Potential anti-genotoxic effect of sodium butyrate to modulate induction of DNA damage by tamoxifen citrate in rat bone marrow cells

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Abstract Sodium butyrate (SB) is one of the histone deacetylase inhibitors (HDACi's) that is recently evidenced to have a prooxidant activity and an ability to reduce hydrogen peroxide-induced DNA damage. Since the majority of estrogen receptor positive breast cancer patients are treated with tamoxifen citrate (TC), which exerts well established oxidative and genotoxic effects, thus the basic objective of this study is to determine whether SB could ameliorate or curate tamoxifen citrate-induced oxidative DNA damage and genotoxic effect in vivo through up-regulation of some antioxidant enzymes. The individual and combined effects of SB and TC have been examined on rat bone marrow cells, using Micronucleus assays (MN), Comet assay, DNA fragmentation, expression of some antioxidant genes using Real time-PCR and finally, oxidative stress analysis. SB significantly increased the mitotic activity ($P < 0.05$), while TC induced marked micronuclei and oxidative DNA damage, in the SB post-treatment group, the combination of SB (300 mg/kg) and TC (40 mg/kg) was able to decrease the induction of MN and oxidative DNA damage through up-regulation of *Cat*, *Sod* and *Gpx1* genes significantly at ($P < 0.05$) more efficiently than that in

the SB pre-treatment one. Therefore, we postulate that SB can be used therapeutically in combination with TC treatment to modulate TC genotoxic effect by reducing its oxidative stress, and thus being an appropriate agonist agent to combine with TC than each compound alone.

Keywords Comet assay · Micronucleus assays · Oxidative stress · Sodium butyrate · Tamoxifen · qRT-PCR

Introduction

Tamoxifen citrate (TC), [Z-(1-(4-(2-dimethylaminoethoxy)phenyl)-1,2-diphenyl-1-butene)], the non-steroidal anti-estrogen drug, was approved by the Food and Drug Administration of the USA in 1977 as a drug that is utilized in the treatment of estrogen receptor-positive breast cancer (Wood and Osborne 1998), and recently as a long-term chemopreventive agent in women with high susceptibility of developing breast cancer (Jordan 2003; Dunn et al. 2005). Moreover, tamoxifen can hinder the growth of estrogen-receptor negative breast cancer cells (Yang et al. 2012). According to an American study, tamoxifen consumption seems to increase as a therapeutic agent not only among women with breast cancer but also among women with a family history of breast cancer (Rea et al. 1998). Since the optimal duration of tamoxifen treatment has not been fully

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established yet, it seems that the treatment will continue for long periods in both groups of women. However, studies have demonstrated that the danger of developing endometrial cancer may increase in two to seven fold after long-term use of tamoxifen (Singh et al. 2007; Brown 2009).

Tamoxifen also has been found to be a powerful hepatocarcinogen agent in laboratory rodents (White 1999). It forms DNA adducts in the liver of rats, hamsters, and mice, in a dose and time dependent manner, which leads to liver tumors, acting as initiator and promoter (Phillips 2001). Moreover, recent studies have reported several genotoxic effects of tamoxifen in vivo. In *Drosophila*, wing spot test demonstrates the induction of mutation and recombination events by TC in somatic cells at concentrations 0.66, 1.66 and 3.33 mM (Heres-Pulido et al. 2004). Al-Jassabi et al. reported the motivation of oxidative DNA damage in Balb/c male mice and some other cellular sections by tamoxifen (Al-Jassabi et al. 2011). In bone marrow cells, Rodrigues et al. demonstrated the induction of micronuclei, and reduction of polychromatic erythrocytes formation, in female Wistar-Hannover rats (Rodrigues et al. 2012; Yilmaz et al. 2014). Moreover, bone marrow depression when taking tamoxifen is a well reported symptom (Sloane 2002; Gail and Wilkes 2016). All previously mentioned studies confirm the genotoxicity of long term administration of tamoxifen.

