อันตรกิริยาของสารพฤกษเคมีองค์ประกอบกำหนดความสามารถของสารสกัดรำข้าวไรซ์เบอร์รี่ในการป้องกันการตายของเซลล์ประสาท SK-N-SH จากภาวะเครียดออกซิเดชั่น

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บทคัดย่อ

ภาวะสูงวัยนำไปสู่ความบกพร่องของการทำหน้าที่ของไมโตคอนเดรียและความเสียหายจากภาวะเครียดออกซิเดชั่นซึ่งมีความสำคัญต่อพยาธิสภาพของโรคความเสื่อมของระบบประสาท เนื่องจากเซลล์สมองมีอัตราเมแทบอลิซึมสูงแต่มีความสามารถสร้างเซลล์ใหม่ได้น้อยจึงเสี่ยงต่อการเกิดความเสียหายจากอนุมูลอิสระค่อนข้างสูง การศึกษานี้มีจุดมุ่งหมายเพื่อตรวจสอบผลของสารสกัดจากรำข้าวไรซ์เบอร์รี่ (RBBE) ต่อความเป็นพิษในเซลล์ประสาทจากสารไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ที่ทำให้เกิดภาวะเครียดออกซิเดชันในเซลล์ SK-N-SH โดยการวัดจำนวนเซลล์มีชีวิต ระดับอนุมูลอิสระ (ROS) ในเซลล์ และความต่างศักย์เมมเบรนไมโทคอนเดรียน (MMP) ผลจาก co-treatment บ่งชี้ว่าในภาวะเครียดออกซิเดชันจาก H_2O_2 ขนาดสูง 400 μM สารสกัดจากรำข้าวและสารไซยานิดินออกฤทธิ์เป็นโปรออกซิแดนท์ จึงเลือกใช้วิธี pre-treatment ในการศึกษา พบว่า H_2O_2 ที่ความเข้มข้น 800 ไมโครโมล ทำให้ระดับ ROS ในเซลล์สูงขึ้นเซลล์ตายมากขึ้นในขณะที่ MMP มีแนวโน้มลดลง เมื่อให้ RBBE ความเข้มข้น 5 มิลลิกรัมต่อมิลลิลิตร ก่อนให้ H_2O_2 สามารถปกป้องเซลล์โดยลดอนุมูลอิสระและเพิ่มจำนวนเซลล์มีชีวิตถึงระดับใกล้เคียงกับเซลล์ควบคุม นอกจากนี้ระดับ MMP ยังเพิ่มขึ้นสูงกว่าเซลล์ควบคุม ส่วนสารไซยานิดินในขนาดที่มีอยู่ใน RBBE ความเข้มข้น 5 มิลลิกรัมต่อมิลลิลิตร ไม่สามารถลดความเสียหายต่อเซลล์ประสาทจากภาวะเครียดออกซิเดชัน ผลการศึกษาอาจชี้แนะว่า pre-treatment เซลล์ด้วย RBBE ลดการตายของเซลล์ได้เป็นผลจากอันตรกิริยาของสารผสมของสารพฤกษเคมีองค์ประกอบที่มีอยู่ในสารสกัดจากรำข้าวไรซ์เบอร์รี่

คำสำคัญ: โรคความเสื่อมของระบบประสาท ภาวะเครียดออกซิเดชัน ไรซ์เบอร์รี่ ไซยานิดิน เซลล์ SK-N-SH

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Interaction of Phytochemical Components Determine the Protective Effect of Riceberry Rice Bran Extract against Oxidative-Induced Neuronal Cell Death in SK-N-SH Cells

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Abstract

Aging leads to a progressive impairment of mitochondrial function and/or increased oxidative damage has been suggested to play critical roles in the pathogenesis of the neurodegenerative diseases. Brains have a high metabolic rate and relatively low capacity for cellular regeneration, nerve cells in brain area are particularly susceptible damaged by ROS. This study aimed to investigate the effects of Riceberry rice bran extract (RBBE) on H_2O_2-induced oxidative neuronal toxicity in SK-N-SH cells by measurement of cell viability, intracellular ROS, and mitochondria membrane potential (MPP). Co-treatment results indicated that RBBE and cyanidin acted as pro-oxidants at high level of oxidative stress induced by 400 μM H_2O_2. Therefore, the pre-treatment method was chosen for this study. High dose H_2O_2 800 μM resulted in a statistically significant increase in ROS level, and cell death with a decreasing MMP trend. Pretreatment with RRBE at concentration of 5 mg/ml before exposure to H_2O_2 showed neuroprotective ability by increasing the percentage of cell viability and decreasing the ROS level to the level not different from control. Moreover, the MMP was raised to the level higher than control. However, cyanidin in the dose that contained in 5 mg/ml RBBE did not improved the oxidative neuronal damage. These results might suggest that pre-treatment cells with RBBE attenuated the neuronal cell death resulted from the interaction of mixed phytocemical components in RBBE.

