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Menadione/Ascorbate Induces Overproduction of Mitochondrial Superoxide and Impairs Mitochondrial Function in Cancer: Comparative Study on Cancer and Normal Cells of the Same Origin

SEVERINA SEMKOVA[1,2,3], ZHIVKO ZHELEV[3,4], THOMAS MILLER[5], KIMIHIKO SUGAYA[1,2], ICHIO AOKI[1,2], TATSUYA HIGASHI[2] and RUMIANA BAKALOVA[1,2,6]

[1]Quantum-state Controlled MRI Group, Institute for Quantum Life Science, National Institutes for Quantum and Radiological Science and Technology (QST), and [2]Functional and Molecular Imaging Team, Department of Molecular Imaging and Theranostics, National Institute of Radiological Sciences (NIRS), QST, Chiba, Japan;
[3]Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[4]Medical Faculty, Trakia University, Stara Zagora, Bulgaria;
[5]IC-MedTech Co., San Diego, CA, U.S.A.;
[6]Medical Faculty, Sofia University, Sofia, Bulgaria

Abstract. *Background/Aim: The menadione/ascorbate (M/A) combination has attracted attention due to the unusual ability of pro-vitamin/vitamin combination to kill cancer cells without affecting the viability of normal cells. The aim of this study was to elucidate the role of M/A in targeting cancerous mitochondria. Materials and Methods: Several cancer and normal cell lines of the same origin were used. Cells were treated with different concentrations of M/A for 24 h. The cell viability, mitochondrial superoxide, mitochondrial membrane potential, and succinate were analyzed using conventional analytical tests. Results: M/A exhibited a highly specific suppression on cancer cell growth and viability, without adversely affecting the viability of normal cells at concentrations attainable by oral or parenteral administration in vivo. This effect was accompanied by: (i) an extremely high production of mitochondrial superoxide in cancer cells, but not in normal cells; (ii) a significant dose-dependent depolarization of mitochondrial membrane and depletion of oncometabolite succinate in cancer cells. Conclusion: The anticancer effect of M/A is related to the induction of severe mitochondrial oxidative stress in cancer cells only. Thus, M/A has a potential to increase the sensitivity and vulnerability of cancer cells to conventional anticancer therapy and immune system.*

Correspondence to: Rumiana Bakalova, Ph.D., DSci, Department of Molecular Imaging and Theranostics, National Institute of Radiological Sciences, QST/NIRS, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan. Tel: +81 8081088136, e-mail: bakalova.rumiana@qst.go.jp

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The combination menadione/ascorbate (M/A; termed also Apatone®, with a ratio of 1/100 mol/mol menadione to ascorbate), has attracted the attention of researchers for more than 20 years due to its unusual ability to kill cancer cells without affecting the viability of normal cells (1-6). Although M/A has often been referred to as a pro-vitamin/vitamin-based therapeutic strategy, it is a misnomer. It should be specified that M/A is not a vitamin. The anticancer effects of M/A do not appear to rely on the vitamin activities of these compounds. Rather, M/A is most correctly termed a combination drug.

The choice of this combination was determined by experimental studies suggesting that menadione and ascorbate exert synergistic anticancer and antifibrotic effects *in vitro* and *in vivo* (7-13). *In vivo*, this could occur at relatively low plasma concentrations, attainable by oral administration, or at higher pharmacological concentrations, attainable by parenteral administration [intravenous (i.v.) or intraperitoneal (i.p.)] (1, 13-21).

In pilot early-phase clinical trials, orally administered M/A did not cause serious drug-related side-effects even at 100 mg/10 g per day (22). Several independent clinical studies suggest that M/A is safe and potentially effective in humans, including: (i) patients with prostate cancer at advanced stages with bone metastases and resistance to hormone therapy (23) and (ii) patients with postoperative pain, or pseudotumor following total joint arthroplasty (24).

Animal studies have reported that oral and parenteral M/A potentiates the efficiency of conventional chemotherapy and radiotherapy of cancer *in vivo* and inhibits invasion and metastasis (13-15, 17, 18, 20, 21).

