Mycotoxin Research (2019) 35:309–320
https://doi.org/10.1007/s12550-019-00358-8

ORIGINAL ARTICLE

Alterations in global DNA methylation and metabolism-related genes caused by zearalenone in MCF7 and MCF10F cells

Ecem Fatma Karaman[1] · Sibel Ozden[1]

Received: 28 November 2018 / Revised: 20 March 2019 / Accepted: 21 March 2019 / Published online: 5 April 2019

Abstract

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* fungi. ZEN has endocrine disruptor effects and could impair the hormonal balance. Here, we aimed at investigating possible effects of ZEN on metabolism-related pathways and its relation to epigenetic mechanisms in breast adenocarcinoma (MCF7) and breast epithelial (MCF10F) cells. Using the MTT and neutral red uptake (NRU) cell viability tests, IC_{50} values of ZEN after 24 h were found to be 191 μmol/L and 92.6 μmol/L in MCF7 cells and 67.4 μmol/L and 79.5 μmol/L in MCF10F cells. A significant increase on global levels of 5-methylcytosine (5-mC%) was observed for MCF7 cells, correlating with the increased expression of DNA methyltransferases. No alterations were observed on levels of 5-mC% and expression of DNA methyltransferases for MCF10F cells. Further, at least threefold upregulation compared to control was observed for several genes related to nuclear receptors and metabolism in MCF7 cells, while some of these genes were downregulated in MCF10F cells. The most notably altered genes were *IGF1*, *HK2*, *PXR*, and *PPARγ*. We suggested that ZEN could alter levels of global DNA methylation and impair metabolism-related pathways.

Keywords Zearalenone · DNA methylation · Metabolism-related genes · Nuclear receptor genes · MCF7 cells · MCF10F cells

Abbreviations

5-mC	5-Methylcytosine
AhR	Aryl hydrocarbon receptor
Caco-2	Human colorectal adenocarcinoma cell line
CHO-K1	Chinese hamster ovary cell line
DNMT1	DNA methyltransferase 1
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT2	Glucose transporter 2
HK2	Hexokinase 2
IC_{50}	50% of inhibitory concentration
IGF1	Insulin-like growth factor 1
L-FABP	Liver fatty acid-binding protein
MCF7	Human breast adenocarcinoma cell line
MCF10F	Human breast epithelial cell line
MGMT	O^6-Methylguanine-DNA methyltransferase
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole]
NRU	Neutral red uptake
PPARɣ	Peroxisome proliferator-activated receptor gamma
PXR	Pregnane X receptor
SH-SY5Y	Human neuroblastoma cell line
SREBP1c	Sterol regulatory element-binding protein 1

✉ Sibel Ozden
stopuz@istanbul.edu.tr

[1] Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Istanbul University, 34116-Beyazit, Istanbul, Turkey

Introduction

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* species that contaminates cereals and other crops (Battorico 1998). ZEN binds to estrogen receptors (ER) and causes alteration of hormone production and reproductive toxic effects (Shier et al. 2001; Frizzell et al. 2011). Exposure to ZEN results in inhibition of protein and DNA synthesis, and triggers endoplasmic reticulum and mitochondrial stress, lipid peroxidation, oxidative damage, and apoptosis (Abid-Essefi et al. 2004; Ayed-Boussema et al. 2007; Bouaziz et al. 2008; Banjerdpongchai et al. 2010; Cai et al. 2019; Cheng et al. 2019; Kowalska et al. 2019; Wang et al.

2019). Moreover, ZEN has immunotoxic, hepatotoxic, and nephrotoxic properties (Maaroufi et al. 1996; Bouaziz et al. 2008; Gao et al. 2013; Lee et al. 2013; Hueza et al. 2014; Jia et al. 2014; Pistol et al. 2015; Islam et al. 2017; Gao et al. 2018; Zhang et al. 2018). There is limited evidence for the carcinogenicity of ZEN in experimental animals and it is classified as Group 3 (IARC 1993).

DNA methylation, one of the most studied epigenetic modifications, plays crucial roles in aging, cell proliferation, and various diseases such as cancer and diabetes (Baylin 1997; Richardson and Yung 1999; Robertson and Wolffe 2000; Moggs et al. 2004; Dean et al. 2005; Ulrey et al. 2005; Jones and Baylin 2007; Kulis and Esteller 2010; Anderson et al. 2012; Bansal and Pinney 2017). DNA methylation contributes to alterations in gene expression of key molecular pathways in several ways, including global genomic DNA hypomethylation, hypomethylation of individual genes, and tumor suppressor gene silencing through hypermethylation of CpG islands of genes (Baylin et al. 1986; Watson and Goodman 2002). Especially, DNA methyltransferase 1 (DNMT1) manages maintenance of methyltransferase activity that conserves the methylation state across DNA replication (Pradhan et al. 1999; Das and Singal 2004). In this case, O^6-methylguanine-DNA methyltransferase (MGMT) repairs one of the most mutagenic alkylations at the O^6-position of guanine nucleotide to cancer prevention by transfer of the methyl group from guanine to a cysteine residue (Pegg et al. 1995). It appears that *DNMT1* and *MGMT* have crucial roles in cell cycle process, cell proliferation, and DNA repair (Kleihues et al. 1983; Pfohl-Leszkowicz and Dirheimer 1986; Jaenisch and Bird 2003; Guo et al. 2004; Sabharwal and Middleton 2006; Pathania et al. 2015; Wang and Li 2017). Although ZEN has genotoxic properties in some test systems (JECFA 2000), reveals DNA adduct formation in treated mice (Pfohl-Leszkowicz et al. 1995a; Grosse et al. 1997), and causes DNA damage, it has been suggested that mutagenic and carcinogenic properties of ZEN were still controversial (Ouanes-Ben Othmen et al. 2008; Abassi et al. 2016; Mandal et al. 2018). However, there are few studies that have limited relevance to epigenetic modifications including global or gene-specific DNA methylation and histone modifications caused by ZEN (Kouadio et al. 2007; Zhu et al. 2014a; Zhu et al. 2014b; Han et al. 2015; Ren et al. 2015; Zhang et al. 2017).

