

Research Article

Purple Sweet Potato Anthocyanin Inhibits the Proliferation of Human Retinal Pigment Epithelial Cell by Blocking Cell Cycle and Inducing Apoptosis

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Abstract: Purple Sweet Potato Anthocyanin (PSPA), a class of naturally occurring anthocyanins derived from purple sweet potato storage roots, possesses unique color and multiple bioactivities. This study investigated the anti-proliferative effect of PSPA in human Retinal Pigment Epithelial (RPE) cells, the proliferation of which accounts for Proliferative Vitreoretinopathy (PVR). Blueberry Anthocyanin (BBA) was used as a contrast. PSPA and BBA inhibited RPE proliferation time- and dose-dependently through blocking the cell cycle in G0/G1 phase and inducing apoptosis via ROS accumulation, DNA damage and caspase 3/7 activation. Meanwhile, PSPA showed stronger potential than BBA in inhibiting RPE growth. Hence, we highlighted the importance of dietary supplementation of anthocyanins in PVR prevention and the application of PSPA in health industry.

Keywords: Apoptosis, cell cycle, proliferation, purple sweet potato anthocyanin, retinal pigment epithelial

INTRODUCTION

The Retinal Pigment Epithelium (RPE), interposed between the neural retina and the choroidal blood, is a monolayer responsible for maintaining the health of the retina by providing structural and nutritional support (Adijanto and Philp, 2014; Sparrrow *et al.*, 2010; Strauss, 2009). *In vivo*, highly differentiated RPE cells have a limited proliferation capacity (Hecquet *et al.*, 2002). However, several pathological insults may induce RPE cells to dedifferentiate and proliferate, which accounts for Proliferative Vitreoretinopathy (PVR), a major cause of failure in retinal detachment surgery (Garweg *et al.*, 2013). The inhibition of the proliferation of RPE cells may be helpful in preventing the recurrence of retinal detachment (Hou *et al.*, 2013). *In vitro*, cultured RPE cells can be used to investigate PVR related pathogenesis and the inhibition of RPE cell proliferation has been achieved by using statins (Wu *et al.*, 2011), daunorubicin (Wang *et al.*, 2002) and hypericin (Zhou *et al.*, 2007), indicating their potential to prevent PVR. However, the most economic and convenient way to gain the potential to prevent PVR is from food rather than medicine. Therefore, it's more practical and meaningful to identify protective components with no side effects in daily diet.

Polyphenolic compounds are the most abundant antioxidants people can get from dietary and they are known to be protective to retina (Hanneken *et al.*,

2006). Meanwhile, anthocyanins are the most available flavonoid constituents of fruits and vegetables. The daily intake of anthocyanins is estimated to be 9-fold higher than that of other dietary flavonoids (Wang and Stoner, 2008). Anthocyanins have been reported to inhibit the proliferation of human umbilical vein endothelial cells and human retinal microvascular endothelial cells (Matsunaga *et al.*, 2010a, 2010b; Tanaka *et al.*, 2012), but their effect on RPE cells hasn't been proven. Besides, the anthocyanins in these researches are limited to nonacylated anthocyanins. Although nonacylated anthocyanins contributed more than acylated anthocyanins in food intake (Wu *et al.*, 2006), acylated anthocyanins are more promising in food processing industry for their better stability (Netzel *et al.*, 2007). Purple sweet potato is a good source of acylated anthocyanins. Mainly constituted by cyanidinacylglucosides and peonidinacylglucosides, Purple Sweet Potato anthocyanin (PSPA) possesses multiple bioactivities including attenuating the proliferation of hepatic stellate cells (Choi *et al.*, 2011) and inhibiting Sarcoma S180 cell growth in ICR mice (Zhao *et al.*, 2013). Furthermore, PSPA can be absorbed directly into rat and human in intact acylated forms (Harada *et al.*, 2004; Oki *et al.*, 2006; Suda *et al.*, 2002) and anthocyanins can pass the blood-brain barrier and blood-retinal barrier and accumulate in eyes, as observed *in vivo* (Kalt *et al.*, 2008; Matsumoto *et al.*, 2006).

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In view of all these considerations, the purpose of the present study was to evaluate the anti-proliferative effects of PSPA in RPE cells. Blueberry Anthocyanin (BBA), well-known to promote vision health (Wang *et al.*, 2015) and mainly constituted by nonacylated anthocyanins, was used as a contrast in this study. This investigation was aimed at providing a new source for preventing PVR from dietary and increasing the application of acylated anthocyanins from purple sweet potato in health industry.

