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3,4-Dihydroxyphenylacetic acid is a potential aldehyde dehydrogenase inducer in murine hepatoma Hepa1c1c7 cells

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3,4-Dihydroxyphenylacetic acid (DOPAC) is one of the major colonic microflora-produced catabolites of quercetin glycosides, such as quercetin 4′-glucoside derived from onion. Here, we investigated whether DOPAC modulates the aldehyde dehydrogenase (ALDH) activity and protects the cells from the acetaldehyde-induced cytotoxicity *in vitro*. DOPAC was shown to enhance not only the total ALDH activity, but also the gene expression of ALDH1A1, ALDH2 and ALDH3A1 in a concentration-dependent manner. DOPAC simultaneously stimulated the nuclear translocation of NFE2-related factor 2 and aryl hydrocarbon receptor. The pretreatment of DOPAC completely protected the cells from the acetaldehyde-induced cytotoxicity. The present study suggested that DOPAC acts as a potential ALDH inducer to prevent the alcohol-induced abnormal reaction.

Key words: 3,4-dihydroxyphenylacetic acid; aldehyde dehydrogenase; acetaldehyde; Nrf2; Hepa1c1c7 cells

3,4-Dihydroxyphenylacetic acid (DOPAC) is one of the major colonic microflora-produced catabolites of quercetin glycosides, such as quercetin 4′-glucoside,[1)] rutin,[2)] and hyperoside (quercetin 3-galactoside).[3)] Human fecal bacteria have the ability to catalyze the formation of DOPAC,[4)] and an excretion of DOPAC was increased in human urine after the digestion of polyphenols from chocolate,[5)] suggesting the actual occurrence of DOPAC in humans. We have recently identified DOPAC as a predominant antioxidative catabolite of quercetin glycosides.[6)] DOPAC also inhibited the hydrogen peroxide-induced cytotoxicity in hepatocytes.[6)] In addition to its antioxidant-related activities, DOPAC inhibited the secretion of pro-inflammatory cytokines from peripheral blood mononuclear cells.[7)]

Two enzymes, alcohol dehydrogenase and aldehyde dehydrogenase (ALDH), are basically involved in the physiological metabolism from ethanol into acetic acid.[8)] The mutation of certain ALDH genes or ALDH polymorphism results in the enhanced acetaldehyde accumulation, which causes serious damage to the liver and abnormal reaction like vasodilation and facial flushing.[9,10)] Therefore, the strategy for enhancement of the liver ALDH activities by the consumption of food phytochemicals is most likely to prevent humans from an alcohol-induced abnormal reaction.

In this study, to explore the possibility of DOPAC as a potential enhancer of the ALDH activity, we examined the protective effect of DOPAC on the acetaldehyde-induced cytotoxicity *in vitro*. Our results showed that DOPAC increased the total ALDH activity as well as nuclear protein levels of the transcriptional factor NFE2-related factor 2 (Nrf2) and aryl hydrocarbon receptor (AhR). Furthermore, the pretreatment of DOPAC increased the resistance to the acetaldehyde-induced cytotoxicity.

Materials and methods

Chemicals and antibodies. DOPAC was obtained from Sigma Aldrich (St. Louis, MO, USA). Antibodies against ALDH2, AhR, Nrf2, Nuclear factor κB (NF-κB), lamin B1, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ALDH1A1 and ALDH3A1 were purchased from Cell Signaling Technology (Beverly, MA, USA) and ABCAM (Cambridge, MA, USA), respectively. Trizol reagent and α-minimum essential medium (α-MEM) were purchased from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). ReverTra Ace was purchased from Toyobo Co., Ltd. (Osaka, Japan). Taq polymerase was purchased from Takara Bio, Inc. (Kusatsu, Japan). β-Nicotinamide-adenine dinucleotide, oxidized form (NAD^+) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from Wako

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Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; Nrf2, NFE2-related factor 2; AhR, aryl hydrocarbon receptor; ALDH, aldehyde dehydrogenase; NF-κB, nuclear factor κB; NAD^+, β-nicotinamide-adenine dinucleotide, oxidized form.