In vitro studies have demonstrated that M/A (over 5/500 μM/μM) induces significant cytotoxicity in cultured cancer cells (16, 25, 26). This is thought to be the result of induction of apoptosis, necrosis, and of a specific form of cell death termed "autoschizis" (8, 10, 11, 27, 28). It is generally accepted that the combination M/A causes cancer cell death by induction of oxidative stress and subsequent replicative stress (3, 4, 18, 19, 29-34). However, the primary source (the trigger) of reactive oxygen species (ROS) and induction of severe oxidative stress in M/A-treated cancer cells has not yet been convincingly established.

Two sources of M/A-induced oxidative stress have been discussed in the literature: (i) extracellular; and (ii) intracellular – cytosolic. Both mechanisms are based on the assumption of overproduction of hydrogen peroxide (*via* superoxide) due to ascorbate-driven one-electron redox-cycling of menadione (Figure 1) (10, 29-31, 34-37). All these events are reported at high concentrations of M/A (>5/500 μM/μM) and conclusions are based on indirect evidence such as: (i) effects of catalase, metal chelators, antioxidants, and end-products of oxidative stress in M/A-treated cells (10, 29-31, 34-36); or (iii) production of superoxide and/or hydrogen peroxide in cells treated with menadione or ascorbate alone (37-41). Based on our knowledge, there are no data about the direct production and degradation of superoxide and/or hydrogen peroxide in M/A-treated cells. Moreover, in the cells, NAD(P)H-dehydrogenase quinone 1 (NQO1) catalyzes the two-electron reduction of menadione to menadiol (Figure 1) (42, 43). NQO1 is considered as a menadione detoxification enzyme, which is up-regulated (overexpressed) in various types of cancer (44, 45). Therefore, the overproduction of ROS by ascorbate-driven one-electron redox-cycling of menadione is disputable if NQO1 is not inhibited. This controversy points to other sources and mechanisms for severe oxidative stress in M/A-treated cells, outside of cytosolic and extracellular ROS.

An interesting fact is that ascorbate and menadione are known to interfere with the mitochondrial electron transport chain (ETC). Studies have demonstrated that menadione and other quinones affect mitochondrial respiration directly and even provide insights in the molecular mechanisms for this mitochondrial interference (46-48). For example, it has been demonstrated that menadione bypasses Complex-I deficiency (46, 48). It has also been shown that pharmacological ascorbate and menadione are beneficial in the treatment of mitochondrial diseases (48, 49). The combination of ascorbate and menadione is included in the List of Dietary Supplements for Primary Mitochondrial Disorders by the U.S. Department of Health and Human Services, National Institute of Health (NIH). It has been used clinically to bypass complex-III deficiency of the ETC (48, 49). Menadione and ascorbate have been applied as a dietary supplement in combination with coenzyme Q10 (CoQ10), niacin, riboflavin, and thiamin to bypass Complex-I and Complex-III of the ETC (50). Since menadione (in high concentrations) is hepatotoxic, it is no longer used in dietary supplements in U.S., but is still in use in other countries and common in animal feed, including diets for laboratory animals.

In our study, we attempted to answer the question: "Are mitochondria involved in the M/A-mediated overproduction of ROS and induction of severe oxidative stress in cancer and how specific and targeted is this mechanism?" Experiments were designed to compare the effects of M/A on viability and mitochondrial homeostasis of cancer and normal cells of the same origin.

Materials and Methods

Chemicals. Sodium L-ascorbate and menadione were purchased from Sigma-Aldrich (Weinheim, Germany). Other chemicals and kits were purchased from various suppliers. All reagents, used in the experiments, were of analytical grade or HPLC-grade.

Cells and treatment protocol. The experiments were performed on: (i) lymphocytes – leukemic cells (Jurkat; RIKEN Bioresource Center, Saitama, Japan) and normal cells (Human Peripheral Blood Cells; Cell Applications Inc., San Diego, CA, USA); (ii) colon epithelial cells – cancer (Colon26) and normal (FHC) cells (Cell Applications Inc.); (iii) breast epithelial cells – cancer (MFC7) and normal (MCF10A) cells (Cell Applications Inc.). Normal lymphocytes were also isolated in our Lab from peripheral blood of clinically healthy donors using Lymphosepar-I (Immune-Biological Laboratories Co., Fujioka, Japan) and multiple washings of the lymphocyte fraction by phosphate-buffered saline solution (PBS). Multiple washings by PBS are obligatory to avoid contaminations with free- and heme-iron as a result of haemolysis during isolation. Any contaminations with transition metals in the cell fraction can compromise the results due to induction of Fenton's reactions in the presence of M/A.