Breast cancer is one of the most common diseases in women, and obesity is one of the currently known risk factors of breast cancer (Lorincz and Sukumar 2006; Székely et al. 2010). Liu and Lin (2004) have also shown that commercial form of ZEN was able to transform human normal breast epithelial cell and increase cell proliferation in a dose-dependent manner. In the view of these data, it has been thought that ZEN exposure could be related to mechanisms of breast cancer progression. We aimed to investigate the global levels of DNA methylation and the related enzymes (*DNMT1* and *MGMT*) to observe the role of epigenetic alterations in the ZEN toxicity. Moreover, we showed effects of ZEN on expression levels of metabolism-related genes and nuclear receptor genes in human breast adenocarcinoma (MCF7) and human breast epithelial (MCF10F) cell lines.

Materials and methods

Chemicals

ZEN (99% purity) was obtained from Sigma-Aldrich (St Louis, Missouri, USA). A stock solution of ZEN (1000 μmol/L) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) in a sterile glass volumetric flask and kept at − 20 °C. Cell culture media and all other supplements were purchased from Wisent Bioproducts (Saint-JeanBaptiste, QC, Canada) and sterile plastic materials were purchased from Nest Biotechnology (Jiangsu, China). DNA, RNA isolation kits, and cDNA synthesis kit and syber green master mix were obtained from Roche Life Sciences (Penzberg, Upper Bavaria, Germany). 5-Methylcytosine (5-mC) DNA ELISA kits were purchased from Epigentek (Farmingdale, NY). Primers for gene expressions were obtained from Sentromer DNA Technologies (Istanbul, Turkey).

Cell culture and treatments

The human breast adenocarcinoma MCF7 (ATCC® HTB-22™) and human breast epithelial MCF10F cell lines (ATCC® CRL-10318™) were obtained from American Type Culture Collection. MCF7 cells were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM F-12) medium containing 0.01 mg/mL human recombinant insulin, 10% heat-inactivated fetal bovine serum (FBS), and penicillin–streptomycin (100 U–100 μg/mL) at 37 °C in a humidified atmosphere with 5% CO_2. MCF10F cells were cultured in DMEM F-12 Ham (Sigma, D8900) medium containing 0.006 g/L $CaCl_2{\cdot}2H_2O$ (Sigma-Aldrich, C8106) and 1.2 g/L $NaHCO_3$ (Sigma, S5761) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 ng/mL insulin, 500 ng/mL hydrocortisone (Sigma, H4001), and penicillin–streptomycin (100 U–100 μg/mL) at 37 °C in a humidified atmosphere with 5% CO_2. Subculturing was performed when the cells reached 70–80% confluence (every 2–3 days) using trypsinization. Exposure to ZEN was observed at the 8th to 16th round of subculture for both cell lines.

For gene expression and DNA methylation analysis, 1×10^6 were cultured in a 25-cm^2 culture flask for 24 h in CO_2 incubator prior to treatment. ZEN was treated in the

concentrations of 1, 10, and 50 μmol/L in MCF7 cells and 0.1, 1, and 10 μmol/L in MCF10F cells, and 1% DMSO (exposure concentration in culture media) was used as solvent control in both cells. Cells were trypsinized, collected, and counted by Luna cell counter (Virginia, USA) with trypan blue staining. For all concentrations, it was tested in triplicates and each test was repeated twice.

Based on our cytotoxicity results and also previous studies in different cell types (Venkataramana et al. 2014; Sang et al. 2016; Tatay et al. 2014; Xie et al. 2017; Zhou et al. 2017), in the present study we selected 1, 10, and 50 μmol/L and 0.1, 1, and 10 μmol/L exposure concentrations of ZEN for 24 h for MCF7 and MCF10F cells, respectively. We could not apply the highest dose for the MCF10F because of the high cell death; therefore, we chose the 0.1 μmol/L concentration for the lowest and third concentration for the exposure of ZEN in MCF10F cells.

Cell viability

Effects of ZEN on cell viability were assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole] and neutral red uptake (NRU) cytotoxicity tests. In the principle of the MTT test, yellow MTT is reduced to purple formazan in the mitochondria of living cells by the enzyme succinate dehydrogenase and it measures cell viability and proliferation (Mosmann 1983; Alley et al. 1988). The NRU test is a cell viability test method based on reduction in the uptake of neutral red dye into the lysosomes of cells (Borenfreund and Puerner 1985; Repetto et al. 2008).

