (dm), and 515 nm barrier filter (bf) for Alexa Fluor 488; 330-385 nm excf, 400 nm dm, and 420 nm bf, for Hoechst 33342. Images were acquired with a QICAM Fast 1394 Digital Camera (QImaging, Surrey, BC, Canada) and processed with Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA). Manual cell counting was performed in samples from three independent experiments by two independent operators.

Mitotic index

Cell samples on glass coverslips were prepared and treated with TAM, TAM+air or TAM+O₃ as described in the previous subsection (untreated MCF7 cells were used as controls). 24 h after treatment, the cells were fixed with 70% ethanol for 30 min, washed in PBS and stained for DNA with 0.1 µg/mL Hoechst 33342 (Abcam) in PBS for 10 min. The samples were finally placed on glass slides and mounted in PBS/glycerol (1:1). The percentage of mitotic cells was assessed in 30 randomly selected fields (40x) for each experimental condition. An Olympus BX51 microscope (Olympus Italia S.r.l.) equipped with a 100W mercury lamp was used under the appropriate light excitation and emission conditions for Hoechst 33342. Images were recorded with a QICAM Fast 1394 Digital Camera (QImaging) and processed with Image-Pro Plus software (Media Cybernetics, Inc.). Manual cell counting was performed in samples from three independent experiments by two independent operators.

Wound healing assay

For the wound-healing assay, 3 x 10⁵ cells were seeded onto 24 mm x 24 mm glass coverslips. After 24 h, the confluent cell monolayers were scratched with a 200 µL sterile pipette tip and then treated with TAM, TAM+air or TAM+O₃ (control samples were left untreated). FBS was then omitted from the medium to exclude the effect of cell proliferation. To evaluate cell migration, images were taken at 0, 6, and 24 h post-treatment using an inverted microscope (Leica DMIL, Leica Microsystems S.r.l., Buccinasco, MI, Italy) equipped with a camera (Optika Microscopes, Ponteranica, BG, Italy). The scratched area was measured in 4 randomly chosen fields (4x) in three independent experiments, for a total of 12 fields/experimental condition. The value of the cell-free area was expressed as a percentage of the value at time 0 (considered as 100%).

Western blot

Western blot for Nrf2 was carried out on MCF7 cell pellets obtained as described above and stored at -80°C after 30 min from the treatment. The pellets were lysed in 1% Triton X-100 solution supplemented with 100 mM phenylmethylsulfonyl fluoride (PMSF), and a cocktail of protease inhibitors (Sigma). Protein quantification was then performed with Pierce BCA Protein Assay Kit (Thermo Fisher, Milan, Italy) according to the manufacturer's protocol. Then, equal amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was saturated with 5% bovine serum albumin (BSA, Sigma) and 0.05% of sodium azide, washed in PBS Tween 0.1% and incubated overnight with anti-Nrf2 antibody (Abcam) diluted 1:500 in PBS and anti-alpha-tubulin antibody (Merck Millipore, Milan, Italy) diluted 1:1000 in PBS. The membrane was then washed and incubated with the appropriate horseradish peroxidase-conjugated sec-

ondary antibodies (Perkin-Elmer, Milan, Italy) diluted 1:20,000 in PBS for 1 h at room temperature. Western blot images were acquired at ChemiDoc (BioRad, Hercules, CA, USA) and further analyzed using ImageLab (BioRad) software. The tests were performed in three independent experiments and untreated cells were used as control.

Statistical analysis

Data for each variable were pooled according to the experimental condition and presented as mean ± SEM. Statistical significance was assessed by the one-way analysis of variance (ANOVA) test (for S-phase evaluation and mitotic index) and by the Kruskal-Wallis test (for MTT assay, wound healing assay, and Western blot). In case of significance, the Mann Whitney test was used for pairwise comparisons. Statistical significance was set at $p \leq 0.05$.

Results

MTT assay

MTT assay was used to assess the mitochondrial activity as a measure of cell viability after treatment with TAM or O₂-O₃ mixtures or after their simultaneous administration. To test the combined effects of TAM and O₃, it was necessary that TAM efficiently reduced cell viability at a suitable extent to enable the experimental procedures. Therefore, a TAM concentration able to reduce cell viability by about 40% in comparison with the untreated control cells at 24 h was used. Therefore, MCF7 cells were treated with different concentrations of TAM (Figure 1), and the concentration of 25 µM was selected (control vs 25 µM TAM, $p=0.04$) for all the experiments.

