Extracts of *Chrysanthemum zawadskii* attenuate oxidative damage to vascular endothelial cells caused by a highly reducing sugar

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Abstract Endothelial cells are considered candidates for involvement in the pathogenesis of diabetic vascular complications, and prevention of endothelial cell damage may be important in pharmacological attempts to prevent such complications. In the present study, I explored whether extracts of *Chrysanthemum zawadskii* (CZE) could prevent oxidative damage and dysfunction of a vascular endothelial cell line caused by the highly reducing sugar, 2-deoxy-D-ribose (dRib), and dysfunction of a vascular endothelial cell line. Vascular endothelial cells were treated with dRib in the presence or absence of CZE. Cell viability was monitored using a cell counting kit, and the induction of apoptosis was evaluated with a cell death kit. Prostaglandin E2 and cyclooxygenase-2 levels were measured using enzyme-linked immunosorbent assay kits. Mitochondrial membrane potential [$\Delta\Psi(m)$] was determined using a JC-1 kit. Intracellular oxidative stress was measured by fluorometric analysis of dichlorofluorescein oxidation using 2',7'-dichlorofluorescein diacetate as the probe. The expression levels of genes encoding antioxidant enzymes were analyzed by real-time polymerase chain reaction. dRib reduced cell survival and the $\Delta\Psi(m)$ and markedly increased

intracellular levels of reactive oxygen species and apoptosis. However, pretreatment of cells with CZE attenuated all these dRib-induced effects. The antioxidant *N*-acetyl-L-cysteine (NAC) also prevented dRib-induced oxidative cell damage. CZE attenuated the dRib-induced production of the inflammatory mediators cyclooxygenase-2 and Prostaglandin E2. NAC also exhibited anti-inflammatory effects and treatment with CZE caused transcriptional elevation of genes encoding antioxidant enzymes. Taken together, the results suggest that CZE may exert an antioxidant action that reduces dRib-induced cell damage to vascular endothelial cells and may thus aid in preventing diabetes-associated microvascular complications.

Keywords *Chrysanthemum zawadskii* · Flavonoid · 2-Deoxy-D-ribose · Oxidative stress · Vascular endothelial cells

Introduction

Oxidative stress is caused by a persistent imbalance between antioxidant defense levels and the concentrations of highly reactive oxygen species (ROS) (Robertson et al. 2003). Chronic hyperglycemia triggers oxidative stress, which, in turn, enhances the progression of pancreatic beta cell deterioration and the development of diabetic complications (Robertson 2004). Endothelial cells cultured at high glucose

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concentrations exhibit reduced proliferation rates (Doyle et al. 1997; Rojas et al. 2003) and prolongation of the cell cycle (Lorenzi et al. 1987). The addition of antioxidants returns endothelial cell growth to near-normal, showing that high-level glucose may delay endothelial cell replication via generation of free radicals (Curcio and Ceriello 1992; Recchioni et al. 2002; Wu et al. 1999). Some studies have shown that oxidative stress triggers apoptosis of endothelial cells grown under high-glucose conditions; this is inhibited by antioxidants (Recchioni et al. 2002; Wu et al. 1999; Du et al. 1998).

Sugars containing aldehyde groups that are oxidized to carboxylic acids are classified as reducing sugars and produce ROS via autooxidation and protein glycosylation (Thornalley et al. 1984; Kaneto et al. 1996; Bunn and Higgins 1981). 2-deoxy-D-ribose (dRib) is a strongly reducing sugar that is highly reactive with proteins (Koh et al. 2005, 2010). As glucose is the least reactive of the reducing sugars, long-term exposure to elevated glucose levels is required before cellular oxidative stress is induced (Bunn and Higgins 1981). I chose dRib as a surrogate for glucose in terms of induction of oxidative damage to the bovine pulmonary artery endothelial cells (BPAEs) used in the present study. dRib promotes apoptosis of pancreatic β -cells (Koh et al. 2005, 2010; Lee et al. 2010; Suh et al. 2012) and osteoblastic cells (Choi and Kim 2008; Lee and Choi 2008; Suh et al. 2009) by increasing oxidative stress.

