



Nicotine effects on enzymatic antioxidant defenses in human breast and ovarian cell lines

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ABSTRACT

Introduction: Nowadays, the prevalence of nicotine abuse among women has increased dramatically. In the current study, we aimed to investigate the effect of nicotine exposure on breast MCF-7 and ovarian OVCAR-3 cell lines for assessing the toxicity of nicotine in the cells of these organs.

Methods: The MCF-7 and OVCAR-3 cells were treated with increasing nicotine concentrations ranging from 0 (control), 10^{-11} , 10^{-8} and 10^{-6} M for 24h. Effect of nicotine treatments on major antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), cellular levels of glutathione (GSH) and malondialdehyde (MDA) were monitored.

Results: We showed that the CAT activity in MCF-7 cells increased only at 10^{-6} M dose of nicotine. The GPx and GR activity was decreased at 10^{-8} and 10^{-6} M of nicotine in MCF-7 cells, but in OVCAR-3 cells, this decrease was significant only at 10^{-6} M dose of nicotine. Reduced GSH decrease was statistically significant only at 10^{-8} and 10^{-6} M of nicotine in MCF-7 cells; otherwise, in OVCAR-3 cells, this decline was significant only at 10^{-6} M of nicotine. Nicotine at 10^{-8} and 10^{-6} M concentration caused a significant increase in MDA levels in MCF-7 cells.

Conclusion: This study showed that breast MCF-7 cells are more vulnerable than ovarian OVCAR-3 cells against nicotine-induced oxidative toxicity.

Keywords:

Nicotine
Antioxidant enzymes
Oxidative stress
MCF-7 cells
OVCAR-3 cells

Introduction

Nicotine, the substantial toxic constituent of cigarette smoke, has various side effects on our body's cellular functions (Delijewski et al., 2014; Zal et al., 2020). It has been shown that nicotine through induction of reactive oxygen species (ROS) shows its toxicity in human cells (Yarahmadi et al., 2017). These ROS initiate and promote oxidative damage to cells in the form of lipid

peroxidation, changes in antioxidant enzyme status and finally, cell death (Muthukumaran et al., 2008; Yarahmadi et al., 2018). The human body's defense against ROS harmful effects are mediated through enzymatic and non-enzymatic antioxidants. The enzymatic antioxidant defenses in human cells consist of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Aghagolzadeh et

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al., 2017; Hasanpour et al., 2018). Non-enzymatic antioxidants, including glutathione (GSH), antioxidant vitamins such as A, E and C, are other defense mechanisms for protecting cells against the harmful effect of ROS (Alamdari et al., 2020a; Birben et al., 2012).

Previous *in vitro* and *in vivo* experiments proved that nicotine administration resulted in a prooxidant-antioxidant imbalance in cellular and animal models (Sudheer et al., 2005; Sudheer et al., 2007). Increased oxidative stress and consequent lipid peroxidation has been linked to have a significant role in nicotine related diseases like cancer (Bartsch and Nair, 2006; Hecht, 2002). It has been demonstrated that smoking is associated with an increased risk of cancers such as lung, breast, ovary, bladder and so on (Dasgupta et al., 2009; Djordjevic et al., 2000). It is estimated that nearly 250 million women worldwide smoke cigarettes and more than a million uses smokeless tobacco products containing nicotine (Sieminska and Jassem, 2014). Because of the increasing use of cigarettes among women globally, we decide to examine the adverse effects of nicotine on breast MCF-7 and ovarian OVCAR-3 cells for assessing the toxicity of nicotine in the cells of this organ. The purpose of the current study was to evaluate the effect of nicotine on enzymatic antioxidant defenses and also malondialdehyde (MDA) as lipid peroxidation maker in human MCF-7 and OVCAR-3 cells in an *in vitro* model.

Material and methods

Materials

Nicotine was obtained from Sigma Chemical Co (Poole, Dorset, UK); cell culture material including RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin were from Gibco-BRL (Paisley, UK). BSA total protein assay kit was purchased from Bio-Rad (Hercules, California, USA). Nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA) and other material were from sigma unless mentioned.

