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Exogenous coenzyme Q10 modulates MMP-2 activity in MCF-7 cell line as a breast cancer cellular model

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Abstract

Background/Aims: Matrix Metalloproteinases 2 is a key molecule in cellular invasion and metastasis. Mitochondrial ROS has been established as a mediator of MMP activity. Coenzyme Q₁₀ contributes to intracellular ROS regulation. Coenzyme Q₁₀ beneficial effects on cancer are still in controversy but there are indications of Coenzyme Q₁₀ complementing effect on tamoxifen receiving breast cancer patients.

Methods: In this study we aimed to investigate the correlation of the effects of co-incubation of coenzyme Q10 and N-acetyl-L-cysteine (NAC) on intracellular H₂O₂ content and Matrix Metalloproteinase 2 (MMP-2) activity in MCF-7 cell line.

Results and Discussion: Our experiment was designed to assess the effect in a time and dose related manner. Gelatin zymography and Flowcytometric measurement of H₂O₂ by 2',7'-dichlorofluorescein-diacetate probe were employed. The results showed that both coenzyme Q10 and N-acetyl-L-cysteine reduce MMP-2 activity along with the pro-oxidant capacity of the MCF-7 cell in a dose proportionate manner.

Conclusions: Collectively, the present study highlights the significance of Coenzyme Q₁₀ effect on the cell invasion/metastasis effector molecules.

Introduction

Matrix Metalloproteinases (MMPs) belong to a multi-gene family of enzymes that are mainly involved in physiological alterations of Extracellular Matrix (ECM). MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) are key enzymes in the degradation of ECM collagen, therefore ECM remodeling. MMPs are secreted in the form of inactive zymogens, which proceed to activation by various pathways. Both expression and activity of MMPs are regulated by diverse endogenous and exogenous stimuli (e.g. signals from cell-to-cell interaction and cellular microenvironment) [1].

The function of MMPs (especially MMP-2) is extensively studied because of their pivotal role in pathogenesis of certain diseases. MMP-2 has long been

established as a marker in predicting phenotypes of tumor including growth, progression and metastasis as well as the dysregulated angiogenesis that is associated with these events. As a result, MMPs have come to represent important therapeutic and diagnostic targets for the therapy and diagnosis of human cancers in which their increase presages invasive phenotypes [2] and [3].

MMPs are expressed in form of latent MMPs (proMMP or zymogen) in response to exogenous signals, such as growth factors, cytokines, chemical agents like phorbol esters, physical stress, oncogenic transformation, cell-cell and cell-matrix interactions. ProMMPs are activated by disruption of the cysteine-zinc bond (switch) or by cleavage of the propeptide by proteinases such as plasmin, trypsin, kallikrein, chymase, and mast cell tryptase. Some latent MMPs can also be activated by another MMP. The disruption of the cysteine switch by different organic and inorganic compounds, like

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organomercurials, SH-reactive agents, reactive oxygen and detergents results in autocatalytic cleavage of the propeptide and a conformational change into catalytically active form [4].

As a consequence of the use of oxygen in aerobic respiration, oxygen radicals are naturally produced in all mammalian cells. Superoxide is generated within the mitochondria and is sequentially reduced to hydrogen peroxide (H₂O₂) and hydroxyl radicals. These radicals damage DNA, producing the mutations that initiate tumors and sustain progression.

Human tumor cell lines *in vitro* produce ROS at a far greater rate. Markers for the constitutive oxidative stress have been detected in samples from *in vivo* breast carcinomas and cell lines. 8-Hydroxy-2'-deoxyguanosine, one of the major oxidatively modified DNA base products, is almost ten times more prevalent in invasive ductal breast carcinoma cells than in normal control samples from the same patient [5]. The observed increase of ROS in transformed cells is not only the incendiary of further oxidative damage but also a mediator of certain pathological signal transduction pathways. Several important signal transduction pathways such as MAPK, PI3K, Rho-GTPase and Smads cascades, are known to mediate transcriptional regulation of metastasis-related genes such as integrins and MMPs [6].

