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Induction of the Nrf2-driven antioxidant response by tert-butylhydroquinone prevents ethanol-induced apoptosis in cranial neural crest cells

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ABSTRACT

Previous studies have shown that ethanol exposure causes apoptosis in cranial neural crest cells (NCCs), an ethanol-sensitive cell population implicated in Fetal Alcohol Spectrum Disorders (FASD). Additionally, induction of endogenous antioxidants through activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) has been shown to prevent oxidative stress and apoptosis in ethanol-exposed mouse embryos. The objective of this study was to test whether tert-butylhydroquinone (tBHQ), an Nrf2 inducer, can protect NCCs against ethanol-induced apoptosis. Ethanol exposure was shown to cause a moderate increase in the protein expression of Nrf2 and its downstream antioxidants in the NCCs. Treatment of NCCs with tBHQ alone significantly increased the protein expression of Nrf2 and its downstream antioxidants and also significantly increased the activities of the antioxidant enzymes. In NCCs exposed to ethanol, the tBHQ-mediated antioxidant response prevented oxidative stress and apoptosis. These results clearly demonstrate that the activation of Nrf2 signaling confers protection against ethanol-induced apoptosis in NCCs.

1. Introduction

Prenatal alcohol (ethanol) exposure results in a range of structural and functional birth defects. These comprise the Fetal Alcohol Spectrum Disorder (FASD), with full-blown Fetal Alcohol Syndrome (FAS) representing the severe end of the spectrum. Notably, prenatal alcohol exposure is considered to be the leading known cause of mental retardation in the Western world [1,2].

There are a number of factors that contribute to the genesis of the birth defects that ethanol causes, including the vulnerability of selected cell populations to ethanol-induced cytotoxicity [3–7]. As shown using rodent and avian animal models, among the vulnerable cell populations are cranial neural crest cells (NCCs) [3,5,8–10]. NCCs are progenitors of multiple cell types, including the skeletal and connective tissues of the face [11–13]. Ethanol has been shown to diminish NCC populations via the induction of apoptosis, a result that appears to contribute heavily to subsequent facial abnormalities [3,5,6,8,10].

Substantial evidence supports a critical role for oxidative stress in ethanol-induced apoptosis and subsequent malformations [8,14–17]. Reactive oxygen species (ROS) generation has been observed in mouse embryos exposed to ethanol both *in vitro* and *in vivo* [15,17] with various embryonic cell populations, including NCCs, being involved [8,14,18,19]. These findings provided a framework for studies employing exogenous antioxidants to reduce ethanol's teratogenicity. In this regard, superoxide dismutase (SOD) has been shown to diminish ethanol-induced superoxide anion generation, lipid peroxidation and cell death, as well as the incidence of neural tube defects in cultured mouse embryos [17]. *In vivo* studies have also shown that, in mice, maternal treatment with an SOD and catalase mimetic, EUK-134, reduces ethanol-induced apoptosis in selected cell populations in the developing limb buds and subsequent limb defects [20]. However, while promising for human application, exogenous antioxidants alone are not as effective in reducing ethanol's teratogenicity as desirable.

Another strategy for prenatal protection from ethanol-induced oxidative injury entails chemically mediated upregulation of endogenous antioxidants. In this light, recently, Nrf2 has been demonstrated to be a critical transcription factor that regulates the induction of phase 2 detoxifying and antioxidant genes [21,22]. Under basal conditions, Nrf2 is anchored mainly in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (Keap1), which in turn facilitates the ubiquitylation and subsequent proteolysis of Nrf2. When challenged by oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus where it forms a heterodimer with its partner, Maf and elicit the antioxidant response by the induction of a battery of gene products, including antioxidant genes and phase 2 detoxification enzymes [23,24].

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A wide range of natural and synthetic small molecules with diverse chemical backgrounds are potent inducers of Nrf2 activity [25–27]. Among these Nrf2 inducers are isothiocyanates, 1,2-dithiole-3-thiones (D3T) and tert-butylhydroquinone (tBHQ) [27–29]. Of these, tBHQ, which is approved for human use, is of particular interest. It is a metabolite of the widely used food antioxidant butylated hydroxyanisole that increases Nrf2 protein stability through inhibition of the Keap1-mediated ubiquitination [30–32]. It has been suggested that tBHQ directly acts on the thiol group of Keap1 by a C151-dependent mechanism [33].