Cell culture procedure and treatment

The MCF-7 and OVCAR-3 cell lines were obtained from the cell bank of Pasteur Institute of Iran. The cells were cultured in the following condition: RPMI-1640 medium, 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin-streptomycin and then put in 5% CO₂ humidified incubator at 37°C. All experiments were done only at

cells with a minimum of 70% confluences. Then 5×10⁵ cells were seeded in a culture flask (25cm²) and incubated with increasing nicotine concentrations ranging from 10⁻¹¹ to 10⁻⁶ M for 24h.

Cell viability assay

To assess the viability of MCF-7 and OVCAR-3 cells treated with different nicotine concentrations, an assay was carried out using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as previously described by Mosmann (1983). The MTT assay is a colorimetric assay that relies on the conversion of yellow tetrazolium bromide (MTT) to purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. First, cells in a 96-well plate were incubated with 0, 10⁻¹², 10⁻¹⁰, 10⁻⁸, 10⁻⁶, 10⁻⁴ and 10⁻² M nicotine for 24h at 37°C. Then, cells were incubated with MTT (0.5mg/ml) dissolved in serum-free medium. After 3.5h incubation, 100µl DMSO was added to dissolve the formazan crystals and then, absorbance was determined at 570/650nm wavelength using an ELISA reader (Bio-Rad, USA). Cell viability was determined as the ratio of absorbance of treated cells to that of untreated cells that served as a control.

Measurement of CAT activity

For the determination of CAT activity, we used a method previously described by Aebi (1984). In this method, we spectrophotometrically assessed the decomposition of H₂O₂ to H₂O and O₂. Then, enzyme activity was shown as mmol H₂O₂ consumed/min per mg MCF-7 and OVCAR-3 cell lysate protein by a molar absorptivity of 43.6 mol L⁻¹ per cm.

Measurement of GPx activity

The GPx activity has been evaluated by the method described by Fecondo and Augusteyn based on monitoring continuous substitution of GSH from its oxidized form G-S-S-G in the presence of enzyme GR (Fecondo and Augusteyn, 1983). We also used Na₂ salt of NADPH to assess GPx activity according to a previous publication (Mostafavi-Pour et al., 2008). Furthermore, the GPx enzyme activity in the MCF-7 and OVCAR-3 cell lysate was showed as µmol of NADPH oxidized/min/mg of cell protein using a molar absorptivity of 6.22×10⁶ M⁻¹cm⁻¹ for NADPH. One unit of GPx is determined as U/mg of cell protein.

Measurement of GR activity

The enzyme GR activities were estimated through a method formerly reported by Racker and Carlberg with little changes (Carlberg and Mannervik, 1985; Racker, 1955). For determination of GR activity, 60 μ M buffer, 5mM EDTA with pH 8.0, 0.033M GS-SG, 2mM NADPH and a sample of MCF-7 and OVCAR-3 cell lysate in a final volume of 1000 μ l were used. The reduction in absorbance shows the oxidation of NADPH through the reduction of GS-SG by enzyme GR activity in the MCF-7 and OVCAR-3 cell lysate, which has been monitored for 3min at 340nm by a Shimadzu Spectrophotometer. Finally, the results were reported through a molar absorptivity of $6.22 \times 10^6 \text{M}^{-1} \text{cm}^{-1}$ for NADPH. One unit of GR is defined as U/mg cell protein.

Determination of GSH

Assessment of GSH with 5, 5-dithio-bis (2- nitrobenzoic) acid (DTNB) was performed according to standard Ellman's method (Mashhoody et al., 2014; Zal et al., 2014). We used 1mM solutions of reduced GSH to draw a Standard curve. The GSH amount was assayed in MCF-7 and OVCAR-3 cell lysate. For measurement of GSH level, 2.3ml potassium phosphate buffer 0.2M with PH 7.6 was added to 500 μ l of DTNB (0.001M) solution and then mixed with 200 μ l of MCF-7 and OVCAR-3 cell lysate. Finally, the above solution's absorbance was read after 5min by a Shimadzu Spectrophotometer at 412nm.

Determination of MDA

The MDA was measured according to a colorimetric procedure. First, a mixture of the following solution was prepared: 2ml TBA 0.37%, 15% trichloroacetic acid, 0.25 mol/l HCL and 500 μ l MCF-7 or OVCAR-3 cell lysate. Then, the above mixture was put in a water bath at 95°C for 30min and after rapid cooling, it was centrifuged at 8000g for 15min. After centrifugation, the supernatant's absorbance was measured by a spectrophotometer at 532nm. The MDA level was calculated based on tetraethoxypropane as standard and expressed as nmol/mg cell protein.