MATERIALS AND METHODS

Materials: The human retinal pigment epithelial (RPE) cell line (no. D407) was purchased from the Animal Experiment Center of Sun Yat-sen University (Guangzhou, China). D407 is a spontaneous immortalized cell line retaining many of the metabolic and morphologic characteristics of RPE cells in vivo (Davis *et al.*, 1995) and the same cell line was also used in other research conducted by Xu *et al.* (2010). PSPA used in the study were supplied by Huludao Maohua Biology Co., Ltd. (Liaoning, China), the major components of PSPA by HPLC-MS analysis are cyanidinacylglucosides and peonidinacylglucosides (Sun *et al.*, 2015). BBA were supplied by Tianjin Jianfeng Natural Product R and D Co., Ltd (Tianjin, China), the major components of BBA are cyanidin and petunidin glucosides (Peng *et al.*, 2012). Dulbecco's modified Eagle's Medium (DMEM), penicillin, streptomycin, 0.5 % (vol/vol) trypsin/EDTA and Fetal Bovine Serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFDA), Hoechst 33342 (HOE) and Propidium iodide (PI) were bought from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Muse™ count and viability assay kit, cell cycle kit, Ki67 proliferation kit, Annexin V and dead cell kit, oxidative stress kit, multicolor DNA damage kit and Caspase-3/7 kit were purchased from Merck Millipore (Billerica, MA, USA).

Cell culture and treatment: The RPE cells were grown in whole culture medium, namely, DMEM with 10 FBS and a 1% antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were incubated at 37°C under a humidified 5% CO₂ atmosphere. Before treating with anthocyanins, the cells were incubated in FBS-free DMEM medium for 1 day. PSPA and BBA were dissolved in DMEM at a concentration of 125 µg/mL (Cyanidin 3-O-glucoside equivalent) as a stock solution and stored at -20°C. Before all experiments, the stock solution was sterilized by processing through a 0.1 µm filter and then it was diluted with DMEM to certain concentrations.

Evaluation of proliferation: RPE cells were seeded in 96-well plates at a concentration of 2×10^5 cells/mL and

allowed to attach for 1 day. The cells were then incubated in FBS-free DMEM medium for another day followed by anthocyanins treatments for 1, 2 and 3 days at concentrations ranging from 6.25 to 18.75 µg/mL. The control groups were processed exactly the same, just with 0 µg/mL anthocyanins. Then MTT assay (Wu *et al.*, 2009) was used to detect the Optical Density (OD) values of each group and the proliferation was calculated as % of the control group in each day.

Assay of cell viability: RPE cells were seeded in 6-well plates at a concentration of 5×10^5 cells/mL and allowed to attach for 1 day. The cells were then incubated in FBS-free DMEM medium for another day followed by 12.5 µg/mL anthocyanins treatments for 1, 2 and 3 days. (The same process was used in the following measurements, except where noted.) Then the cells were digested and collected. The viabilities were performed according to the count and viability assay kit user's manual, using Muse™ Cell Analyser (Merck-Millipore, Germany), 2, 000 cells were acquired for each sample. The viable cells and total cells were each counted and viability was expressed as a percentage of the viable cells.

Assessment of apoptosis and necrosis: Morphological changes in cells treated with PSPA and BBA were assessed by double staining with HOE and PI. Having been incubated on cover slips with anthocyanins for 2 d, the cells were washed twice with PBS and then 0.5 mL HOE (10 µg/mL) was added to each slip and incubated for 10 min at 37°C, followed by another 10-min incubation with PI (10 µg/mL) in total darkness. Then the cells were washed twice with PBS and observed by fluorescence microscopy (Olympus CKX41, Japan).

Muse™ Annexin V and dead cell kit was also used to measure the percentages of apoptosis and necrosis after 12.5 µg/mL anthocyanins treatment for 1, 2 and 3 days according to the user's manual.

Analysis of cell cycle: After being treated with 12.5 µg/mL anthocyanins for 1 and 2 days, RPE cells were collected to analyze cell cycle distribution using Muse™ cell cycle kit. 20, 000 cells were recorded for each sample. The results were analyzed using Modfit 3.2 (Verity Software House, USA).

Detection of Ki67 expression: RPE cells treated with 12.5 µg/mL anthocyanins for 1, 2 and 3 days were conducted the assay of Ki67 according to the Muse™ Ki67 proliferation kit user's guide with the Muse™ Cell Analyser.

Observation of ROS generation: The generation of Reactive Oxygen Species (ROS) in the cells with anthocyanins treatment for 2 days was observed by fluorescence microscopy using DCFDA assay (Lu *et al.*, 2011). The production of ROS was also evaluated

by Muse™ oxidative stress kit after incubating with anthocyanins for 1, 2 and 3 days as described in user's guidance.