For cytotoxicity tests, cells (1×10^4 in 100 μL medium) were seeded in 96-well plates and exposed to ZEN in the range of 7.81–250 μmol/L and 3.12–100 μmol/L of concentrations for MCF7 and MCF10F, respectively, and 1% DMSO (exposure concentration in culture media) was used as solvent control for 24 h. The absorbance of formed colored solution was measured at 590 nm for MTT test and 540 nm for NRU test using a microplate spectrophotometer system (Biotek-Epoch, Winooski, USA). The cytotoxicity results were calculated as a relative percentage to the control cells and expressed as 50% of inhibitory concentration (IC_{50}) of the compound that caused 50% inhibition of the enzyme activity in the cells.

Global DNA methylation analysis

Genomic DNA was isolated from MCF7 and MCF10F cells using the High Pure PCR Template Preparation kit (Roche Applied Science, Mannheim, Germany) according to the instructions provided by the manufacturer. 5-mC analysis was performed using the MethylFlash™ Methylated DNA Quantification kit (Epigentek, Farmingdale, NY) according to the instructions provided by the manufacturer using 100 ng of input genomic DNA. DNA samples were treated with binding solution and incubated at 37 °C. Plate was washed with buffer. DNA samples were incubated with anti-5-mC monoclonal antibody and detection antibody. After addition of enhancer and developer solution, the absorbance was read at 450 nm using a microplate spectrophotometer system (Biotek-Epoch, Winooski, USA).

Gene expression analysis

Gene expressions of DNA methyltransferase genes including *DNA methyltransferase 1* (*DNMT1*), *O-6-methylguanine-DNA methyltransferase* (*MGMT*), and metabolism-related genes including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), glucose transporter 2 (*GLUT2*), insulin-like growth factor 1 (*IGF1*), liver fatty acid-binding protein (*L-FABP*), sterol regulatory element-binding protein 1 (*SREBP1c*), hexokinase 2 (*HK2*), and nuclear receptor genes including pregnane X receptor (*PXR*), aryl hydrocarbon receptor (*AhR*), peroxisome proliferator activated receptor gamma (*PPARγ*), estrogen receptor alpha (*ERα*), and estrogen receptor beta (*ERβ*) were performed by real-time PCR analysis. Total RNA was extracted from control and ZEN-treated groups in MCF7 and MCF10F cell lines using a High Pure RNA Isolation kit (Roche Life Science) according to the instructions provided by the manufacturer. First-strand cDNA was prepared from 500 ng of total RNA with the mixture of anchored-oligo(dT) and random hexamer primers by Transcriptor First Strand cDNA Synthesis kit (Roche Life Science). Real-time PCR reactions were performed using LightCycler® 480 Sybr Green master mix (Roche, Mannheim, Germany) under the following cycling conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 40 s, melting curve, and cooling. The primer sequences and the annealing temperatures of the genes are provided in Table 1. Ct of real-time PCR specific for nuclear receptor and metabolism-related genes and the reference gene (*β-actin*) were determined. The relative expression was evaluated by the comparative Ct method.

Statistical analysis

Global methylation levels (5-mC%) and cytotoxicity results were represented as mean ± standard deviation (SD). Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test using SPSS version 21.0 for Windows (IBM Analytics, New York, USA). *P* values less than 0.05 and 0.001 were selected as the levels of significance.

Table 1 Primers used real-time PCR analysis of metabolism-related genes and DNA methyltransferases and the corresponding annealing temperatures

Gene name	Forward (5′–3′)	Reverse (5′–3′)	T_a (°C)	Reference
DNMT1	CCTCCAAAAACCCAGCCAAC	TCCAGGACCCTGGGGATTTC	60	Ahmadnejad et al. 2017
MGMT	TGCACAGCCTGGCTGAATG	GGTGAACGACTCTTGCTGGAA	58	Lai et al. 2008
GAPDH	GGCCTCCAAGGAGTAAGACC	AGGGGTCTACATGGCAACTG	57	Hao et al. 2014
GLUT2	ACAGCCTATTCTAGTGGCAC	TTGCTAAAGCAGCAGGACGT	57	Reimer et al. 2004
IGF1	CTCTTCAGTTCGTGTGTGGAGAC	CAGCCTCCTTAGATCACAGCTC	58	Srinivasa et al. 2016
L-FABP	TGTCGGAAATCGTGCAG	GATTATGTCGCCGTTGAGTT	53	Wang et al. 2005
SREBP1c	CGCGGAGCCATGGATTGC	GGGCTGGGGTAGCCTAAC	59	Reimer et al. 2004
HK2	CAAAGTGACAGTGGGTGTGG	GCCAGGTCCTTCACTGTCTC	60	Zhao et al. 2013
PXR	CATGAGGGGGGTAGCAAAGC	TGCAGGGGATCTCCCTCTTC	59	Ayed-Boussema et al. 2011
AhR	TGGACAAGGAATTGAAGAAGC	AAAGGAGAGTTTTCTGGAGGAA	54	Ayed-Boussema et al., 2011
PPARγ	CTGAATGTGAAGCCCATTGAA	GTGGAAGAAGGGAAATGTTGG	54	Harada et al. 2005
ERα	CGACGCCAGGGTGGCAGAGAAAGATT	GGCCAAAGGTTGGCAGCTCTCATGTC	65	Jang et al. 2015
ERβ	TAGTGGTCCATCGCCAGTTAT	GGGAGCACACTTCACCAT	56	Mollerup et al. 2002
β-Actin	AACTACCTTCAACTCCAT	TGATCTTGATCTTCATTGTG	48	Rosa et al. 2009