Butyric acid is a microbiota-derived short-chain fatty acid (SCFAs), produced from microbial fermentation of dietary fibers in the large intestine (Pryde et al. 2002; Roy et al. 2006). It increases the relaxation of chromatin structure which leads to reversible chromatin decondensation (Vashishta and Hetman 2014). SCFAs act on the gene level by increasing the accessibility of different transcription factors to DNA through deacetylation inhibition of histones, and thus their names “histone deacetylase inhibitors” (HDA-Ci’s) (Xu et al. 2007). They play a vital role in growth inhibition, cell differentiation, cell cycle progression and apoptosis in several in vitro systems (Johnstone et al. 2002; Piekarz and Bates 2004; Wang et al. 2006). HDACi’s are considered as promising anticancer drugs and already have shown anticancer effects in both pre-clinical and clinical settings. They are gaining increasing attention because of their therapeutic effectiveness in selectively killing cancer cells and their mild toxicity profile (Kumar et al. 2015; Gajer et al. 2015).

Although the production of reactive oxygen species (ROS) is involved in several physiological processes, including defense against pathogenic microorganisms, cell proliferation control and apoptosis induction (Nose 2000; Thannickal and Fanburg 2000), an overproduction or a reduction in the removal of ROS from cells may contribute to destruction of the cell membrane by inducing lipid peroxidation. DNA damage may be induced as a result of attacking by exogenous agents or endogenous stress factors such as products of lipid peroxidation (Ames and Gold 1990). Recent evidences have demonstrated that butyrate ameliorates oxidative stress in the colonic mucosa of healthy humans (Hamer et al. 2009), and reverses the induced oxidative damage in the cerebral area of rats and mice (Steckert et al. 2013; Sun et al. 2015). In addition, β -hydroxybutyrate, an endogenous histone deacetylase inhibitor, can suppress the oxidative stress in HEK293T cells (Shimazu et al. 2013). Simultaneously, lack of butyrate causes inhibition of *Bax* gene expression, an anti-apoptotic gene and consequently, increases apoptosis in non-transformed colon cells in vivo (Hass et al. 1997). Abrahamse et al., has demonstrated that butyrate is able to decrease DNA damage induced by H_2O_2 in rat colon cells by modulating the oxidative stress (Abrahamse et al. 1999). It should be mentioned that some studies have evidenced a pro-oxidant activity of butyrate (Hockenbery et al. 1993; Klaunig et al. 1998; Chénais et al. 2000), against undifferentiated tumor cell lines; therefore this is not inconsistent with the protective role of butyrate.

These accumulated findings, regarding the anti-cancer and antioxidant activities of histone deacetylase inhibitors, motivated us to study whether sodium butyrate (SB) has protective and/or curative effects against TC-induced oxidative DNA damage in vivo, and consequently, may be an appropriate agonist agent to combine with TC to potentiate its anticancer effect.

Materials and methods

Chemicals

Tamoxifen, marketed as Nolvadex [tamoxifen citrate (20 mg)] was obtained from AstraZeneca (Cairo, Egypt). Sodium butyrate and all other chemicals were of analytical grade and were purchased from Sigma-Aldrich

(St. Louis, MO, USA). Kits for all biochemical parameters were purchased from Bio-diagnostic Company (Giza, Egypt). Other molecular kits are listed elsewhere.

Animals

Adult male wistar rats (*Rattus norvegicus*), aged 3–4 months, weighing 250–300 g were obtained from National Research Center (NRC, Giza, Egypt). All procedures dealing with rats followed the specifications recommended in The Guide for the Care and Use of Laboratory Animals (Kilkenny et al. 2014). Animals were maintained under standard conditions of humidity, temperature (25 ± 2 °C) and light (12-h light/12-h dark). Animals were allowed free access to tap water and standard pellet diet. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Science, Cairo University, Egypt (CUFS/F/PHY/45/15).

Experimental design

All chemicals were administrated orally; TC (40 mg/kg) and SB (300 mg/kg) were dissolved in saline. The chosen dose of SB has been previously reported to reduce the malondialdehyde (MDA) level and inhibit the expression of *Tnf- α* and *Il6* genes (Hu et al., 2014). The study included forty-nine male Wistar rats divided into three main groups: negative group (7 rats), SB pre-treated and SB post-treated groups (21rats/group).

Negative group (Control): rats received saline orally for 21 days.

SB pre-treatment group: animals were divided into three subgroups (7 rats/group): *Subgroup I* (SB-1): rats received SB for 7 days and then received saline for 14 days. *Subgroup II* (TC-1): rats received saline for 7 days and then received TC for 14 days. *Subgroup III* (SB-TC): rats received SB for 7 days and then received TC for 14 days.