In order to confirm the safety of the selected low O₃ concentrations on MCF7 cells, cell samples were exposed to O₂-O₃ mixtures at concentrations of 10 µg O₃/mL O₂ or 20 µg O₃/mL O₂ (which are usually administered in clinical practice), and the cell viability was evaluated 24 and 48 h post-treatment (Figure 2). The values were compared to those of control cells and to those of cells undergoing the same handling as O₃-treated samples but exposed only to air.

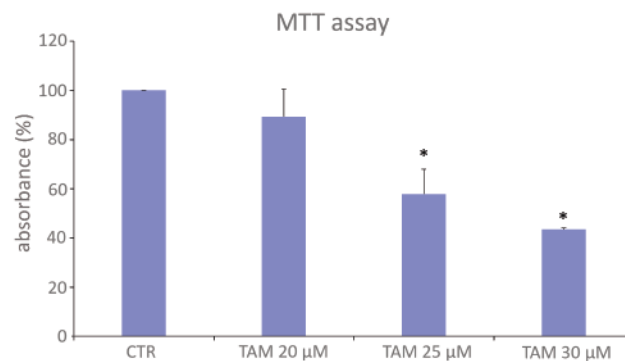


Figure 1. MTT mean values ± SEM (as a measure of cell viability) in MCF7 control cells (CTR) and cells treated with increasing concentrations of tamoxifen (TAM). The absorbance values were normalized to the control, which was fixed as 100. *Statistically significant difference in comparison with control.

Both O₃ concentrations did not affect cell viability at 24 h ($p=0.06$ and $p=0.40$, respectively) and 48 h ($p=0.75$ and $p=0.92$ respectively) post-treatment. In order to reveal the possible interference between low O₃ concentrations and TAM cytotoxic effect, MCF7 cells were treated with both TAM and O₂-O₃ mixtures and the resulting cellular viability was assessed after 24 h and 48 h from the concomitant treatment (Figure 3). After 24 h, a statistically significant decrease in cell viability was observed in TAM+air, TAM+10 µg O₃ and TAM+20 µg O₃ samples in comparison with control ($p=0.03$ for all treatments), and such a decrease was comparable to that obtained after TAM treatment alone. The same results were observed also after 48 h ($p=0.02$ for all treatments).

S-phase evaluation

5-Bromo-2'-deoxyuridine (BrdU) is a thymidine analogue that, being incorporated into newly synthesized DNA, labels S-phase cells and allows their detection and quantification after specific immunostaining. In our samples, BrdU-positive cell nuclei were green-fluorescing, while nuclear DNA was counterstained in blue with Hoechst 33342 (Figure 4). After 24 h from the gas exposure, a significant decrease in the percentage of BrdU-positive cell nuclei was observed in TAM and TAM+air treated samples in comparison with control (untreated) cells ($p<0.001$ for both samples). No significant differences were observed between controls and cells treated with TAM+O₃ 10 µg ($p=0.32$) or TAM+O₃ 20 µg ($p=0.09$).

Mitotic index

Proliferation rate of control samples and TAM, TAM+air- and TAM+O₃-treated MCF7 cells was assessed also by evaluating the mitotic index (*i.e.*, the number of mitotic figures/total cell nuclei × 100) after DNA staining with Hoechst 33342 (Figure 5). All treated samples showed a significant decrease in the percentage of mitotic cells compared to controls (TAM, $p=0.01$; TAM+air, $p=0.04$; TAM+10 µg O₃, $p=0.04$; TAM+20 µg O₃, $p=0.04$).

Wound healing assay

The migration capability of TAM, TAM+air- and TAM+O₃-treated cells with respect to control was evaluated using a wound-healing assay (Figure 6).

After 6 h, cells treated with TAM+10 µg O₃ showed a significant decrease in the percentage of cell-free area ($p=0.03$), which further decreased after 24 h ($p=0.003$). The other treatments, namely TAM, TAM+air and TAM+20 µg O₃, did not significantly influenced the cell capability to migrate with respect to control at both 6 h ($p=0.69$, $p=0.94$ and $p=0.38$, respectively) and 24 h ($p=0.69$, $p=0.81$ and $p=0.47$, respectively).

Western blot

Given the pivotal role of Nrf2 as a critical regulator of the cellular stress response and, in particular, as the lead activator of O₃-driven antioxidant response, its enhancement was evaluated by Western blot. The expression of Nrf2 significantly increased in all treated samples in comparison to control (TAM, $p=0.03$; TAM+air, $p=0.04$; TAM+10 µg O₃, $p=0.04$; TAM+20 µg O₃, $p=0.04$) (Figure 7), but values of treated samples were statistically similar with each other ($p>0.05$ for all comparisons).