As a consequence to the general knowledge of ROS implications in cancer, several studies have examined the effect of individual and combinational antioxidant dietary supplements on cancer outcome [7]. Despite equivocal conclusions in certain areas, there are supportive publications that antioxidant regimens would complement tamoxifen therapy in breast cancer patients in various aspects [8].

Enthusiasm for introducing new phytochemicals and supplements as potential therapeutic or preventive agents for cancer and other pathologic conditions has propelled researchers to study the behavior of key components of malignancy in the presence of these antioxidants. Therefore, evidence is rapidly mounting on the effect of various novel antioxidants on MMPs activity/expression in such studies [9] and [10]. However, the majority of recently introduced antioxidants are hydrophilic. This property delimits certain antioxidant applications, namely membrane passage and membrane protections.

Among four major groups of natural lipid-soluble antioxidants (carotenoids, tocopherols, estrogens and coenzyme Q) only Coenzyme Q is mostly present in reduced form. Coenzyme Q, in addition to membrane lipids, protects proteins and DNA due to its ubiquitous nature [11]. A lipid-soluble, endogenous, membrane-protective antioxidant, Coenzyme Q₁₀ has been frequently employed in studies on vascular and atherosclerotic

diseases. Consequently, its multiple roles in health and disease are still under vast examination and review [12]. Its multiple-reductive enzymatic mechanisms and presence in mitochondrial membrane endows Coenzyme Q₁₀ an important place in the natural antioxidant defense system. Since oxidative stress is a predominating event in most pathological conditions, it is assumed that in those conditions, Coenzyme Q amount increases as an adaptive response to neutralize the pathogenic generated ROS. Moreover, in pathologic conditions, the majority of coenzyme Q is in reduced form, showing that the reductive enzymatic mechanism is not a limiting factor [13].

In addition to the properties mentioned above, recent findings in coenzyme Q₁₀ capabilities to improve the survival of progressed cancer patients had made coenzyme Q₁₀ a potential candidate in our study [14].

Several authors have focused on effects of coenzyme Q₁₀ on breast cancer patients generally supporting the idea of ameliorating effects of coenzyme Q₁₀ in the disease [15,16] and [17]. Alternatively, studies show that baseline plasma Coenzyme Q₁₀ levels are a powerful and independent prognostic factor that can be used to estimate the risk for pancreatic and melanoma progression [18] and [19]. Coenzyme Q₁₀ effect on MMPs has not been documented previously. Therefore, in this study we aim to elucidate the effect of exposure of a breast cancer cell line to exogenous Coenzyme Q₁₀ on expression/activity of MMP in the microenvironment. To investigate this effect, we employed MCF-7, a renowned model of cancerous breast ductal epithelium [20].

Parallel to coenzyme Q₁₀, we manipulated monothiol antioxidant content. It has been proven that supplementation of certain thiol antioxidants has inhibitory effect on MMP activity [21]. Therefore, we investigated the effects of alterations in monothiol antioxidant content by adding NAC and Buthionine Sulfoximine (BSO), a substantial competitive inhibitor of γ -glutamylcysteine synthetase (γ -GCS). BSO is a specific inhibitor of γ -GCS and is commonly employed to deplete the GSH content of a cell [22] and [23]. We assumed that this would also help us to obtain a better understanding of the regulatory effects of monothiol antioxidants (GSH and NAC) on MMP-2 gelatinolytic action in the MCF-7 breast cancer cell line. To our knowledge, there is a paucity of publication in which the effect of BSO on MMP-2 was investigated.

Methods

Cells and reagents

MCF-7 cell lines derived from pleural metastasis of a ductal human breast carcinoma were obtained from the Iranian National Cell Bank. DMEM, L-glutamine, FCS

and antibiotics were purchased from Gibco. 2',7'-dichloro-fluorescein-diacetate, Sodium bicarbonate, NAC, BSO and Coenzyme Q10 were from Sigma. The liquids were from Merck. After culturing HT1080 in a serum-free medium for 48 hours, we collected the supernatants and used them as a molecular weight marker of pro-MMP-2 and MMP-2 as described in literature [24].