Leukemic lymphocytes were cultured in RPMI-1640 medium (Sigma-Aldrich, Weinheim, Germany), containing antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) (Sigma-Aldrich). Normal lymphocytes were cultured in RPMI-1640 without antibiotics and used for experiments within 10 days of their isolation. Colon26 and MCF7 cells were cultured in DMEM (Sigma-Aldrich). FHC and MCF10A cells were cultured in DMEM-F12 (Sigma-Aldrich) and DMEM, respectively, both supplemented with growth factors. All media were supplemented with 10% FBS (heat-inactivated) (Sigma-Aldrich). All cell lines were grown in an incubator at 37˚C and a humidified atmosphere, saturated with 5% CO_2.

Twenty four hours before the experiment, the cells were placed in fresh medium without antibiotics. To detach the adherent cells from the plates, we used a trypsin-EDTA solution (0.5% of trypsin, 0.2% of EDTA) and subsequent washings with PBS. The cells were sedimented by centrifugation (1000 × *g*/10 min for non-adhesive or 800 × *g*/5 min for adhesive).

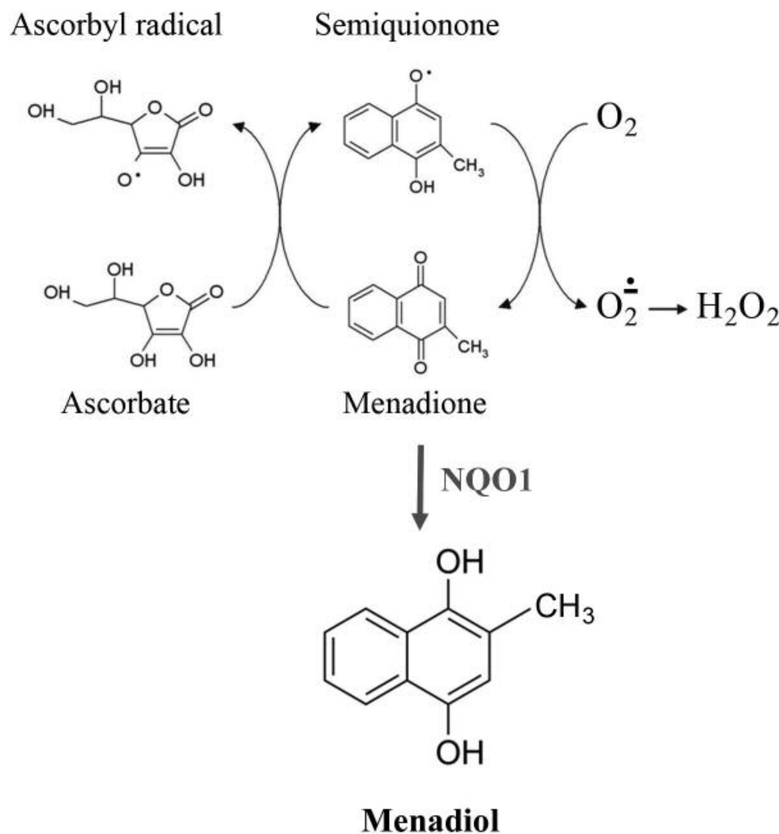

Figure 1. *Schematic representation of ascorbate-driven one-electron redox-cycling of menadione with production of ROS (superoxide and hydrogen peroxide). In the cells, NQO1 maintains menadione in its reduced form (menadiol) and thus depletes the menadione required for one-electron redox-cycling mechanism. NQO1: NAD(P)H-dehydrogenase quinone 1.*

The cells were incubated with ascorbate and menadione for different time-intervals and at each time-interval, aliquots were used for analyses.

Ascorbate was dissolved in PBS (10 mM, pH 7.4). Menadione was dissolved in DMSO to 10 mM stock solution and then several working solutions in PBS were prepared. The final concentration of DMSO in the cell suspension was below 1%. At this concentration, DMSO did not influence cell viability.

Cell proliferation and viability assay. Cell viability and proliferation were analyzed by using CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA). The analysis is based on generation of a luminescent signal from luciferin/luciferase reaction, which is proportional to the amount of ATP synthesized in live cells (51).