Chrysanthemum zawadskii (CZE), a species of the chrysanthemum genus, has traditionally been used to treat various diseases. Extracts of chrysanthemum plants have been shown to have various medicinal properties, including antimicrobial (Sassi et al. 2008), antioxidant (Liu et al. 2012; He et al. 2011), and antimycotic activities (Marongiu et al. 2009). Extracts of CZE reduced inflammation and oxidative stress in murine RAW 264.7 macrophage cells (Wu et al. 2011). More recently, CZE has been shown to attenuate dRib-induced oxidative cell damage in osteoblastic cells by upregulating the transcription of genes encoding antioxidant enzymes, suggesting that CZE may be useful in the treatment of diabetes-associated bone disease (Suh et al. 2013).

Although the beneficial pharmacological effects of CZE are attributable to its antioxidant activity, little is known about the effects of CZE on the functions of vascular endothelial cells. In the present study, I

investigated the effects of CZE on oxidative stress-induced damage to and cellular dysfunction of BPAEs (Bos taurus pulmonary artery/endothelium).

Materials and methods

Preparation of CZE

CZE was kindly provided by Dr. Kwang Sik Suh (Research Institute of Endocrinology, Kyung Hee University Hospital, Seoul, Korea) from dried aerial parts of *Chrysanthemum zawadskii* (1 kg). These were refluxed with 80% ethanol (in water, v/v) at room temperature. The extract was filtered, evaporated to dryness at 50 °C in a rotary vacuum evaporator, and finally lyophilized. The extract (total yield 26.7%) was dissolved in dimethyl sulfoxide (DMSO) and diluted to appropriate concentrations with culture medium [the final DMSO concentration was 0.05% (v/v)]. High-performance liquid chromatograph (HPLC) profiling, performed as previously described, showed that the lignan content was 4.05 ± 0.27 w/w % (Suh et al. 2013).

Cell culture

BPAEs were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in medium 199 (Invitrogen, CA, Carlsbad, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Corp., Logan, UT, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco BRL Co., Grand Island, NY, USA). Cultures were maintained at 37 °C in a humidified atmosphere under 5% (v/v) CO₂ and sub-cultured by trypsinization with 0.05% trypsin-0.02% (both w/v) EDTA in Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS) when confluence attained about 80%.

Assessment of cell viability

Cell viability was determined by measuring metabolic activity using a CCK-8 kit (Dojindo Co., Kumamoto, Japan) (Suh et al. 2010). The kit uses WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] to produce a water-soluble formazan dye after reduction in the presence of an electron carrier. The level of yellow

formazan dye generated by cellular dehydrogenase activity is directly proportional to the number of living cells. BPAEs were plated in 24-well culture plates at a density of 2×10^4 cells per well. At the end of the culture period, 50 μ L of CCK-8 solution was added to each well, which contained 500 μ L of medium. After 4 h of incubation, absorbance was measured using a multi-scan GO detector (Thermo Fisher Scientific, Vantaa, Finland) at 450 nm employing a 650 nm filter as a reference. Cells incubated with culture medium alone were used to estimate full viability and were included as controls in all experiments to allow estimation of the percentage viabilities of test samples.

Assessment of apoptosis via ELISA

A cell-death ELISA kit was used to measure apoptosis, according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The kit quantitatively measures cytosolic histone-associated DNA fragments. Briefly, BPAEs were seeded at a density of 2×10^4 cells in 24-well culture plates. The culture conditions used were the same as those described above for the cell viability assay. After incubation, cells were lysed, and intact nuclei were pelleted by centrifugation. Aliquots of the supernatants were added to streptavidin-coated wells of a microtiter plate to which antigens were allowed to bind. The plates were next exposed to a secondary anti-DNA monoclonal antibody coupled to peroxidase. Nucleosome levels were quantified by determining the levels of peroxidase retained in the immunocomplexes. Peroxidase activity was determined photometrically at 405 nm using ABTS (2,2'-azino-di[3-ethylbenzthiazolin-sulfonate]) as substrate and a Zenyth 3100 multimode detector.