SB post-treatment group: animals of this group were divided into three subgroups (7 rats/group): *Subgroup I* (SB-2): rats received saline for 14 days and then received SB for 7 days. *Subgroup II* (TC-2): rats received TC for 14 days and then received saline for 7 days. *Subgroup III* (TC-SB): rats received TC for 14 days and then received SB for 7 days.

At the end of the experiment, rats were euthanized under chloroform vapor and sacrificed after having been fasted overnight. Blood samples from each group

were collected without anticoagulant in centrifuge tubes. The two femurs were removed and immediately one of them was flushed with saline and divided into two parts: one part was mixed with mincing buffer and stored at -80 °C for comet assay; the other part was used for RNA extraction, biochemical measurements and DNA fragmentation, while the other femur was flushed with fetal calf serum for micronucleus test.

Detection of genomic DNA damage

Bone-marrow micronucleus assay

Bone marrow smears were prepared according to the method described by Schmid (1975). The dried smears were fixed in methanol for 5 min, and stained with May–Grunwald and Giemsa at pH 6.8. The slides were blindly coded, and screened with an oil-immersion objective (Jenaval microscope; Carl Zeiss, Jena, Germany). The number of micronucleated polychromatic erythrocytes (MNPCEs) were recorded among 1000 polychromatic erythrocytes (PCEs) per animal. Additionally, 1000 PCEs and normochromatic erythrocytes (NCEs) were analyzed to calculate PCEs/NCEs ratio for each animal to evaluate bone-marrow suppression rat for each treated group.

Alkaline comet assay

Bone marrow cells of treated or non-treated groups were tested for DNA damage using alkaline (pH > 13) comet assay, according to Tice et al. (2000). Conventional frosted microscopic slides were dipped into hot 1.0% normal melting point agarose, and the underside of the slide was wiped to remove agarose. 10 μ l aliquot of bone marrow cells homogenized with cold mincing solution (Hanks balanced Salt Solution (HBSS) was mixed with 75 μ l of 0.5% low melting point agarose, and coverslips were applied to spread the samples. After solidification, slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, NaOH to pH 10.0, 1% Triton-100 and 10% DMSO) at 4 °C for 24 h in the dark. Slides were soaked in a coupling jar containing a freshly-made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) to unwind for 20 min and electrophoresed at a constant current of 300 mA, for 20 min. By then, the slides were neutralized with Tris–HCl buffer (pH 7.5) by three washes for 5 min, followed by fixation in

100% cold ethanol and dried in air. Finally, the slides were stained with ethidium bromide (2 mg/ml) and covered with coverslips. The electrophoretic patterns were analyzed with an epifluorescence microscope (Zeiss epifluorescent) (400×) equipped with an image analysis system. Images of 50 isolated comets were randomly selected, and measured for comet tail length, %DNA in tail and tail moment using Comet Assay IV software (Perceptive Instruments, Suffolk, UK).

Qualitative DNA fragmentation assay

DNA fragmentation was determined according to the standard protocol described by Sambrook et al. (1989). Bone marrow cells were suspended in 100 µl lysis buffer containing 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA and 0.2% triton-100×, for 20 min on ice before centrifugation, then incubated with 50 µg/ml proteinase K at 56 °C overnight, centrifuged at 10,000 rpm for 30 min, and finally precipitated in 0.1 volume of 5 M NaCl. Soluble DNA in the resulting supernatant was precipitated with 600 isopropyl alcohol at –20 °C overnight. The DNA pellet was then rinsed with 70% ethanol, and dissolved in TE buffer, and incubated with 100 µg/ml RNase A at 56 °C for 1 h. Electrophoresis was carried out at 80 Volt (Power Supply Biorad (Hercules, CA, USA), Model 200/2.0), in a 1.5% agarose gel containing ethidium bromide, and photographed under UV light transilluminator (Stratagene, La Jolla, CA, USA) to visualize DNA fragmentation (laddering), characteristic of apoptosis.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

To assess the expression of *Cat*, *Sod* and *Gpx1* genes, total cellular RNA was extracted from bone marrow cells of control and experimental animals using PAX gene Bone Marrow RNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and was stored at –80 °C. The quantity of RNA was measured using Nanodrop Spectrophotometer, while RNA quality and degree of degradation was evaluated by electrophoresis using agarose gel (1%). For cDNA preparation, 1 mg single-stranded RNA was reversed transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA).