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Cell culture. The mouse hepatoma Hepa1c1c7 cells, from the American Type Culture Collection, were grown at 37 °C in a 5% CO_2 atmosphere in α-MEM supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Hepa1c1c7 cells (1.2×10^6) were cultured on 60-mm dishes at 37 °C in a 5% CO_2 atmosphere for 24 h, then treated with or without the different concentrations of DOPAC in 0.2% dimethyl sulfoxide (DMSO) for 6 h.

Assay of ALDH activity. The ALDH activity was measured as previously described by Moreb et al.[10] Briefly, the cell pellet was dissolved in 1 mL lysis buffer (50 mM Tris (pH 8), 25 mM EDTA, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% sarcosyl) and centrifugated at 10,000 rpm for 10 min at 4 °C. A two-hundred-μL supernatant was used as the enzyme samples to catalyze 20 μL of 5 mM propionaldehyde, and 20 μL of 5 mM NAD^+ was used as a cofactor to start this assay. NADH was measured by the change in absorbance at 340 nm over 5 min. One unit was defined as the amount of enzyme activity that catalyzed 1 μmol NAD^+ to NADH per minute. The ALDH activity was normalized to the protein concentration and expressed as the change in nanomoles of NADH formed per min per mg of protein.

RNA extraction and RT-PCR. Hepa1c1c7 cells (5.0×10^5) were precultured in 6-well plate for 6 h and then treated with or without the different concentrations of DOPAC. Total RNA was extracted using Trizol reagent according to the manufacturer's manual. Total RNA was reverse transcribed to cDNA using ReverTra Ace. PCR amplification was then performed with Taq polymerase. Primers used in PCR amplification are as follows: mALDH1A1, (F) 5′-gACAggCTTTCCA-gATTggCTC-3′ and (R) 5′-AAgACTTTCCCACCATT-gAgTgC-3′; mALDH2, (F) 5′-TgAAgACggTTACTg TTACTgTCAAAgTgC-3′ and (R) 5′-AgTgTgTgTgg CggTTTTTCTC-3′; mALDH3A1, (F) 5′-gATgCCCAT TgTgTgTgTTCg-3′ and (R) 5′-CCACCgCTTgATgTC TCTgC-3′; mβ-actin, (F) 5′-gCTCTTTTCCAgCCTTC CTT-3′ and (R) 5′-CTTCTgCATCCTgTCAgCAA-3′. Cycles and annealing temperatures used in PCR amplification are as follows: mALDH1A1, 20 cycles, 57 °C; mALDH2, 20 cycles, 57 °C; mALDH3A1, 25 cycles, 56 °C; mβ-actin, 16 cycles, 65 °C. The PCR product were separated on an agarose gel (3% or 4%), stained with ethidium bromide, and visualized under UV light. The relative densities of bands were measured using Image J Software Program.

Western blotting. The total cell lysate containing 30 μg of protein and nuclear fraction containing 10 μg of protein were prepared for SDS-PAGE as previously reported.[11] After the SDS-PAGE purification, proteins were electrophoretically transferred to PVDF or nitrocellulose membranes. Antibody binding was visualized using a Chem-Lumi One Super. The densitometric analysis of the bands was carried out using the Image J Software Program.

Cell viability assay. The cells were seeded in a 96-well plate at a density of 2×10^5 for 24 h. After preculture, the cells were pretreated with 10 μM DOPAC for 6 h, then washed twice and treated with different concentrations of acetaldehyde for 3 h with the complete medium. Finally, an MTT solution was added to each well, and the absorbance was measured at 570 nm by a microplate reader (Benchmarkplus, Bio-Rad laboratories, Hercules, CA, USA) after a 2-h incubation at 37 °C. The cell viability results were expressed as a percentage compared to that of the control.

Statistical analysis. All values were expressed as means ± SD. The statistical significance was analyzed by one-way ANOVA followed by Tukey's HSD using XLSTAT software. A p-value of 0.05 was regarded to be statistically significant.