Results

Effects of ZEN on cell viability in MCF7 and MCF10F cells

To assess the effects of ZEN on cell viability, cells were treated with 7.81–250 μmol/L and 3.12–100 μmol/L of concentrations for MCF7 and MCF10F, respectively, for 24 h then analyzed by MTT and NRU tests. IC_{50} value of ZEN was determined as 191 and 92.6 μmol/L in MCF7 cells and 67.4 and 79.5 μmol/L in MCF10F cells for 24 h by MTT and NRU tests, respectively. Figure 1a and b shows that treatments of ZEN for 24 h decreased the cell viability of MCF7 cells and MCF10F cells in comparison to the vehicle control group for MTT and NRU tests, respectively.

Effects of ZEN on global DNA methylation in MCF7 and MCF10F cells

Fifty micromoles per liter of ZEN exposure resulted in a significant increase in 5-mC% status (8.14-fold, $p < 0.001$) compared to control group in MCF7 cells for 24 h (Fig. 2a). However, we found no changes in levels of 5-mC% in MCF10F cells after exposure to ZEN for 24 h (Fig. 2b). Figure 3a and b shows an increase on the expression levels of *DNMT1* (> 4.28-fold) and *MGMT* (> 4.72-fold) genes significantly after 24-h exposure to ZEN in MCF7 cells. However, expression levels of *DNMT1* and *MGMT* showed no changes in MCF10F cells (Fig. 3c, d).

Effects of ZEN on gene expression in MCF7 and MCF10F cells

In Fig. 4a, our data showed that exposure to ZEN significantly increased expression levels of *GAPDH* (> 5.19-fold), *IGF1* (> 11.2-fold), *L-FABP* (> 6.19-fold), *HK2* (> 19.46-fold), *PXR* (> 6.4-fold), *PPARγ* (> 5.05-fold), *ERα* (> 3.25-fold), and *ERβ* (> 5.72-fold), while expression levels of *GLUT2*, *SREBP1c*, and *AhR* did not show any changes in MCF7 cells. However, expression levels of *GAPDH* (< 5.88-fold), *AhR* (< 3.03-fold), and *PPARγ* (< 5.88-fold) significantly decreased while *IGF1* (> 8.72-fold) and *HK2* (> 2.26-fold) increased after ZEN exposure for 24 h in MCF10F cells (Fig. 4b). ZEN did not change expression levels of *L-FABP*, *SREBP1c*, *PXR*, and *ERβ* genes in MCF10F cells for 24 h. Additionally, *ERα* and *GLUT2* were not expressed in MCF10F cells, even in control samples.

Discussion

ZEN is a non-steroidal estrogenic mycotoxin produced by *Fusarium* species. It has been reported that ZEN was well known as an estrogenic exposure source in the environment and it could be accepted as one of the important endocrine disruptors. Main source of exposure to ZEN consists of food such as grain, breakfast cereals, bread, wine, beer, and dried fruits; therefore, ZEN affects human and animal health through the food chain (Kriszt et al. 2012; EFSA 2017). It has been shown that ZEN could induce various health problems such as alteration of hormone levels, reproductive and

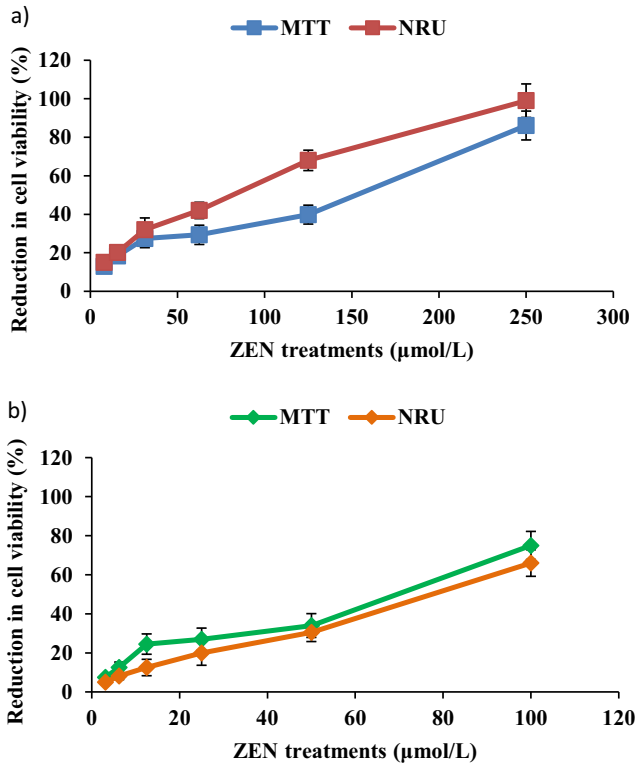

Fig. 1 Effects of ZEN (7.81, 15.62, 31.25, 62.5, 125, 250 μmol/L) on cell viability by MTT and NRU in MCF7 cells (**a**) and MCF10F cells (**b**) after 24-h exposure. Data are presented as mean ± SD

developmental disorders, modulation of cell cycle control, inflammation, and cancer (Fink-Gremmels and Malekinejad 2007; Zinedine et al. 2007; Escrivá et al. 2015; Kowalska et al. 2016; Gao et al. 2018; Zhang et al. 2018; Wang et al. 2019).

