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Modulation of autophagy enhances the apoptotic cell death in human T lymphoma cells treated with Anthocyanin

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ABSTRACT

In the present study, the effect of modulation of autophagy induced by anthocyanin on cell death of Human T lymphoma cells (Jurkat) was studied. Anthocyanin was abstracted from dry petals of *Hibiscus sabdariffa*, aquous solution of anthocyanin was added to cells at different concentrations then cell viability was determined by trypan blue exclusion method. Autophagy induced by anthocyanin was inhibited by 5 mM NH_4CL which halts lysosomal enzymes and accordingly preventing autolysosomes formation. On the other hand, Autophagy was enhanced by glucose starvation. In both experiments of autophagy inhibition and enhancement, cell viability was studied to investigate the effect of autophagy modulation on cell viability. The results of this work revealed that both inhibition and enhancement of autophagy induced by anthocyanin lead to massive cell death. Immuno-detection of active caspase 3, one of the major hallmark of apoptotic cell death revealed remarkable increase of active caspse3 upon autophagy inhibition or enhancement. In conclusion, modulation of autophagy induced by anthocyanin lead to increasing of apoptotic cell death.

Keywords: modulation of autophagy- human tlymphoma cells - anthocyanin

INTRODUCTION

Anthocyanins are plant secondary metabolites (Harborne and Williams, 2000), function as pigments (Mohr and Schopfer, 1995) commonly found in the red, blue, and purple colors of fruits, vegetables, flowers, and other plant tissues or products. Their amounts are especially high in berries (Mateus *et al*., 2001). Anthocyanins are generally accepted as the largest and most important group of water soluble pigments in nature (Harborne, 1998). The word anthocyanin derived from two Greek words: anthos, which means flowers, and kyanos, which means dark blue (Horbowicz *et al*., 2008). They belong to the family of compounds known as flavonoids, (Brouillard, 1982; Mazza and Miniati, 1993; Mazza *et al*., 2004; Mazza, 2007). recently, numerous studies have shown that anthocyanins display a wide range of biological activities (Mazza, 2000; Kong *et al*., 2003) including antioxidant (Wang *et al*., 1997; Tsuda *et al*., 1998; Fukumoto and Mazza, 2000; Mazza *et al*., 2002), and anti inflammatory (Wang and Mazza, 2002; Youdim *et al*., 2002). During the last decade, it has been shown that anthocyanins may have beneficial effects on various pathologies to reduce the risks of cancers with anti-inflammatory, antioxidant and chemoprotective properties (Jang *et al*., 1997; Middleton *et al*., 2000). Several investigations have compared the antiproliferative effects of anthocyanins on normal vs. cancer cells and found that they selectively inhibit the growth of cancer cells with relatively little or no effect on the growth of normal cells (Hakimuddin *et al*., 2004; Galvano *et al*., 2004). Recent evidence suggests that various anthocyanins should exert remarkable cytotoxic effects on malignant cells (Yeh and Yen, 2005;

Ding *et al*., 2006; Hafeez *et al*., 2008). Induction of apoptosis by anthocyanins is a pivotal mechanism of their cytotoxic effects on various malignant cells *in vitro* (Hou *et al*., 2003; Yeh and Yen, 2005). For example, anthocyanins induced apoptosis in human colon cancer HCT-116 cells (Shin *et al*., 2009), and in human stomach cancer cell line (Hayashi *et al*., 2006). Also, it was recently reported that the cytotoxic effects of anthocyanins on various cancer cell lines were well correlated with autophagy induction (Tsuyuki *et al*., 2012; Choe *et al*., 2012). Longo *et al*. (2008) were reported for the first time a novel function of antocyanins; the induction of autophagy, in various cancer cell lines. Depending on these studies, anthocyanins induced both programmed cell deaths; apoptosis and autophagy. The mechanism of induction of both cell death types and relationship between them are still need clarification. In the present study, the relationship between apoptosis and autophagy induced by anthocyanins is investigated.