Results and discussion

As shown in Fig. 1(A), DOPAC significantly and concentration-dependently enhanced the total ALDH activity after a 6-h treatment. The total ALDH activity in the cells treated with 10 μM DOPAC, the minimal concentration for a significant enhancement, was 1.7-fold greater than that of the control. The ALDHs consist of a large family of enzymes catalyzing the conversion of different aldehydes into their corresponding acids. Among the 19 genes in the ALDH family, ALDH1A1 and ALDH2 have been reported to express at a high level in the liver tissue of humans and play a major role in the acetaldehyde metabolism.[12,13] ALDH3A1 has also been suggested to assist ALDH2 in the metabolism of acetaldehyde and ethanol *in vivo*.[14] Thus we examined the effect of DOPAC on the gene expression of ALDH1A1, ALDH2 and ALDH3A1. As shown in Fig. 1(B)–(D), DOPAC significantly increased each gene expression of ALDH1A1, ALDH2 and ALDH3A1, respectively. Furthermore, as shown in Fig. 2, DOPAC also significantly increased the protein levels of ALDH1A1, ALDH2 and ALDH3A1. Therefore, DOPAC acts as a potential inducer of the total ALDH activity, possibly through the transcriptional regulation of not only ALDH2 and ALDH1A1, but also ALDH3A1.

A previous study using a DOPAC click chemistry probe indicated that the signaling pathway of Nrf2 and/or AhR is attributable to the gene expression of the phase 2 drug-metabolizing enzymes.[6,15] NF-κB is known as the nuclear factor that interacts positively or negatively with Nrf2 and AhR.[16] To investigate whether Nrf2, AhR and NF-κB, are involved in the ALDH expression, we examined their protein expression. As shown in Fig. 3(A), DOPAC showed the tendency to increase the total protein expression of Nrf2

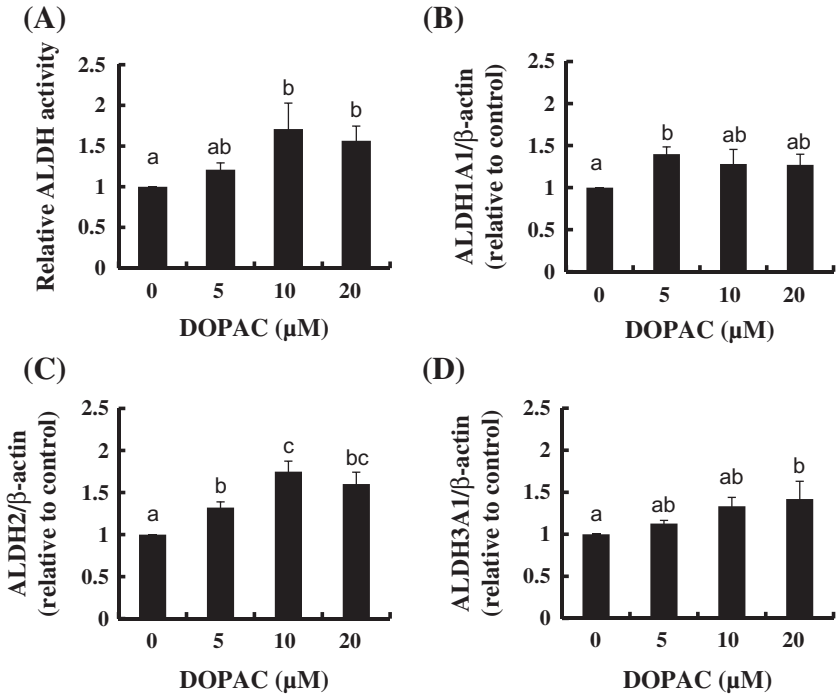

Fig. 1. Modulating effects of DOPAC on the activity and gene expression of ALDHs.