Synthesized cDNA was used for analyzing the expression of the tested genes by RT-PCR. Each gene was amplified in a separate 25 mL reaction using DreamTaq Green PCR Master Mix (2×) (Thermo Scientific). The reaction was performed using thermal cycler PCR (Techne TC-3000, Burlington, NJ, USA) at the following conditions: 94 °C for 5 min followed by 25 cycles at 94 °C for 30 s, (59 °C for the tested antioxidant genes and 57 °C for *Gapdh*) for 30 s, 72 °C for 1 min and final extension. The primers were designed using NCBI Primer blast and synthesized by Invitrogen (Carlsbad, CA, USA) (Table 1), where Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as an internal control gene.

Quantitative real time (qRT-PCR)

qRT-PCR was carried out using SYBR green-based real-time PCR and was detected with 7500 Fast systems (Applied Biosystem, Foster City, CA, USA) as follow: an initial heat activation step at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and elongation at 60 °C for 1 min. Total volume for each PCR reaction was 20 mL according to the manufacturer's protocol. The primer sequences were the same used for RT-PCR (Table 1). Each tissue sample was analyzed in triplicate for each gene. To normalize the expression level of the studied genes, *Gapdh* gene was used. Relative quantification of gene expression was performed using the Comparative Ct method ($\Delta\Delta C_t$) by comparing the number of cDNA copies in different samples among each other. $\Delta\Delta C_t = \Delta C_t \text{ target} - \Delta C_t \text{ Gapdh}$. The relative quantification of mRNA was calculated for statistical analysis $RQ = 2^{-\Delta\Delta C_t}$. The results were demonstrated in a graph as mean $\pm$ Standard Error (SE) values of relative change compared to the control group using Prism (Graph Pad Software, San Diego, CA, USA, version 5.01).

ROS production

Bone marrow cells were collected in tubes containing 1.5 ml fetal calf serum, centrifuged with ice-cold PBS (pH 7.4). Bone marrow cells were harvested by centrifugation, washed twice with cold PBS, and finally resuspended in PBS. Malondialdehyde (MDA) (Ohkawa et al. 1979), glutathione (GSH) (Beutler et al. 1963), glutathione peroxidase (GPX1) (Paglia and Valentine 1967), catalase (CAT) (Aebi 1984),

Table 1 Primer sequences for *Gapdh*, *Sod*, *Cat* and *Gpx1* cDNAs

Gene	Sense 5-3	Antisense 5-3	Product size (bp)
<i>Gapdh</i>	GTATCGGACGCCTGGTTAC	CTTGCCGTGGGTAGAGTCAT	128
<i>Sod</i>	AGGGCGTCATTCACCTCGAG	CCTCTCTTCATCCGCTGGAC	194
<i>Cat</i>	GGTAACTGGGACCTTGTGGG	GCCATTCATGTGCCGATGTC	222
<i>Gpx1</i>	AGTGCGAGGTGAATGGTGAG	TCGATGTCGATGGTGCGAAA	226

superoxide dismutase (SOD) (Nishikimi et al. 1972) and nitric oxide (NO) (Montgomery and Dymock 1961) were determined using Bio-diagnostic assay kits according to the manufacturer's instructions.

Statistical analysis

Unpaired student *T* test was used to test the significant difference between groups. Statistics were carried out with the statistical analysis systems (SAS) program (SAS, 2005). $P < 0.05$ was considered statistically significant. The following formula has been used to calculate the % of improvement:

$$\% \text{ of improvement} = \frac{\text{Treated group} - \text{TC group}}{\text{Control group}} \times 100$$

Results

Micronucleus test

Figure 2 shows statistically significant increase ($P < 0.05$) of MNPCEs frequencies in TC groups when compared with their negative control and SB groups. On the other hand, there was a significant decrease in the MNPCEs frequencies ($P < 0.05$) in the SB-TC group in comparison with the control group, and in the SB group in comparison with the TC group. This indicates that SB is more effective as a therapeutic agent than being a protective one in reducing MNs induced by TC (Fig. 1).

In addition, there was an observable increase in PCEs/NCEs ratio in the SB groups when compared with their controls, indicating its efficiency in ameliorating the mitotic activity of bone marrow cells (Fig. 2). This ratio significantly decreased in all TC treated groups and SB-TC group in comparison with controls, except TC-

Fig. 1 Photomicrograph showing normochromatic erythrocytes (NCEs), polychromatic erythrocytes (PCEs) and micronucleated polychromatic (MNPCEs) induced by TC in rat bone marrow cells

SB group which showed a significant increase of PCEs/NCEs ratio when compared to TC groups.