Measurement of total protein

After 24h treatment with nicotine, MCF-7 and OVCAR-3 cells were washed with PBS four times and lysed with the lysis buffer containing 50mM Tris-

HCl (pH 7.4), 0.1% sodium deoxycholate, 0.1% SDS, 0.1mM EDTA, 1.0% Triton X-100 and 50mM sodium fluoride. Lysates were incubated at 4°C for 20min and centrifuged at 20000g for 10min. Then, supernatants were collected in Eppendorf tubes for protein and enzymatic measurements. Protein content was determined using BSA as a standard with Bio-Rad total protein assay kit according to manufacture protocols (Bio-Rad, Hercules, CA, USA).

Statistical analysis

GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA) and SPSS18 software (SPSS, Chicago, IL, USA) were used for group comparison. The data presented here were analyzed by the Kruskal-Wallis test, followed by Dunn's multiple comparisons test and a difference with P -value ≤ 0.05 was used as the level of significance.

Results

In the current study, to determine the toxic effect of nicotine exposure on antioxidant defense systems in both MCF-7 and OVCAR-3 cell lines, the cellular activities of CAT, GPx, GR and also the level of reduced GSH and MDA were assayed after 24h of treatment. MCF-7 and OVCAR-3 cell lines were exposed to nicotine doses ranging from 10^{-11} , 10^{-8} , and 10^{-6} M for 24 h, respectively.

Effect of nicotine treatment on the viability of MCF-7 and OVCAR-3 cells

The viability of cultured MCF-7 and OVCAR-3 cells exposed to different nicotine concentrations are presented in Figure 1. As shown in Figure 1, all the nicotine concentrations below 10^{-4} M are safe for *in vitro* uses compared with the control group.

Effect of nicotine on CAT activity in MCF-7 and OVCAR-3 cells

It has been demonstrated that the activity of CAT in the MCF-7 cell line increased with rising concentrations of nicotine. The treatment of MCF-7 cells with 10^{-11} , 10^{-8} and 10^{-6} M concentrations of nicotine made an increase in CAT activity by 34%, 30.77% and 44.46% respectively as compared to controls, which was significant only at 10^{-6} M dose of nicotine (Figure 2A). The CAT activity in OVCAR-3 cells after exposure to doses 10^{-11} , 10^{-8} and 10^{-6} M of nicotine was increased by 9.71%, 21.99% and

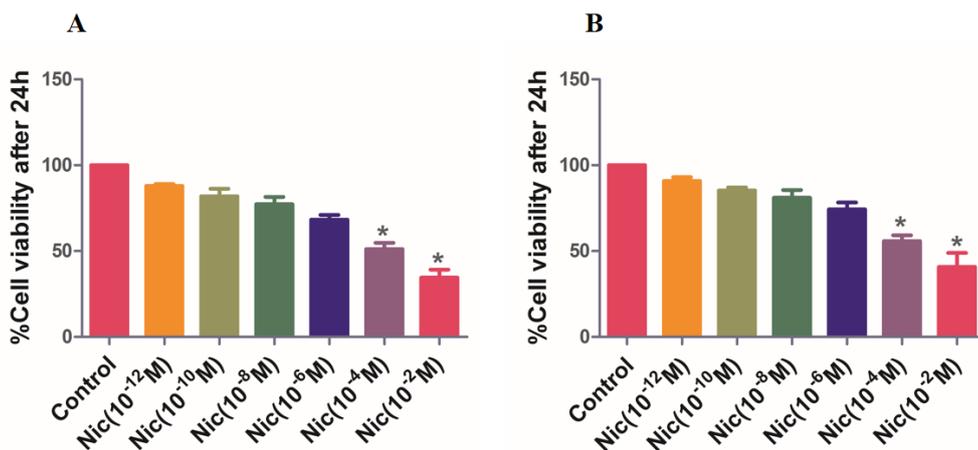


FIGURE 1. Effect of nicotine on the viability of A- MCF-7 and B- OVCAR-3 cells after 24h treatment using the MTT assay. Data are presented as mean±SD (n=4). All data are presented as a percentage with respect to control (100% cell viability). *Shows a significant difference compared to the respective control group ($P<0.05$).