Determination of DNA damage: Treated with 12.5 µg/mL PSPA and BBA for 1, 2 and 3 days, DNA damage of RPE cells in each group was determined by multicolor DNA damage kit at the end of each day.

Examination of caspase-3/7 expression: The activation of Caspase-3/7 in RPE cells treated with anthocyanins was examined by Muse™ Caspase-3/7 kit, utilizing a novel Muse™ Caspase-3/7 reagent NucView™ for the detection.

Statistical analysis: All experiments were performed in triplicate; results were given in means ± standard deviation. One-way ANOVA with Duncan's multiple comparison tests were performed with SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA). Results were considered statistically significant at $p < 0.05$.

RESULTS

Inhibitory effects of anthocyanins on RPE cell proliferation and viability: The inhibition in RPE cell proliferation induced by anthocyanins was assayed following treatment with different doses of PSPA and BBA for 1~3 days (Fig. 1A). Both anthocyanins exhibited inhibitory effects in the dose-and time-dependent manner, as observed at each time point analyzed. PSPA showed an obviously stronger activity than BBA at the same concentrations (cyanidin 3-O-glucoside equivalent). Anthocyanins at the concentration of 12.5 µg/mL had significantly better inhibitory effect than that of 6.25 µg/mL, but there were no very

dramatic differences between the groups of 12.5 and 18.25 µg/mL. Thus, 12.5 µg/mL anthocyanins were selected for the following study.

When given the individual cell rather than cell population, viabilities of RPE cells treated with 12.5 µg/mL PSPA and BBA were detected by counting the numbers of living and dead cells (Fig. 1B). The control group maintained 84.73% living cells after 3-day incubation, while the viabilities of PSPA and BBA groups decreased marvelously during the treatment. Notably, the preserved percentages of viability (Fig. 1B) were higher than that of proliferation (Fig. 1A) at the end of each day's treatment with anthocyanins, which suggested that the loss of cell viability partially accounted for the decrease in proliferation treated by anthocyanins.

Effects of anthocyanins on RPE cell apoptosis and necrosis: Double staining with Hoechst 33342 and PI was used to observe the morphological changes in cells treated with anthocyanins for 2-day (Fig. 2A). Anthocyanins treated cells showed more early apoptotic cells (intensive bright-blue fluorescence) and late apoptotic cells (blue-violet fluorescence). BBA treatment resulted in more apoptotic cells than PSPA treatment, showing the typical apoptosis-like morphological changes like chromatin condensation and fragmentation (Fig. 2A). The percentages of apoptosis and necrosis after 12.5 µg/mL anthocyanins treatment for 1, 2 and 3 days were also measured by Annexin V and dead cell kit (Fig. 2B). Numbers of late apoptotic and dead cells increased sharply after PSPA and BBA treatment for 2 days. BBA had better apoptosis-inducing effect than PSPA, while PSPA led to more cell death in all at the end of third day (47.76% vs 38.98%).

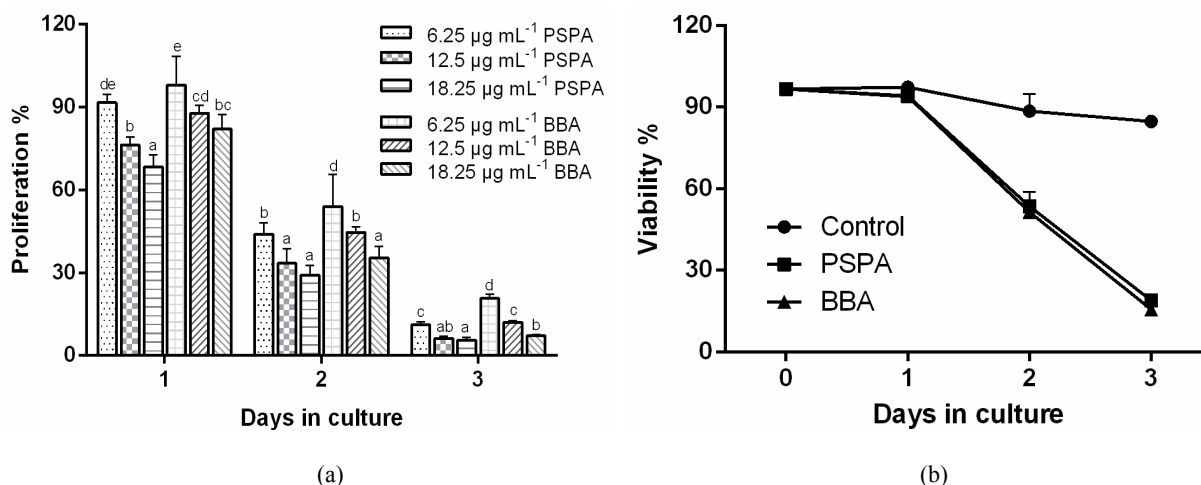


Fig. 1: PSPA and BBA inhibited RPE cell proliferation and viability; (a): Time-dependent and dose-dependent effect of PSPA and BBA on cell proliferation assayed by MTT; (b): time course of 12.5 µg/mL PSPA and BBA inhibition in cell viability determined by count and viability assay kit. The means of the values marked with different lowercase letters are significantly different ($p < 0.05$) relative to others in each day of incubation