Comet assay

TC groups showed statistically significant increase ($P < 0.05$) of all DNA damage parameters (tail length, %DNA in tail and tail moment) in comparison with the negative control group and SB groups as shown in Fig. 4, while SB-TC and TC-SB groups showed a significant decrease ($P < 0.05$) in these parameters when compared with TC groups. In addition, tail length was significantly higher in the SB-TC group ($P < 0.05$) when compared with its negative control or with the SB group, indicating its lower efficiency as a protective agent than being a therapeutic one (Fig. 3, 4).

DNA fragmentation assay

DNA fragmentation is a marker of late stage apoptosis. As shown in Fig. 5, the SB group does not induce any

Fig. 2 Effects of SB on TC-induced micronucleated polychromatic erythrocytes (MNPCEs) and polychromatic to normochromatic (PCEs/NCEs) ratio in rat bone marrow cells. Results are expressed as mean $\pm$ SE, (asterisk) significant

difference with negative control at $P < 0.05$ using T -test, (number sign) statistically compared with SB, and (dollar sign) statistically compared with TC group

Fig. 3 Representative photo of damaged DNA induced by TC (**B**) compared with undamaged DNA (**A**)

Fig. 4 DNA damage represented as tail length, % DNA in tail and tail moment in different treatment groups in rat bone marrow cells. Results are expressed as mean $\pm$ SE, (asterisk) significant difference with negative control at $P < 0.05$ using T -

test, (number sign) statistically compared with the SB, (dollar sign) statistically compared with the TC group and (circumflex accent) statistically compared with the SB-TC group

DNA fragmentation as it has banding pattern similar to that of the negative control group (C), with neither a DNA ladder nor a smear on the gel. On contrary, DNA fragmentation of bone marrow cells by the TC groups

was evidenced by the appearance of fragmented DNA in the form of both laddered at 500 bp and 300 bp and smeared DNA fragments. TC-induced DNA damage was markedly reduced by co-administration of SB

Fig. 5 DNA fragmentation assay showing DNA isolated from bone marrow cells of different experimental groups of rats. *Lane (1)* DNA marker. *Lane (2)* intact DNA of control group (C).

Lane (3) intact DNA of SB group. *Lane (4)* slight smear of SB-TC group. *Lane (5)* smeared and ladder DNA in TC group. *Lane (6)* slight smear of TC-SB group

in the SB-TC and the TC-SB groups as indicated by the disappearance of laddering with slight existence of smeared DNA.

Levels of *Cat*, *Sod* and *Gpx1* genes expression

Changes of the gene expressions by treatment of SB, TC and their combinations are shown in Fig. 6. We examined the expression of *Cat*, *Sod* and *Gpx1* genes at the mRNA level by conventional semiquantitative RT-PCR (Fig. 6a) and results were confirmed by real time PCR (Fig. 6b). Results showed that the expression levels of *Sod* and *Gpx1* genes were significantly ($P < 0.05$) up-regulated in the SB-treated groups when referred to the control group. While *Cat*, *Sod* and *Gpx1* genes revealed significant increase ($P < 0.05$) in their expression levels in the SB-TC and the TC-SB groups when compared with TC groups, and reached nearly the expression level in the control. In addition, a significant increase ($P < 0.05$) in the expression of *Cat*, *Sod* and *Gpx1* genes was noticed in the SB post-treatment group when compared with the SB pre-treatment group, indicating SB efficiency in up-regulating the tested antioxidant genes.

Measurements of oxidative stress biomarkers

There was an increase in the level of MDA subsequent to TC administration either in the SB-TC or in the TC-SB groups, as compared to the corresponding control groups (Fig. 7). On the other hand, GSH, CAT, SOD, GPX1 and NO levels decreased significantly

($P < 0.05$) subsequent to TC administration either in the SB pre-treatment or in the SB post-treatment groups, as compared to the corresponding control groups (Fig. 7). Sodium butyrate administration caused a significant decrease ($P < 0.05$) in MDA concentration and a significant increase ($P < 0.05$) in bone marrow GSH, CAT, SOD, GPX1 and NO levels either in the SB pre-treatment or the SB post-treatment groups when compared to the TC group, with better % changes of MDA, GSH, GPX1 and CAT levels in the SB -TC group than those in the TC-SB group (Fig. 7).