MATERIALS AND METHODS

Jurkat cell line was purchased from VacSera, Alagoza, Algeza, Egypt. RPMI (with L-glutamine) growth medium, FBS (fetal bovine serum) and anti biotic mix (penicillin and streotomycin) were purchased from Lonza, Belgium. RPMI-1640 (with L-glutamine, without D-glucose) growth medium was purchased from Biochrome Ltd, Cambridge UK. Cell culture dishes and glassware were purchased from high quality local suppliers. Monodansylcadaverine (MDC), Trypan blue dye, Nonidet-P40, TritonX-100, Na dioxycholate, Leupeptin/pepstatin mix, Tween-20, and Skim milk (fat free milk powder) were purchased from Sigma (St. Louis, MO, USA). Antibodies from Santa Cruz biotechnology, and Protein markers from Jena bioscience, Germany. SuperSignal West Pico Chemiluminescent Substrate From Pierce. Other chemicals were purchased from high qualified local suppliers if not mentioned elsewhere.

Anthocyanin preparation

Anthocyanin pigment was prepared from dry petals of *Hibiscus sabdariffa* which obtained from the farmers directly and not from grocery. Petals were socked in methanol over night, and then using chloroform and water 1:1:0.5 (v/v), the organic phase was aspirated. The resulted solution was left to dry over a clean glass slap in dark at room temperature and the pigment was scratched out and immediately kept in sealed tubes at 4 °C. 1 µg/ µl water solution of anthocyanin was prepared.

Cell Culture

Jurkat cell line (human T lymphoma cells) was cultured in RPMI-1640 (with L-glutamine) growth medium supplemented with 10% (v/v) (FBS), and 1% anti biotic mix (10.000 u penicillin / ml, and 10.000 u streptomycin / ml) at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cells are harvested 48h after inculcation, and then seeded onto 24-well plates in suitable cell density.

Visualization of Monodansycadaverine (MDC)-labeled autophagic vacuoles

Autophagic vacuoles were labeled with MDC by incubating cells with 0.05 mM MDC in PBS at 37°C for 10 minutes. After incubation, cells were washed four times with PBS and immediately analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TE 300, Germany) equipped with a filter system (excitation filter V-2A: 380-420 nm, barrier filter: 450 nm). Images were obtained with a CCD camera (Orca I, Hamamatsu). Number of vacuoles were counted in 10 cells at 3 different fields for each treatment.

Cell Viability: Trypan blue-exclusion method

50µl of cell suspension (at a concentration of approximately 10^6 cells

per ml) was mixed with 50 µl of 0.4% trypan blue in 1 ml Eppendorf tube, and incubated at room temperature for about 3 min. The mixture of 1:1 cell suspension and trypan blue was loaded into a hemocytometer. Using brightfield optics, viable (clear) cells were counted in the four large corner squares. Cell viability was estimated after addition of different anthocyanin concentrations as 0 (control), 0.1, 0.2, 0.3, and 0.4 mg/ml anthocyanin to (50 x10^3 cells/ml). Autophagy was blocked using 5 mM of ammonium chloride solution (NH_4Cl) 1 h before addition of different concentrations of anthocyanin. Auto-phagy was induced by glucose starvation by harvesting cells in RPMI-1640 (with L-glutamine, without D-glucose) growth medium before anthocyanin treatment.

Immunoblotting

Cells (10^6/sample) were lysed in ice-cold 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 30 mM Hepes, pH 7.4, with protease and phosphatase inhibitors. After 10min, SDS-sample buffer was added, the samples were boiled and proteins were separated by 10% SDS-PAGE. After transfer to nitrocellulose sheets, they were probed with mouse anti-caspse 3 and goat anti-mouse IgG peroxidase. The membranes were reprobed, after stripping, with mouse anti-actin IgG. Immunoreactive bands were visualized with the chemiluminescent substrate and quantified densitomerically by Image J software.

Statistical Analysis

Statistical significance of differences throughout this study was analyzed by One-way ANOVA test. *P* value <0.001 was considered to be statistically significant.