Measurement of ROS

The fluorescent probe chloromethyl-2,7-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR, USA) was used to measure intracellular ROS levels (Suh et al. 2010). BPAEs were cultured for 24 h in medium 199 containing 0.5% (v/v) FBS, rinsed twice with DPBS, and next treated with 10 μ M DCFDA for 1 h. The cells were rinsed, scraped off their substrate, and their fluorescence was measured (excitation 485 nm, emission 515 nm) using a Zenyth 3100 multimode detector.

Cyclo-oxygenase-2 (COX2) and prostaglandin E2 (PGE2) determinations

Cells were seeded at a density of 2×10^4 /ml in 24-well plates. The culture conditions used were the same as those described above for the cell viability assay. All analyses were performed in line with the instructions of the ELISA kit (Mybiosource, Inc., San Diego, CA, USA). We plotted curves of optical densities against standard concentrations. The levels of COX2 and PGE2 in each sample were derived using these standard curves.

Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ values of cells were measured using a JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo cyanine iodide)-based kit detecting mitochondrial membrane potential (Cayman Chemical Co., Ann Arbor, MI, USA). Cells were incubated with the mitochondrial membrane potential-sensitive fluorescent dye JC-1 for 20 min at 37 °C and washed twice with DPBS; the levels of red (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) were measured using a Zenyth 3100 multimode detector. Mitochondrial depolarization (i.e., loss of mitochondrial membrane potential) was indicated by a decrease in the red/green fluorescence ratio.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen Corp.). After isolation, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cDNAs were synthesized using a Transcriptor First-Strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) and stored at -70 °C prior to further processing. All procedures followed the manufacturers' instructions. Real-time PCR was used to verify the differential expression of selected genes; we used a Roche Light Cycler 480 system (Roche Diagnostics GmbH) and the Taqman method of the Roche Universal Probe Library (UPL) kit to this end. Relative gene

expression levels were determined employing the comparative CT method. All reactions were performed in a total volume of 20 μ L containing 10.0 μ L 2X UPL master mix, 1.0 μ L 5' primer (10 pmol/ μ L), 1.0 μ L 3' primer (10 pmol/ml), a 0.2 μ L UPL probe, 1.0 μ L cDNA, and 6.8 μ L sterile water. The thermal cycling conditions were: initial denaturation for 10 min at 95 $^{\circ}$ C, which was followed by 40 cycles of 94 $^{\circ}$ C for 10 s and 60 $^{\circ}$ C for 30 s. The primers were designed using the Roche ProbeFinder tool. Primer sequences were as follows: SOD1, sense: 5'-TCAGGAGAATAT TCCATCATTTGG-3' and antisense 5'-TGCCCAAGT-CATCTGGTTTT-3'; SOD2, sense: 5'-TTCTATTGA AAATTGCTCTGTTGG-3' and antisense: 5'-CGGG GTGGTGAC TATCAGAA-3'; SOD3, sense: 5'-TGC TCTGTGCCTCTGTGG-3' and antisense: 5'-ATC TG CTGCTGGATCTGGTC-3'; GPX1, sense: 5'-CAACC AGTTTGGGCATCAG-3' and antisense: 5'-GGACG-TACTTCAGGCAATTCA-3'; GPX4, sense: 5'-TGC CTATGACGGTCTGTCTG-3' and antisense: 5'-AG TGCACGCCAGGTTCTC-3'; 18SrRNA, sense: 5'-A TCCATTGGAGGGCAAGTC-3' and antisense: 5'-GCTCCCAAGATCCAACACTACG-3'. Real-time PCR analysis featured duplicate PCR for each cDNA. Negative controls (except for templates) were included in all PCR reactions to ensure that amplification was specific. LightCycler 480 software version 1.2 (Roche, Mannheim, Germany) was used to quantitatively analyze the PCR data. The sample values were normalized to those of 18SrRNA. The gene expression levels in all experimental groups were compared with those of the control group.

Statistical analysis

All results are expressed as mean $\pm$ SDs. Statistical analysis featured one-way ANOVA with subsequent Tukey's multiple comparison testing. A p value < 0.05 constituted the threshold of statistical significance. All statistical analyses were conducted with the aid of SAS software (SAS Inc., Cary, NC, USA).

