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Research Article

Blueberry Anthocyanin Promotes the Growth of Human Retinal Pigment Epithelial Cells

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Abstract: Blueberry Anthocyanin (BBA) has been proved to be beneficial to eyes and it could protect human Retinal Pigment Epithelial (RPE) cells, which perform essential functions for visual process, from light and oxidative damages. However, fundamental information about the effects of BBA on the growth of normal RPE cells is scarce. In the present study, the influences of BBA on the growth morphology, viability, cell cycle, Ki67 and PI3K/MAPK expression of RPE cells were investigated. RPE cells treated with 50 μg/mL BBA demonstrated a predominant polygonal morphology in mosaic arrangement, a better platform growing status, statistically higher viability, an increase in S-phase and more Ki67+cells. But neither pAkt nor pERK was detected in both groups. In conclusion, BBA could maintain high cell viability, boost DNA synthesis and preserve high percent of continuous cycling cells to promote cell survival and division without changing cellular morphology. The growth promotion effect of BBA might play a role in its damage-protective activities.

Keywords: Blueberry anthocyanin, morphology, proliferation, retinal pigment epithelial cells, survival

INTRODUCTION

In vivo, the RPE cells tightly packed to form a compacted monolayer resting on a basement membrane above Bruch's membrane and perform highly specialized roles in maintaining retinal homeostasis and visual functions (Wimmers *et al*., 2007; Chen *et al*., 2014a). Due to their unique functions and position, the RPE cells are suffering from oxidative stress (Arnouk *et al*., 2011) and light exposure (Sui *et al*., 2013). And RPE dysfunction leads to many devastating retinal diseases, such as Age-related Macular Degeneration (AMD) (Zhou *et al*., 2014), Retinitis Pigmentosa (RP) (Van Soest *et al*., 1999). Maintaining certain phenotype, keep the structural integrity and normal physiological functions of RPE cells is crucial for retinal health. Thus the RPE cells are suitable candidates for investigating the protection on human vision health and they have been used in many *in vitro* studies (Chen *et al*., 2014b; Su *et al*., 2014).

Anthocyanins, the most abundant polyphenolic compounds people can get from dietary, are considered to be protective to vision health and retinal cells (Hanneken *et al*., 2006; Kalt *et al*., 2010; Tanaka *et al*., 2011). And Buleberry Anthocyanin (BBA) is one of the most widely used anthocyaninsin these relevant studies (Liu *et al*., 2011). It's reported that Blueberry Anthocyanin suppressed RPE cell ageing and apoptosis and protected them from visible-light induced damage (Liu *et al*., 2012; Wang *et al*., 2015). However, few studies have shown the influences of BBA on normal RPE cellular morphology, survival and proliferation without light or oxidative damages, which might have effects on the resistance of RPE cells to damages.

In view of all these considerations, the purpose of the present study was to explore whether BBA could exhibit influences on the growth characteristics like cellular morphology, survival and proliferation of RPE cells, in order to replenish the fundamental research to elucidate the role of BBA on human vision health.

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). 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% trypsin/EDTA and Fetal Bovine

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Serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Sigma-Aldrich, Inc. (St. Louis, MO, USA).96-well plates, 6-well plates and 25 cm^2 flasks were purchased from Corning Incorporated (NY, USA). Muse^{TM} Count and Viability Assay Kit, Cell Cycle Kit, Ki67 Proliferation Kit and PI3K/MAPK Dual Pathway Activation Kit were purchased from Merck Millipore (Billerica, MA, USA).

Cell culture and BBA treatment: The RPE cells were grown in whole culture medium, namely, DMEM with10% FBS and containing a 1% antibiotic mixture of penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were incubated at 37°C under a humidified 5% CO_2atmosphere. When the cells were confluent, they were detached with 0.5% trypsin/EDTA after a rinse with 0.1 mol/L Phosphate Buffered Saline (PBS).

The BBA was dissolved in DMEM without FBS supplement at a concentration of 500 μg/mL as a stock solution and stored at -20°C. Before experiments, filter sterilization was used to process the stock solution through 0.1 μm filter and then it was diluted with DMEM to certain concentrations. 10% FBS was added to BBA culture medium.