RESULTS

Autophagy induction by anthocyanin and its modulation

Monodansylcadaverine (MDC) is an autofluorescent compound that has been reported to specifically label autophagic vacuoles *in vivo* and *in vitro* conditions. Therefore, we studied the incorporation of MDC in Jurkat cells after treatment with anthocyanin. In non-treated (control) cells (Fig. 1a) very little staining was observed, with only weak staining of the cell wall and/or plasma membrane at all time points. The MDC-labeled vesicles were small and less intensely stained vesicles. After addition of 0.1 mg/ml of anthocyanin (Fig 1a) there was an obvious MDC-labeled vesicles distributed in the cytoplasm to verify that anthocyanin induce autophagy. For 0.5 mg/ml anthocyanin treated cells (Fig. 1a) the number of MDC-labeled vesicles was increased and more intensely stained when compared with 0.1 mg/ml of anthocyanin treated cells. NH_4Cl inhibit autophagy at a later stage by preventing the fusion of autophagosomes and lysosomes. After autophagy inhibition by 5 mM NH_4Cl, the accumulation of MDC-labeled vesicles in NH_4Cl-0.1 mg/ml antho-cyanin treated Jurkat cells was increased. MDC-labeled vesicles were obvious and increased compared with 0.1 mg/ml anthocyanin treated cells (Fig. 1b). For autophagy enhancement, Jurkat cells were incubated in RPMI-1640 (with L-glutamine, without D-glucose) (starved cells). After two hours, starved cells (Fig. 1b) showed an increase in the number of vesicles as well as in their size compared with non-treated Jurkat cells (Fig. 1b), indicating that starvation induced the formation of the MDC-labeled autophagic vacuoles. After one hour of 0.1 mg/ ml anthocyanin addition to starved cells (one hour starvation), the MDC-labeled vacuoles was decreased (Fig 2i) compared with two hours starved cells (Fig. 1b) and 0.1 anthocyanin treated cells.

Autophagy inhibition by NH_4CL leading to massive cell death

It is well known that anthocyanin lead to autophagy induction and simultaneously apoptotic cell death.

Addition of 0.1, 0.2, 0.3, 0.4, and 0.5mg/ml of anthocyanin to Jurkat cells at density of 50 X 10^3 cells/ml for 24hours markedly decreased cell viability as 74.90%, 45.30%, 13.48%, 5.86%, and 0%, respectively, compared with control (Fig. 2a). After 48 hours viability of Jurkat cells continued in decreasing as 69.01%, 42.08%, 4.62%, 0%, and 0% for 0.1, 0.2, 0.3, 0.4, and 0.5mg/ml of anthocyanin, respectively, compared with control (Fig. 2a). Abrogation of autophagy process by 5mM NH_4Cl has a great effect on cell viability. NH_4CL alone has a little effect on cell viability after 24 hours cell viability reaches 88.25% compared to the control. Cell viability for cells treated with 5mM NH4CL and different concentrations 0.1, 0.2, 0.3, 0.4, and 0.5mg/ml of anthocyanin for 24 hours were 63.18%, 22.31%, 1.58%, 0% and 0% compared with control (Fig. 2b). After 48 hours cell viability recorded the following measures: 71.59%, 37.80%, 5.70%, 0%, 0%, and 0% in 0.1, 0.2, 0.3, 0.4, and 0.5mg/ml anthocyanin-NH_4Cl treated group, respectively, compared with control (Fig. 2b).

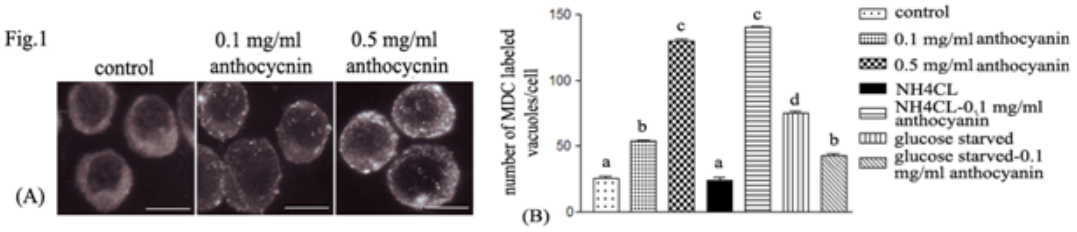

Fig. 1: Induction of autophagy by anthocyanin. Autophagic vacuoles is visualized by MDC auto fluorescent stain using fluorescent microscopy, MDC labeled autophagic vacuoles are shown at control, 0.1 and 0.5 mg/ml anthocyanin, bar equals 50 µm (A). mean number of MDC labeled autophagic vacuoles/cell at different treatments, data with different letters are significantly different (p<0.001) (B).