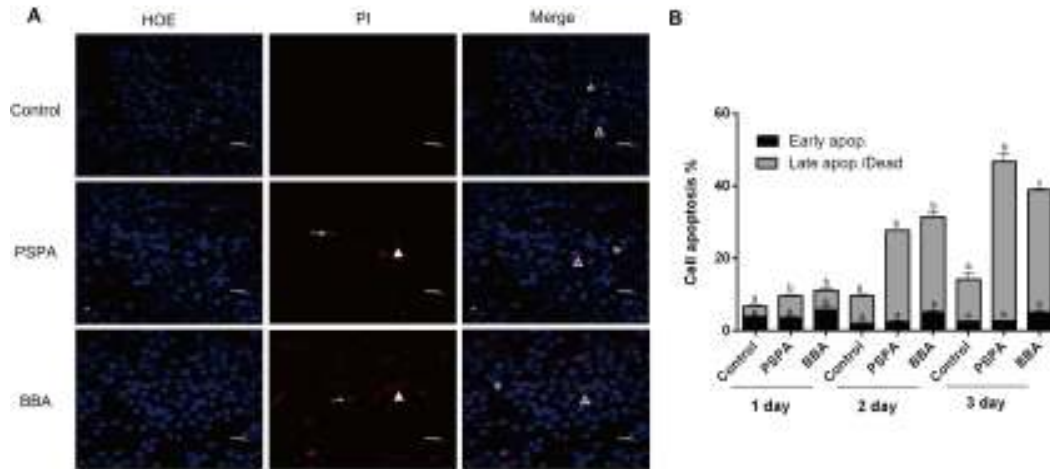


Fig. 2: PSPA and BBA induced apoptosis and necrosis in RPE cells; (A): Fluorescence micrographs stained with Hoechst 33342 and PI of RPE cells treated with 12.5 $\mu\text{g/mL}$ PSPA and BBA for 2-day. Scale bar: 25 μm . * intensive bright-blue fluorescence, early apoptotic cells; Δ blue-violet fluorescence, late apoptotic cells; \rightarrow chromatin condensation; and \blacktriangle fragmentation; (B): apoptosis of RPE cell assessed by Annex in V and dead cell kit. The means of the values marked with different lowercase letters are significantly different ($p < 0.05$) relative to others in each day of incubation