Notes: Hepa1c1c7 cells were treated with the indicated concentrations of DOPAC for 6 h. The total ALDH activity was measured using propionaldehyde as a substrate (A) and a RT-PCR analysis for each gene was carried out; (B) ALDH1A1, (C) ALDH2, and (D) ALDH3A1. All values are expressed as means ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

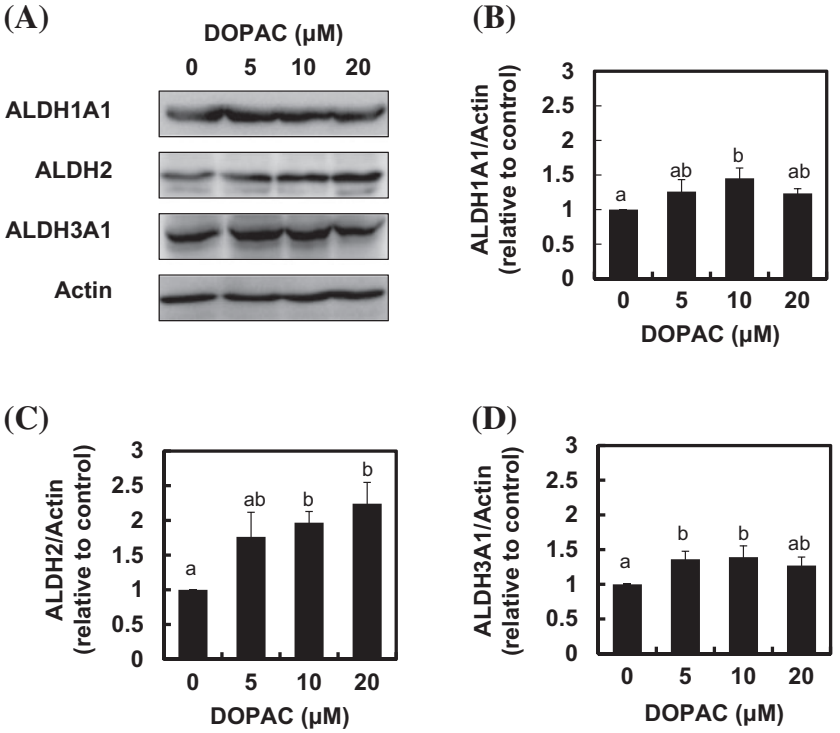

Fig. 2. Modulating effects of DOPAC on the protein expression of ALDHs.

Notes: Hepa1c1c7 cells were treated with the indicated concentrations of DOPAC for 6 h. A Western blotting analysis for each protein was carried out; representative blots (A) and quantitative data for ALDH1A1 (B), ALDH2 (C), and ALDH3A1 (D). All values are expressed as means ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

and AhR, but not NF-κB. We next checked the nuclear translocation of these transcriptional factors. The nuclear levels of Nrf2 and AhR significantly increased after the DOPAC treatment, whereas the nuclear level of NF-κB was attenuated by the DOPAC treatment (Fig. 3(B)). These results suggested that DOPAC is