Using an *in vivo* FASD model, recent studies have shown that maternal ethanol treatment increases both Nrf2 protein levels and Nrf2–ARE binding in mouse embryos. Ethanol exposure also resulted in a moderate increase in the mRNA expression of Nrf2 downstream target detoxifying and antioxidant genes as well as an increase in the expression of antioxidant proteins. Pretreatment with the Nrf2 inducer, D3T, significantly increased Nrf2 protein levels and Nrf2–ARE binding, and strongly induced the mRNA expression of Nrf2 downstream target genes. In addition, maternal D3T pretreatment resulted in a significant decrease in ethanol-induced ROS generation and apoptosis in the embryos [15]. These results demonstrate that Nrf2 signaling is involved in the induction of an antioxidant response in ethanol-exposed mouse embryos.

While the studies of intact embryos have contributed significantly to our base of knowledge regarding Nrf2 activation in mouse embryos following ethanol exposure, for a more complete understanding of the role Nrf2 signaling in ethanol-induced teratogenesis, studies focused on vulnerable cell populations are needed. To this end, the current study employed cultured NCCs to elucidate the molecular mechanisms involved in ethanol-induced Nrf2 activation in NCCs, and to determine whether the Nrf2 inducer, tBHQ, can provide protection against ethanol-induced oxidative stress and apoptosis in NCCs.

2. Materials and methods

2.1. Animal care

C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were mated for 2 h early in the light cycle. The time of vaginal-plug detection was considered 0 days, 0 h of gestation (GD 0:0). Mice were maintained on an *ad libitum* diet of breeder chow and water. Pregnant mice were killed on GD 10.5. The embryos were removed from the uterus and processed for NCC culture as described below. All protocols used in this study were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

2.2. Primary NCC culture

NCC cultures were established according to the methods of Zhao et al. [34] with modification. Briefly, embryos collected on gestational day 10.5 (GD 10.5) were placed in a Petri dish containing Dulbecco's Phosphate Buffered Saline (DPBS). From each embryo, the first branchial arches were dissected and digested at 37 °C in DPBS with 0.05% collagenase (Sigma, St. Louis, MO, USA). The first arch tissue was pipetted up and down to yield a single cell suspension. The cells were washed with fresh culture medium to stop digestion and were then plated onto fibronectin coated 6-well plates and maintained in Dulbecco's Modified Eagle Medium (DMEM) with Chicken Embryo Extract at 37 °C in 5% CO_2/95% air. The cells were used for experiments when they reached 70% confluence.

2.3. Ethanol exposure and tBHQ treatment

For ethanol exposure, NCCs were cultured in the presence of 100 mM ethanol (Sigma, St. Louis, MO, USA) for 24 h. This ethanol concentration was chosen because previous studies have shown that at early developmental stages in mice, acute administration of ethanol that yields peak maternal blood ethanol concentrations of 400–500 mg/dl (approximately 85–105 mM) is required to induce excessive cell death and subsequent malformations that are characteristic of FAS [4,5,10]. Although this ethanol concentration is high, it is not beyond that which can, and does occur in chronic alcoholics [35]. Evaporation of ethanol was prevented by tightly sealing the culture plates with Optical Tape (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Parallel cultures of control cells were treated in the same manner as the ethanol-exposed cells with respect to sealing of the culture plates. For tBHQ treatment, NCCs were incubated with 10 μM tBHQ (Sigma, St. Louis, MO, USA) alone for 16 h, followed by 24 h of concurrent exposure to tBHQ and 100 mM ethanol. The concentration of tBHQ used in the present study was based on the efficacy of tBHQ as established in cell culture systems by other investigators [28,31].