Result and discussion

I explored the effects of CZE on dRib-induced oxidative damage using a vascular endothelial cell culture model. One mechanism by which diabetes-

Fig. 1 Protective effects of CZE on dRib-induced BPAEs ► viabilities. **A** Effect of dRib on cell viability in BPAEs. Viabilities of cells treated with different concentrations of dRib. **B** Cells were cultured with an increasing concentration of CZE in the presence or absence of 20 mM dRib. Cells were preincubated with CZE or 10 mM NAC for 30 min at the indicated concentrations and then cultured with 20 mM dRib for 24 h. Cell viabilities were determined using CCK-8 assay. **C** Morphological changes were photographed by inverted microscope (*upper left* control, *upper right* dRib, *middle left* dRib + 0.005 mg/ml CZE, *middle right* dRib + 0.01 mg/ml CZE, *lower left* dRib + 0.05 mg/ml CZE, *lower right* dRib + 0.1 mg/ml CZE). The data are expressed as the mean $\pm$ SD of four independent determinations, each performed in quadruplicate. * $p < 0.05$ versus untreated control, # $p < 0.05$ versus dRib-treated cells

related vascular complications develop may feature a direct effect of oxidants on endothelial cells, as suggested by studies showing that hyperglycemia delays endothelial cell replication via generation of free radicals (Curcio and Ceriello 1992; Recchioni et al. 2002; Wu et al. 1999). This indicates that ROS may play a major role in vascular endothelial cell dysfunction, triggering diabetic vascular complications. Earlier studies found that dRib damaged pancreatic β -cells and osteoblastic cells by increasing the level of oxidative stress and the extent of protein glycation (Koh et al. 2005, 2010; Lee et al. 2010; Suh et al. 2009, 2012, 2013; Choi and Kim 2008; Lee and Choi 2008).

To evaluate the effect of dRib on BPAE survival, cell viability was measured using the CCK-8 assay. As shown in Fig. 1A, a dose-dependent decrease in cell viability was evident in cells exposed to various concentrations of dRib for 24 h. Based on these results, BPAEs were treated with 20 mM dRib in later assays. At this concentration, the reduction in cell viability was approximately 50% over 24 h under our experimental conditions.

To evaluate the effect of CZE per se on BPAE survival, cells were incubated in medium 199 containing 0.5% (v/v) FBS and increasing concentrations of CZE (0.001–0.1 mg/ml), for 24 h, and cell viabilities were determined. At these concentrations, CZE had no effect on cell viability (Fig. 1B). To determine whether CZE affected the dRib-induced decrease in cell survival, BPAEs were preincubated with CZE for 30 min and then cultured for a further 24 h with 20 mM dRib. Cell survival assays showed that CZE

(0.01–0.1 mg/ml) partially reversed the dRib-mediated reduction in cell viability in a dose-dependent manner (Fig. 1B). Therefore, I used the highest non-

toxic concentrations of CZE (0.05 and 0.1 mg/ml) in all subsequent cell culture experiments. The antioxidant N-acetyl cysteine (NAC) was also used to explore

the mechanism of dRib-induced cell damage. Pretreatment of BPAEs with 10 mM NAC almost completely inhibited dRib-induced cytotoxicity. Such findings suggest that the dRib-induced cytotoxicity was likely attributable to oxidative stress. The antioxidant NAC almost completely inhibited dRib-mediated reductions in the viabilities of pancreatic beta cells (Lee et al. 2010; Suh et al. 2012) and osteoblastic cells (Suh et al. 2009, 2013). I used an inverted microscope to compare morphological changes between dRib-induced and control cells. Control cells were flat, polygonal in shape, and arranged in monolayers. After exposure to dRib for 24 h, the cells had degenerated and were spindle-shaped in appearance. CZE inhibited the morphological changes in BPAEs induced by dRib (Fig. 1C). My data are consistent with those of previous work suggesting that CZE protects osteoblastic cells from dRib-induced oxidative damage by functioning as an antioxidant (Suh et al. 2013).