Preparation of the reagents

NAC was dissolved in DMEM in serial concentrations from 2 mM to 0.5 mM. Coenzyme Q₁₀ was dissolved in 40° ethanol and added to DMEM in a sublethal concentration of ethanol in 1×, 0.5 ×s and 0.25 ×s of 122 μM concentrations. DMEM with similar concentrations of ethanol was prepared for the control wells.

Preparation of MCF-7 samples

MCF-7 cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, antibiotics and 2 mM L-glutamine at 37°C in a 7% CO₂-humidified atmosphere. Cells were routinely passaged twice a week at a 1:10 split ratio. From the 48 h cultured cells, 2 × 10⁵ cell were seeded into each well of a 24-well culture plate in the presence of 5% FCS, and incubated for 6, 15, 24 and 42 hours. By completion of the incubation time, the media over the cells were aspirated and underwent flowcytometric analysis. A portion of the media was kept in -70°C for further analysis.

Fluorescent Measurement of H₂O₂ content

Hydrogen peroxide as a marker of cellular ROS production was examined by a method dependent on intracellular deacylation and oxidation of 2', 9'-dichlorodihydrofluorescein diacetate (DCHF-DA) to the fluorescent compound 2', 7'-dichlorofluorescein (DCF). This probe is highly reactive with H₂O₂ and has been used in evaluating ROS generation in mammalian cells [25].

To assess the levels of intracellular ROS, flow cytometric analysis was performed using the oxidative-sensitive probe (DCFH-DA) as described [26]. Cells were incubated for 30 minutes at 37°C in the presence of 5 mM DCFH-DA. After incubation, the cells were transferred to an ice bath, and the formation of 2', 7'-dichlorofluorescein (DCF) was analyzed by flowcytometry using a Becton Dickinson Facscan with excitation and emission settings of 495 and 525 nm, respectively. Ten thousand viable cells from quadruple samples were analyzed.

Gelatin Zymography

To assess the expression/activation of MMP-2, Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) substrate-embedded enzymography (zymography) was carried out by a modification of the

method [27]. Briefly, SDS-PAGE gels were prepared for mini-gels from 30:1 acrylamide/bis with the incorporation of gelatin (1 mg/ml) before casting. The gelatin gels were routinely 7.5% acrylamide. Various denatured but non-reduced samples and standards were then run into the gels at constant voltage of 150 V under non-reducing conditions. When the dye fronts reached a point approximately 0.5 cm from the bottom of the gels, the gels were removed and subjected to the following washing protocol: twice for 30 minutes each time in 50 mM Tris buffer (containing 1 mM Ca²⁺ and 0.5 mM Zn²⁺) with 2.5% Triton X-100; once for 5 minutes in Tris buffer alone and finally overnight in Tris buffer with 1% Triton X-100 [28].

The molecular weight marker of pro-MMP-2 and MMP-2 and inhibitors were added to the overnight wash. Then the gels were stained with Coomassie Brilliant Blue 250-R. After destaining, zones of enzyme activity showed up as regions of negative staining. Relevant controls included samples incubated with EDTA (MMP inhibitors), PMSF (serine proteinase inhibitor), E64 (cysteine proteinase inhibitor) and pepstatin (aspartic proteinase inhibitor). HT1080 culture supernatant was used as the molecular weight marker of pro-MMP-2 and MMP-2 [29].

Statistical analysis

Values were expressed as MMP-2 activity percentage of control samples, means and standard errors. For effectiveness and dose-response studies, differences among groups were evaluated with two-way analysis of variance (ANOVA). If the F-value was significant, groups were then compared at each dose by one-way analysis of variance (ANOVA) followed by Dunnett's C test (non-homogenous variances) and Schaffe (homogenous variances) Post Hoc analysis. Bivariate correlation one-tailed Spearman tests were performed for the determination of correlation between H₂O₂ vs. supplement concentration and H₂O₂ vs. MMP-2 activity. If a p value was less than 0.05, a difference was considered significant. Statistical analysis was carried out using SPSS 16.0 program.