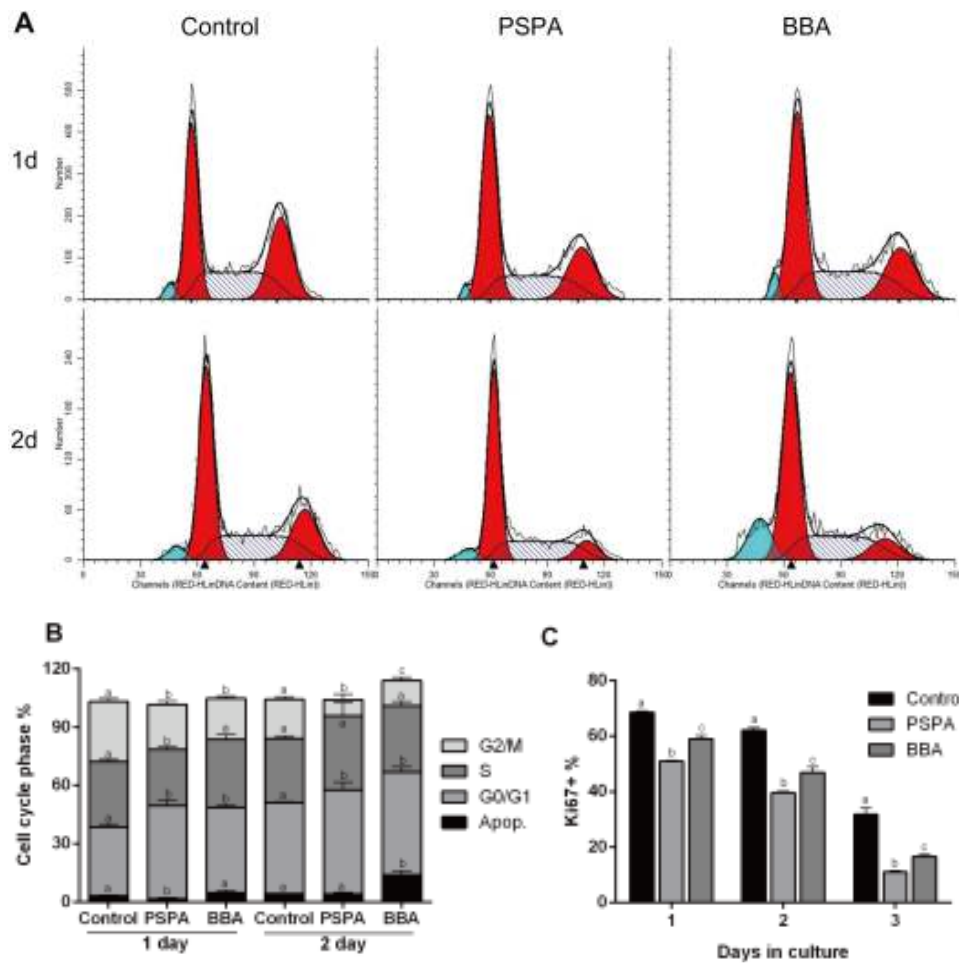


Fig. 3: PSPA and BBA induced cell cycle arrest in RPE cells. (A, B) Cell cycle distribution of RPE cells treated with PSPA and BBA analyzed by cell cycle kit; (C): percentages of Ki67-positive cells treated with PSPA and BBA for 1~3 days, The means of the values marked with different lowercase letters are significantly different ($p < 0.05$) relative to others in each day of incubation

Effects of anthocyanins on RPE cell cycle and Ki67 expression: Cell cycle analysis revealed the RPE cell cycle distribution after anthocyanins treatment (Fig. 3A and B). PSPA and BBA incubation both resulted in striking increases of G1/G0 stage cells whereas the percentages of G2/M phase decreased significantly at the end of the first day. Similar changes accompanied by declines in the relative amount of S-phase cells were recognized on the second day. Altogether, anthocyanins blocked PRE cells in G0/G1 phase and led to the cell cycle arrest. And it was remarkable that an obvious sub-G1 phase was detected after 2-day treatment by BBA, which indicating the noteworthy apoptosis in BBA group. This finding was in line with the results in HOE-PI staining (Fig. 2A) and Annexin V and dead analysis (Fig. 2B).

Ki67 is a prototypic cell cycle-related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2, M phases), but it is absent in the resting G0 phase (Scholzen and Gerdes, 2000). In Fig. 3C, anthocyanins treatment diminished the percentages of Ki67+ cells observably in each day, which meant fewer cells were undergoing mitosis after incubating with anthocyanins. And PSPA exhibited a more intense effect than BBA in giving rise to G0 phase cells. With the extension of incubation time, an evident decline of Ki67+ percentages in the control group was also found. Ki67 assay together with cell cycle analysis revealed that anthocyanins induced a decrease of continuously cycling cells and a blockage in G0/G1 phase, contributing to cell proliferation inhibition.

Effects of anthocyanins on ROS generation in RPE cells: Anthocyanins induced ROS generation in RPE cells could be seen in fluorescence microscopical

photos (Fig. 4A). More green fluorescence stained cells were observed in anthocyanins treated groups. The data measured by Muse oxidative stress kit (Fig. 4B) also proved that the production of ROS was significantly ($p < 0.05$) increased by anthocyanins treatment, especially from the second day of incubation. In addition, PSPA led to a larger augmentation of the ROS generation than BBA did on the third day (44.31% vs 34.38%, $p < 0.05$). Notably, the control group also showed a sharp rise in ROS generation on the third day.

Effects of anthocyanins on DNA damage in RPE cells: Ataxia-Telangiectasia Mutated kinase (ATM) is a DNA damage-inducible protein kinase (Tichy *et al.*, 2010) and once activated, ATM phosphorylates a number of downstream factors, including histone H2A.X (Burma *et al.*, 2001). The activation of ATM was easily detected in anthocyanins treatment in time course of incubation, while phospho-H2A.X didn't change a lot (Fig. 5). And there were significant differences between PSPA and BBA treatment, resulting in 17.6% p-ATM in PSPA group and 9.57% in BBA group on the third day.