Keywords: Neurodegenerative disease, Oxidative stress, Riceberry, Cyanidin, SK-N-SH cells

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Introduction

Neurodegenerative diseases are typically defined by specific protein accumulations and anatomic vulnerability. Neurodegenerative diseases share many fundamental processes associated with progressive neuronal dysfunction and death, such as proteotoxic stress and its attendant abnormalities in ubiquitin–proteasomal and autophagosomal/ lysosomal systems, oxidative stress, programmed cell death, and neuroinflammation. The neuropathology of Alzheimer's disease (AD) is a mixed proteinopathy that includes the presence of both Aβ deposits in the parenchyma as amyloid or senile plaques, as well as neuronal tau inclusions as neurofibrillary tangles (NFTs)[1]. Apoptosis or programmed cell death is a major cell death pathway for neurons that execute through signals, either extrinsically or intrinsically. In the extrinsic pathway, death receptors are activated through the binding of extracellular ligands, whereas in the intrinsic pathway, internal stimuli, consists of DNA damage, activate p53 and the up-regulation of pro-apoptotic factors in the Bcl-2 family. Both pathways alter the inner mitochondrial membrane permeability, which eventually lead to the release of pro-apoptotic factors including cytochrome C, Smac/DIABLO, HtrA2/Omi and apoptosis-inducing factors (AIFs) from mitochondria into the cytosol[2]. Mitochondrial membrane potential (MMP) declines control matrix remodeling prior to cytochrome c release to the intermembrane space of mitochondria during apoptosis[3]. Aging leads to a progressive impairment of mitochondrial function and/or increased oxidative damage has been suggested to play critical roles in the pathogenesis of the neurodegenerative diseases[4].

Hydrogen peroxide (H_2O_2) is used to generate reactive oxygen species (ROS) in the investigation of neuronal oxidative stress due to being the main source of the highly reactive hydroxyl radical (OH•) in the brain. H_2O_2 induced apoptotic cell death through both extrinsic and intrinsic pathways[5-6]. Because of very low level of antioxidant enzymes, high levels of iron and unsaturated lipids, the nerve cells are suffered from mitochondrial dysfunctions as well as excitotoxicity and finally apoptosis. Brain obviously needs nutritional antioxidants against oxidative stress[7].

Rice bran is a by-product of rice milling obtained from outer layer of the brown (husked) rice kernel during milling. Red and purple bran rice have significantly higher total polyphenol, flavonoid concentrations and antioxidant capacities than light coloured bran rice due to their higher concentrations of proanthocyanidins and anthocyanins. Riceberry rice contains

high nutrients and bioactive phytochemicals that include cyanidin, a major flavonoid anthocyanin in coloured grains[8]. Cyanidin is a well-known natural anthocyanin and possesses antioxidant and anti-inflammatory properties[9]. Riceberry was also demonstrated to prevent memory impairment and hippocampal neurodegeneration by decrease hippocampal acetylcholinesterase activity and lipid peroxidation product formation in rats with memory deficit and cholinergic lesions[10]. The objective of the study is to investigate the effects of Riceberry bran extract (RBBE) on H_2O_2-induced cytotoxicity in neuroblastoma SK-N-SH cells.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, glutamax, non-essential amino acid, penicillin/ streptomycin, fetal bovine serum (FBS), phosphate-buffered saline (PBS) and trypsin-EDTA (0.05%) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Hydrogen peroxide (30%), dimethyl sulfoxide (DMSO) and ethanol were purchased from Merck (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7′-Dichlorofluorescin diacetate (DCFH-DA), cyanidin-3-glucoside, and rhodamine 123 (Rh123) were purchased from Sigma (MO, USA).