2.4. Western blotting

Western blots were performed as described previously [15]. Briefly, NCCs were washed once in phosphate-buffered saline (PBS) and lysed for 30 min in RIPA lysis buffer (PBS, 0.5% Sodium Deoxycholate, 1% NP-40, 0.1% SDS, 1 mM Dithiothreitol) with 1 mM PMSF and protease cocktail inhibitors (Roche Applied Science, Indianapolis, IN, USA). The samples were centrifuged at 16,000 × *g* for 10 min at 4 °C. The collected supernatants were then used for Western blotting. The protein concentration in each sample was determined using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. Western blots were performed by standard protocols. The levels of Nrf2, catalase, and SOD1 and SOD2 proteins were analyzed with the following antibodies, respectively: rabbit polyclonal anti-Nrf2 IgG (1:500, Santa Cruz, Santa Cruz, CA, USA), mouse monoclonal anti-catalase antibody (1:500; Sigma, St. Louis, MO, USA), rabbit polyclonal anti-SOD1 IgG (1:1000; Assay Designs, Ann Arbor, MI, USA), and rabbit polyclonal anti-SOD2 IgG (1:500; Assay Designs, Ann Arbor, MI, USA). The membranes were scanned on a Bio-Rad Versa DocTM Imaging System (Model 4000) and the intensity of the protein bands was analyzed using Bio-Rad Quantity One software (version 4.5.1).

2.5. Measurement of antioxidant enzyme activities

The activities of antioxidant enzymes were determined as described previously [15]. Total superoxide dismutase (SOD) activity was measured with an SOD Assay Kit from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD, USA) according to the manufacturer's instruction. Catalase activity was determined with an Amplex Red Catalase Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol.

2.6. Measurement of ROS generation

ROS generation was measured using a 2′,7′-dichlorodihydro-fluoroscein diacetate (DCHFDA) assay as described previously [15]. Briefly, the NCCs were washed twice in assay buffer (130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris–HCl, 30 mM glucose) and then homogenized in 100 μl of the buffer. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatant, which was equivalent to 40 μg of protein, was transferred to a 96-well micro-plate. Assay buffer and 2 μl of 2′,7′-dichlorodihydro-fluoroscein diacetate (DCHFDA; Molecular Probes, Inc., Eugene, OR, USA) (5 mM in DMSO) were added to the micro-plate to give a total reaction volume of 200 μl. The samples were incubated at 37 °C for 15 min. The fluorescence

intensity was monitored for 30 min after excitation at 485 nm and emission at 535 nm using a CytoFlor fluorescence plate reader (Perseptive Biosystems, Foster City, CA, USA). The protein concentration was measured using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The ROS levels were expressed as relative fluorescence intensity units.

2.7. Flow-cytometric analysis of apoptosis

Determination of apoptosis was performed using an Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. In brief, NCCs were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. Then 100 μl of the solution (1×10^5 cells) was transferred to a 5 ml culture tube and 5 μl of Annexin V was added. The cells were incubated for 15 min at room temperature in the dark and then analyzed in a Dako CyAn flow cytometer (Beckman-Coulter Dako CyAn ADP).

2.8. Statistical analysis

Statistical analyses were performed using StatView software (SAS Institute, Inc., Cary, NC). Data are expressed as mean ± SEM of three separate experiments. Statistical comparisons between groups were analyzed by a One-way ANOVA. Multiple comparison post-tests between groups were conducted using Bonferroni's test. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Ethanol exposure and treatment with tBHQ significantly increased Nrf2 protein expression in NCCs

To determine whether ethanol exposure and treatment with tBHQ can induce Nrf2 expression in NCCs, Nrf2 protein level was examined in NCCs that were exposed to ethanol or tBHQ alone, or in combination. Western blot analysis revealed an increase in the level of Nrf2 protein in NCCs exposed to ethanol or tBHQ alone. As illustrated in Fig. 1, NCCs treated with 10 μM tBHQ alone showed a 2.8-fold increase in Nrf2 protein expression as compared to control cultures. Exposure to 100 mM ethanol for 24 h also increased Nrf2 protein expression, though to a lesser extent. The combination of the tBHQ treatment and ethanol exposure yielded a 4.5-fold increase in Nrf2 protein expression in NCCs as compared to the control group.