Results

In order to determine the effect and correlation of the dose of NAC, BSO and coenzyme Q₁₀ on H₂O₂ content variations, we measured the extracellular H₂O₂ of maximum, half and quarter-maximum dose on MCF-7 by the DCHF flowcytometric method. The experiment was repeated for different periods of exposure. Results were then compared to the control (null drug) by subtracting the emission of controls (null drug) from the emission of the samples to calculate the variations of H₂O₂ content.

To study the effect of different doses of NAC and coenzyme Q₁₀ in different time courses on MMP-2 activity of MCF-7 cell line, standard gelatin zymography was performed, and the density of the developed bands were measured and compared with the control sample in each dose and exposure time via post hoc test. Finally, we measured the correlation between H₂O₂ content and MMP-2 activity, and drug dose and MMP-2 activity by the Spearman correlation test.

In the case of NAC, correlation was observed between NAC concentration as a thiol antioxidant and the amount of H₂O₂ content in different exposure time courses. Flowcytometric measurements of H₂O₂ by emissions of DCHF revealed large correlation (-0.710 in the first 15 hours -0.775 after 24 hours all $p < 0.05$) with the amount of thiol antioxidant NAC. Results for 6 and 42 hours show a large correlation, however they were not statistically significant.

In 6 and 15 hours of supplementation, mean MMP-2 activity showed significant ($p < 0.05$) difference in all groups compared to the control group. In full dose NAC vs. control, MMP-2 activity showed a difference of 58.97%, 52.4% and 28.16% in 6, 15 and 42 hours respectively. Coefficients of -0.946, -0.621 and -0.650 correlation coefficient in 6, 15 and 42 hour respectively ($p < 0.05$) were calculated by correlation analysis. Also a positive medium correlation was observed between content and MMP-2 activity ($p < 0.05$).

Following Ortega et al experiments on cell lines, in this study, we employed 0.2 mM concentration for BSO to effectively inhibit γ -GCS and to decrease the intracellular content of GSH [30].

The results showed that merely in the first 6 hours of exposure, BSO was effective to increase the content of the cell. At 6 hours, the flowcytometric analysis showed a positive 0.798 correlation coefficient between H₂O₂ increase and dosage ($p < 0.05$). In these samples, only the concentration of 0.1 mM had a significant increase (20%, $p < 0.05$) of MMP-2 activity in comparison with control samples by Post Hoc analysis. Although incubation for 6 and 15 hours showed significant increase ($p < 0.05$) on MMP-2 activity in ANOVA tests, incubation for more than 15 hours had no significant effect of MMP-2 gelatinolytic activity. (Samples incubated for 24 and 42 hours did not show significant dose-responsive correlation with either H₂O₂ content or MMP-2 activity.)

Coenzyme Q₁₀

In this study we used 122 μ M of Coenzyme Q₁₀, which according to pilot studies had shown no apoptotic or cytotoxic effect on the cells. DCF fluorescence measurements revealed that there is a strong negative correlation between Coenzyme Q₁₀ dose and H₂O₂ content.

Accordingly, 24 and 42 hours of MCF-7 cells exposure to coenzyme Q₁₀, illustrated a significant decline of H₂O₂ content with a correlation coefficient of -0.926 and -0.739 (p value < 0.05) respectively (Figure 1).

Samples supplemented merely with Coenzyme Q₁₀ for 24 and 42 hours showed significant change in MMP-2 gelatinolytic activity ($p < 0.05$) (Figure 2). In 24 hours of supplementation, full and quarter dose showed significant ($p < 0.05$) differences compared to control group mean. Post hoc analysis of samples after 42 hours of incubation revealed significant ($p < 0.05$) decrease in mean MMP-2 activity when full dose and quarter dose Coenzyme Q₁₀ was used. Interestingly, full dose in 42 hours was the only group among Coenzyme Q₁₀ supplemented samples that showed less MMP-2 activity compared to control group mean. In correlation analysis, both 24 hours and 42 hours showed large negative correlations (-0.650 and -0.946, $p < 0.05$) respectively (Figure 2. Panel A).