Observation of cellular growth morphology: RPE cells suspended in whole culture medium and 50 μg/mL BBA culture medium were seeded on cover slips at a density of 5×10^5 cells/mL respectively. Specimens cultured with each medium for 2-day and 4-day were viewed by phase contrast microscopy without further processing.

Determination of cell growth curve: To obtain the growth curves of RPE cultured with or without BBA, RPE cells suspended in whole culture medium and 50 μg/mL BBA culture medium were seeded in 96-well plates at a density of 2×10^5cells/mL respectively. Cells incubated with each medium for 6 days and OD values were detected by MTT assay (Wu *et al*., 2009) at each day.

Assay of cell viability: The viability assay was performed according to the user's manual, using Muse^{TM}Cell Analyser (Merck-Millipore, Germany), 2000 events were acquired for each sample. The viable cells and total cells were counted respectively and the viability was expressed as a percentage of the viable cells.

Analysis of cell cycle: Cell cycle analyses were performed with the Muse^{TM} Cell Analyser according to

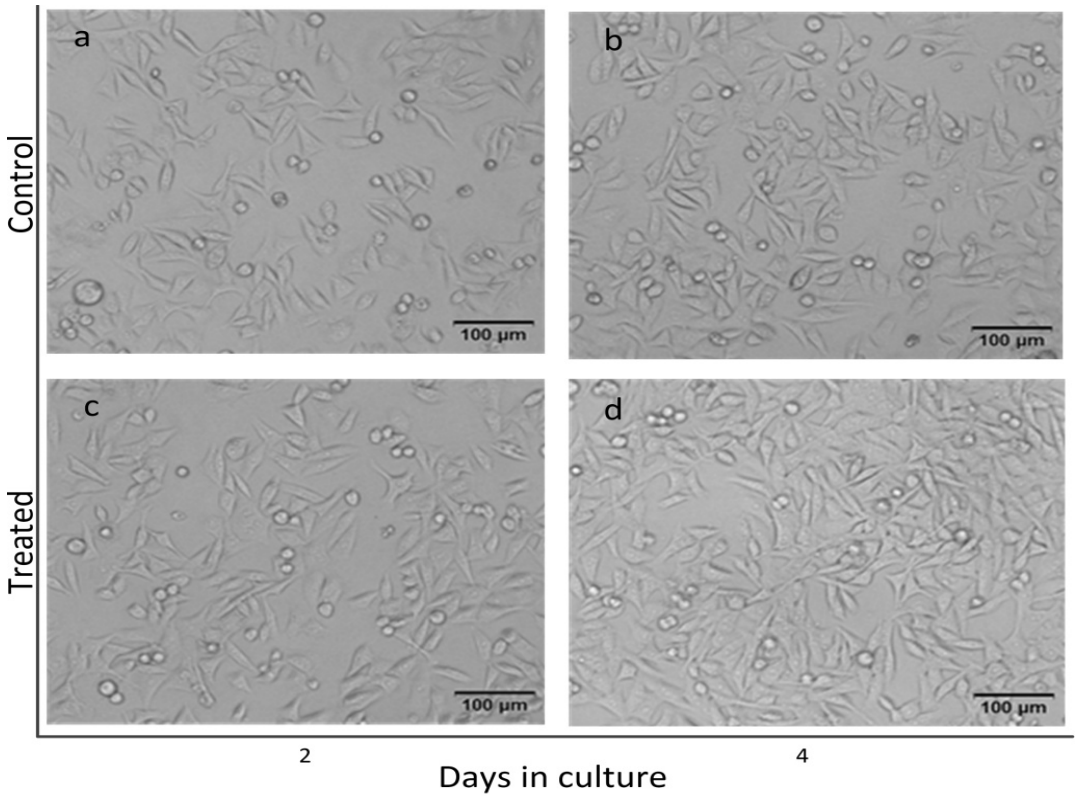


Fig. 1: Phase-contrast micrographs of RPE cells cultured without BBA (control) for 2-day; (a) and 4-day; (b) and with 50 μg/mL BBA (treated) for 2-day; (c) and 4-day; (d) respectively; Scale bar: 100μm

the Muse™ Cell Cycle Kit user's guide after RPE cells were cultured in whole culture medium and 50 μg/mL BBA culture medium for 1~3 days. 20000 events were recorded for each sample. The results were analyzed using Modfit 3.2 (Verity Software House, USA).