RBBE

One hundred grams of rice bran was extracted with 1000 mL of 70% v/v ethanol by soaking and stirring for 12 hr. at room temperature, following by a filtration through Whatman No.2 filter paper. Solvent was removed from filtrate by rotary evaporator. Crude extract was lyophilized overnight, and then was re-dissolved with sterile water and extracted with hexane to remove fat residual. Evaporation to remove hexane and overnight lyophilization of the aqueous phase of extract were performed again. Finally, the extract was aliquoted into microtubes for storage at -30 ºC until used. In the experiments, RBBE was dissolved in the medium containing 0.1% dimethyl sulfoxide (DMSO) before filtering through a 0.22 µm membrane filter and collected as a stock solution. Prior to each experiment, RBBE was diluted to final concentrations in the serum free medium (DMEM) supplemented with 1% non-essential amino acids, 1% glutamate, 1% sodium pyruvate, and 1% penicillin-streptomycin).

Cyanidin

Cyanidin 3-O-glucoside chloride (Sigma-Aldrich, USA) was prepared as a

1 mg/mL stock solution in 0.1% DMSO and stored at -20°C until use. A series of working solution of cyanidin was also diluted with serum free medium immediately before use.

Cell culture

Human neuroblastoma SK-N-SH cells were maintained in complete media DMEM supplemented with 10% FBS, 1% non-essential amino acids, 1% glutamate, 1% sodium pyruvate, and 1% penicillin-streptomycin. The cells were maintained at 37 ^{o}C in a humidified incubator in the presence of 5% CO_2.

Treatment conditions

SK-N-SH cells were seeded in a 96-well plate at a density of 5× 10^4 cells per well and cultured for 24 hr prior to commencing each experiment. For carrying out the experiments, two treatment conditions were adopted: the co- and pretreatment. The co-treatment method comprised administration with both RBBE or cyanidin together with H_2O_2 or serum free medium for 24 hr. In pretreatment, the cells were pre-treated with either RBBE or cyanidin for 3 hr. followed by exposure to H_2O_2 or serum free medium for 3 hr.

Assessment of cell viability

After co- or pretreatment, the medium was carefully aspirated from each well, then 200 µl of serum free medium was added. After that, 50 µL of 2 mg/mL MTT was added to each well, to achieve MTT final concentration of 0.5 mg/mL and the plate was incubated for 1 hr at 37 ^{o}C. The medium containing MTT was removed from each well and replaced with 100 µL of DMSO to dissolve the formazan crystals. The absorbance was measured on an Infinite M200 microplate reader (Tecan, Switzerland) at the wavelength of 570 nm and reference wavelength at 630 nm. The experiments were repeated three times. Percentage of cell viability were calculated as follows: % cell viability = (Absorbance of treated cells x 100) / Absorbance of control

Measurement of intracellular ROS

Intracellular ROS production was measured by incubating the cells with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This assay is based on the properties of DCFH-DA that can freely diffuse through the cell membrane, then be recognized and hydrolyzed by cellular esterases. The non-fluorescent DCFH molecule that has been oxidized becomes the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of peroxides. The cells were seeded at a density of 5 x 10^4 cells per well in a black 96-well plate for 24 hr. The medium was removed and Hank's balanced salt solution

(HBSS) containing 20 µM of DCFH-DA was added. After 30 min incubation at 37 °C in a CO_2 incubator, DCFH-DA containing medium was removed and cells were washed once with HBSS prior subjecting them to co-treatment or pretreatment experiment. The fluorescence signal of ROS was measured using a fluorescence microplate reader (Tecan infinite F200) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The ROS production was determined using the following formula: % ROS production = (Fluorescence intensity of treated cells x100) / Fluorescence intensity of control

Detection of oxidative damage to mitochondrial membrane

Mitochondrial membrane potential is a key indicator of cellular viability, because it reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation. Mitochondrial membrane potential ($\Delta\Psi m$) was monitored using a Rhodamine123 (Rh123). Cells were seeded in a black 96-well plates at a density of 5×10^4 cells per well and incubated at 37 °C, 5% CO_2 for 24 hr before receiving co- or pretreatment. After co- and pre-treatment, the cells were incubated with 10 µg/mL Rh123 in the loading medium at 37 °C for 30 min. Then, cells were washed twice with PBS and analysed by microplate reader with an excitation wavelength of 485 nm and emission wavelength of 530 nm. The results were expressed as a percentage of fluorescence intensity in treated cells relative to control, calculated as follows:

Rh123 fluorescence intensity = (Fluorescence intensity of treated cells x 100) / Fluorescence intensity of control

Statistical analysis

All experiment data were presented as mean ± standard error of mean (SEM) from 3 independent experiments. Data were analyzed using the GraphPad Prism version 5 software. Significant differences between treatments were determined using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A value of $P< 0.05$ was considered statistically significant.