Discussion

TC is an anticancer agent, it is a drug of choice for the prophylactic treatment of breast cancer (Bush 2007). Recently, many side effects have been denoted including increased colorectal and endometrial cancer risk (Brown 2009), which may indicates its genotoxic effects. This may be attributed to the drug action at DNA level, through its ability to induce DNA adducts in leucocytes and various tissues (Phillips et al. 1994a; Phillips 2001; da Costa et al. 2001; Umemoto et al. 2002). In the present study, TC induced significant DNA damage, apoptosis and micronuclei accomplished with lowering of the proliferation rate of PCEs. These findings are in agreement with many reports that have tested tamoxifen for its induction of micronucleus in metabolically competent human cells (Crofton-Sleigh et al. 1993; Phillips et al. 1994b; Styles et al. 1994; Vijayalaxmi and Rai 1996),

Fig. 6 Comparison of the effects of SB, TC and their combinations on the expression of *Cat*, *Sod* and *Gpx1* mRNA in bone marrow cells in the different experimental groups of rats. **A** Representative images of semiquantitative RT-PCR to amplify *Cat*, *Sod*, *Gpx1* and *Gapdh* genes. **B** Quantitative real time PCR was performed to *Cat*, *Sod* and *Gpx1* after normalization to the *Gapdh* gene. The mRNA ratios of *Cat*, *Sod*, *Gpx1* to *Gapdh* were calculated using the $\Delta\Delta C_t$ method

after normalization to *Gapdh* gene. Results are expressed as mean $\pm$ SE of nine independent experiments for each bar, (asterisk) significant difference with respect to the negative control at $P < 0.05$ using *T*-test, (number sign) statistically compared with the SB, (dollar sign) statistically compared with TC group and (circumflex accent) statistically compared with the SB-TC group

aneuploidy (Styles et al. 1994; Sargent et al. 1994, 2013), mutations in the *lacI* reporter and *P53* genes in rat hepatocytes (Davies et al. 1997; Vancutsem et al. 1994) and chromosomal aberrations, DNA damage in several cell types and animal organs (Mizutani et al. 2004; Carthew et al. 2001; Wozniak et al. 2007). Nevertheless, Wilson et al. reported that 50 μ M tamoxifen decreased human lymphocyte proliferation but it did not exert genotoxic activity (Wilson et al. 1995). Taken together with its ability of DNA adduct formation, these findings can indicate that tamoxifen, at least in some circumstances, shows the properties of a genotoxin.

Although, the mechanism of chromosomal aberration and MN induction by tamoxifen is not known, it is concluded that the clastogenic effects caused by tamoxifen may be due to the covalent DNA adducts produced by tamoxifen metabolites, as previously demonstrated by many researches. It has been well recognized that if the repair mechanisms could not remove the DNA adducts, they subsequently may produce mutations in genes required for growth control and chromosome aberration (Brown 2002; White 2001). 3,4-dihydrotamoxifen, 4-hydroxytamoxifen, tamoxifen N-oxide were found to be the major epoxide metabolites in rat, mouse and humans when tamoxifen is incubated with liver microsomes

Fig. 7 Effect of sodium butyrate (SB) on oxidative stress markers in bone marrow cells of male rats having received tamoxifen citrate (TC). Values are expressed as mean $\pm$ SE, and were statistically compared with the negative control (asterisk), the SB (number sign), with the TC group (dollar sign) at $P < 0.05$ and (circumflex accent) statistically compared with the SB-TC group

(Lim et al. 1994), in which 4-Hydroxy tamoxifen (OHT) was the predominant one as observed in mouse liver (Lyman and Jordan 1985). Furthermore, assessment of the percentage of DNA in the comet tail, tail length and tail moment showed that TC induced significant DNA damage in rat bone marrow cells. Additionally, TC was found to cause apoptosis as indicated by the appearance of laddered and smeared DNA. This is in contrast with the reports stated that OHT inhibited proliferation, induced DNA damage and apoptosis in several types of ER-positive and ER-negative breast cancer cells, in the cancer cells, non-cancerous cells, and was also cytotoxic to the non-cancerous MCF10A cells (Mandlekar and Kong 2001; Salami and Karami-Tehrani 2003; Yaacob and Ismail 2014). Taking together, these results indicate that accumulation of DNA damage is involved in TC

induced cytotoxicity. Indeed, in vitro assays and studies in mouse and rat models have shown that formation of DNA single and double strand breaks and DNA adducts by tamoxifen, were thought to be through chromosomal breaks and generation of free radical species (Phillips 2001; Poirier and Schild 2003; Mizutani et al. 2004; Wozniak et al. 2007).