In the present study, we aimed to investigate the effects of ZEN on the expressions of the genes which are related to metabolism pathways in breast cell lines; in addition, we observed the effects of ZEN on global DNA methylation. The MTT test converts yellow MTT tetrazolium salt to purple formazan crystals by mitochondrial succinate dehydrogenase

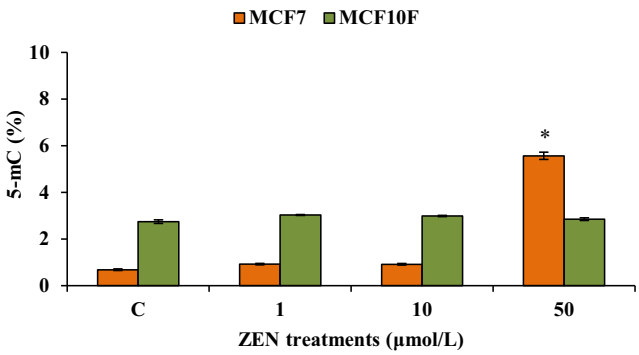

Fig. 2 Effects of ZEN (1, 10, and 50 μmol/L) on the levels of 5-mC% in MCF7 cells and MCF10F cells after 24-h exposure. Genomic DNA was extracted, then 5-mC% levels were detected using the ELISA kit. Data are presented as mean ± SD. Statistical analysis was performed by ANOVA–Dunnett post hoc test. Statistically significant changes are indicated by an asterisk ($p < 0.001$)

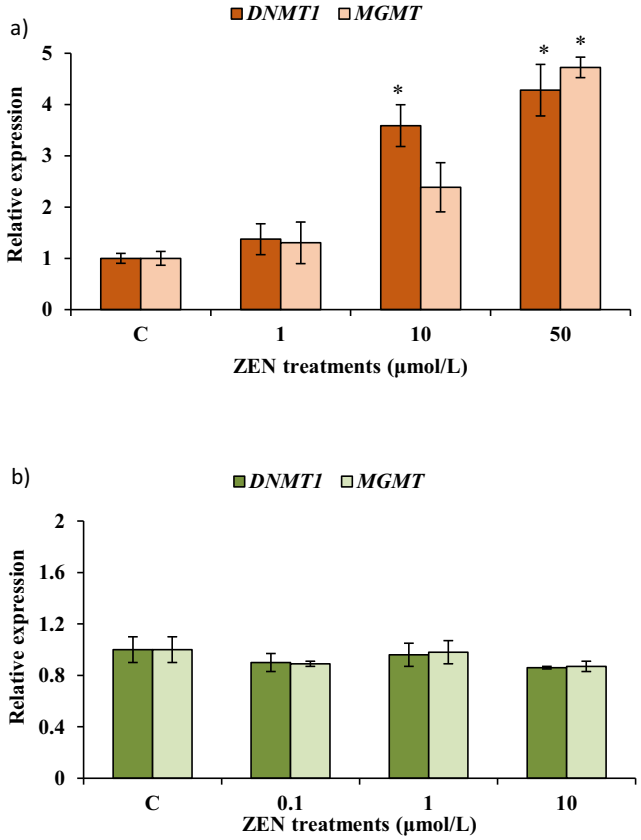

Fig. 3 Effects of ZEN (1, 10, and 50 μmol/L) on gene expressions of *DNMT1* and *MGMT* by real-time PCR in MCF7 (**a**) cells after 24-h exposure. Effects of ZEN (0.1, 1, and 10 μmol/L) on gene expressions of *DNMT1* and *MGMT* by real-time PCR in MCF10F cells (**b**) after 24-h exposure. Data are presented as mean ± SD. Statistical analysis was performed by ANOVA–Dunnett post hoc test. Statistically significant changes are indicated by asterisks ($p < 0.001$)

in viable cells (Mosmann 1983) while the NRU test determines the incorporation of neutral red dye into lysosomes of uninjured cells (Borenfreund and Puerner 1985). ZEN decreased cell viability in the concentrations more than 31.25 μmol/L and 25 μmol/L in MCF7 and MCF10F, respectively. IC_{50} value of ZEN was determined as 191 and 92.6 μmol/L in MCF7 cells and 67.4 and 79.5 μmol/L in MCF10F cells for 24 h by MTT and NRU tests, respectively. Venkataramana et al. (2014) have observed effects of 1–200 μmol/L concentrations of ZEN on cell viability in SH-SY5Y human neuroblastoma cell line for 24 h. After 24, 48, and 72 h of ZEN exposure (12.5–100 μmol/L) in ovarian CHO-K1 cells, it has been shown that the IC_{50} value was > 100 μmol/L for 24 h (Tatay et al. 2014). In the other study, ZEN (3–300 μmol/L) treated in HEK-293 human embryo kidney cells for 24 h and the IC_{50} value has been determined as 80 μmol/L by WST-8 assay (Sang et al. 2016).

Investigating epigenetic alterations such as DNA methylation and histone modifications has been useful biomarkers for the toxicity assessment of endocrine-disrupting chemicals (Zhang and Ho 2011; Greally and Jacobs 2013; Casati et al.