Discussion

This study explored the combined effects of TAM and O₃ on the human breast cancer cell line MCF7, aiming to contribute to the debate on the possible cytoprotective effects of low O₃ concentrations on cancer cells under antitumor treatment. The simplified

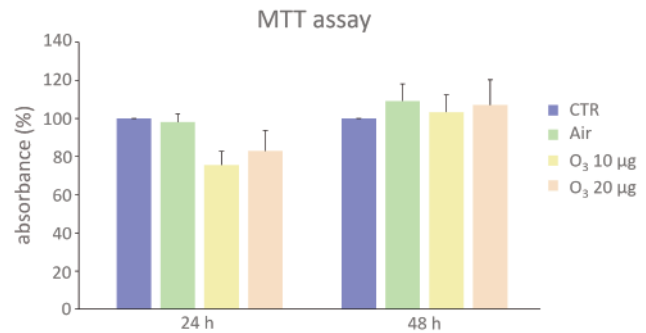


Figure 2. MTT mean values ± SEM (as a measure of cell viability) in MCF7 control cells (CTR) and cells treated with air or O₂-O₃ mixtures after 24 h and 48 h from gas exposure. The absorbance values were normalized to the control, which was fixed as 100. No statistically significant difference was found in comparison with control.

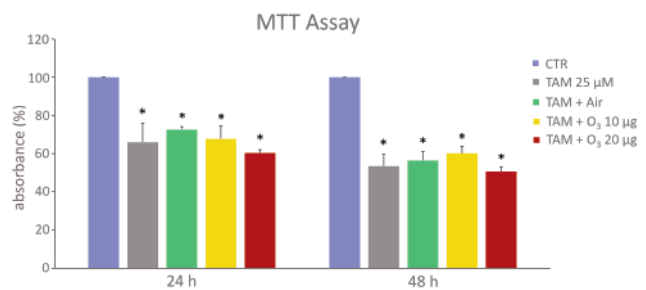


Figure 3. MTT mean values ± SEM of MTT absorbance (as a measure of cell viability) in control (CTR) and treated MCF7 cells after 24 h and 48 h from treatment. The absorbance values were normalized to the control, which was fixed as 100. TAM, tamoxifen; *statistically significant difference in comparison with control.

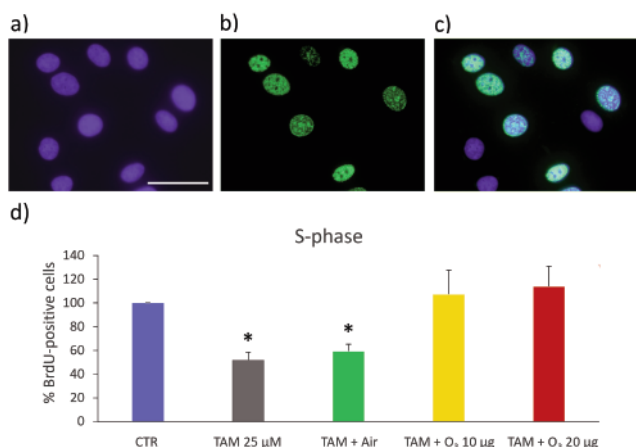


Figure 4. a-c) Representative fluorescence microscopy images of MCF7 cells stained for DNA with Hoechst 33342 (blue) (a), immunolabelled for BrdU (green) (b), and merged (c); scale bar: 50 µm. **d)** Mean values ± SEM of percentages of BrdU-positive cells 24 h after treatment. The values were normalized to the control, which was fixed as 100. TAM, tamoxifen; *statistically significant difference in comparison with control (CTR).

in vitro model chosen for this investigation cannot obviously give a conclusive answer, because the complexity of a living organism and the peculiarity of the tumor microenvironment are not fully reproduced;^{30,31} however, these experiments, performed under defined experimental conditions, provide basic cellular and molecular data that may contribute to mechanistically elucidate the reported positive effects of the O₃ administration to oncologic patients.¹⁴⁻¹⁶ It is well-established that TAM induces cell death and reduces proliferation in MCF7 cells (see *e.g.*,^{2,32-34}). Consistently, we found a concentration-dependent reduction in MCF7 cell viability following treatment with TAM. This allowed us to select a TAM concentration suitable to significantly reduce cell viability while maintaining an appropriate number of living cells to perform our investigation.