Detection of Ki67 expression: RPE cells were cultured in whole culture medium and 50 μg/mL BBA culture medium for 1~3 days and then the Ki67 proliferation assay was performed according to the Muse^{TM}Ki67 Proliferation Kit User's Guide with the Muse^{TM}Cell Analyser.

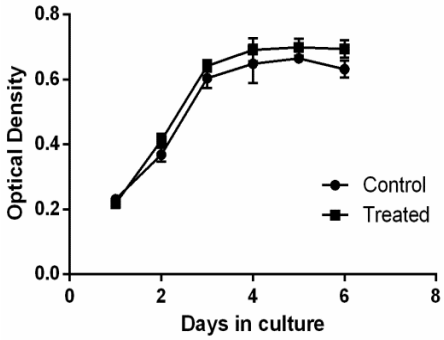
Examination of PI3K/MAPK expression: RPE cells suspended in whole culture medium and 50 μg/mL BBA culture medium were cultured for 1~3 days. The Muse™ PI3K/MAPK Dual Pathway Activation Kit was used to assess the activation of both the PI3K and MAPK signaling pathways simultaneously according to the user's guide.

Statistical analysis: All experiments were performed in triplicate; results were given in means ±standard deviation. Unpaired Student t tests were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) for all assays. Results were considered statistically significant at $p<0.05$.

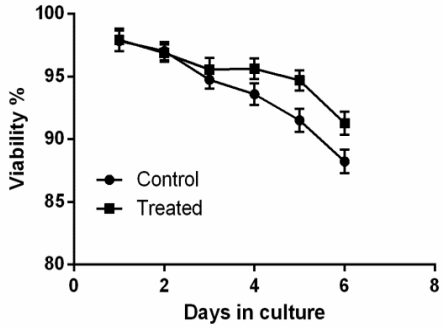
RESULTS AND DISCUSSION

Cell morphology of RPE cells treated with BBA: An epithelioid phenotype of RPE cells with polygonal morphology and colony-like distribution suggested that the cells maintained their specific cell functions (Srivastava *et al*., 2011; Singh *et al*., 2014). Therefore, we observer the growth morphology of RPE cells cultured with or without 50 μg/mL BBA for 2 and 4 days (Fig. 1) to see whether BBA could help to preserve the certain phenotype of RPE cells. There was no notable difference in cell growth morphology, adhesion and distribution between BBA group and the control group, with both groups exhibiting polygonal morphology and mosaic arrangement of RPE cells. RPE cells treated with BBA for 4 days had better growth than control group, since more cells could be seen in Fig. 1d than in Fig. 1b. Therefore, we thought BBA treatment could promote RPE cell growth without changing its morphology.

Effects of BBA on the growth curve of RPE cells: To determine whether BBA has influences on the growth rhythm of RPE cells, a line plot graph was plotted (Fig. 2a). The growth curves clearly showed that the RPE cells incubated with BBA had the similar growth rhythm to control group. But cells cultured with BBA had higher OD values compared to control group from the fourth day of incubation. These results led to the assumption that BBA might have effects on both cell



(a)



(b)

Fig. 2: Effects of BBA on RPE cell growth curve and viability. RPE cells without BBA treatment were defined as control, the effect of BBA on RPE cell growth curve and viability was evaluated at a dose of 50μg/mL. Figure 2a, the growth curve of each group, expressed as optical density; the viability of each group were showed in Fig. 2b, respectively (n = 3)

division and survival, when given the cell population and individual cell as well. The potential to promote individual RPE cell survival and longevity can be supported by reports on anti-aging effects of BBA. BBA was reported to suppress ageing in replicatively senescent RPE cells by extending their lifespan, reducing the number of β-galactosidase-positive cells (Liu *et al*., 2012). It was also demonstrated that BBA could prolong the lifespan of *Drosophila melanogaster* (Peng *et al*., 2012). The maintenance of a specific number or density of healthy RPE cells *in vivo* is more important than turnover of the cell population for normal vision (Defoe and Grindstaff, 2004), whereas the reports on RPE cell survival have been limited. Our study might be one of the early trials to demonstrate the protective effects of BBA on RPE cell survival.