Effects of anthocyanins on the expression of caspase 3/7: The effect of anthocyanins on the expression of caspase 3/7 was demonstrated in Fig. 6. Increases of caspase 3/7 activation in early apoptotic RPE cells were only observed when cells were treated with anthocyanins for 3 days, with percentages less than 3% in each group. While the expression of caspase 3/7 in late apoptotic or dead cell elevated prominently by

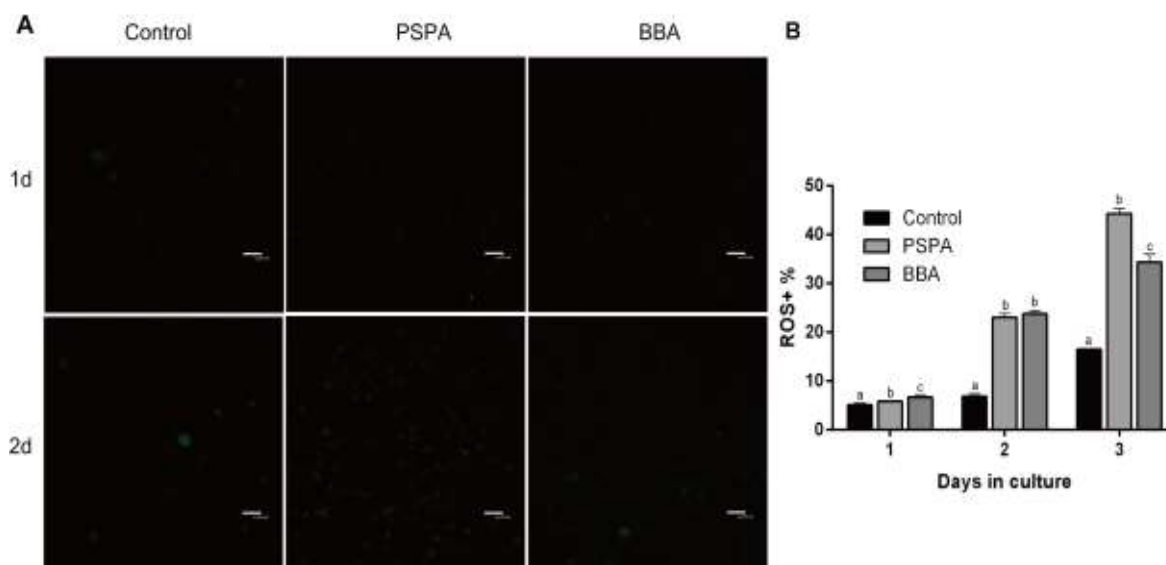


Fig. 4: PSPA and BBA induced ROS generation; (A): Fluorescence micrographs stained with DCFDA of RPE cells treated with 12.5 $\mu\text{g/mL}$ PSPA and BBA for 1 and 2-day. Scale bar: 25 μm ; (B): ROS generation after 12.5 $\mu\text{g/mL}$ PSPA and BBA treatment detected by oxidative stress kit. The means of the values marked with different lowercase letters are significantly different ($p < 0.05$) relative to others in each day of incubation