Oxidative stress caused by dRib was evaluated by measuring apoptosis and ROS generation. Oxidative stress may initiate the transition to mitochondrial permeability, an early step in cellular apoptosis. When cells were treated with 20 mM dRib, ROS generation and apoptosis increased, whereas further addition of CZE (0.1 mg/ml) attenuated all the dRib-induced effects (Fig. 2A, B). I used the antioxidant NAC to investigate the effects of oxidative stress. NAC prevented dRib-induced ROS production and apoptosis, consistent with data from previous studies showing that NAC protected HIT-T15 pancreatic beta cells (Lee et al. 2010; Suh et al. 2012) and MC3T3-E1 osteoblastic cells against oxidative stress, as shown by reductions in ROS generation and apoptosis (Suh et al. 2009, 2013). These findings indicate that CZE can function as an antioxidant protecting BPAEs from dRib-induced oxidative damage.

Excess ROS production associated with inflammation plays a key role in endothelial cell dysfunction, which causes various diabetic complications. COX2 and PGE2 are important mediators of inflammation in endothelial cells (Li and Shah 2004). Recent studies have shown that certain phytochemicals attenuate high-glucose- or cytokine-induced increases in COX2 and PGE2 levels in human umbilical vein endothelial cells (Abbasi et al. 2014; Chao et al. 2011; Park et al. 2014; Sheu et al. 2008). In the present study, CZE attenuated the dRib-induced production of the inflammatory mediators COX2 and PGE2. NAC also exerted

significant anti-inflammatory effects (Fig. 3), supporting a previous study indicating that the thiol-reducing antioxidant NAC attenuated cadmium-induced PGE2 production and COX2 expression in brain endothelial cells (Seok et al. 2006). These results suggest that the antioxidative effect of CZE and the associated restoration of cellular function explain the anti-inflammatory effect of the material in vascular endothelial cells.

The membrane-permeant JC-1 dye was used to assess $\Delta\Psi_m$, an indicator of the mitochondrial oxidative phosphorylation level in a variety of cell types. JC-1 permeates both the plasma and mitochondrial membranes. A low JC-1 ratio indicates that the aggregated form of JC-1 accumulates to only low levels in mitochondria, which is associated with high concentrations of ROS (Szilágyi et al. 2006). Oxidative stress may initiate the transition to mitochondrial permeability, an early indication of the commencement of apoptosis. This process is typically considered to reflect the collapse of the electrochemical gradient across the mitochondrial membrane, as measured by the change in $\Delta\Psi_m$ (Salido et al. 2007). Mitochondrial dysfunction is a consequence of oxidative damage caused by increased oxidant levels. Therefore, reducing oxidant level, and oxidative damage, should inhibit mitochondrial impairment. In the present study, pretreatment of cells with CZE caused a marked decrease in dRib-induced $\Delta\Psi_m$ (Fig. 4). These results indicate that CZE protects BPAEs mitochondrial function by acting as an antioxidant.

Excess ROS must be promptly eliminated; a variety of antioxidant defense mechanisms are usually in play. Cellular antioxidant enzymes and other redox molecules eliminate ROS generated within the cell. Superoxide dismutase (SOD), which catalyzes conversion of the superoxide anion to hydrogen peroxide and molecular oxygen, is one of the most important antioxidant enzymes (Zelko et al. 2002). The SOD enzymes are classified into three types: SOD1 is located in the cytoplasm, SOD2 is located in the mitochondria, and SOD3 in the extracellular fluid. Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxides, by reduced glutathione and also protects the cell from oxidative damage. Glutathione peroxidase 1 (GPx1) is the most abundant form of the enzyme, as it is present in the cytoplasm of nearly all mammalian tissues; its

Fig. 2 Effect of CZE on dRib-induced apoptosis (**A**) and ROS production (**B**) in BPAEs. Cells were preincubated with 0.1 mg/ml CZE or 10 mM NAC for 30 min and then cultured with 20 mM dRib for 24 h. The data are expressed as the mean $\pm$ SD

of three independent determinations, each performed in quadruplicate. * $p < 0.05$ versus untreated control. # $p < 0.05$ versus dRib-treated cells