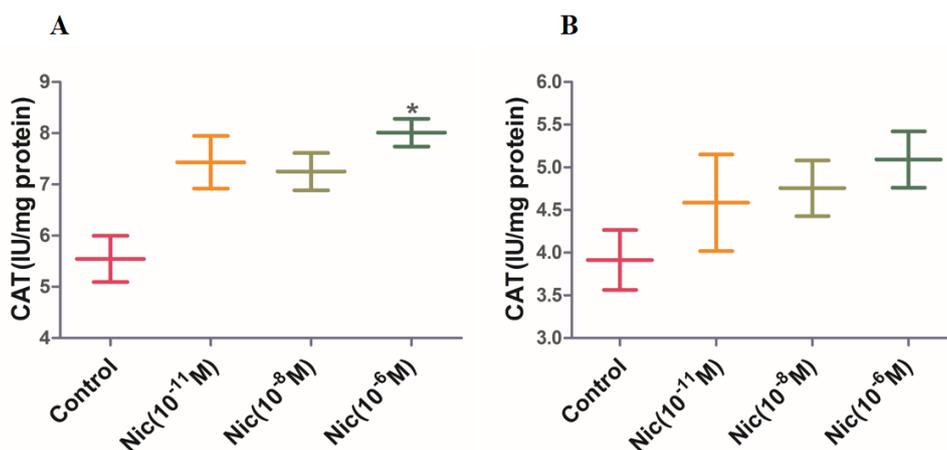


FIGURE 2. Effect of nicotine on CAT activity in A- MCF-7 and B- OVCAR-3 cells. Sample size (n=3). * $P<0.05$ for significant changes compared to the control (no treatment). CAT: Catalase.

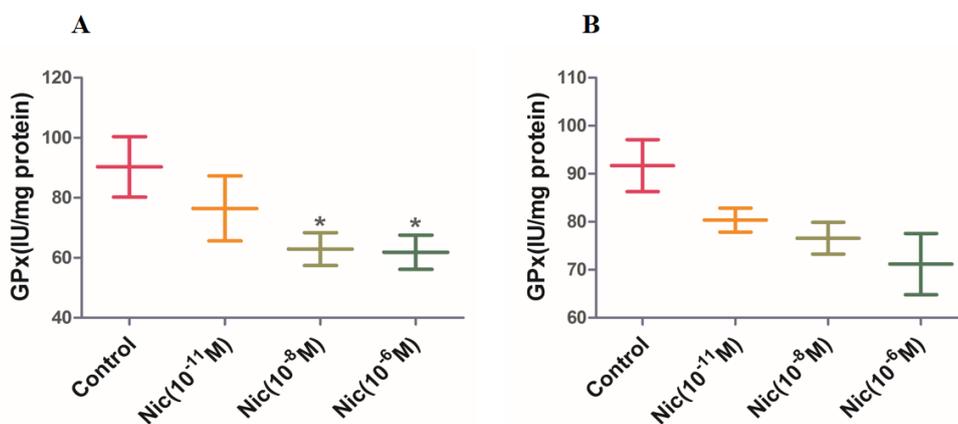


FIGURE 3. Effect of nicotine on GPx activity in A- MCF-7 and B- OVCAR-3 cells. Sample size (n=3). * $P<0.05$ for significant changes compared to the control (no treatment). GPx: Glutathione peroxidase.

30.17%, respectively, but this increase was not statistically significant (Figure 2B).

Effect of nicotine on GPx activity in MCF-7 and OVCAR-3 cells

In MCF-7 cells, the intracellular GPx activity decreased by 15.31%, 30.32% and 31.48 % for cells treated with 10^{-11} , 10^{-8} and 10^{-6} M nicotine concentrations, respectively (Figure 3A). This decrease was significant only at 10^{-8} and 10^{-6} M nicotine concentrations compared to the control group ($P < 0.05$). In the case of OVCAR-3 cells, treatment of this cell with the doses 10^{-11} , 10^{-8} and 10^{-6} M of nicotine resulted in a decline in GPx activity by 12.37%, 16.47% and 22.25%, respectively. However, these decreases were not significant in all groups

compared to the control group (Figure 3B).