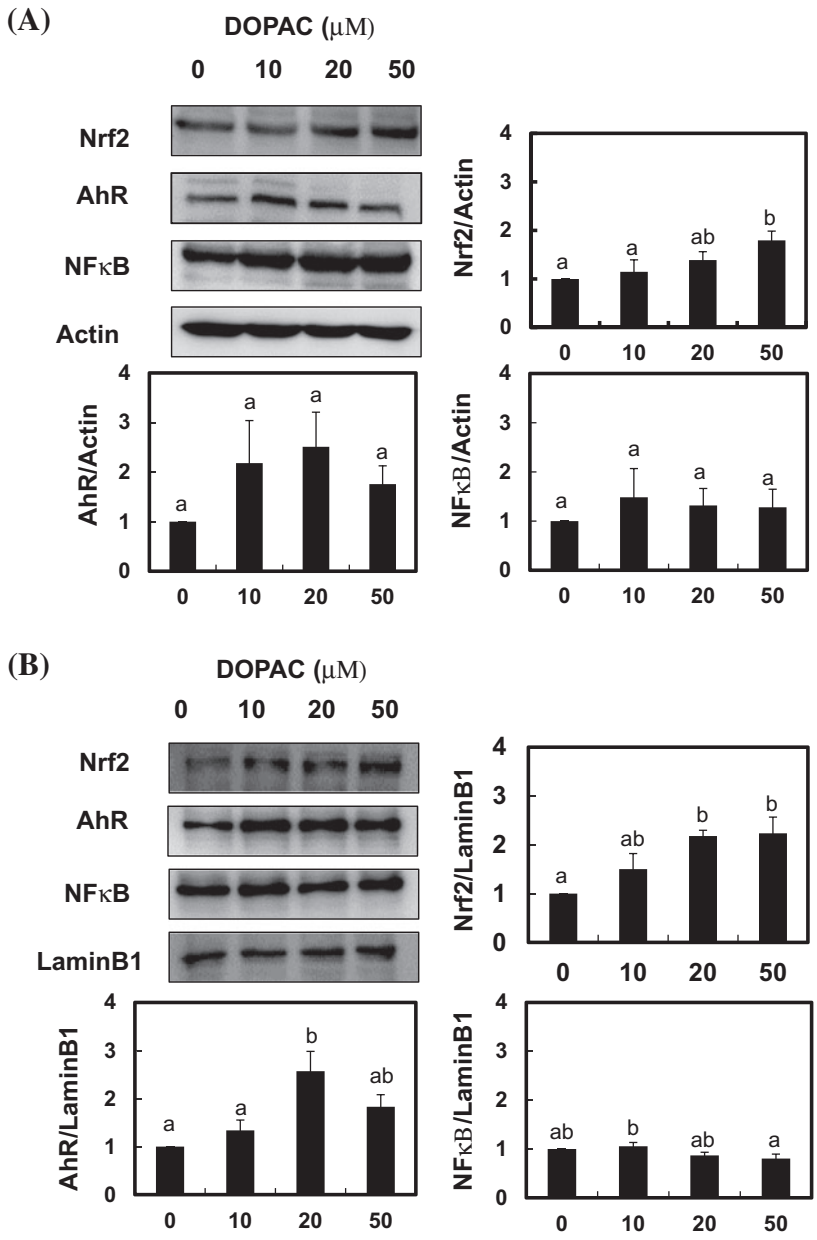

Fig. 3. Modulating effects of DOPAC on the protein expression and nuclear translocation of Nrf2, AhR and NF-κB.

Notes: Hepa1c1c7 cells were treated with DOPAC for 6 h, and nuclear fractions as well as total cell lysates were subjected to a Western blot analysis; (A) the protein levels of Nrf2, AhR and NF-κB in the whole lysate; (B) the nuclear accumulation of Nrf2, AhR and NF-κB. All values are expressed as means ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

able to activate the signaling pathways of both Nrf2 and AhR, but rather inhibit the NF-κB pathway.

Acetaldehyde, the primary metabolite of ethanol in human body, covalently binds to a variety of thiols, such as GSH and protein sulfhydryls, and consequently induces lipid peroxidation, thereby altering the liver function and structure.[8] We finally examined the effect of the DOPAC pretreatment on the acetaldehyde-induced cytotoxicity. Although the 6-h treatment of DOPAC at concentrations up to 200 μM did not significantly influence the cell viability (Fig. 4(A)), the 3-h incubation with acetaldehyde at each concentration significantly decreased the cell viability (Fig. 4(B)). The pretreatment of 10 μM DOPAC for 6 h completely impaired the acetaldehyde-induced cytotoxicity (Fig. 4(B)), suggesting that DOPAC at the concentration required for the inducible expression of the ALDHs actually exhibited a cytoprotective effect.

Nrf2 has been reported as a transcriptional factor involved in the inducible expression of ALDH1,[17] ALDH2,[18] and ALDH3A1.[19] In this study, we observed that DOPAC significantly increased the nuclear translocation of Nrf2 (Fig. 3(A) and (B)). A previous study demonstrated that the knockdown of AhR reduced the total ALDH activity by 80% in Hs578T cells.[20] The gene expression of ALDH3 was shown to be mediated by an AhR/xenobiotic response element axis.[21] AhR has been identified as a potential target of covalent modification by DOPAC.[15] Expectedly, DOPAC significantly increased the nuclear level of the AhR proteins (Fig. 3(A) and (B)). Although the promoter region of the ALDH3A1 gene is also reported