Fig. 3 Effects of CZE on dRib-induced PGE2 and COX2 production. Cells were preincubated with CZE and then cultured with 20 mM dRib. PGE2 and COX2 production were assessed as described in “Materials and methods” section. The data are expressed as the means $\pm$ SD of three independent determinations, each performed in quadruplicate. * $p < 0.05$ versus untreated control, # $p < 0.05$ versus dRib-treated cells

Fig. 4 Effect of CZE on dRib-induced mitochondrial membrane potential in BPAEs. Cells were preincubated with CZE for 30 min and then cultured with 20 mM dRib for 24 h. The data are expressed as the mean $\pm$ S.D. of three independent determinations, each performed in quadruplicate. * $p < 0.05$ versus untreated control, # $p < 0.05$ versus dRib-treated cells

preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPx4) exhibits a high preference for lipid hydroperoxides. Various flavonoids are known to

increase the activities of antioxidant enzymes in endothelial cells. Vaccarin, a major flavonoid glycoside of vaccarial semen (Xie et al. 2015), and genistein, the primary isoflavone of soybeans (Zhang et al. 2013), protected human umbilical vein endothelial cells (HUVECs) from injury caused by hydrogen peroxide-induced oxidative stress by enhancing SOD activity. Rhein, an active component of rhubarb, increased the viability of hydrogen-injured HUVECs by increasing both SOD and GPX activities, allowing recovery from oxidative cell damage (Zhong et al. 2012).

In the present study, I investigated the expression of genes encoding several antioxidant enzymes. The reducing sugar dRib profoundly inhibited expression of the genes encoding SOD1, SOD2, SOD3, GPx-1, and GPx-4. However, when BPAEs were treated with CZE in the presence of 20 mM dRib, the expression levels of the genes encoding SOD2, SOD3, and GPx4, but not SOD1 or GPx-1, increased significantly (Fig. 5). These increases in antioxidative enzyme levels enhance the endothelial ROS-mediated stress response and may reduce the risk of endothelial cell dysfunction.

I conclude that CZE protects BPAEs from the oxidative cell damage induced by a highly reducing

sugar via both antioxidant and anti-inflammatory mechanisms, which may promote effective endothelial cell functioning in patients with diabetes-related vascular diseases.

The various pharmacologic effects of CZE have been demonstrated in cultured 3T3-L1 preadipocytes (Park et al. 2016), murine RAW 264.7 macrophage cells (Wu et al. 2011), osteoclastic cells (Gu et al. 2013), osteoblastic cells (Suh et al. 2013) and mouse models of type II collagen-induced arthritis (Kim et al. 2016).

Linarin, the main active compound of CZE was identified by chromatography (Han et al. 2002; Suh et al., 2013). Linarin has a polyphenolic structure, which may be related to the antioxidant property of linarin. It has recently been shown that linarin inhibits ischemia–reperfusion injury through activating an important regulator for anti-oxidation, nuclear factor erythroid 2-related factor 2 (Yu et al. 2017). Linarin increases osteoblast differentiation *in vitro* and protect against oxidative stress-induced toxicity, which may promote bone recovery under inflammatory bone disease (Kim et al. 2011).

Although there is some evidence that the beneficial effect of CZE is due to a decrease in oxidative stress, the action of individual compounds of CZE was not identified in the present study. Further studies are needed to investigate which ingredient primarily contributes to the antioxidative effect of CZE and to identify the underlying mechanism responsible for this action on the oxidative stress-induced cellular damage in vascular endothelial cells.

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Fig. 5 Effect of CZE on dRib-induced gene expression involved in anti-oxidant enzymes. Total RNA was extracted from BPAEs and the mRNA levels for SOD1, SOD2, SOD3, GPX1 and GPX4 were assessed by real-time PCR as described in “Materials and methods” section. The data are expressed as the mean $\pm$ SD of three independent determinations. * $p < 0.05$ versus untreated control, # $p < 0.05$ versus dRib-treated cells

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