Effect of nicotine on GR activity in MCF-7 and OVCAR-3 cells

As demonstrated in Figure 4A, GR enzymes' activities in the MCF-7 cell line were significantly decreased in cells treated with 10^{-8} and 10^{-6} M of nicotine by 37.34% and 42.36%, respectively, in comparison with the control group. In OVCAR-3 cells, treatment with 10^{-11} , 10^{-8} and 10^{-6} M concentration of nicotine decreased the activity of GR by 7.36%, 6.27% and 17.37%, respectively, as compared with the control (no treatment), but this decrease was significant only at 10^{-6} M dose of nicotine ($P < 0.05$, Figure 4B).

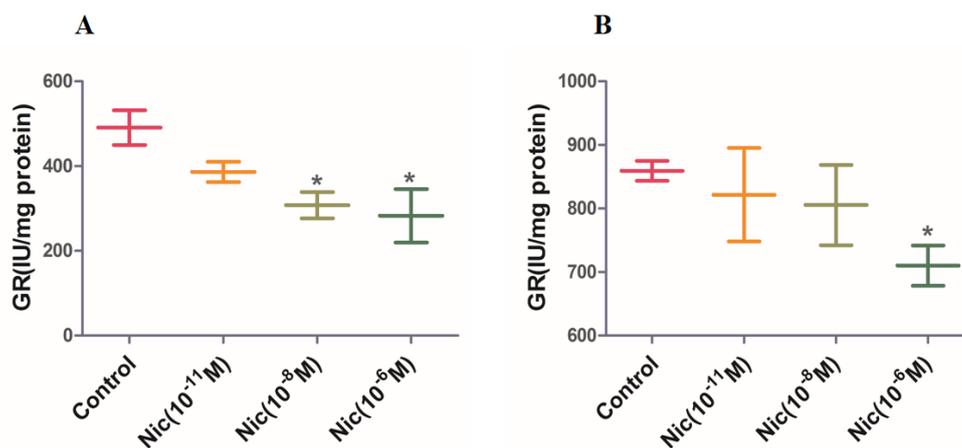


FIGURE 4. Effect of nicotine on GR activity in A- MCF-7 and B- OVCAR-3 cells. Sample size (n=3). * $P < 0.05$ for significant changes compared to the control (no treatment). GR: Glutathione reductase.

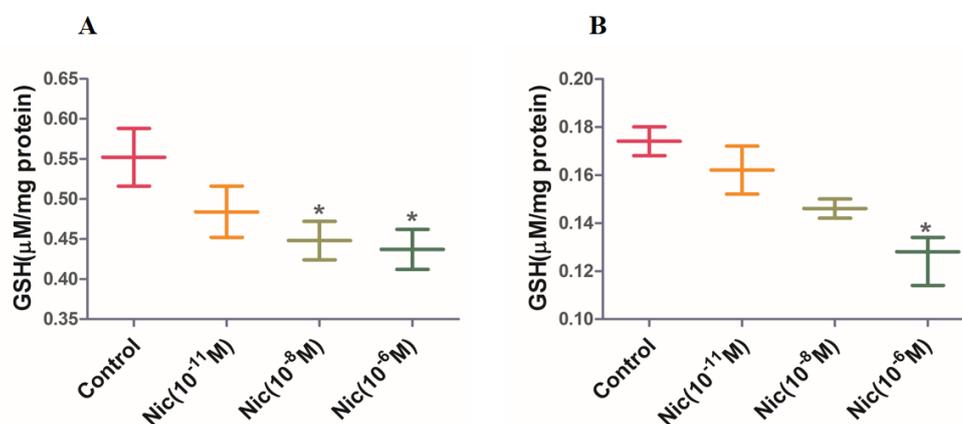


FIGURE 5. Effect of nicotine on GSH level in A- MCF-7 and B- OVCAR-3 cells. Sample size (n=3). * $P < 0.05$ for significant changes compared to the control (no treatment). GSH: Glutathione.