Results and Discussion

Effect of co-treatment of RBBE or cyanidin on H_2O_2-induced oxidative stress in SK-N-SH cells

RBBE and cyanidin did not exert cytotoxic effects when incubated for 24 hr using the highest concentration (8 mg/mL and 15 µg/mL) as cell viability (always >90%) was not significantly affected (data not shown). Cells were co-treated with

different concentrations of RBBE or cyanidin and H_2O_2 for 24hr. Figure 1 (a) showed that H_2O_2 at 200 µM did not affect the viable of cell. Co-treatment with RBBE at 2 mg/mL exhibited the significantly higher cell viability compared with control and H_2O_2 alone whereas 0.25 mg/mL RBBE and 1 µg/mL cyanidin gave the significantly lower cell viability compared to control. Figure 1(b) demonstrated that at the highest concentration 400 µM H_2O_2, the cell viability was markedly 40% lower than control. All doses of RBBE (except concentration of 2 mg/mL) or cyanidin diminished viable cells lower than control.

The results from co-treatment suggested the possibility that cyanidin and bioactive compounds in RBBE might act as pro-oxidants through increasingly oxidised in present higher concentration of H_2O_2. Previous evidence indicated some phenolic substances can display pro-oxidant activities under certain conditions such as in the high concentrations of transition metal ions, alkali pH and the presence of oxygen molecule[11]. The pro- oxidant action of phenolic substances are induced by ROS such as $^{\cdot}OH$, O^{-} and resulting in the enhancement to produce reactive oxygen species including H_2O_2 or autoxidation process of polyphenol[12]. Anthocyanins can change from antioxidants to pro-oxidants in the presence of transition metals, resulting in ROS generation. Catechol B-ring anthocyanins including cyanidin and delphinidin, possess lower reduction potentials and more pro-oxidant activities[13]. At 400 µM H_2O_2, the viability of cells treated by all doses of RBBE and cyanidin reduced by lower than H_2O_2 treatment alone and 2 mg/mL no longer enhanced the cell proliferation.The results from co-treatment suggested the possibility that cyanidin and bioactive compounds in RBBE might act as pro-oxidants at high level oxidative stress.

Therefore, we changed the method to pre-treatment to reduce the interaction between the oxidant H_2O_2 and phytochemicals in RBBE or cyanidin. The cells were pre-treated with RBBE or cyanidin before induction of oxidative stress by exposure to H_2O_2.

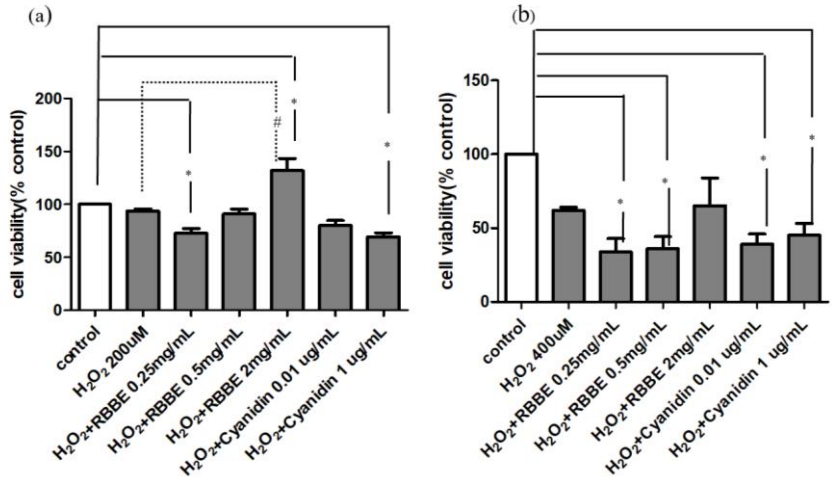


Figure 1. Effect of RBBE or cyanidin co-treatment on H_2O_2-induced cytotoxicity in neuroblastoma SK-N-SH cells. Cells were co-treatment with various concentrations of RBBE or cyanidin and H_2O_2 (a) 200 µM (b) 400 µM for 24 hr. Values are mean ± SEM (n=3-6). Significant $P < 0.05$. Tukey's Multiple Comparison Test compare between control and treatment groups. *Significant difference from control. #Significant difference from H_2O_2 treatment alone.