Effects of BBA on viability of RPE cells: Cell proliferation is, by definition, a balance between cell division and cell death (Uebersax *et al*., 2000). So at first we examined the viability of RPE cells incubated with or without BBA, which was independent of cell

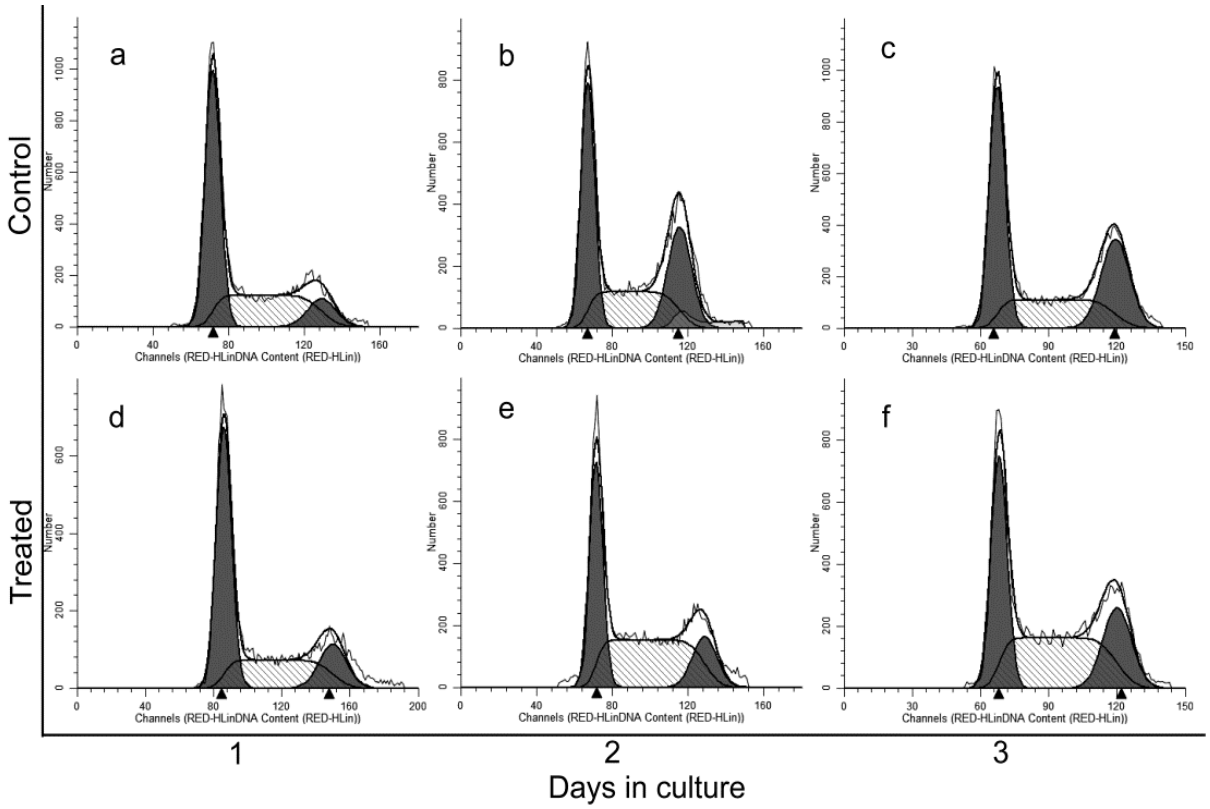


Fig. 3: Cell cycle distribution of RPE cells without BBA treatment (control) for 1~3 days (a-c) and RPE cells cultured with 50 μg/mL BBA (treated) for 1~3 days (d-e)

division by counting the viable cells in every 2000 events of each sample. As shown in Fig. 2b, RPE cells with equal viability were seeded in each group, then the viability in both groups decreased day by day, but BBA treatment exhibited a gentler descent than control group from the fourth day of incubation ($p<0.05$). It suggested that BBA could preserve the survival and viability of RPE cells. This assay also provided further evidence for that RPE cells treated with BBA could have better platform growing status and activity, which was in line with the growth curve in Fig. 2a.