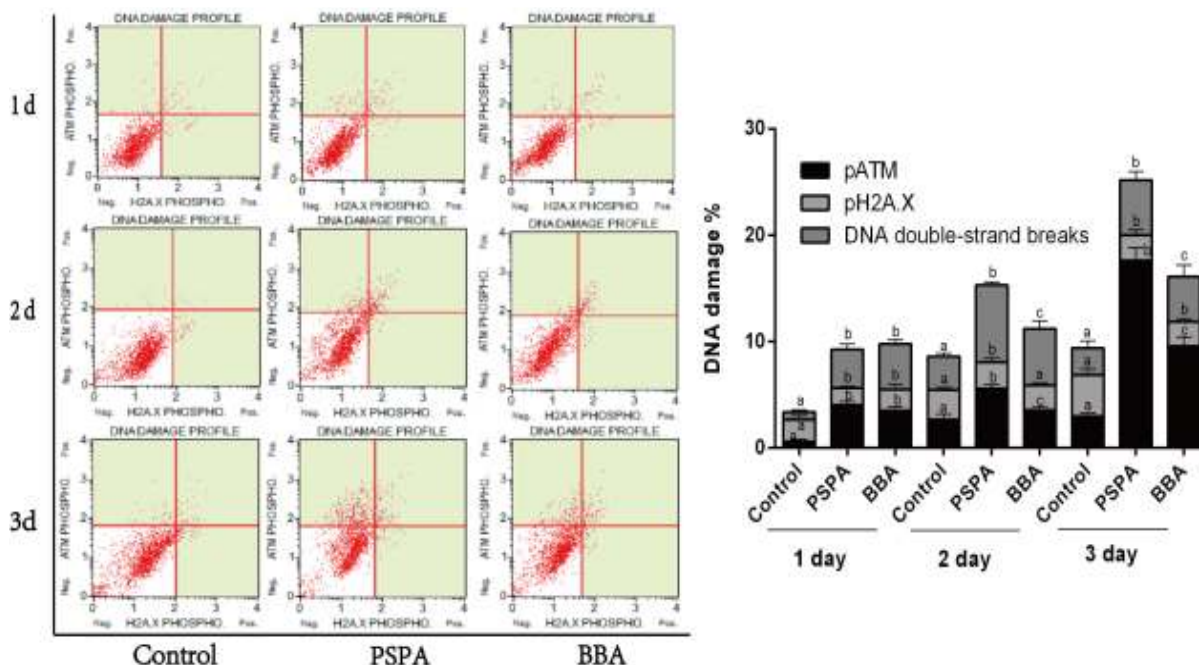


Fig. 5: PSPA and BBA induced DNA damage. The distribution of phosphor-ATM, H2A.X and double-strand breaks measured by multicolor DNA damage kit after 12.5 µg/mL PSPA and BBA treatment. The means of the values marked with different lower case letters for each indicator are significantly different (p<0.05) relative to others in each day of incubation

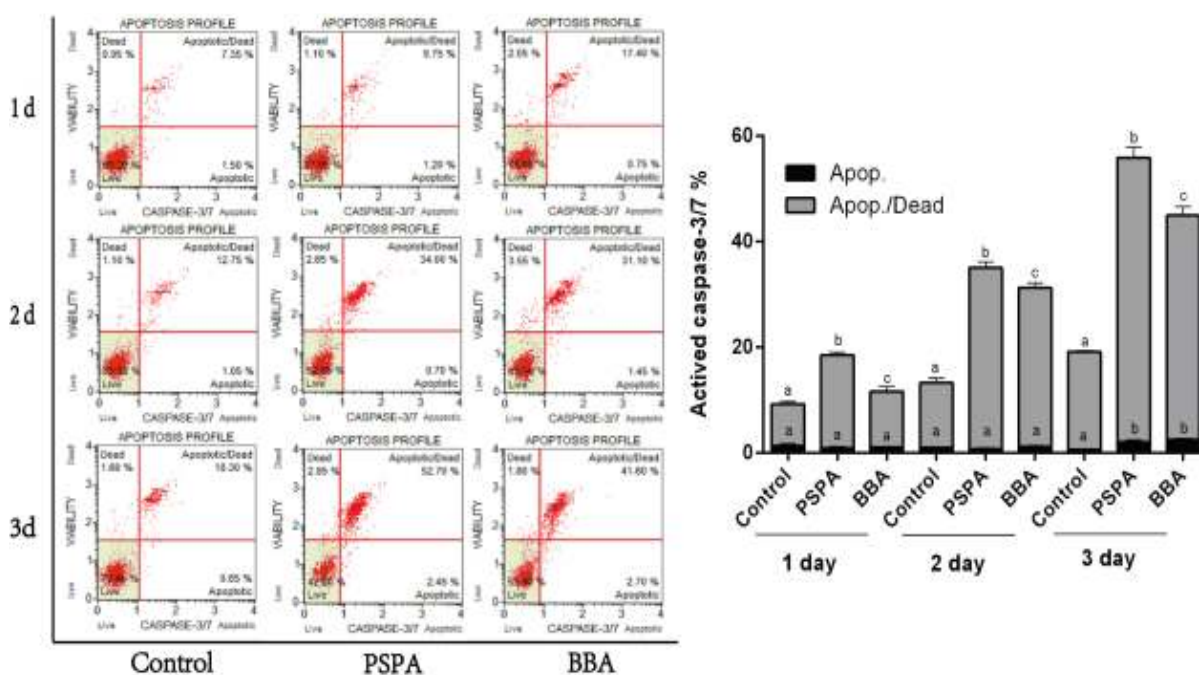


Fig. 6: PSPA and BBA induced caspase 3/7 activation. The caspase 3/7 activation in RPE cells treated by 12.5 µg/mL PSPA and BBA was examined by Caspase-3/7 kit. The means of the values marked with different lowercase letters for each indicator are significantly different (p<0.05) relative to others in each day of incubation

anthocyanins treatment, reaching the percentage of 30% at the end of the second day. PSPA showed a distinct stronger enhancement in caspase 3/7 activation than BBA.

DISCUSSION

Pure anthocyanins and anthocyanin-rich extracts from fruits and vegetables have exhibited anti-

proliferative activity towards multiple cell types *in vitro* (Choi *et al.*, 2011; Matsunaga *et al.*, 2010a; Shih *et al.*, 2005; Tanaka *et al.*, 2012; Wang and Stoner, 2008; Zhang *et al.*, 2005). The non-toxic anthocyanins from purple sweet potato and blueberry used in this study demonstrated potent growth inhibitory properties in RPE cells, the proliferation of which might cause PVR. Both PSPA and BBA showed the time- and dose-dependently anti-proliferation effect through cell cycle arrest and induced cell apoptosis.