Finally, statistical analysis of H₂O₂ content and MMP-2 activity revealed that there are good correlations (24 hours: 0.772; 42 hours: 0.804, $p < 0.05$) between H₂O₂ content and MMP-2 gelatinolytic activity (Figure 2. Panel B). In other words, this shows that when supplemented with Coenzyme Q₁₀, extracellular activity of MMP-2 in MCF-7 cell line is proportionate to H₂O₂ concentration.

After 24 and 42 hours of exposure to Coenzyme Q₁₀, both groups at full dose showed significant ($p < 0.05$) 22 and 24% decrease compared to control group (illustrated in Figure 3).

Discussion

In the current study, MMP-2 activity as a marker of malignancy was investigated in the presence of various substances that have been shown to play a pivotal role in maintaining the redox status of the cell (namely coenzyme Q₁₀ and GSH). Hydrogen peroxide, as an abundant ROS in the cell, was the target of manipulation and measurement.

Nelson et al have carefully reviewed aspects of MMPs activation and regulation by redox status. ROS is considered one of the major routes of MMP regulation through oxidation of the zinc-cysteine switch. It is clear that ROS, particularly H₂O₂, play fundamental roles in modulating the various kinase cascades, phosphatase activity, transcription factor binding and MMP latency. Although there are many likely candidates, the specific signaling molecules that are directly modified by ROS and lead to MMP expression have yet to be clearly determined [31].

Sandhya and Mishra had used a concentration of 100 μ M for NAC directly on MCF-7 as a suitable non-cytotoxic concentration. In the present experiment, we

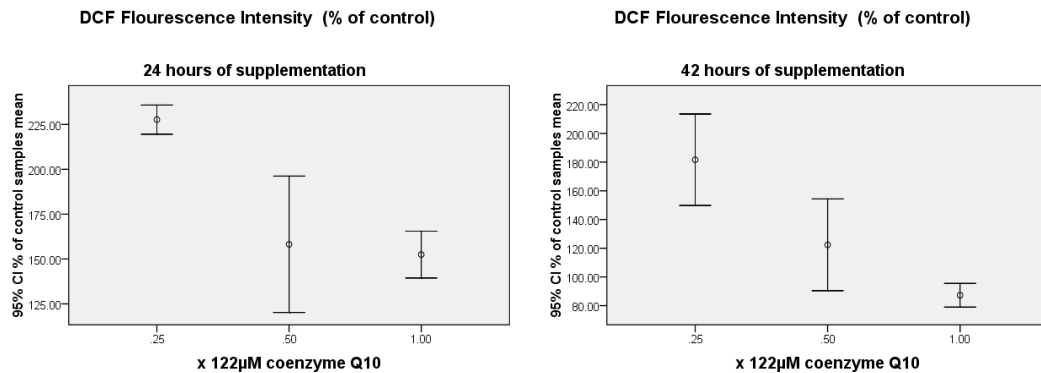


Figure 1 Measurements of DCF fluorescence as a marker of Hydrogen Peroxide content in the medium after incubating for 24 and 42 hours with serial dilutions of Coenzyme Q₁₀. The numbers represent the percentage of control samples mean.

used serial dilutions of 100 µM to study the dose dependency and the correlation between supplement doses vs. H₂O₂ concentration [32].

In almost all exposure times, large correlation was found between NAC and H₂O₂ concentrations (15 and 24 hours: $p < 0.05$, 6 and 42 hours: $p > 0.05$). ROS was decreased in dose-dependent fashion. This finding was majorly consistent with previous studies. MMP-2

gelatinolytic activity showed a significant ($p < 0.05$) decrease in all incubation times; and according to our observations this decrease was also in a dose-responsive manner in almost all of the incubation times.