Effects of RBBE and cyanidin pre-treatment on H_2O_2-induced oxidative stress in neuroblastoma SK-N-SH cells

No significant loss of cell viability (<90%) were observed after 3 hr pretreatment with the highest concentrations 50mg/mL RBBE and 1mg/mL cyanidin (data not shown). RBBE used in this study was analysed for cyanidin, the important bioactive substance of Riceberry rice. Concentration of cyanidin in the RBBE extract was 2.45 mg/g extract. Before H_2O_2 exposure, SK-N-SH cells were pre-treated 3 hr with the non-toxic concentration of RBBE (0.0005, 5 mg/mL) and 1ng/mL, and 0.01 mg/mL cyanidin that contained in the 0.0005 and 5 mg/mL RBBE , respectively.

The significant decrease in cell viability to 60% of control was found in cells treated with 800 µM H_2O_2. Pre-treatment with 0.0005 mg/mL RBBE or 1 ng/mL and 0.01 mg/mL cyanidin did not improve the number of viable cells. However, pre- treatment with 5 mg/ mL RBBE before exposure to 800 µM H_2O_2 gave the %cell viability comparable to control and although statistical significance data were not presented, showed a clear trend of increasing compared to H_2O_2 treatment alone (Figure2). In consistent with cell viability, cells exposed to 800 µM

H_2O_2 showed significantly increase of intracellular ROS compared with control. Pre-treatment with 0.0005 mg/mL RBBE or 1 ng/mL and 0.01 mg/mL cyanidin before exposure to 800 µM H_2O_2 did not reduce intracellular ROS generation compared with cells treated with H_2O_2 alone. Pre-treatment with RBBE at 5 mg/mL significantly inhibit H_2O_2-induced intracellular ROS generation, the ROS level was not different from control cells (Figure 3).

Mitochondria are the main source of ATP, responsible for cell functions include force generation, biosynthesis, folding and degradation of proteins, and maintenance of membrane potentials. The inner membrane of mitochondria is a tight diffusion barrier selective for a particular ion or molecule that results in the electrochemical membrane potential across these inner membranes. A set of membrane protein complexes generate oxidative phosphorylation that create the electrochemical gradient across the inner membrane, and ATP synthesis[14]. A decrease in MMP causes a reduction in ATP production, which could compromise cell viability. MMP was monitored using the fluorescent dye Rhodamine 123. Depolarization of MMP results in the loss of Rhodamine 123 from mitochondria and the reduced fluorescence[15]. Figure 4 showed that the fluorescence intensity of Rhodamine 123 in 800 µM H_2O_2 treatment was decreased to 76% relative to control, pre-treatment with 5mg/ml of RBBE raised the rhodamine signal to 117% (significantly higher than control). Cyanidin did not alter the reduced MMP of H_2O_2 treatment. A previous study performed in SH-SY-5Y cells demonstrated that H_2O_2 treatment increased the percentage of Rhodamine 123 negative cells, pretreatment with germinated brown rice protected cells against H_2O_2-induced lowering of MMP, by decreasing Rhodamine negative cells[16]. ROS such as H_2O_2 and superoxide can cause cytochrome c release from mitochondria and induction of apoptosis through the mitochondrial pathway.

In all experiments our control cells were in serum free medium. Normally the oxidizing or reducing conditions in the extracellular environment can be drastically altered according to the presence or absence of serum. Cell culture media by themselves are oxidizing in that they promote thiol auto-oxidation and associated peroxide generation within cells. Addition of serum provides glutathione, albumin that change media to a reducing condition[17]. The measured background intracellular ROS of our control cells were higher than those of cells that cultured in medium supplemented with 10% fetal bovine serum (data not

shown). The loss of MMP had been demonstrated concomitantly with the oxidative state of serum-deprived retinal ganglion cells[18]. The results indicated that control cells might have impaired oxidative status and reduced MMP due to serum deprivation. These could indicate that RBBE had capability to improve cellular oxidative stress and MMP disruption induced by both external H_2O_2 exposure and serum deprivation.