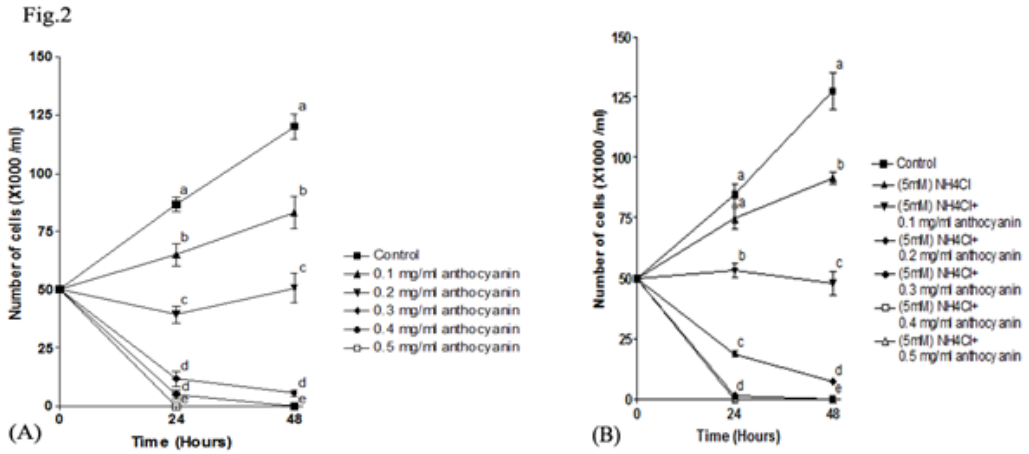

Fig 2: Cell viability after autophagy abrogation: Jurkat cells were inoculated at 50 X 10^3 cell/ml then left untreated or treated with different concentrations of anthocyanin as shown with or without 5mM of NH_4CL or cells were inoculated in RPMI 1640 without glucose for the indicated time, then viable cell count carried out with trypan blue exclusion method, data with different letters are significantly different (p<0.001). Mean viable Jurkat cells ± SE at 24 and 48 hours after addition of different concentrations of anthocyanin (a). Mean viable Jurkat cells ± SE at 24 and 48 hours after addition of different concentrations of anthocyanin with autophagy inhibition by 5mM of NH4Cl (b).

Autophagy induction by glucose starvation enhances cell death

After 24 and 48 hours of glucose starvation cell viability significantly decreased (P<0.001) by (50.42%) at 24 hours and (23.63%) at 48 hours in glucose starved group versus control group (Fig. 3a). After 24 hours of anthocyanin addition to glucose starved Jurkat cells, cell viability decreased as 41.30%, 28.31%, 8.98%, 0% and 0% in 0.1, 0.2, 0.3, 0.4, and 0.5mg/ml of anthocyanin compared with the control (Fig. 3a). After 48 hours cell viability recorded the following measures: 12.03%, 6.41%, 2.57%, 0% and 0% in 0.1, 0.2, 0.3, 0.4, and 0.5mg/ml of anthocyanin compared to the control (Fig. 3a). Cell viability after 48 hours of autophagy inhibition and enhancement are summarized in (Fig. 3b).