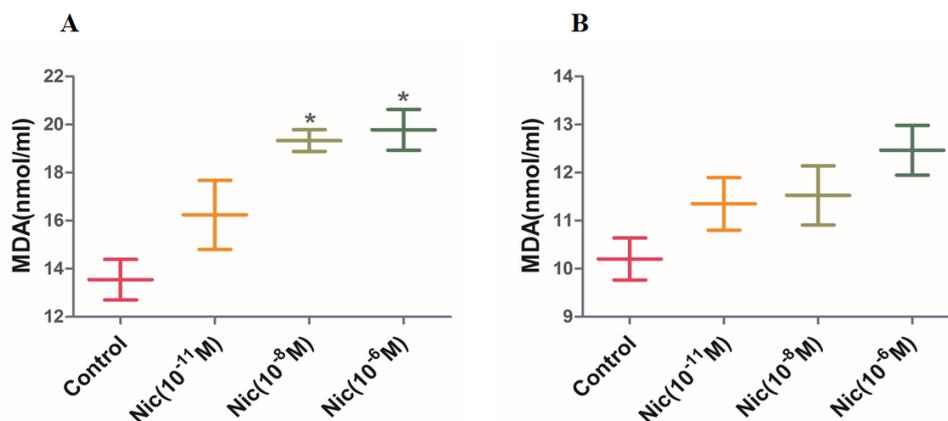


FIGURE 6. Effect of nicotine on MDA level in A- MCF-7 and B- OVCAR-3 cells. Sample size (n=3). * $P < 0.05$ for significant changes compared to the control (no treatment). MDA: Malondialdehyde.

Effect of nicotine on GSH level in MCF-7 and OVCAR-3 cells

The GSH level in the MCF-7 cells after 24h of treatment with 10^{-11} , 10^{-8} and 10^{-6} M nicotine showed a reduction by 12.32%, 18.85% and 20.84%, respectively in comparison to the control group, but this decrease was significant only at 10^{-8} and 10^{-6} M nicotine concentration (Figure 5A). Similarly, in OVCAR-3 cells, the GSH level in cells after 24h of treatment with 10^{-11} , 10^{-8} and 10^{-6} M of nicotine showed a decline by 6.9%, 16.1% and 26.44% as compared with the control group, but these declines were significant only at 10^{-6} M nicotine concentration (Figure 5B).

Effect of nicotine on MDA level in MCF-7 and OVCAR-3 cells

As shown in Figure 6A in MCF-7 cells, nicotine at 10^{-8} and 10^{-6} M concentration provoked a significant increase in MDA level by 42.02% and 45.56%, respectively, compared to the control group ($P < 0.05$). Comparably, in OVCAR-3 cells, nicotine increased MDA level by 11.07%, 11.86%, 18.72 % in comparison with the control group, but these increases were not significant in all three groups (Figure 6B).

Discussion

The purpose of the current study was to evaluate the toxic effects of nicotine on breast MCF-7 and ovary OVCAR-3 cells in an *in vitro* experimental model system as a means to understand the toxicity of nicotine in these cells. We showed that nicotine at doses below 10^{-4} M is safe for MCF-7 and OVCAR-3 cells, which is con-

sistent with our previous studies on nicotine effects on human HepG2 and endometrial stromal cells (Totonchi et al., 2016; Yarahmadi et al., 2017). Hence, we used 10^{-11} , 10^{-8} and 10^{-6} M nicotine to treat MCF-7 and OVCAR-3 cells to assess nicotine effects on the antioxidant enzyme status. The ROS are byproducts of normal cell metabolism and rise in many drug exposure. Depending on their cellular concentration, these free radicals could have beneficial or harmful effects on human cells and tissues (Circu and Aw, 2010). At limited quantities, ROS function like redox messengers in many signaling and regulatory pathways in cells. Nevertheless, the over-production of ROS induces oxidative toxicity and severe damages to basic macromolecules in the cells, such as DNA, proteins and lipids, which destroys normal proteins and lipids function and leads to cell death (Delijewski et al., 2014; Hwang et al., 2008). Many studies also showed that the over-production of ROS disturbers cellular signaling and finally, leads to cancer occurrence (Pelicano et al., 2004). Normal cell function depends on a balance between the production and destruction of free radicals, known as oxidant-antioxidant balance (Alamdari et al., 2020b; Halliwell, 2007). Human body cells consist of a complex antioxidant defense system include enzymatic defense CAT, SOD, GPx and GR and non-enzymatic antioxidant GSH, vitamin C and E. This system is responsible for protecting the destructive effects of ROS (Circu and Aw, 2010). The enzyme SOD functions as the primary cellular defense against the destructive effects of free oxygen radicals by the dismutation of superoxide anion ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Then, sufficient activity of en-

zymes CAT and GPx are necessary to degrade H_2O_2 to H_2O and O_2 . The function of enzyme GR is needed to maintain the GSH level sufficient for the activity of GPx (Matés et al., 1999).