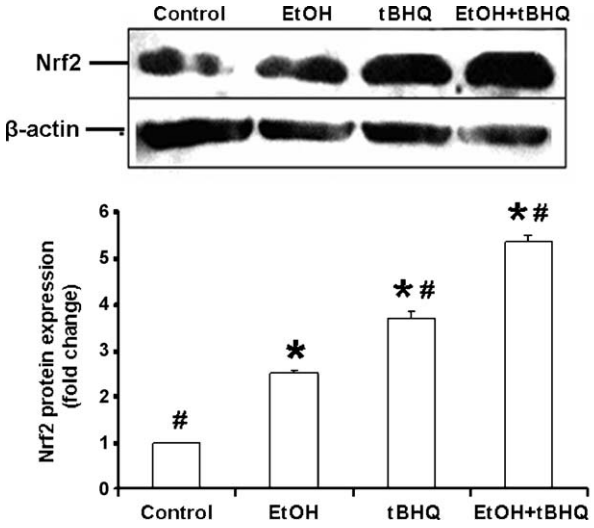

Fig. 1. Treatment with ethanol, tBHQ, and a combination of these agents increased the level of Nrf2 protein in NCCs. Western blot was performed to analyze the level of Nrf2 protein. Cell lysates were prepared from NCCs cultured in control medium (Control), treated with 100 mM ethanol (EtOH) or 10 μM tBHQ (tBHQ) alone, or treated with both ethanol and tBHQ (EtOH + tBHQ). Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. $^*p < 0.05$ *vs.* control. $^\#p < 0.05$ *vs.* EtOH.

3.2. tBHQ treatment significantly increased antioxidant protein expression in ethanol-exposed NCCs

Western blot analysis was also performed to examine whether over-expression of Nrf2 protein is accompanied by increased expression of Nrf2 downstream antioxidant proteins in this model system. Exposure to ethanol alone was found to increase the expression of cytoplasmic superoxide dismutase (SOD1), mitochondrial superoxide dismutase (SOD2) and catalase. However, for each of these antioxidants, less than a 1-fold change occurred. This result is consistent with that of previous *in vivo* studies that have shown that ethanol can only induce a moderate increase in antioxidant protein expression in mouse embryos treated with ethanol [15]. In contrast, treatment with tBHQ alone, or along with ethanol, significantly increased the protein expression of SOD1,

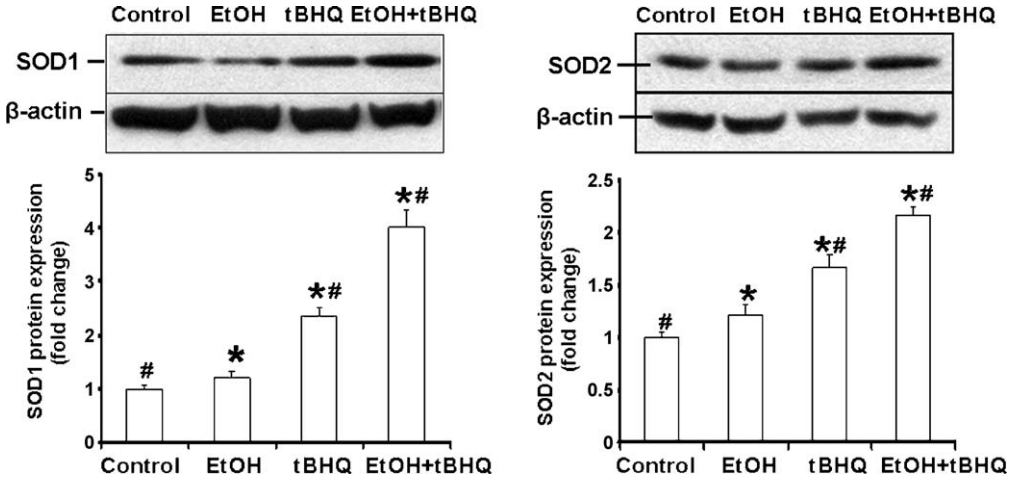

Fig. 2. Treatment with ethanol and tBHQ increased the SOD protein expression in NCCs. Western blot was performed to analyze the level of SOD protein in neural crest cells. Cell lysates were prepared from NCCs cultured in control medium (Control), treated with 100 mM ethanol (EtOH) or 10 μM tBHQ (tBHQ) alone, or treated with both ethanol and tBHQ (EtOH + tBHQ). Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. $^*p < 0.05$ *vs.* control. $^\#p < 0.05$ *vs.* EtOH.