Briefly, 90 μl aliquots of cell suspensions (1×10^6 cells/ml for non-adhesive cells and 5×10^5 cells/ml for adhesive cells) were placed in 96-well plates and incubated with 10 μl of M/A (at different concentrations) for 24 hours, in a humidified atmosphere (at 37˚C, 5% CO_2). Hundred μl of CellTiter-Glo reagent (containing luciferin and luciferase) were added to each well and incubated as recommended by the manufacturer. The luminescence, produced by the luciferase-catalyzed conversion of luciferin into oxyluciferin in the presence of ATP, was detected using a microplate reader (TECAN Infinite® M1000, Vienna, Austria).

Analysis of mitochondrial superoxide. MitoSOX™ Red Mitochondrial Superoxide Indicator (MitoSOX; Molecular Probes, Invitrogen, Eugene, Oregon, USA) is a fluorogenic probe for highly selective detection of superoxide in the mitochondria of live cells. The probe is cell-penetrating and locates in the mitochondria. Once in the mitochondria, MitoSOX is oxidized by superoxide and exhibits red fluorescence (52). The probe is not oxidized by other ROS/RNS and its oxidation is prevented by superoxide dismutase (52).

Briefly, MitoSOX was dissolved in DMSO to 5 mM stock solution, which was diluted with Hank's Balanced Salt Solution (HBSS, containing Ca^{2+} and Mg^{2+}) to prepare 5 μM MitoSOX™ Red working solution on the day of the experiment. One mL of cells (1×10^6 cells/ml) was collected by centrifugation and the pellet was re-suspended in 1 ml of 5 μM MitoSOX. The samples were incubated for 30 min at room temperature, protected from light, washed three times with PBS using centrifugation, and finally re-suspended in 1 ml of PBS. The fluorescence intensity was detected immediately at λ_{ex}=510 nm and λ_{em}=605 nm, using a microplate reader (TECAN Infinite® M1000) or fluorescence confocal microscope (live cell imaging; magnification 60×) (Olympus DP73, Tokyo, Japan).

Analysis of mitochondrial membrane potential. Mitochondrial membrane potential was analyzed using tetramethylrhodamine methyl ester (TMRE) as described in Levraut *et al*. (2003) (53), with slight modifications. TMRE is cell-penetrating, cationic fluorophore, which accumulates in the mitochondrial matrix based on mitochondrial membrane potential. The fluorescence intensity is proportional to the mitochondrial potential and decreases upon depolarization of the mitochondrial membrane.

Briefly, 1000 μl of cells (1×10^6 cells/ml) were placed in 12-well plates. Five μl of TMRE (from 40 μM stock solution in DMSO) were added to each well. The samples were incubated at 37˚C for 30 min, washed twice with PBS using centrifugation, and finally re-suspended in 500 μl of PBS. The fluorescence intensity was detected immediately at λ_{ex}=550 nm and λ_{em}=575 nm, using a microplate reader (TECAN Infinite® M1000).

Succinate assay. Succinate level was analyzed using Succinate Assay Kit (Colorimetric) (Abcam, Tokyo, Japan). The analysis is based on a coupled enzyme reaction, which results in a colour product with maximum absorbance at 450 nm, proportional to the succinate concentration in the sample. Succinate was used as a standard.

Briefly, cells (1×10^6 cells per sample) were lysed in succinate assay buffer as described in the manufacturer's instruction booklet. Fifty μl (in duplicates) of each cell lysate were placed in 96-well plate and incubated with 50 μl of reaction mix-1 or 50 μl of reaction mix-2 (without succinate converter; blank sample) for 20 min at 37˚C, in the dark. Absorbance at 450 nm was recorded, using a microplate reader (TECAN Infinite® M1000). A blank sample was included to correct the NADH-dependent background absorbance.

Statistical analysis. All results are expressed as means±standard deviation (SD). Comparisons between the groups were performed using Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results and Discussion

The combination menadione/ascorbate markedly decreased the proliferation of cancer cells in a dose-dependent manner (Figure 2A, C, E – black columns). The effect was cytostatic at low/tolerable concentrations of M/A (≤3/300 μM/μM) and cytotoxic at high concentrations (≥5/500 μM/μM). M/A was not cytotoxic towards normal cells up to 5/500 μM/μM, but decreased their viability at high concentrations, especially at 20/2000 μM/μM (Figure 2A, C, E – gray columns). The cytotoxic effects of high concentrations of M/A on normal cells were much less pronounced than on cancer cells of the same origin. This is evidence for a targeted anticancer effect of M/A, as well as a clear cytostatic effect at doses that are absolutely harmless for normal cells and tissues.