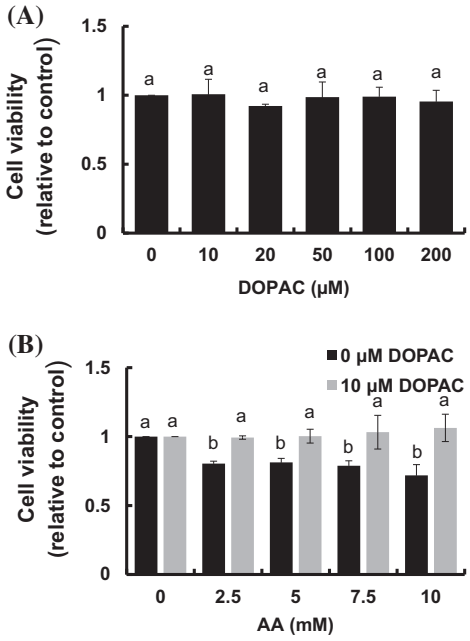

Fig. 4. Mitigating effect of DOPAC on the acetaldehyde-induced cytotoxicity in Hepa1c1c7 cells.

Notes: Hepa1c1c7 cells (2×10^5) were seeded in a 96-well plate and incubated for 24 h. (A) Effect of DOPAC on cell viability of Hepa1c1c7 cells. Cells were treated with different concentrations of DOPAC for 6 h. (B) Inhibitory effect of DOPAC pretreatment on the acetaldehyde-induced cytotoxicity. After the pretreatment of 10 μM DOPAC for 6 h, the cells were treated with different concentrations of acetaldehyde for 3 h. After the acetaldehyde stimulation, an MTT assay was carried out. All values are expressed as means ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

to bind with NF-κB,[22] DOPAC did not increase its nuclear level (Fig. 3(B)). Taken together, DOPAC might activate both the AhR and Nrf2-dependent pathways, but not the NF-κB pathway, to increase the expression of the ALDH genes. Keap1 is postulated as a plausible target of electrophiles that facilitate the depression of Nrf2.[23] Electrophilic quinones have also been reported to have capability to covalently bind to and activate AhR.[24] Our group has successfully detected the direct modification of Keap1 and AhR by DOPAC using its click chemistry probe and the pull down assay.[15] These results indicated that covalent modification of Keap1 and AhR by DOPAC might be involved in the activation of these pathways and up-regulation of ALDH genes in Hepa1c1c7 cells. DOPAC not only induced the gene expression of ALDHs, but also the phase 2 genes including glutathione S-transferase,[25] heme oxygenase-1 and glutamate-cysteine ligase, catalytic subunit,[6] both of which play an effective role in protection against oxidative stress.[23] Oxidative stress is also suggested to be involved in the toxic mechanism of acetaldehyde.[8] Therefore, the DOPAC-induced phase 2 enzymes cannot be ruled out in the cytoprotective mechanism against acetaldehyde. Acetaldehyde can impair the protein functions and gene expression by covalent binding formation with proteins and DNA.[26] Glutathione is also a binding target for acetaldehyde and inhibits its electrophilic reactivtiy.[8,26] However, the treatment of Hapa1c1c7 cells with lower than 100 μM of DOPAC did not exhibit a significant alteration of the intracellular level of reduced form of glutathione.[6] Therefore, the glutathione-dependent mechanism might be unattributable to the DOPAC-induced cytoprotection against acetaldehyde.

In conclusion, we identified a major catabolite of quercetin glycosides, DOPAC, as an ALDH activity enhancer. The DOPAC pretreatment also increases the resistance to the acetaldehyde-induced cytotoxicity, possibly through the transcriptional regulation of the ALDH genes by Nrf2 and AhR. Since DOPAC is a phenolic acid catabolite of dopamine as well as quercetin with a much lower cytotoxicity (Fig. 4(A)), DOPAC has some advantages for application as a food chemical to prevent humans from an alcohol-induced abnormal reaction. Future efforts will be concerned with further understanding the signaling pathway of the ALDH induction as well as *in vivo* significance of the protective effect of DOPAC against the alcohol-induced toxicity.

Author contributions

Y. L., A. K. and S. N. performed the experiments. B. Z., S. M., T. N., and Y. M. assisted with the experiments and contributed to discussions. Y. L. and Y. N. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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