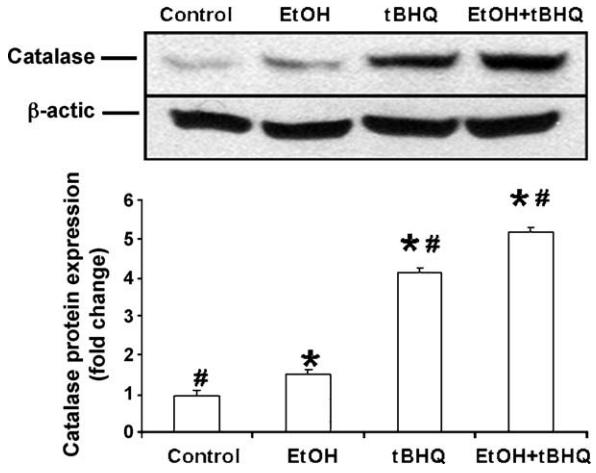

Fig. 3. tBHQ treatment resulted in significantly increased catalase protein expression in NCCs. Western blot was performed to analyze the level of catalase protein in NCCs. Cell lysates were prepared from NCCs cultured in control medium (Control), treated with 100 mM ethanol (EtOH) or 10 μM tBHQ (tBHQ) alone, or treated with both ethanol and tBHQ (EtOH + tBHQ). Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. $^{*}p < 0.05$ *vs.* control $^{\#}p < 0.05$ *vs.* EtOH.

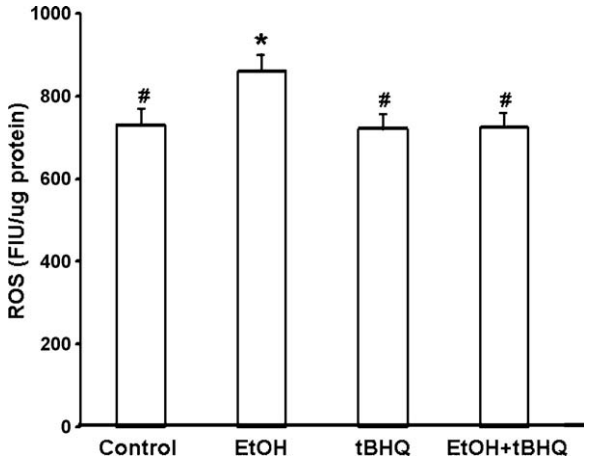

Fig. 5. tBHQ treatment significantly reduced ROS generation in ethanol-exposed NCCs. ROS generation was measured in NCCs cultured in control medium (Control), treated with 100 mM ethanol (EtOH) or 10 μM tBHQ (tBHQ) alone, or treated with both ethanol and tBHQ (EtOH + tBHQ). Data are expressed as relative fluorescence intensity units and represent the mean ± SEM of three separate experiments. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.05$ *vs.* EtOH.

SOD2 and catalase. The expression of SOD1 and catalase was increased 3.0- and 4.4-fold, respectively, in the co-treated NCCs as compared to controls (Figs. 2–3).

3.3. Treatment with tBHQ increased the activity of the antioxidant enzymes, SOD and catalase, in NCCs

The activities of SOD and catalase were analyzed to test whether upregulation of Nrf2 by ethanol or tBHQ results in an increase in the catalytic activity of antioxidant enzymes in NCCs. As shown in Fig. 4, while ethanol exposure alone resulted in increased SOD and catalase activity, treatment with tBHQ in combination with ethanol resulted in significantly greater increases in the activities of these enzymes, confirming the corresponding changes in the activities of major antioxidant enzymes that accompany protein induction.

3.4. tBHQ treatment significantly reduced ROS generation in ethanol-exposed NCCs

To test whether the activation of Nrf2 and induction of Nrf2 downstream antioxidant proteins reduce ethanol-induced oxidative stress in NCCs, a 2′,7′-dichlorodihydro-fluoroscein diacetate (DCHFDA) assay was used. The results of these experiments confirmed previous findings showing that ethanol exposure significantly increases ROS generation in NCCs. As expected, treatment with tBHQ was found to significantly reduce the ethanol-induced ROS generation (Fig. 5).

3.5. Treatment with tBHQ significantly reduced ethanol-induced apoptosis in NCCs

With the above findings and recognizing that ethanol-induced excessive NCC apoptosis is a prominent pathogenic feature in FASD models, the potential of tBHQ-mediated Nrf2-dependent antioxidant response to diminish this endpoint was tested. As shown in Fig. 6, flow cytometric analysis revealed a significant increase in apoptosis in the ethanol-exposed NCCs as compared to controls. tBHQ treatment significantly reduced the ethanol-induced apoptosis.