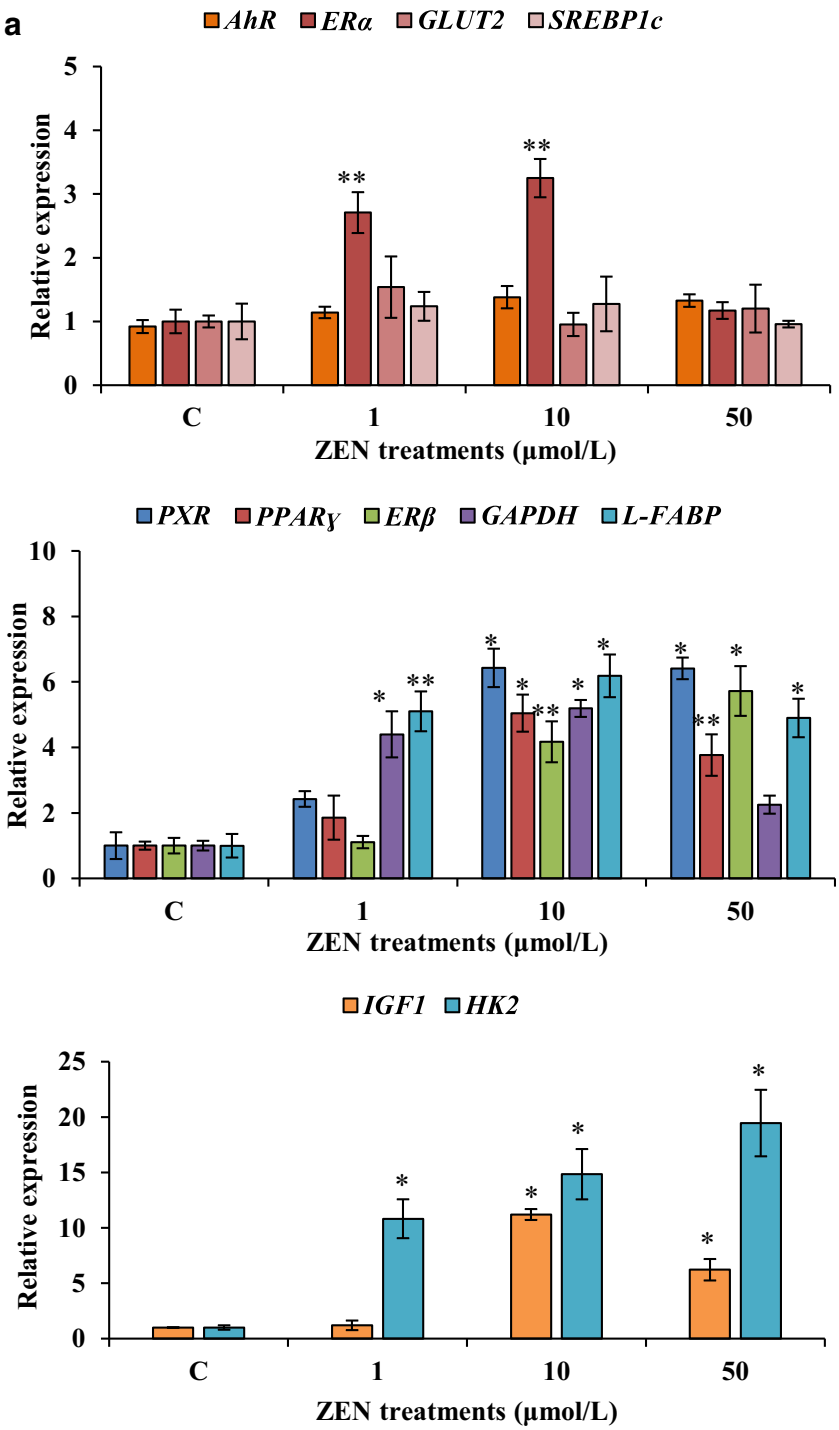

Fig. 4 Quantification of metabolism-related genes and nuclear receptor genes expressions by real-time PCR. Statistical analysis was performed by ANOVA + Dunnett post hoc test. (*$p < 0.001$, **$p < 0.05$). (**a**) MCF7 cells were treated with 1, 10, and 50 μmol/L of ZEN for 24 h prior to extraction of total RNAs. (**b**) MCF10F cells were treated with 0.1, 1, and 10 μmol/L of ZEN for 24 h prior to extraction of total RNAs

2015; Maqbool et al. 2016). However, there have been limited studies on epigenetic modifications of ZEN. Our results showed that 50 μmol/L of ZEN exposure for 24 h increased the levels of 5-mC% (8.14-fold) in MCF7 cells. Similar to our results, Zhu et al. (2014a) showed that ZEN increased global DNA methylation level in the high-dose group (50 μmol/L) of mouse oocytes for 12 h. Kouadio et al. (2007) studied the effects of combinations of *Fusarium* mycotoxins (ZEN, deoxynivalenol, fumonisin B1) on the global DNA methylation and found that ZEN increased the 5-mC% levels in Caco-2 human intestinal cell line DNA at 40 μmol/L for 24 h. In ZEN-treated porcine oocytes, levels of 5-mC% increased at