Interestingly, regarding MMP-2 activity change with NAC pretreatment, highest mean difference between groups and the largest correlation (-0.946 , $p < 0.05$) was observed in the first 6 hours of incubation. This

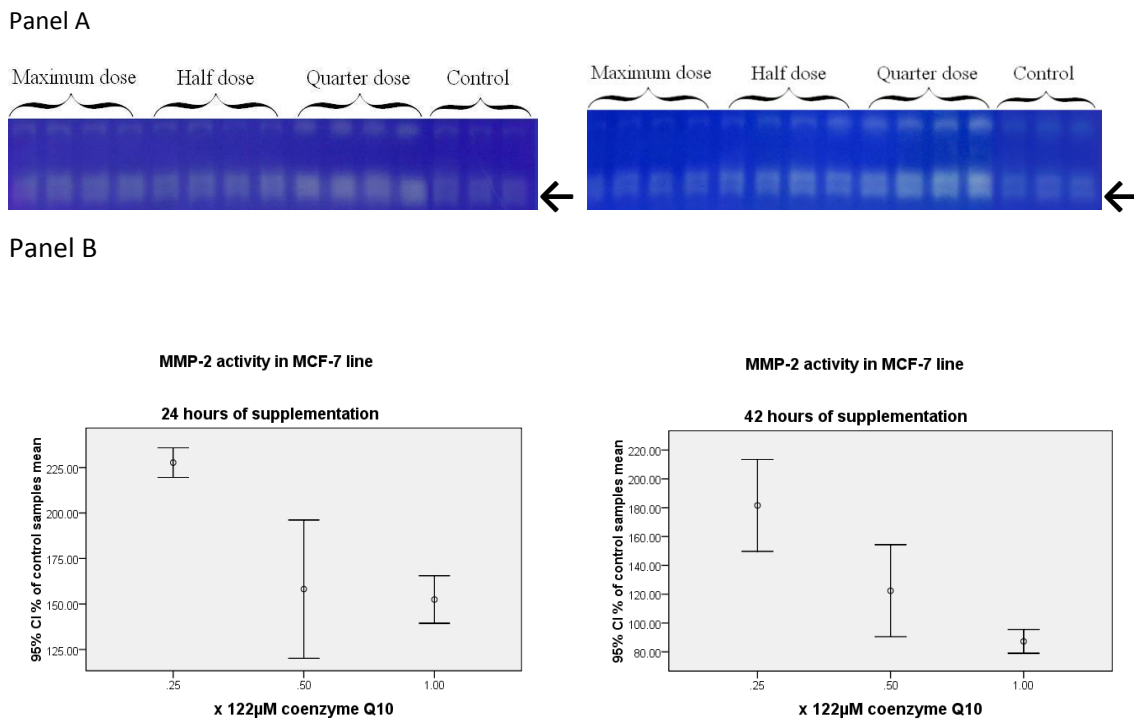
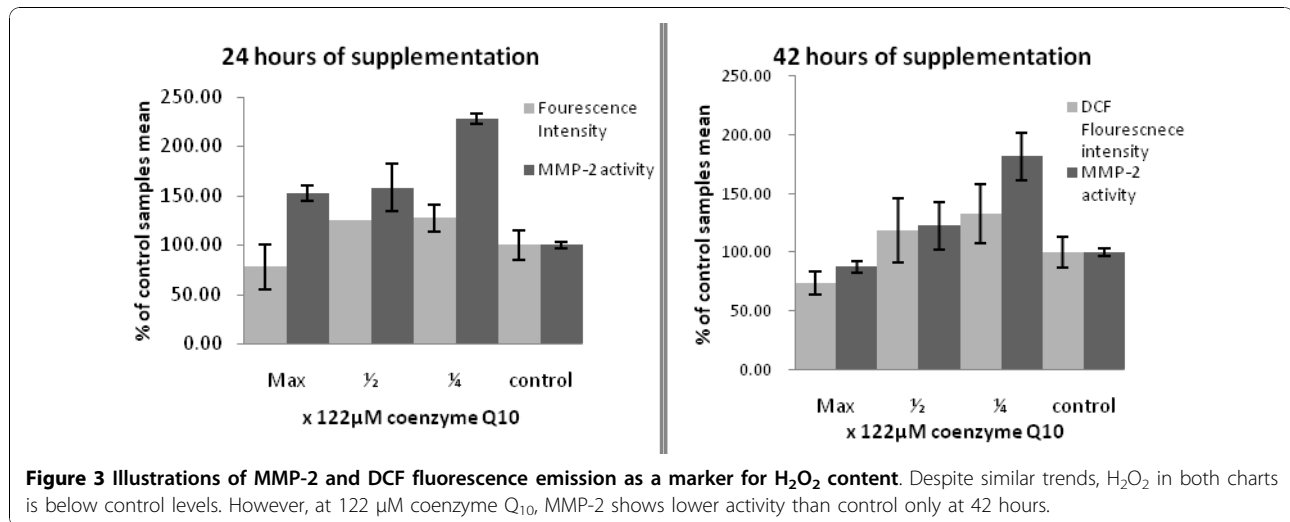