Effects of BBA on cell cycle of RPE cells: Cell cycle analysis shed light upon the cells' current cell cycle stage due to BBA treatment. There were no differences in cycle distribution after 1-day treatment (Fig. 3a and 3d), but after 2-day, BBA incubation resulted in a striking decrease of G1/G0 stage cells (36.45±0.13% vs 40.42±0.34%; $p<0.05$) whereas percentage of cells cycling in S phase increased significantly (47.29±2.54% vs 31.14±0.48%; $p<0.05$) (Fig. 3b and 3e).Besides, obvious decline was also observed in the relative amount of G2/M-phase cells (16.28±2.41% vs28.43±0.47%; $p<0.05$) and similar changes on the third day were obtained (Fig. 3c and 3f). Melding this phenomenon with RPE cellular morphology (Fig. 1) and viability (Fig. 2b) treated with 50 μg/mL BBA for 2 to 4 days, which had shown more living cells with higher viability compared to control group respectively, it manifested that BBA promoted DNA synthesis which took place in S-phase rather than inducing a cell cycle blockage. Cells are undergoing synthesizing DNA in S-phase and DNA synthesis rates are determined as S-phase or growth fraction to indicate cell proliferation (Gao *et al*., 2009; Thieltges *et al*., 2011). And an increase in the percentage of RPE cell population in the S phase of the cell cycle was also found in thrombin induced RPE cell proliferation (Parrales *et al*., 2010). It also explained why there were no dramatic increases in OD values until the fourth day of incubation with 50 μg/mL BBA from cell growth curves as seen in Fig. 2a, more active cell mitosis took place after higher level of DNA synthesis.

Effects of BBA on Ki67 expression of RPE cells: There are several markers expressed during cell proliferation, including Ki67, which is tightly associated with proliferation. 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 is absent in the resting G0 phase (Scholzen and Gerdes, 2000). It was proved that Ki67 could be detected as a growth fraction marker to offer good representative characteristics of proliferation status (Le Pessot *et al*., 2001; Jakobsen and Sørensen, 2013). The Ki67 assay showed that the percentage of Ki67 + cells

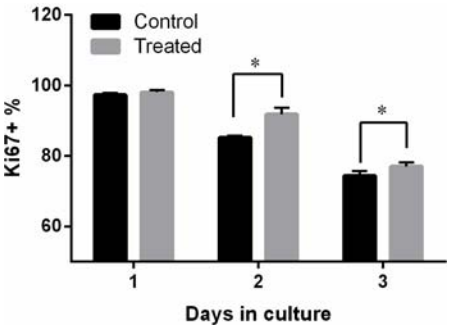


Fig. 4: Ki67 expression of RPE cells treated without BBA (control) and with 50μg/mL BBA (treated) for 1~3 days. (n = 3); *Statistical comparisons between BBA treated groups and controls were carried out on each day of incubation and mean values were significantly different ($p<0.05$; Unpaired Student t-test)

was higher when RPE cells cultured with 50 μg/mL BBA for 2-day (Fig. 4), which meant less cells in G0 phase treated by BBA. It was noteworthy that the percentage of Ki67+ cells resulted in a dramatic decline during the incubation, which indicated that more and more cells wouldn't undergo mitosis with the extension of incubation time. It was supposed that contact inhibition contributed to this decline in dividing cells. Hou *et al*. (2013) revealed that post confluent ARPE-19 cells rarely displayed Ki67 staining compared with sub-confluent cells. Higher percent of Ki67 + cells treated with BBA indicated that more cells passed through G1-phase to initiate DNA synthesis which also contributed to the increase in S-phase. Ki67 assay data together with cell cycle analysis data revealed that incubation of cells in BBA resulted in an increase of continuously cycling cells and a promoted DNA synthesis boosting cell proliferation.

Effects of BBA on PI3K/MAPK expression of RPE cells: It has been reported that PI3K and MAPK signaling pathways are involved in the control of cell proliferation and cell survival (Defoe and Grindstaff, 2004; Parrales *et al*., 2011, 2013). Herein we carried out the PI3K/MAPK dual pathway activation assay to see whether the proliferation of RPE cell boosted by BBA is mediated by Akt or/and ERK activation. As shown in Fig. 5, neither pAkt nor pERK was detected in both groups in our experiments. It was supposed that there're other signaling pathways involved in RPE cell survival and division incubated by BBA. The importance of integration of multiple intracellular signaling pathways in the regulation of RPE cell survival and longevity was also highlighted by Defoe and Grindstaff (2004). Parrales *et al*. (2010) results showed that ERK activation was necessary but not sufficient for the induction of cyclin D1 expression and