Fig.3

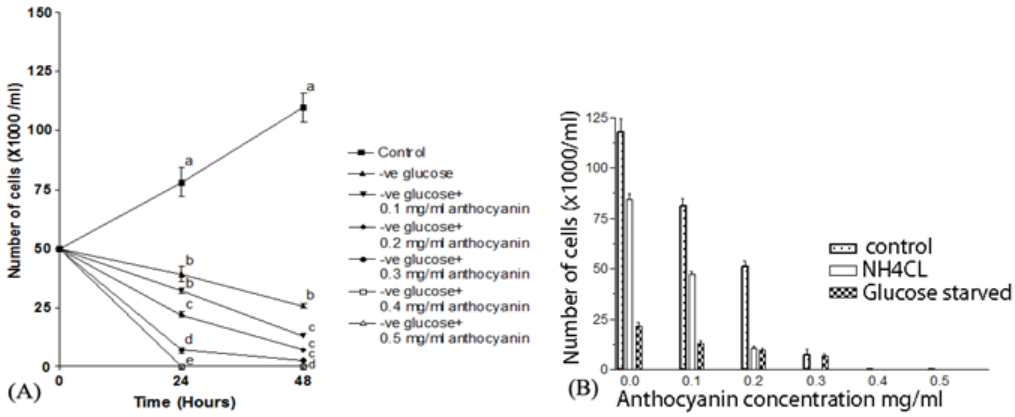

Fig. 3: Cell viability after autophagy induction: Jurkat cells were inoculated at 50 X 10^3 cell/ml in RPMI 1640 without glucose for the indicated time, then viable cell count carried out with trypan blue exclusion method. Mean viable Jurkat cells ± SE at 24 and 48 hours after addition of different concentrations of anthocyanin to the glucose starved cells, data with different letters are significantly different (p<0.001) (a). Mean viable Jurkat cells ± SE at 48 hours after different treatments (b).

Inhibition or enhancement of autophagy lead to increasing of apoptotic cell death changes in protein levels of active caspase-3 in different treatments (Figure 4a). The level of active caspase-3 was increased (291.10%) after addition of 0.1mg/ml of anthocyanin versus those of untreated (control) cells (100%) as calculated from denistomitric measurement. After 0.5mg/ml of anthocyanin addition the level of active caspase-3 continued in increasing (833.90%) comparing with untreated (control) cells (Figs. 4a and b). The inhibition of autophagy by 5mM of NH_4CL not significantly (P>0.001) increased (134.75%) the level of active caspase-3 in Jurkat cells versus those of untreated (control) cells. The level of active caspase-3 was markedly increased (961.44%) by addition of 0.5mg/ml anthocyanin to Jurkat cells after autophagy inhibition by NH_4CL compared with control cells. No change in active caspase-3 level was observed (99.58%) after autophagy induction by glucose starvation comparing with untreated (control) cells. However, significant (P<0.001) increase of active caspase-3 level was observed in Jurkat cells after autophagy induction by glucose starvation and anthocyanin addition (0.5mg/ml). The increase in active caspase-3 level was 1023.73%

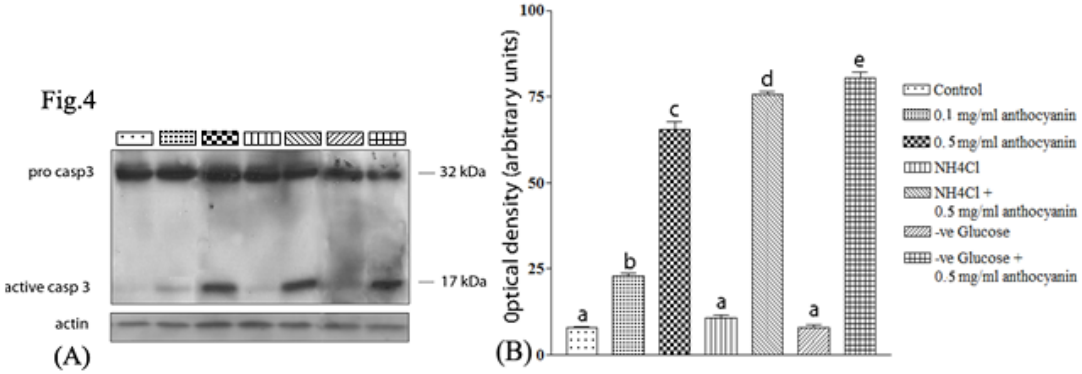

Fig. 4: Level of active caspase3 after autophagy modulation. 10^6 cells were lysed in lysis buffer and proteins were separated by 10% SDS polyacrylamide gel, caspse3 was immune-detected by anti-caspase3 IgG antibody and anti-mouse HRP conjugated goat IgG antibody, bands of pro and active caspase3 were visualized by ECL substrate, actin is shown for equal loading of protein samples (A). Bands from (A) were quantified denistomitrically using Image J software, data with different letters are significantly different ($p<0.001$) (B).