Figure 2 Panel A. Zymogram charts representing gelatinolytic activity measured by standard gel zymography in 24 and 42 hours of exposure of cells to coenzyme Q₁₀. The arrows point to the row of bands of gelatin digestion. Double bands represent 72 KD MMP-2 and 92 KD proMMP-2. **Panel B**. Statistical analysis presentation of corresponding zymograms: numbers on the y axis represent the percentage of control samples mean.



observation as well as the large negative correlations between NAC and H₂O₂ could indicate that in MCF-7 cell the effect of NAC tends to be more radical scavenging rather than biological inhibiting or inducing. This idea could be supported by the observed positive correlation between H₂O₂ and MMP-2 activity ($p < 0.05$). These findings are partly consistent with reports of Voronkina et al who stated that the effect of NAC on the activities of MMP secreted by normal (3T3) and transformed (3T3-SV40) mouse fibroblasts by supplementation with NAC for 2-6 hours completely inhibited MMP-2 and MMP-9 activity in both cell lines. They speculated that the inhibition was independent of NAC concentration at the range of 1-10 mM [33].

Similarly, the decline of MMP-2 activity in the presence of NAC could be due to the protection of cysteine switch by this monothiol. This postulation is congruent with observations of Bogani et al who used 5 mM NAC [34].

As the most important thiol antioxidant components, glutathione is a tri-peptide involved in converting H₂O₂ to water. It functions very closely to NAC which is itself a precursor of glutathione. Its roles have undergone extensive research in areas of prevention and therapy of cancer; it has been shown that MMP-1 mRNA and MMP-2 and -9 activities are inhibited by glutathione in human fibroblasts and liver allografts, respectively. Glutathione has been shown to induce MMP-1 and -2 expressions in human heart fibroblasts. Conversely, in transformed fibroblasts, glutathione inhibited MMP-1 and -2 expressions and increased TIMP-2 expression. Altogether these findings advocate the role of oxidative stress in cancer [35]. N-acetylcysteine has been reportedly able to block the TNF- and high-glucose mediated induction of MMP-9 mRNA and protein as well as the activities of MMP-2 and -9 in human fetal membranes [36].

To investigate its GSH depleting effect on activity/expression of MMP-2, we pretreated cells with BSO (a glutathione synthesis inhibitor). BSO prooxidant behavior was only significant in the first 6 hours of incubation (BSO vs. H₂O₂). Also in this period, a significant 20% increase ($p < 0.05$) of MMP-2 activity was observed. Incubations more than 6 hours did not show any significant change in either H₂O₂ or MMP-2 activity. It is possible that after 6 hours, GSH synthesis had resumed by the translation of enzyme γ -glutamylcysteine synthetase or compensation by increasing the rate of antioxidant recycling or other antioxidants synthesis.