Notably, enhanced reductions in proliferation rate (Fig. 1A) and Ki67 positive percentage (Fig. 3C), more pronounced cell cycle arrest (Fig. 3A and B); ROS generation (Fig. 4); DNA damage (Fig. 5) and Caspase 3/7 activation (Fig. 6) were found in PSPA group when compared to BBA group at the same concentration. And BBA treatment resulted in more remarkable early apoptosis than PSPA (Fig. 2 and 3A). Since the anthocyanins used in this study are compounds, it's difficult to put the discrepant effect between PSPA and BBA treatment down to the structure differences of their aglycone anthocyanidins. But the acylation of aglycon in PSPA might responsible for the discrepancy partly. Compared to BBA, cyanidindiacyl glucosides and peonidindiacyl glucosides in PSPA have higher molecular weights, which makes the PSPA group received more anthocyanins than BBA group did when the concentration was calculated as cyanidin 3-O-glucoside equivalent. Furthermore, coffeoyl and feruloyl in PSPA molecules might contribute to the anti-proliferative effect as well, which could be supported by the inhibitory effect of caffeic and ferulic acid in cancer cells (Eitsuka *et al.*, 2014; Rajendra Prasad *et al.*, 2011; Serafim *et al.*, 2011).

As reported, anti-proliferation activity in RPE cells is mainly regulated by inhibition in growth and induction of apoptosis and many signaling pathways are involved (Sawada *et al.*, 2014; Sun and You, 2014; Wu *et al.*, 2011). Our present study tried to shed light on the role of ROS mediated pathway in PSPA induced growth inhibition and apoptosis. ROS have crucial roles in normal physiological processes; either too low or too high level of ROS could cause health problems (Brieger *et al.*, 2012). Anthocyanins are known to act as ROS scavengers in many bioactivities, whereas they also could lead to accumulations of ROS and subsequent apoptosis in certain cells (Cvorovic *et al.*, 2010; Feng *et al.*, 2007). The paradoxical effect of anthocyanins might due to different cell redox state and the double-edged sword property of ROS in determining cell fate (Wang and Yi, 2008). Increased ROS generations were observed in anthocyanins treated RPE cells, especially from the second day of incubation (Fig. 4). Many studies have demonstrated that ROS is responsible for DNA damage (Dhillon *et al.*, 2014; Lv *et al.*, 2013), while the data in our present work seemed that DNA damage was partially independent on ROS production, since more aggravated increases in DNA damage than ROS generation were observed on the first day of

treatment (Fig. 5). Cleavage of the DNA backbone during DNA base excision repair might account for DNA strand break in this stage (Watt *et al.*, 2007). And anthocyanin might induce oxidative DNA cleavage, as reported by Webb *et al.* (2008). Within DNA damage, the activation of ATM was easily detected in anthocyanins treated RPE cells while histone H2AX phosphorylation and DNA double-strand breaks were limited (Fig. 5). Besides, PSPA treatment led to distinguished phospho-ATM than BBA, accompanied by the severer cell cycle arrest in G0/G1 phase and decrease in Ki67 positive cells. DNA damage and repairmen in addition to ROS generation might responsible for the cell cycle arrest and inhibition of quiescent RPE cells transiting into cell cycle (Surova and Zhivotovsky, 2013).

Although it's been reported that necrosis, but not apoptosis, is a major type of cell death in RPE cells in response to oxidative stress (Hanus *et al.*, 2013), we drew a conclusion here that anthocyanins induced RPE cell death mainly through caspase 3/7 mediated apoptosis, when taken the results from cell apoptosis/necrosis analysis (Fig. 2) and the outcome of caspase 3/7 detection (Fig. 6) together. Caspase 3 activation has been demonstrated to be involved in RPE cell apoptosis (Yang *et al.*, 2007) and anthocyanins induced cell death (Forbes-Hernandez *et al.*, 2014), which aid the reliability of our conclusion. Both ROS and DNA damage could act as intracellular death signals to trigger the intrinsic cell death pathway, and then activate executioner caspases (caspase 3, 6, 7), which finally induce cell apoptosis (Radogna *et al.*, 2015).

To conclude, anthocyanins from purple sweet potato and blueberry inhibited RPE cell proliferation through cell cycle arrest and the induction of apoptosis, which involved in ROS generation, DNA damage and caspase 3/7 activation. The findings from our study have highlighted the important role PSPA can play in inhibiting RPE cell proliferation, which is responsible for the progression of PVR. Dietary supplementation with fruits and vegetables rich in anthocyanins can be an effective strategy for prevention of eye diseases like PVR. And we believe that acylated anthocyanins from purple sweet potato are worth to be popularized and applied in food and health industry.

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