The O₃ concentrations chosen for this study (10 and 20 µg O₃/mL O₂) are widely used in clinical practice and their effects have been investigated in various cell types, demonstrating that they are non-toxic.²²⁻²⁷ These low concentrations proved to be safe also for the MCF7 cell line, excluding any interference with cells' viability and thus enabling their use in our study. When TAM and O₃ were concomitantly administered, the observed lowering in cell viability was similar to that occurring after treatment with TAM alone, thus demonstrating that, under our experimental conditions, both O₃ concentrations were unable to interfere with the cytotoxic effect of the antitumor drug.

The possible interference of O₃ administration with TAM on cell proliferation was evaluated by assessing the rate of DNA-duplicating (S-phase) cells and mitotic cells. A significant decrease in S-phase MCF7 cells after TAM treatment was found, according to previous studies demonstrating that TAM induces an arrest of the cell cycle at G₀/G₁ phase.^{2,35,36} On the other hand, in cell samples treated with TAM+10 or 20 µg O₃ a significant increase of S-phase cells occurred in comparison with samples treated with TAM only, reaching the control values. Looking at the mitotic index, a drastic decrease of mitotic cells was found in TAM-treated samples according to the well-known anti-proliferative effect of this drug^{2,33} and similarly low values were found in TAM+O₃-treated cells, thus excluding a pro-proliferative effect of both low O₃ concentrations. Therefore, the increase in S-phase cells observed in TAM+O₃-treated cells should not be interpreted as a sign of proliferation recovery but as due to an S-phase block in cell cycle progression. Accordingly, in a recent work it has been demonstrated that low O₃ concentrations may induce an increase in the percentage of S-phase cells in human hepatocellular carcinoma cells.³⁷

The migration capability of cancer cells is a key factor for their invasive potential and the combined effect of TAM and O₃ on this functional aspect was taken into consideration. MCF-7 cells are

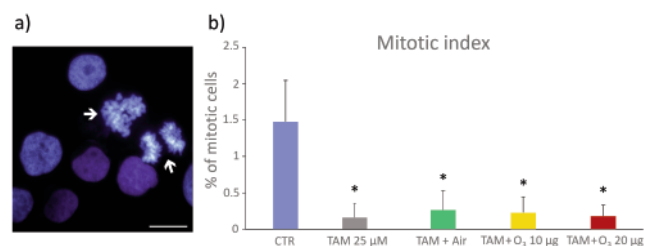


Figure 5. a) Representative fluorescence microscopy image of control MCF7 cells stained for DNA with Hoechst 33342 (blue), the arrows indicate mitotic figures; scale bar: 25 µm. b) Mean values ± SEM of percentages of mitotic cells 24 h after the treatment. TAM, tamoxifen; *statistically significant difference in comparison with control (CTR).

weakly metastatic cells with low invasive potential³⁸ and this is due to their cytoskeletal pattern.³⁹ TAM treatment has been shown to reduce the spreading rate in MCF7 by influencing biomechanical parameters and cytoskeletal organization.⁴⁰ In our study, no statistically significant reduction in cell migration capability was found in TAM-treated MCF7 cells, although a tendency to decline was observed; this discrepancy with other findings in the literature could be due to the different experimental conditions used. Interestingly, when TAM was concomitantly administered with O₃, TAM+20 µg O₃-treated cells showed similar values as TAM-treated cells, whereas TAM+10 µg O₃-treated cells showed a significant

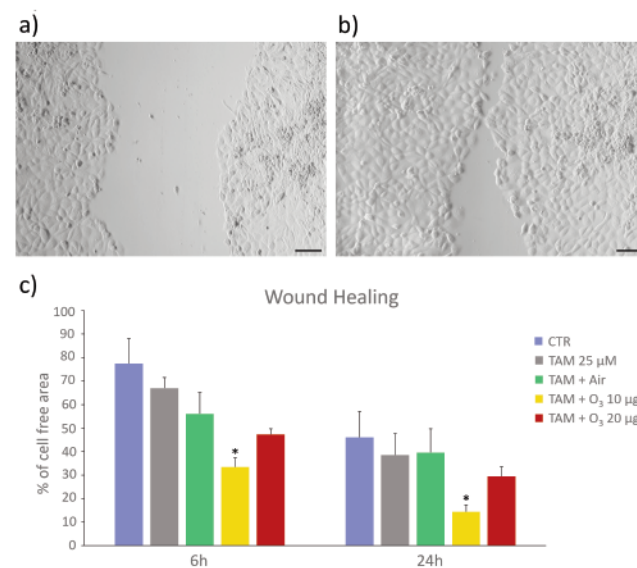


Figure 6. Wound healing assay. a,b) Representative images of MCF7 cells at 6 h (a) and 24 h (b); scale bars: 50 µm. c) Mean values ± SEM of percentages of cell-free areas of control (CTR), and treated cells at 6 h and 24 h. TAM, tamoxifen; *statistically significant difference in comparison with control.