M/A concentration of 5/500 μM/μM is critical for the transition from cytostatic to cytotoxic effect on the analyzed cancer cells. However, it should be noted that the concentration of 5 μM for menadione is considered crucial for its mitochondrial redox-cycling (48, 54). Chan *et al*. have reported that Complex-I bypass and ATP recovery in menadione-treated cells occurs only at concentrations below 5 μM, which is considered a threshold level for its beneficial effects in mitochondrial diseases (48). This suggests that the observed decreased growth and viability of M/A-treated cancer cells, could be the result of suppression of mitochondrial respiration. Cell viability and proliferation assay is based on the generation of a luminescent signal from luciferin/luciferase reaction, which depends to the amount of ATP synthesized in live cells.

The cytotoxicity of M/A on cancer cells was accompanied by impressive dose-dependent increase of mitochondrial superoxide – from 2-3 times (at 2/200 μM/μM of M/A) up to 8-15 times (at 20/2000 μM/μM of M/A) over the baseline level, observed in the respective non-treated cancer cells (Figure 2B, D, F – black columns). The steady-state level of mitochondrial superoxide was analyzed by MitoSOX™ Red Mitochondrial Superoxide Indicator and fluorescence spectroscopy. The dose-dependent overproduction of mitochondrial superoxide in M/A-treated cancer cells was also clearly illustrated by fluorescence confocal microscopy (Figure 3). In normal cells, M/A induced a relatively small dose-independent increase in mitochondrial superoxide (~2 times over the baseline) (Figure 2B, D, F – gray columns). A very good negative correlation was found between cell viability and mitochondrial superoxide in cancer cells (R=–0.85568), while the same correlation was significantly lower in normal cells (R=–0.64566) (Figure 2G, H).

M/A also decreased the mitochondrial membrane potential and succinate levels in cancer cells (Figure 4). Both effects were dose-dependent. The effect of M/A on succinate was clearly observed even at low/tolerable concentrations of M/A and 24-hours incubation. Depolarization of mitochondrial membrane and depletion of succinate in M/A-treated cancer cells is further evidence that mitochondria are the target of this combination.

Studies published so far suggest two mechanisms for the anticancer effect of menadione/ascorbate: (i) extracellular generation of hydrogen peroxide due to one-electron redox-