In our study, as shown in Figure 2, the activity of the enzyme CAT increased in both MCF-7 and OVCAR-3 cells after treatment with different nicotine concentrations. However, this increase was significant at 10^{-6} M nicotine concentration in MCF-7 cells, which is consistent with our previous study on the effects of nicotine on the antioxidant enzyme status in HepG-2 cells. Similarly, Delijewski et al. (2014) showed that CAT activity in melanocytes significantly increased after 24h incubation with different nicotine concentrations. In contrast, Muthukumaran et al. (2008) showed that nicotine induces antioxidant imbalance and reduces CAT activity in the circulation and lung of Wistar rats after 22 weeks of nicotine treatment. This controversy in CAT activity could be explained by the fact that in acute nicotine exposure, cellular CAT activity increases to mitigate nicotine oxidative damages, but in chronic nicotine exposure, depletion in the activity of CAT may be due to decreased synthesis of enzymes or oxidative inactivation of enzyme protein. In Muthukumaran et al.'s study, decreased CAT activity was associated with decreased antioxidant status after chronic nicotine exposure that can be related to insufficient antioxidant potential.

As demonstrated in Figure 3, GPx activity decreased after treatment with all nicotine doses in both MCF-7 and OVCAR-3 cells. However, it was significant only at 10^{-8} and 10^{-6} M in MCF-7 cells, which is consistent with Mahapatra et al. (2009) study on nicotine effects in mice peritoneal macrophages in an *in vitro* model. This decrease in GPx activity along with an increase in CAT activity may show that both pathways of H_2O_2 degradation by enzymes CAT and GPx are involved in the destruction of H_2O_2 during the high concentration of free radicals induced by nicotine toxication in these cells.

The function of enzyme GR is the preservation of GSH content in its reduced form, which is necessary for the activity of GPx. In our study, the activity of GR was significantly decreased in high dose nicotine treated groups in both MCF-7 and OVCAR-3 cells, which was compatible with the results of Erat et al. (2007) that showed nicotine treatment reduces GR activity in the liver, lungs, heart, stomach, kidney and testicles of rats in an *in vivo* and *in vitro* models and also showed that application of

vitamin E could restore the activity of GR.

GSH level is crucial for the preservation of cellular oxidant-antioxidant balance. In our experiment, the level of reduced GSH significantly decreased after treatment with nicotine at doses 10^{-8} and 10^{-6} M in MCF-7 cells and at 10^{-6} M nicotine concentration in OVCAR-3 cells, which is compatible with Balakrishnan and Menon (2007) study that showed nicotine decreased reduced GSH in lung, liver and kidney of rats and hesperidin a polyphenolic compounds which are mainly available in citrus fruits restored GSH level.

Similar to our results, Sener et al. (2005) showed that nicotine-induced oxidative damage results in GSH depletion in the bladder and kidney of rats compared to control and amino acids taurine could significantly enhance reduced GSH in these rats. MDA, an indicator of lipid peroxidation, showed a marked increase in both MCF-7 and OVCAR-3 cells. However, it was significant only in MCF-7 cells at 10^{-8} , and 10^{-6} M nicotine concentration, which is compatible with the results of Al-Malki and Moselhy (2013) that indicated nicotine administration to rats makes a significant elevation in MDA level.

Conclusion

In conclusion, the results of this study showed that the breast MCF-7 cells are more vulnerable than ovarian OVCAR-3 cells to nicotine-induced oxidative toxicity in an *in vitro* model. Further studies are needed to assess different nicotine toxicity patterns in cells in the *in vitro* and *in vivo* models.

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Conflict of interest

The authors have no financial interests related to the manuscript.

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