Sodium butyrate, a naturally existing short-chain fatty acid (SCFA), is normally produced as a result of bacterial fiber fermentation in mammalian intestines, and is one of the most broadly studied HDACi's (Liu et al. 2014; Dong et al. 2014). The present findings have investigated the effect of sodium butyrate alone and in combination with TC. Administration of SB alone showed no micronuclei induction nor DNA damage, indicating its safety at the tested dose. This was in agreement with

Rosignoli et al. who showed no change in the DNA damage level after the incubation of colonocytes and tumor cell lines with butyrate at both tested concentrations (Rosignoli et al. 2001). Importantly, there were no detectable DNA breaks in SB-induced senescent cells as measured by the comet assay (Pospelova et al. 2009). In contrary to the previous studies, our findings demonstrated that administration of SB alone has altered bone marrow erythropoiesis based on the marked increase of PCEs percent in the bone marrow ($3.39 \pm 0.05^*$), that may be attributed to the increased ratio of the anti-apoptotic proteins BCL-XL and MCL-1 to their pro-apoptotic partners BCL-XS and MCL-1SL, respectively, as demonstrated by Castaneda et al. (2005), who found that SB induces erythroid cell progenitor survival and proliferation in vitro through BCL-family antiapoptotic protein expression (Castaneda et al. 2005). On the other hand, Terse et al. showed that SB did not significantly change PCEs percentage, indicating that it did not change bone marrow erythropoiesis at the tested doses (6.25 and 12.5 mM) (Terse et al. 2011). Anyway, the underlying mechanism of the observed hematologic effects at the dose tested is not completely understood.

Interestingly, Rosignoli et al. and Abrahamse et al. studies showed that butyrate (alone or in SCFAs mixtures) displayed a protective effect on the oxidative DNA damage induced by H_2O_2 (Abrahamse et al. 1999; Rosignoli et al. 2001). This supports our results showing reduction of micronuclei and DNA-damage after using SB as a protective or therapeutic agent. Furthermore, apoptosis was initiated during our long term exposure to TC as indicated by DNA fragmentation assay and was abrogated by co-administration of SB.

DNA damage demonstrates a steady-state process between the beginning of DNA damage and its repair by cellular processes (Collins et al. 1996). Thus, SB ability to reduce DNA damage could be through increasing both the chromatin accessibility to DNA repair enzymes (Smith 1986) and DNA excision repair enzymes (Dresler 1985). Furthermore, butyrate could enhance the intracellular antioxidant enzymes (i.e. catalase and glutathione peroxidase) responsible for the reduction of ROS (Yano and Tierney 1989; Sandstrom and Marklund 1990) this has been proven by our findings that revealed up-regulation of *Cat*,

Sod and *Gpx1* genes after SB administration and further confirmed by measuring their concentrations in bone marrow, thus resulting in a reduced level of DNA-damage, as indicated in our results. One other explanation for the protective role of butyrate against DNA damage may contribute to the protection against apoptosis, as demonstrated by Hass et al., who have revealed that the colonic epithelium cells induced apoptosis after absence of butyrate, while addition of butyrate protected against the induction of apoptosis. This reduction of apoptosis contributed to the reduction in the expression of the apoptotic BAX proteins (Hass et al. 1997). It is worth to mention that, this was not the case in the cancerous cells as demonstrated by Li et al. who showed that the sensitivity to the cytotoxic effect of etoposide was increased and the capacity of DSB repair was reduced after sodium butyrate treatment in MCF-7 but not in HEK293 cells (Li et al. 2015). These results introduce Histone deacetylase inhibitors (HDACi's) as promising antigenotoxin drugs alone or in combination with chemotherapy agents due to their ability to reduce ROS and DNA damage induced by TC at the tested dose. TC on the other hand, reduced the proliferation of PCEs cells but the presence of SB partially abrogated this inhibition.