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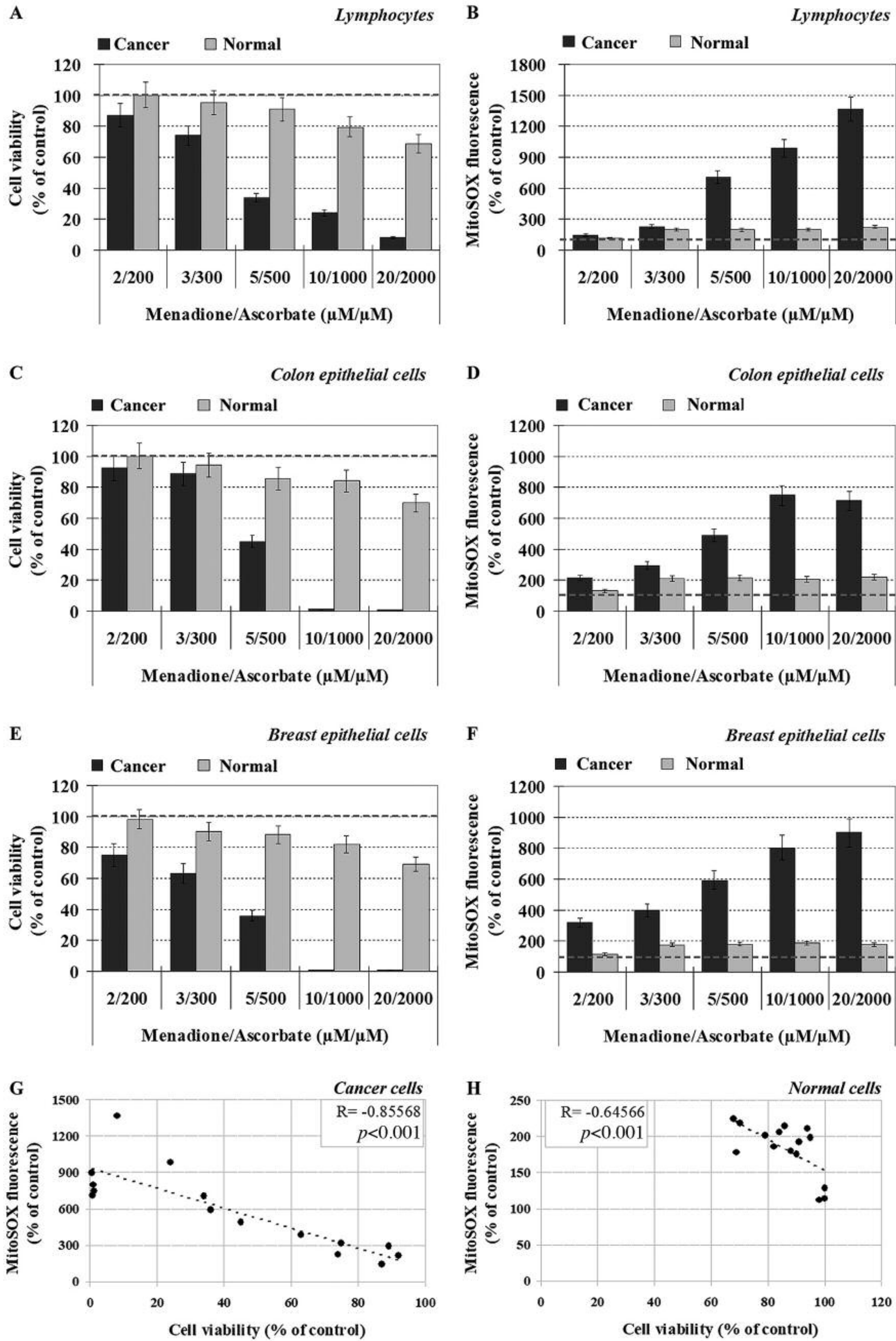
Figure 2. *Effects of menadione/ascorbate (M/A) on cell viability and mitochondrial superoxide after 24-h incubation in humidified atmosphere: (A, B) viability and mitochondrial superoxide in cancer (Jurkat) and normal lymphocytes; (C, D) viability and mitochondrial superoxide in cancer (Colon26) and normal (FHC) colon epithelial cells; (E, F) viability and mitochondrial superoxide in cancer (MCF7) and normal (MCF10A) breast epithelial cells. The number of cells in all samples at the beginning of each experiment was 1×10^6 cells/ml for lymphocytes, and 5×10^5 cells/ml for the other cell lines. Data are means±SD from three independent experiments. The red dotted lines indicate the level of control (non-treated cells). (G, H). Correlation analysis between cell viability and mitochondrial superoxide in M/A-treated cancer cells and normal cells, respectively (two-sample equal variance). R: Correlation coefficient.*


A
Lymphocytes
Cancer
Normal
Cell viability (% of control)
2/200
3/300
5/500
10/1000
20/2000
Menadione/Ascorbate (µM/µM)
B
Lymphocytes
MitoSOX fluorescence (% of control)
C
Colon epithelial cells
D
Colon epithelial cells
E
Breast epithelial cells
F
Breast epithelial cells
G
Cancer cells
R= -0.85568
p<0.001
Cell viability (% of control)
H
Normal cells
R= -0.64566
p<0.001