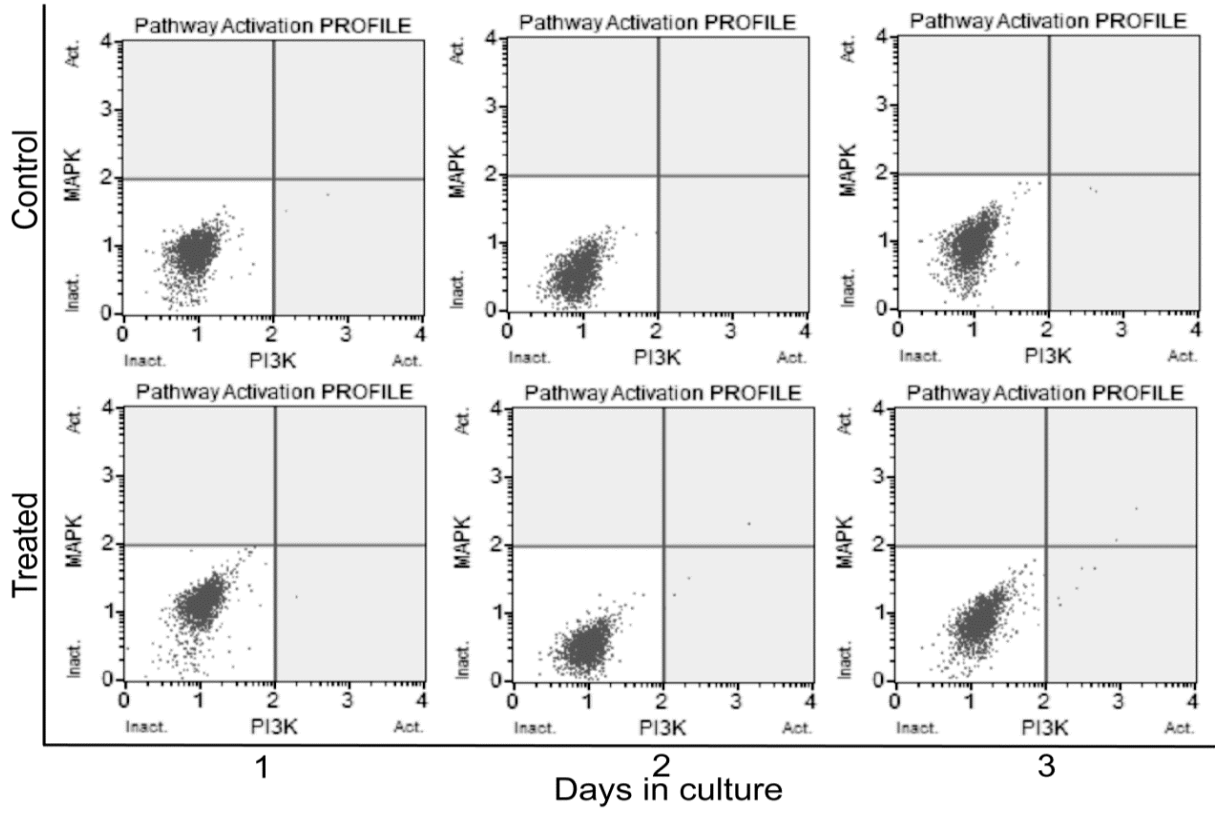


Fig. 5: PI3K/MAPK expression of RPE cells treated without BBA (control) and with 50μg/mL BBA (treated) for 1~3 days

RPE proliferation; Hecquet *et al*. (2002) also demonstrated that MEK/ERK only participated in the signaling involved in cell growth whereas the activation of the Ras/Raf-1 pathway was essential for Fetal Calf Serum (FCS) induced RPE cell proliferation; and PKC was reported to play an important role in the regulation of RPE cell proliferation (Gao *et al*., 2009). Further study on examining more kinases in multiple signaling pathways will be needed in order to elucidate the mechanism of BBA effects on RPE cells.

CONCLUSION

BBA could maintain high cell viability, boost DNA synthesis and preserve high percent of continuous cycling cells to promote cell survival and division without changing cell morphology. These effects showed no involvement of phosphorylation of Akt and ERK. We proved that BBA could have benefits on the growth of RPE cells and this might contribute to damage-protective effects of BBA on RPE cells.

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