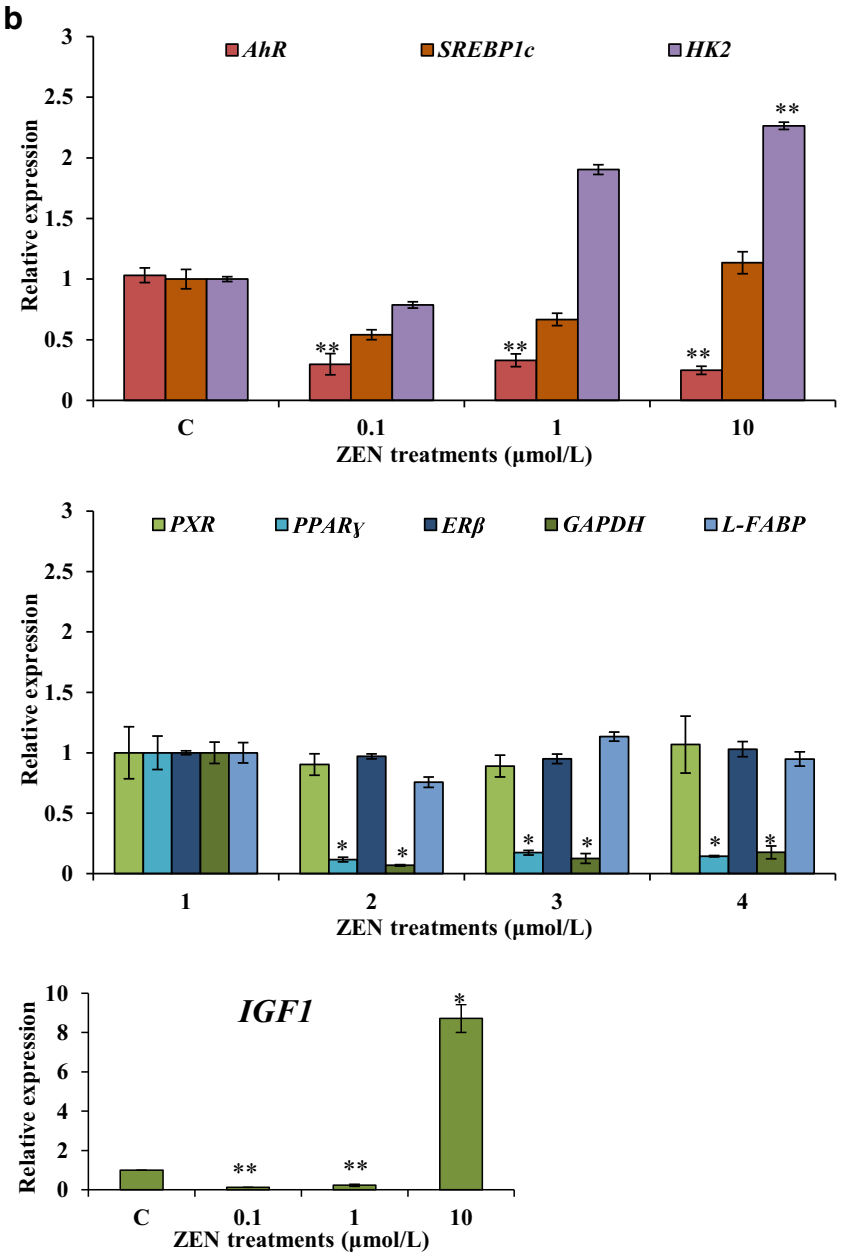

Fig. 4 (continued)

30 μmol/L of ZEN for 30 h (Han et al. 2015). Although we observed increases in the levels of 5-mC status in MCF7 cells, interestingly, we did not find any significant changes after 24-h exposure to ZEN in MCF10F cells. As correlated with global DNA methylation results, ZEN induced expression levels of *DNMT1* and *MGMT* at 50 μmol/L in MCF7 cells; however, expressions of these genes did not change in ZEN-treated MCF10F cells. Similarly to our results in MCF7 cells, Han et al. (2015) have found that ZEN increased global DNA methylation level and mRNA levels of DNA methyltransferases (*DNMT3a* and *DNMT3b*) significantly increased compared to control at 30 μmol/L of ZEN in the oocytes. However, Zhu et al. (2014a) examined gene expressions of DNA methyltransferases (*DNMT1*, *DNMT3a*, *DNMT3b*, and *DNMT3L*) in the ZEN-treated oocytes for 8.5 and 12 h and the *DNMTs* expression levels did not differ from the control group. Therefore, exposure time and different cell culture could affect alterations on global DNA methylation and gene expression levels by ZEN. Additionally, several studies have demonstrated that *DNMT1* and *MGMT* levels were higher in tumors than in their normal tissues (Pfohl-Leszkowicz et al. 1995b; Pieper 1997; Gerson 2004; Sabharwal and Middleton 2006; Sharma et al. 2009; Shi et al. 2012; Roll et al. 2013; Yu et al. 2014). Wang and Li (2017) have observed that *DNMT1* regulates the cell cycle, proliferation, and apoptosis process. Increased methyltransferase activity and higher expressions of

methyltransferases have also been shown in breast cancer cells (Ottaviano et al. 1994; Roll et al. 2013).