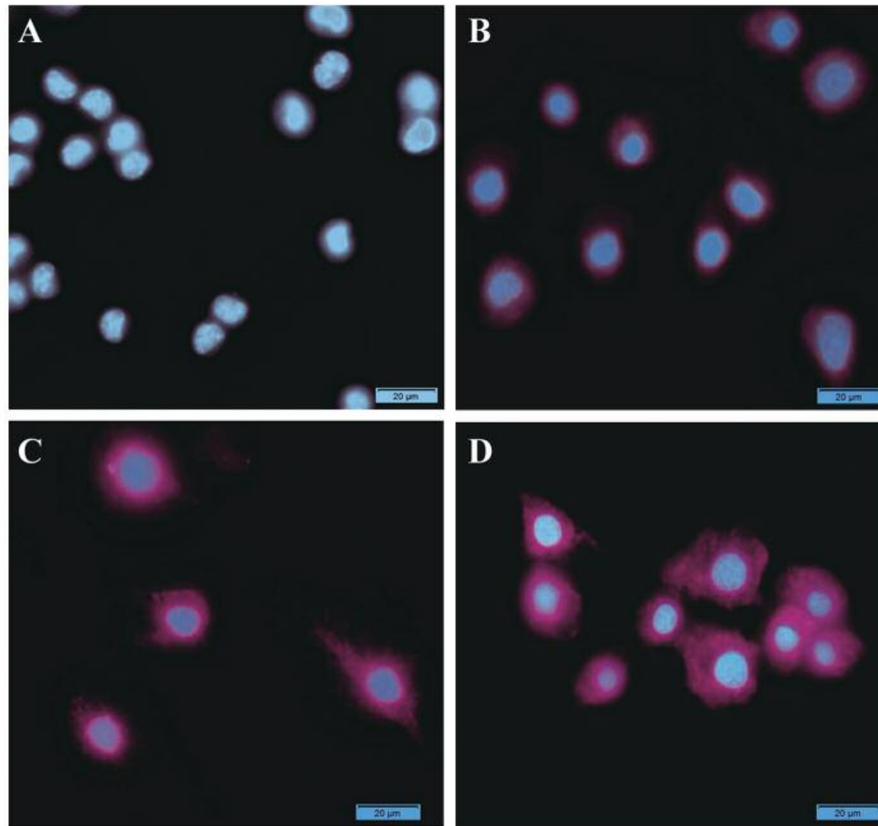


Figure 3. *Fluorescence imaging of mitochondrial superoxide in colon cancer cells (Colon26), analyzed after 24-h incubation, using MitoSOX™ Red Mitochondrial Superoxide Indicator and fluorescence confocal microscopy (live cell imaging): (A) untreated cells (control); (B) 3/300 μM/μM of M/A; (C) 5/500 μM/μM of M/A; (D) 10/1000 μM/μM of M/A. Representative fluorescence images are shown in the figure.*

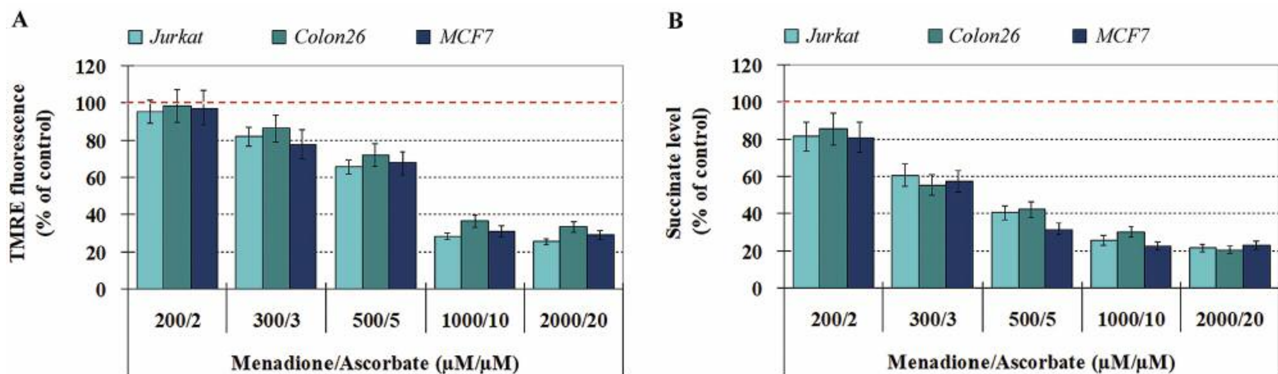


Figure 4. *Effect of menadione/ascorbate (M/A) on mitochondrial membrane potential (A) and steady-state level of succinate (B) in cancer cells, after 24-h incubation in humidified atmosphere. All data were normalized to 1×10^6 cells and presented as a percentage of the respective control (untreated cells), which was considered 100%. The red dotted lines indicate the level of controls. The data are means±SD from three independent experiments. TMRE: Tetramethylrhodamine ethyl ester.*

cycling of menadione/ascorbate and subsequent induction of oxidative stress, accompanied by activation of PARP1, inhibition of glycolysis, depletion of NAD^+ and ATP, and subsequent cell death (4, 30-32, 55); (ii) intracellular ("cytosolic") generation of hydrogen peroxide due to ascorbate/menadione redox-cycling and severe oxidative and replicative stress as a result of Fenton's reactions (34, 36, 37, 56).