To rationalize the marked efficiency of using SB as a therapeutic agent for ameliorating TC-induced genotoxicity than being used as a protective one, it was concluded that using SB before the administration of TC as a protective agent facilitated the accessibility of TC as well as the repair enzymes to the DNA, through modification of chromatin structure. Thus the enhancement of DNA damage and DNA adducts by TC overwhelm the efficiency of the repair mechanism which result in lower protective ability. Moreover, long-term exposure to this drug may induce mutations in critical genes especially those involved in the DNA repair pathways and consequently retarding their action (Yaacob and Ismail 2014). While using SB as a therapeutic agent in the SB post-treatment group facilitated the entrance of the repair enzymes alone, which demonstrated much more curative effects as proven by Smerdon et al. who revealed that normal and (partially) repair-deficient human cells treated with sodium butyrate motivated the initial rate of nucleotide excision repair at concentrations where the histones are extremely hyperacetylated (Smerdon et al. 1982).

Knowing that DNA damage depends on the protective endogenous factors, such as DNA repair efficiency and levels of antioxidant systems (Collins et al. 1996), and because exogenous agents or endogenous stress factors, such as products of lipid peroxidation, may induce oxidative DNA damage by attacking DNA, thus it is important to shed some lights on the possibilities of reducing TC-induced ROS by SB and enhancing the endogenous oxygen scavengers in order to investigate its potential to prevent oxidative DNA damage. ROS have been discovered to play a vital role in anticancer drug-induced toxicity (Parvez et al. 2006). Tamoxifen generates ROS and causes thiol depletion in a dose-dependent manner. The balance between ROS and cellular thiol levels plays a pivotal role in regulating cell cycle progression and apoptosis. This oxidative burst, must be balanced and counteracted by endogenous antioxidants (Ferlini et al. 1999). In accordance with the studies of El Beshbishy et al. and Parvez et al. the present study showed that TC caused a significant increase in the malondialdehyde (MDA) concentration in bone marrow cells (El-Beshbishy 2005; Parvez et al. 2006). Consequently, some intracellular antioxidant enzymes are reduced due to the increase of lipid peroxidation (Diplock et al. 1994). The oxidative stress seen after tamoxifen intoxication was accompanied by decreased SOD, CAT activities and GSH concentration and increased lipid peroxidation (El-Beshbishy et al. 2013). This has been demonstrated by the state of oxidative stress observed during tamoxifen administration that associated with decreased hepatic glutathione content and increased peroxidation (Rizzo et al. 1994). This is in sharp contrast with our data revealed down-regulation of the tested anti-oxidant genes. The functional and the structural integrity of the cell were affected by a cascade of events generated upon the depletion of GSH that enhances generation of reactive oxygen species and oxidative stress (DeLeve et al. 1996). Superoxide dismutase (SOD) and catalase (CAT) act as mutually supportive antioxidative enzymes, which provide protective defense against reactive oxygen species (Cerutti et al. 1994). Furthermore, tamoxifen has been shown to potentiate nitrous oxide production in breast cancer patients through enhancement of nitric oxide synthase II expression (Simeone et al. 2002). The present study demonstrated significant improvements of some antioxidant enzyme levels (SOD, CAT, GPX1) in bone marrow cells upon treatment of TC-intoxicated rats with sodium butyrate

and confirmed by significant upregulation of the expressions of *Cat*, *Sod* and *Gpx1* genes using qRT-PCR. These findings are concomitant with the results achieved by Canani et al. who reported an increase of GSH concentration in tamoxifen-intoxicated rats after SB administration, and thus prove the assumption of DNA damage due to oxidative stress (Canani et al. 2011).

In conclusion, from the current findings we can deduce that SB affords high efficacy therapeutic action than protective one for TC-induced DNA damage and oxidative stress. Moreover, SB could improve the mitotic activity of bone marrow cells and conserve the cellular DNA integrity. Therefore, the use of SB might be capable of reducing the genotoxic effects of long term administration of TC and could improve the quality of life for breast cancer patients. Furthermore, this is the first study that could postulate a mechanism by which SB, as being one of the histone deacetylase inhibitors (HDACi's), can modulate ROS through up-regulation of the anti-oxidant enzymes.

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Author contributions All the work of the present study is my own effort.

Compliance with ethical standards

Conflict of interest There is no potential conflict of interest or competing interest.

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