DISCUSSION

Anthocyanins, which are bioactive phytochemicals, are widely distributed in plants. Anthocyanins not only possess antioxidant ability (Pool-Zobel *et al*., 1999; Tsuda *et al*., 2000), but also mediate other physiological functions related to cancer suppression (Kamei *et al*., 1995; Meiers *et al*., 2001; Nagase *et al*., 1998). Previous studies have shown that Hibiscus anthocyanins (HAs) (which are extracted from the dried calyx of *H. sabdariffa*) possess antioxidant bioactivity both *in vivo* and *in vitro* (Tseng *et al*., 1997; Wang *et al*., 2000). Our results showed that anthocyanin (after 24h) significantly inhibited cell viability of Jurkat (50 X 10^3 cells/ml) cells in dose-dependent manner. These results are in agreement with those of Devi *et al*. (2011) who demonstrated that, with increasing concentrations of red sorghum bran anthocyanins from 0.015mg/ml to 1mg/ml the percentage of growth inhibition of MCF-7 (30 X 10^3 cells per well) cells increased progressively from 11.47% to 86.88% in acidified methanol extract after 24h of anthocyanins exposure. Also, Hogan *et al*. (2010) demonstrated that an anthocyanin-rich extract generated from açai treatment dose-dependently inhibited the growth of C-6 rat brain glioma cells. Moreover, Malik *et al*. (2005) showed that Pomegranate fruit extract, a rich source of anthocyanins, treatment of highly aggressive human prostate cancer PC3 cells resulted in a dose-dependent inhibition of cell growth/cell viability and induction of apoptosis. In the present study, anthocyanin inhibited the growth of Jurkat cells in a time-dependent manner. Also, Lin *et al*. (2007) reported that *H. sabdariffa* extracts treatment affected AGS (human gastric carcinoma) cells viability and showed a dose-dependent and time-dependent inhibitory effect on the growth of AGS cells with IC50 near to 2.5mg/ml in a 24h exposure to *H. sabdariffa* extracts. From the previous data, it may be concluded that anthocyanin have antiproliferative activity on cancer cells *in vitro*, but this activity depend on concentration, time exposure, cell density, origin of cancerous cell lines, and extraction method. Autophagy optimizes nutrient utilization in rapidly growing cells when faced with hypoxic or metabolic stress, similar to therapeutic assault, and removes organelles that are sources or targets of lethal levels of free radical–induced damage (Kim *et al*., 2007). Thus, lack of autophagy for a long period of time results in the accumulation of

intracellular oxidants that enhance the probability of transformation. Paradoxically, however, in some cases autophagy can also promote the survival of cancer cells once tumours have developed. This is attributed to the ability of autophagy to promote cell survival under conditions of poor nutrient supply, as often faced by solid tumours and metastasising cancer cells. In addition, autophagy is frequently upregulated in tumours as a response to therapy and may protect tumours against therapy-induced apoptosis (Brech *et al.*, 2009). These observations lead to the conclusion that the influence of autophagy on the fate of cells is based on the environment or stimuli that cells receive and the type of cells involved (Lin *et al*., 2012). The present study provides evidence to show the induction of both autophagic and apoptotic machineries in Jurkat cells upon treatment with anthocyanin, which are highly promising cancer chemopreventive constituents of several vegetables (Neto, 2007; Nichenametla *et al*., 2006). In most apoptotic processes, caspase-3 has been shown to play a pivotal role in the terminal and execution phase of apoptosis induced by diverse stimuli (Thornberry and Lazebnik, 1998; Ashkenazi, 2002). In the current study, anthocyanin treatment significantly ($P<0.001$) increased expression level of active caspase-3 by 291.10% and 833.90% for 0.2mg/ml and 0.4mg/ml anthocyanin, compared with untreated cells (control), respectively. Cancer cells can respond to drugs by activating autophagy to promote cell survival, thus counteracting apoptosis (Kondo *et al*., 2005), so inhibition of autophagy using chemotherapeutic agents is important for the induction of apoptosis in cancer cells (Longo *et al*., 2008; Chen *et al*., 2011). To elucidate the involvement of the autophagic process in anthocyanin-induced apoptosis, the effects of autophagy inhibitors were tested. NH_4Cl inhibits autophagy at a later stage by preventing the fusion of autophagosomes and lysosomes (Yamamoto *et al*., 1998). Therefore, autophagy inhibition by NH_4Cl markedly increased anthocyanin-induced generation monodansylcadaverine-labeled vacuoles.