It has been suggested that commercial form of ZEN, zeranol (Ralgro), accelerated breast cancer cell growth at low concentrations and induced human normal breast epithelial cell transformation to neoplastic cell (Liu and Lin 2004; Yuri et al. 2006). Xu et al. (2009, 2010, 2011) have found that zeranol had mitogenic activity on breast cancer cells and there was an interaction between leptin and zeranol. It has been suggested that zeranol promoted proliferation in human breast cancer cells and obese individuals could have a higher risk of developing zeranol-induced breast cancer (Xu et al. 2009). In summary, it is well known that interactions between breast cancer and obesity have been studied for many years and zeranol is used as growth promoter for cattle. Therefore, in the present study, for further analysis we aimed to examine the effects of ZEN on metabolism-related genes in human breast epithelial cells.

Altered energy metabolism of cancer cells provides rapid growing for tumor cells than normal cells using higher rate of glucose metabolism (Hanahan and Weinberg 2011). Here, we have observed the effects of ZEN on transformed energy metabolism as a hallmark of cancer in breast cancer cells by analysis of gene expression levels. Our results showed that ZEN exposure altered mRNA levels of *GAPDH*, *IGF1*, *L-FABP*, *HK2*, *AhR*, *PXR*, and *PPARγ* genes significantly for 24 h in both cell lines. Various studies have shown that dysregulations in these genes could disrupt carbohydrate and lipid metabolism and could be associated with both metabolic dysfunctions and cancer progress (Gordon and Lowe 1985; Lee et al. 2010; Ahn et al. 2014; Cave et al. 2016; Lee et al. 2017). We observed that *IGF1* was significantly upregulated in both MCF7 and MCF10F cells after 10 μmol/L of ZEN for 24 h. In the present study, our results have shown that all exposure groups of ZEN significantly increased mRNA levels of *L-FABP* and *HK2* in MCF7 cells for 24 h, whereas only 10 μmol/L of ZEN increased *HK2* expression in MCF10F cells. Song et al. (2012) observed that differentially expressed proteins in tumor tissues compared to normal tissue were related to glycolysis/gluconeogenesis among other pathways, including *GAPDH*. Harami-Papp et al. (2016) found that *GAPDH* expression was higher in the *p53* mutant group between two breast cancer lines. Ter Braak et al. (2017) have reported that *IGF1* signaling axis plays a major role in tumorigenesis and *IGF1* overexpression has a strong and significant proliferative effect and mitogenic potential in mammary gland tumors. Besides, fatty acid synthase has a crucial role in the epithelial–mesenchymal transition of breast cancer cells, related to cell migration, metastasis, and *L-FABP* in its downstream proteins (Li et al. 2014). Furthermore, it has been shown that *L-FABP* was upregulated and could play a key role in the progress of invasiveness and metastasis in human breast cancer (Li et al. 2007). *HK2* performs the first step in most glucose metabolism pathways that is overexpressed in many cancer cell types, and *HK2* upregulation is related to the chemoresistance phenotypes in breast cancer cells (Lyon et al. 1988; Kaplan et al. 1990; Shinohara et al. 1994; Mathupala et al. 2009). Gao et al. (2017) have also demonstrated that *HK2* plays a role in the process of inflammation-driven migration in breast cancer cells.

Interplay between nuclear receptor function and breast cancer has been studied for several years, especially estrogen receptors. After nuclear receptor activation, growth factor signaling pathways could be coordinately activated. Interaction between nuclear receptors and epithelial cell growth is clinically associated with breast cancer (reviewed in Conzen 2008). Overexpressions of *PPARγ* and *ERα* have proliferative and antiapoptotic effects in breast cancer (Harvey et al. 1999; Suzuki et al. 2006). Our results have also shown that ZEN increased mRNA levels of *PPARγ* and *ERα* in MCF7 cells. Besides, it has been reported that *PPARγ* and *ERs* play a role on the regulation of *HK2* expression directly (Onishi et al. 2010; Tennessen et al. 2011; Panasyuk et al. 2012). Moreover, it was observed that after ZEN exposure, *PPARγ* and *AhR* was downregulated in MCF10F cells. Similar to our MCF10F results, downregulation of *PPARγ* and *AhR* in mice has exhibited an increase in mammary adenocarcinomas, ductal hyperplasia, and mammary growth in mammary gland and primary culture of mammary epithelial cells in carcinogen-mediated carcinogenesis (Nicol et al. 2004; Miret et al. 2017).

Consequentially, this is the first study to investigate the effects of ZEN on metabolism-related genes and global DNA methylation levels in MCF7 and MCF10F cells. Our findings could contribute that ZEN might affect epigenetic regulation and could induce progress of breast cancer. Especially, we found significant dose-related changes of *IGF1*, *L-FABP*, *HK2*, *PXR*, and *PPARγ* genes in MCF7 cells. Altered DNA methylation may have resulted in the abnormal gene expression of the key regulator genes which involve ZEN toxicity. Furthermore, we also highlight the role of *IGF1*, *HK2*, *PXR*, and *PPARγ* genes in the mechanism of ZEN toxicity.

Acknowledgements This work was supported in part by the TUBITAK (2211-C/2015-2 grant for E.F.K.) and Scientific Research Projects Coordination Unit of Istanbul University (project no. TOA-2016-20307).

Compliance with ethical standards

Conflicts of interest The authors report no conflicts of interest.

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