However, both mechanisms are nonspecific (general) and cannot explain: (i) why M/A attacks cancer cells but not normal cells; and (ii) why the *in vivo* anticancer effects of M/A are demonstrated at significantly lower plasma concentrations than those inducing cancer cell death *in vitro* (1, 3). Cancer cells have a variety of mechanisms to control oxidative stress and survival, and they are much more resistant than normal cells (57, 58).

Our study demonstrates that M/A suppresses cancer cell growth and viability in a highly specific manner, without adversely affecting the viability of normal cells at pharmacologically attainable concentrations. The cytostatic/ cytotoxic effect of M/A in cancer cells is accompanied by:

- An extremely high production of mitochondrial superoxide in cancer cells, but not in normal cells of the same origin.
- A significant dose-dependent depolarization of mitochondrial membrane.
- A significant dose-dependent depletion of succinate.

The data clearly indicate that the anticancer effect of M/A is a result of a specific mechanism, that is tightly connected to the cancerous mitochondria. It is related to the induction of a severe mitochondrial oxidative stress in M/A-treated cancer cells. Such effect is not observed in normal cells of the same origin. M/A-treated normal cells are characterized by induction of mild oxidative stress, which seems to be controlled. Most likely this mild oxidative stress is a result of extracellular and cytosolic redox-cycling of menadione and ascorbate with production of hydrogen peroxide.

We assume two possible reasons for the specific overproduction of mitochondrial superoxide in M/A-treated cancer cells: (i) a direct impairment of mitochondrial ETC by compromising its functionality [mainly Complex-I and Complex-III that are known to produce superoxide (59, 60)]; and (ii) a specific mitochondrial redox-cycling of both substances, mediated by dysfunctional mitochondria, but not by the mitochondria of non-transformed cells.

The specific cytotoxicity of M/A towards cancer cells only can be also explained by the possibility of normal cells to convert menadione to menaquinone (vitamin K2) *via* UBIAD1-catalyzed prenylation (61). Down-regulation of UbiA prenyltransferase domain-containing protein 1 (UBIAD1), also known as transitional epithelial response protein 1 (TERE1), is a hallmark of majority of cancers (62). Therefore, conversion of menadione to vitamin K2 will be strongly suppressed in cancer cells. Vitamin K2 has been shown to exert two orders of magnitude lower cytotoxicity against cancer cells compared to menadione (63).

An important observation is that low/tolerable doses of M/A possess cytostatic (not cytotoxic) potential, but they induce essential metabolic changes in cancer cells such as: (i) decrease of succinate; (ii) depolarization of mitochondrial membrane; and (iii) specific overproduction of superoxide and severe oxidative stress in cancerous mitochondria only.

Succinate is considered one of the major oncometabolites (64, 65). It has been found that Krebs cycle metabolites (such as succinate, fumarate, itaconate, *etc*.) are coupled with non-metabolic signaling in cancer and immune cells, which is crucial for cancer progression and invasion (66, 67). In support, it has been suggested that suppression of succinate production in cancer cells and modulation of the immune response underlie the anticancer effect of relatively low/tolerable doses of M/A *in vivo*. The decrease of succinate can also explain the anti-inflammatory effect of M/A, described recently (68).

Our study and data published in the literature suggest that M/A has a great potential for beneficial anticancer effects and may increase the sensitivity and vulnerability of cancer to the conventional anticancer therapy, as well as to the immune system.

Conflicts of Interest

No potential conflicts of interest exist regarding this study.

Authors' Contributions

ZZ, RB, and TM conceived the idea for the study. ZZ and RB produced the first draft of manuscript. SS, RB, ZZ and KS conducted the experiments. TM, IA and TH were involved in the critical review of the drafts and final version. All Authors read and approved the final version of the manuscript.

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