In the current study, inhibition of anthocyanin–induced autophagic vacuoles by NH_4Cl, significantly decreased the cell viability when compared with anthocyanin-treated cells. Paglin *et al*. (2001) showed for the first time the possibility of treating cancer cells by autophagy inhibition because cancer cells respond to radiation by inducing autophagy. These and our observations indicate that autophagy protects cancer cells from therapy-induced apoptosis and that autophagy inhibitors strengthen the efficacy of proapoptotic chemotherapeutic strategies. Autophagy is commonly induced by hypoxia and it represents the ultimate nutritional source for tumor cells to survive low-nutrient conditions (Kondo *et al*., 2005). Our results are in agreement with those of Longo *et al*. (2008) who reported that autophagy inhibition helps to enhance the chemopreventive/therapeutic activity of anthocyanin. They found inhibition of *P. lentiscus* anthocyanin–induced autophagy by 3-MA markedly inhibited *P. lentiscus* anthocyanin-induced generation of LC3-II–labeled vacuoles and enhanced anthocyanin-induced cell demise. Increased autophagy is normally induced by environmental cues such as starvation and hormones (Wu *et al*., 2011). In the present study, anthocyanin treatment of glucose-starved cells lead to induction of apoptosis by significantly increases the level of active caspase-3. Our results were in agreement with several studies which reported that autophagy induction appears to facilitate successful therapy-induced killing of tumour cells (Fujiwara *et al*., 2007; Peng *et al*., 2008). Autophagy protects cell from apoptosis when is involved in the degradation of

caspases such as caspase-8 and related proteins (Hou *et al*., 2010), however, if Inhibitor of Apoptosis (IAP) family of proteins are significantly degraded via autophagy, apoptosis will be triggered (Nezis *et al*., 2010). Treatment of glucose starved cells with anthocyanin in the current study leads to decrease of MDC labeled autophagic vacuoles which does not mean the decrease of autophagy. Each Anthocyanin and glucose starvation separately lead to increase of autophagic vacuoles but together lead to decrease of autophagic vacuole may be according to the increase of the lysosomal degradation process so the number of autophagic vacuoles is decreased.

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ARABIC SUMMERY

التحوير في الاتهام الذاتي يعزز موت الخلايا المبرمج في خلايا السرطان اللمفية التائية البشرية المعاملة بالانثوسيانين

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في هذا البحث، تم دراسة تأثير التحور في الالتهام الذاتي الناجم عن الأنثوسيانين على موت الخلايا السرطانية اللمفية التائية البشرية (خط خلايا جوركات). تم استخرج الأنثوسيانين من البتلات الجافة لنبات الكركديه، تم إضافة المحلول المائي من الأنثوسيانين إلى الخلايا بتركيزات مختلفة ثم تم دراسة حيوية الخلايا بطريقة استبعاد صبغ التريبان الأزرق. الالتهام الذاتي الذي يسببه الأنثوسيانين تم تثبيطه بواسطة 5ملي مولار من كلوريد الامونيوم الذي يوقف الإنزيمات الليزوزومية وبالتالي منع تشكيل جسيمات التحلل الذاتي. من ناحية أخرى، تم تعزيز الالتهام الذاتي عن طريق التجويع الجلوكوزي .في كلا التجربتين من تثبيط الالتهام الذاتي وتعزيزه، تم دراسة حيوية الخلايا للتحقق من تأثير تحور الالتهام الذاتي على حيوية الخلايا .وكشفت نتائج هذا العمل أن كلا من تثبيط وتعزيز الالتهام الذاتي الناجم عن الأنثوسيانين تؤدي إلى موت الخلايا بشكل مكثف . الكشف المناعي لإنزيم كاسباس 3، واحدة من السمات المميزة الرئيسية لموت الخلايا المبرمج، اظهر ارتفاعا ملحوظا من مستوى هذا الإنزيم في حالة تثبيط الالتهام الذاتي أو تعزيزه .في الختام، تحور الالتهام الذاتي الناجم عن الأنثوسيانين يؤدي إلى زيادة الموت المبرمج لخلايا السرطان اللمفية التائية البشرية.