4. Discussion

The results of this study show that *in vitro* exposure of a vulnerable embryonic cell type, NCCs, to teratogenic ethanol

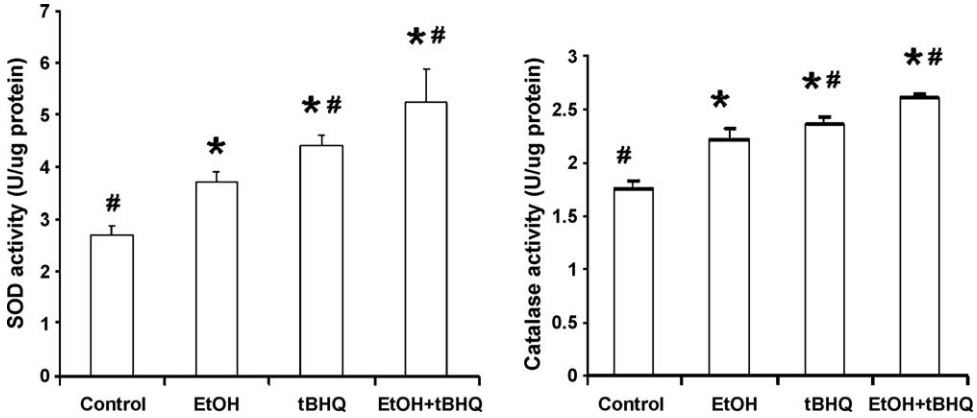

Fig. 4. Ethanol and tBHQ exposure increased the activities of the antioxidant enzymes, SOD and catalase, in NCCs. Cell lysates were prepared from NCCs cultured in control medium (Control), treated with 100 mM ethanol (EtOH) or 10 μM tBHQ (tBHQ) alone, or treated with both ethanol and tBHQ (EtOH + tBHQ). Data represent the mean ± SEM of three separate experiments. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.05$ *vs.* EtOH.

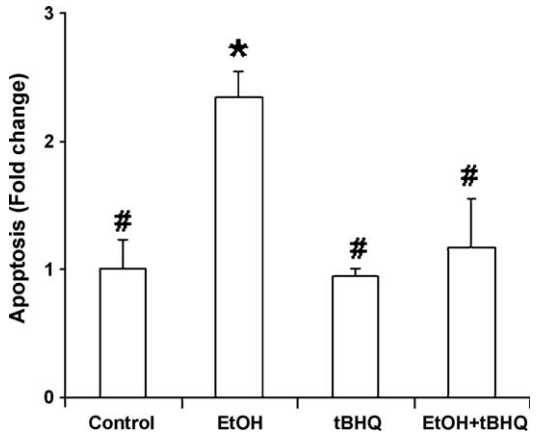

Fig. 6. Flow cytometry of apoptotic NCCs with Annexin V-FITC showed that tBHQ treatment significantly prevents ethanol-induced apoptosis. Apoptosis was measured in NCCs cultured in control medium (Control), treated with 100 mM ethanol (EtOH) or 10 μM tBHQ (tBHQ) alone, or treated with both ethanol and tBHQ (EtOH + tBHQ). Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. $^{*}p < 0.05$ *vs.* control. $^{\#}p < 0.05$ *vs.* EtOH.

concentrations increases the protein expression of Nrf2. It also increases the protein expression and catalytic activity of Nrf2's downstream antioxidants, SOD and catalase. These results provide support for the hypothesis that Nrf2 signaling is involved in the induction of antioxidant response in ethanol-exposed mouse embryos. Importantly, they also, demonstrate that Nrf2 activation and Nrf-2 dependent antioxidant response can be induced in NCCs, an ethanol-sensitive cell population implicated in FASD.