Brennisen and Wank et al have proposed that the inhibition of catalase by aminotriazol (ATZ), inhibition of GSHPx by buthionine sulfoximine (BSO), and blocking the Fenton reaction by the iron chelator desferrioxamine (DFO) in concert led to an increase in steady-state MMP-1 mRNA levels, possibly dependent on intracellular H₂O₂ increase [37]. In a later study, Wenk et al reported increase in basal MMP-1 expression in consequence of H₂O₂ increase as the mediator of cell signaling [38]. From these observations it could be concluded that BSO acts on MMP as a secondary targets via alteration of ROS content of the cell.

Coenzyme Q₁₀ has been suggested as the most important antioxidant against H₂O₂. Since the cytoplasmic membrane is permeable to H₂O₂, it is to be assumed that H₂O₂ measured in the microenvironment represents an approximation of intracellular content of H₂O₂. In addition, supplemented Coenzyme Q₁₀ has been shown to consist a fraction of Mitochondrial Coenzyme Q₁₀ [13]. Moreover, Coenzyme Q₁₀ action against other elements of acute inflammation has been shown in in-vitro studies [39].

To our knowledge, this is the first time that the effect of Coenzyme Q₁₀ on MMP activation/expression has

been published, and in this sense, we have found no other parallel experiment to compare our observation.

According to our observations, supplementation with Coenzyme Q₁₀ increased the H₂O₂ content of cellular environment in 6 and 15 hours of incubations at all doses (data not shown). After 24 and 42 hours of exposure to Coenzyme Q₁₀, both groups at full dose showed significant ($p < 0.05$) 22 and 24% decrease compared to control group (illustrated in Figure 3), respectively. We assume two mechanisms could be accounted for this phenomenon. First, it could be due to oxidative stress caused by high dose exposure of exogenous Coenzyme Q₁₀. As indicated previously in this text, Coenzyme Q₁₀ freely passes through membranes and, to certain extent, would disrupt the delicately tuned superoxide anion/H₂O₂ signaling pathways [40,41] and [42].

Therefore, longer exposures were needed for the cells to adapt to the new redox state. Secondly, it is well known that Coenzyme Q₁₀ possesses pro-oxidant properties which, in turn, greatly contribute to sustaining the physiological redox potential of the cytoplasm and membranes. Accordingly, generating the superoxide anion/H₂O₂ as second messengers in signaling systems are affected [41]. Our data suggest that pro-oxidant behavior of Coenzyme Q₁₀ could have contributed to the increased H₂O₂ content observed. It may be possible that in the case of incubation time over 24 hours, enough time was given to the cells to establish a new equilibrium, thus decrease the overall H₂O₂ content of the ECM. It is to highlight that due to their molecular properties, both Coenzyme Q₁₀ and H₂O₂ would face little hindrance for passing the lipid membranes.

In addition to widely accepted effects of H₂O₂ on MMP activity, causal role of H₂O₂ and quelling role of antioxidants on the expression of MMP mRNAs have been frequently demonstrated in numerous original reports and reviews [37] and [42]. In line with these findings, we assume similar mechanisms may have played a role in the current study. The studied novel modulatory effect of Coenzyme Q₁₀ on MMP activity/expression might be mainly due to Coenzyme Q₁₀ interaction with ROS mediators. However, further expression studies need to elucidate the exact mechanisms of this interaction.

Conclusions

Taken together, the present study highlights the significance of Coenzyme Q₁₀ effect on the cell invasion/metastasis effector molecules.

List of abbreviations

MMP: matrix Metalloproteinase; ROS: reactive Oxygen Species; NAC: N-acetyl cysteine; BSO: buthionine sulfoximine; DCFH-DA: 2', 9'-dichlorodihydrofluorescein diacetate; TGFβ: transforming growth factor β; HGF: hepatocyte growth factor; TPA: 12-O-tetradecanoylphorbol-13-acetate; MAPK: mitogen-activated protein kinases; PI3K: phosphoinositide 3-kinase;

VEGF: vesicular endothelial growth factor; DMEM: dulbecco's minimum essential medium.

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Authors' contributions

MB, Sh Kh and PP: participated in the study design, carried out the analyses and drafted the Manuscript. **MP, MR Kh, HM and SGN** Participated in study design and coordination and Helped to draft manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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