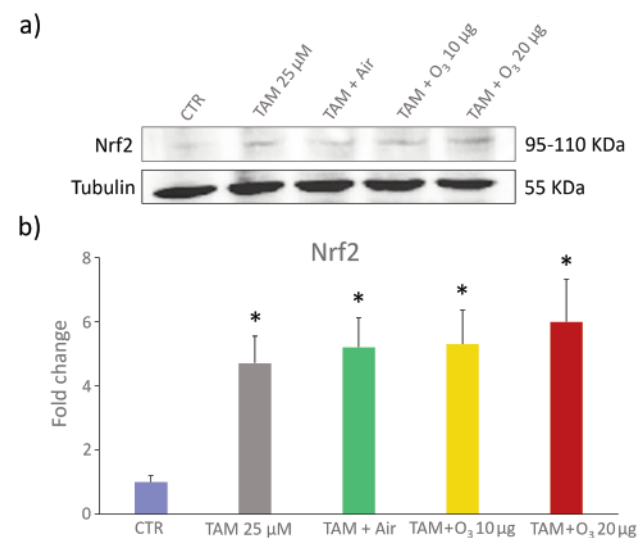


Figure 7. Western blot of Nrf2 protein in control (CTR) and treated MCF7 cells. a) Representative Western blot experiment. b) Mean values ± SEM of Nrf2 levels; data were normalized with respect to a housekeeping protein (tubulin) and expressed as proportional to CTR sample. TAM, tamoxifen; *statistically significant difference in comparison with control.

increase in their migration rate. It has been reported that small local changes in the amount of ROS (as it occurs after exposure to low O₃ concentrations) act on cytoskeletal organization stimulating actin polymerization⁴¹⁻⁴³ and promoting cell adhesion.^{44,45} In particular, 10 µg O₃ induced cytoskeletal reorganization and adhesion in HeLa cells²² as well as the formation of cell surface protrusions in fibroblasts.²⁶ Moreover, low O₃ concentrations proved to increase the membrane deformability of blood cells.^{46,47} All these effects are known to promote cell motility, and may play a role in the increased migration capability observed in MCF7 cells concomitantly treated with TAM and 10 µg O₃.

Since TAM is known to induce marked ROS increase in MCF7 cells² while low O₃ concentrations are able to counteract oxidative stress mainly through Nrf2 activation,^{13,48} it was crucial to clarify the possible interference of O₃ with this antitumor drug by evaluating Nrf2 levels. Generally, breast cancer cells are characterized by a low or undetectable expression of Nrf2 compared with normal mammary epithelial cell lines.^{49,50} According to its strong oxidative potential, TAM has been found to induce increased Nrf2 expression in breast cancer cells *in vivo*.^{51,52} Consistently, in our *in vitro* model, an increase in Nrf2 was found in TAM-treated MCF7 cells, but the addition of low O₃ concentrations to TAM did not change the Nrf2 level, thus excluding the induction of a powerful antioxidant response and a consequent resistance to the antitumor treatment. This finding is also consistent with the results on cell viability and proliferation. Therefore, the concomitant treatment with TAM and O₃ does not mimic what happens in TAM-resistant MCF7, whose unresponsiveness is due to the abnormally high expression of Nrf2, which upregulates the antioxidant capacity, thus improving cell survival.^{52,53} It is worth noting that 10 and 20 µg O₃ did not increase Nrf2 level in microglial cells previously treated with dimethyl fumarate, a Nrf2-activating drug,²⁵ thus suggesting that these low O₃ concentrations are generally unable to overstimulate the Nrf2-pathway in already activated cells.

Taken together, the results of the present *in vitro* study demonstrate that, under our experimental conditions, low O₃ concentrations administered to TAM-treated human breast cancer MCF7 cells do not interfere with the cytotoxic oxidative stress induced by TAM. In fact, cell viability, proliferation and migration were similar in cells treated with TAM alone as in those exposed to the concomitant treatment with TAM and O₃, except from samples treated with 10 µg O₃, which showed higher migration ability. Importantly, low O₃ concentrations proved to be unable to overstimulate the antioxidant response through the Nrf2 pathway, thus excluding a possible O₃-driven cytoprotective effect that would lead to increased cell survival to TAM treatment.

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