The central role of Nrf2 in cell survival has been well established by over a decade of research. Kotlo et al. [36] found that over-expression of Nrf2 protected cell from Fas-induced apoptosis. Activation of Nrf2 was also found to protect against cell death in an *in vitro* model of ischemia/reperfusion [37]. Additionally, nitric oxide-induced apoptosis can be prevented by the activation of Nrf2 [38]. Data have also shown that in response to elevated chromium (VI) and cadmium, mouse embryonic fibroblast cells lacking Nrf2 exhibit increased ROS production and apoptosis [39,40]. However, in this study, even though the activity of Nrf2 downstream antioxidants was increased following ethanol exposure, more than 1-fold increases in NCC apoptosis were observed. This maybe accounted for by the fact that the observed Nrf2, SOD and catalase changes were moderate. These findings are consistent with a previous *in vivo* study showing that the increases in the protein expression of Nrf2, SOD and catalase in mouse embryos exposed to ethanol alone are moderate [15], as well as with previous reports illustrating ethanol-induced apoptosis and subsequent abnormal development of involved tissues [4,5,20]. These results indicate that the ethanol-induced activation of Nrf2 and its downstream antioxidants is an adaptive response that is insufficient to prevent ethanol-induced apoptosis. In contrast, a chemical inducer of Nrf2, D3T, has been shown to potently increase Nrf2 levels, induce Nrf2 downstream antioxidant and detoxifying gene expression and prevent apoptosis in ethanol exposed embryos [15].

In addition to D3T, tBHQ has long been of interest as effective inducer of Nrf2 [22,33,41]. Studies have shown that tBHQ treatment increases Nrf2 activation in both *in vivo* and *in vitro* systems [28,31–33]. *In vitro* studies have shown that tBHQ treatment augments ARE-dependent gene expression in cultured astrogytes and protects neurons from oxidative injury [41–43]. There is abundant evidence that tBHQ is effective in diminishing ROS-mediated damage as a result of its action on Nrf2 [44,45]. Lee et al. [46] observed a protective effect of tBHQ against oxidative stress in wild-type astrocytes. This did not occur in Nrf2-deficient cells. They also found that 98% of tBHQ-inducible Nrf2-dependent genes in wild-type cells were not altered in Nrf2-null astrocytes. Other studies have shown that in $Nrf2^{-/-}$ mice the protective effect of tBHQ on mitochondrial stress was lost [47]. Additionally, microarray analyses of tBHQ-induced gene expression have indicated that the majority of identified genes in glia and neurons are Nrf2 dependent [41]. Consistent with these studies, the current results show that treatment of ethanol-exposed NCCs with tBHQ led to a significant induction of Nrf2 and an increased expression of antioxidant proteins and enzyme activity. tBHQ treatment also significantly reduced ethanol-induced ROS generation and NCC apoptosis. These results clearly demonstrate that the Nrf2 inducer, tBHQ, can induce an antioxidant response and prevent oxidative stress and apoptosis in ethanol-exposed NCCs. However, it is noteworthy that while tBHQ treatment resulted in a 3- to 4-fold increase in SOD and catalase protein expression in NCCs, tBHQ-induced increases in SOD and catalase activities were relatively moderate. It appears that the modest increases in SOD and catalase activities alone might not fully explain the robust anti-apoptotic action of tBHQ. This suggests that other antioxidants induced by Nrf2, which can induce a wide spectrum of antioxidants, may also contribute to tBHQ's antioxidant and anti-apoptotic actions. In addition, other protective actions of tBHQ which have not yet identified maybe involved in its anti-apoptotic effects.

Although FASD can be completely prevented by not drinking any alcohol during pregnancy, given the difficulties in achieving this goal, it is important to discover practical therapeutic approaches to prevent FASD. The results of this study add to the growing evidence that antioxidant supplementation can prevent ethanol induced damage or pathogenesis in certain experimental models of fetal alcohol exposure. The potency of tBHQ in inducing Nrf2 activation and preventing ethanol-induced oxidative stress and apoptosis in NCCs, along with the fact that tBHQ is a metabolite of a widely used food antioxidant which is approved for human use makes tBHQ a promising therapeutic agent for human FASD.

In conclusion, the results of the current study demonstrate that Nrf2 activation and Nrf-2 dependent antioxidant response can be induced in NCCs, an ethanol-sensitive cell population implicated in FASD and that tBHQ-mediated antioxidant response can prevent ethanol-induced oxidative stress and NCC apoptosis. These findings provide insights into the regulatory mechanisms involved in the induction of antioxidant response in ethanol-exposed embryonic cells and provide a new therapeutic perspective for FASD that is based on transcriptional modulation.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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