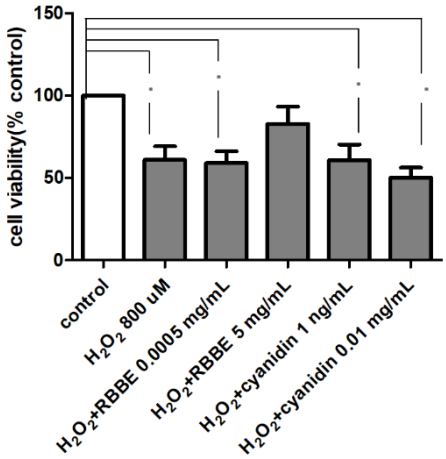


Figure 2. Effect of RBBE or cyanidin pretreatment on H_2O_2-induced cytotoxicity in neuroblastoma SK-N-SH cells. Cells were pre-treatment with various concentration of RBBE or cyanidin for 3 hr. before exposure to H_2O_2 for 3 hr. Values are mean ± SEM (n=6), Significant $P < 0.05$. Tukey's Multiple Comparison Test compare between control and treatment groups. *Significant difference from control.

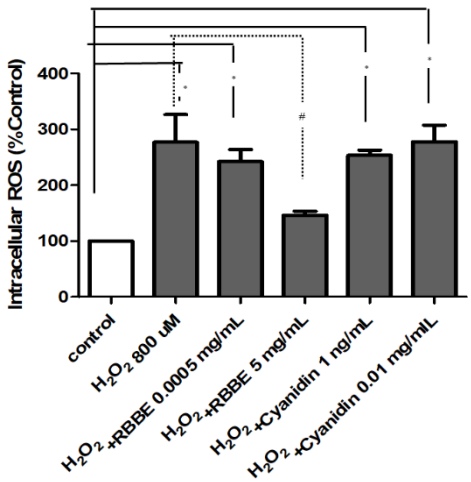


Figure 3. Effect of RBBE or cyanidin pretreatment on H_2O_2- induced in intracellular ROS in neuroblastoma SK-N-SH cells. Cells were pre-treated with RBBE or cyanidin for 3hr. and treated with H_2O_2 for 3 hr. ROS levels were determined by DCFH-DA assay. Values are mean ± SEM (n=3-6),

Significant P < 0.05. Tukey's Multiple Comparison Test compare between control and treatment groups. *Significant difference from control. #Significant difference from H_2O_2 treatment alone.

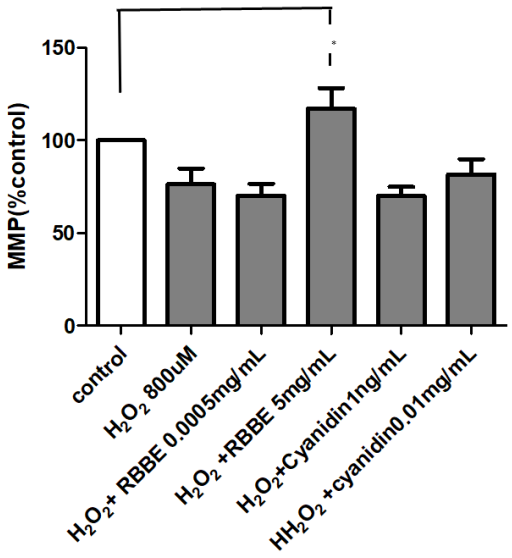


Figure 4. Effect of RBBE or cyanidin pretreatment on H_2O_2- induced reduction of mitochondrial membrane potential in neuroblastoma SK-N-SH cells. Cells were pre-treated with RBBE or cyanidin for 3 hr. and treated with H_2O_2 for 3 hr. before determining Rhodamine 123 fluorescence. Values are mean ± SEM (n=9), Significant P < 0.05. Tukey's Multiple Comparison Test compare between control and treatment groups. *Significant difference from control

Conclusion

In summary, our present study showed that the high dose H_2O_2 induced an obvious increase of ROS and cell death. On the contrary, MMP showed a trend to decreased (76% of control). These results might suggest ROS induced cell death through mitochondrial pathway. Pretreatment only high dose 5 mg/mL RBBE attenuated or restored all of these changes especially MMP that markedly increased to the level higher than those of H_2O_2 treatment and control. Single compound, cyanidin in the dose that received from 5mg/ml RBBE could not attain these results.

A number of studies reported that the overall activity of botanical extracts obtained from mixtures of compounds with synergistic, additive, or antagonistic activity. Biological effects were lost after fractionation to find the active compound[19]. Characterizing the nature of compound interactions and elucidating their potential mechanisms of action may require to discover the complex mixtures that

determine the biological activities of natural substances.

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Conflicts of Interest

The authors declare no conflict of interest.

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