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Blueberry/Bilberry Juice and Blueberry Fruit Supplementation Protects DNA

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Authors' contributions

This work was carried out in collaboration between all authors. Author YTS designed the study, performed the statistical analysis. Authors PYYL and DMKN recruited subjects, performed experiments and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The study was to investigate the protective effect of blueberry/bilberry juice and blueberry fruit on leucocyte DNA against exogenous oxidative stress.
Methodology: The study was divided to two parts which included the in vitro antioxidant effect of blueberry/bilberry juice on lymphocytes and the effect on human leucocytes after supplementation. For in vitro study, lymphocytes from 4 subjects were pre-incubated with diluted blueberry/bilberry juice. Cells were then treated with 50 μM H_2O_2. Comet assay was performed. For in vivo study, lymphocytes of 9 subjects from venous blood were collected before and after taking 200 mL blueberry/bilberry juice and control drinks. Lymphocytes were then stressed with 50 μM H_2O_2 and followed by comet assay. In another experiment, 9 subjects took 40 g blueberry and blood samples before and after 2 hours were irradiated with UV light and DNA damage was measured.
Results: Results showed that blueberry/bilberry juice lowered DNA damage in in vitro study. Supplementation of a single dose of blueberry/bilberry juice was able to decrease DNA damage by

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19% while 40g blueberry fruit lowered DNA damage by 28% within 2 hours.
Conclusion: Blueberry/bilberry juice or blueberry fruit were able to lower cellular DNA damage in white blood cell within 2 hours.

Keywords: Antioxidant; comet assay; DNA damage; bilberry; blueberry; juice.

1. INTRODUCTION

DNA damage plays an important role in the development of many chronic disease, aging and cancers [1,2]. Reactive oxygen species exist normally in living cells since they are formed in metabolic or other biochemical reactions such as mitochondrial respiration and cellular defense mechanism. Excessive ROS can be a key mediator of damage to cell structures, including DNA damage by reacting with nucleotides in DNA [3]. DNA damage induced by ROS includes base modification, base transversion, oxidization of purines and pyrimidines, single strand and double strand DNA breaks. Although there are effective DNA-repair enzyme systems [4], some DNA damage may escape from the repair process and cause permanent change. Reactive Oxygen Species [ROS) scavenger such as anti-oxidative agents are targets for preventing DNA damage.

Fruits and vegetables are good sources of nature antioxidant [5]. Blueberry and bilberry are perennial flowering plants with dark purple fruits of *Vaccinium spp* in the family Ericaceae. It has a widespread genus with over 200 species. Berries of Ericaceae family contain vitamins, organic acids, glycosides and anthocyanins. It is a good natural source of antioxidants. The major species of Vaccinium berries are the North-American highbush (*Vaccinium corymbosum*) and lowbrush (*Vaccinium angustifolium)* blueberries and the native European blueberry, also known as bilberry (*Vaccinium myrtillus*). There are cultivated and wild type of Vaccinium berries. The commonly available in commercial market named as blueberry, which is usually refers to cultivated type (*V. corymbosum* and *V. angustifolium)*. The wild, non-cultivated type is bilberry [6]. Bilberry (*V. myrtillus*) is one of the important species in Northern Europe. It is a small shrub and grows wildly in the Northern European forest. The fruit of bilberry is in single form which is different from cluster form of blueberry's. Many studies have been carried out to investigate various compositions of berries and their antioxidant capacity [7]. Previous studies have shown that blueberry contains high level of anthocyanins and possesses the highest antioxidant capacity among common fruits and vegetables [8]. High diversity of phenolic compounds including flavonols such as quercetin, catechins, phenolic acids, tannins, ellagitannins and anthocyanins are found in bilberry. Anthocyanins are considered as major phytochemical mix in fruit and are responsible for the orange, red, blue, pink and purple color of fruits, vegetables, grains, flower and other plants [9]. Although phytochemicals are not essential for normal cell function, their bioactivity may have impact on health and disease prevention [10]. Anthocyanin belongs to flavonoids class which is under a larger class called polyphenols. Anthocyanin is made with anthocyanidin which acts as core structure and a sugar moiety. Anthocyanins are thought to be potential antioxidants because of their natural electron deficiency structure which makes them particularly reactive towards oxygen radicals [9]. It has been demonstrated that the extract of bilberry contains polyphenol and anthocyanin-rich fraction exhibit high antioxidant activities [11]. It is of interest to explore berry to be a nutraceutical in the prevention of aging and chronic diseases.

The major contributor to cardiovascular diseases is the uptake of oxidized low density lipoprotein cholesterol by macrophages/monocytes and deposited to the blood vessels. The oxidative and inflammatory process damage vascular endothelium [12]. Research on the anti-inflammatory and antioxidant effects of berries have been conducted to investigate the protection on different diseases related to inflammation and oxidative stress. Bilberry protects cardiac tissue against oxidative toxicity in animal model [6]. Oxidative stress is one of the pathogenic factors that cause hepatocyte damage through lipid peroxidation and protein alkylation in which can lead to chronic liver injury and hepatic fibrosis [13,14]. It has been shown that blueberry juice supplements attenuated liver fibrosis in animal model [15]. Oxidative stress is also involved in progression of hypertension and hypertension-induced renal injury [16]. Bilberry and blueberry extracts are available commercially as supplement for promoting vision health.

In this study, we investigated the potential genoprotective effect of bilberry/blueberry juice and blueberry fruit body on human white blood cell DNA against exogenous oxidative stress within a short period of time. Comet assay was used to assess to change of nuclear DNA damage of leucocytes if any.

2. MATERIALS AND METHODS

All the chemicals used in this study were in high purity grade. For comet assay: Histopaque 1077, disodium ethylenediaminetetracetic acid (EDTA) dehydrate, dimethyl sulfoxide (DMSO), Tris[hydroymethyl]aminomethane (Tris), type VII low melting point (LMP) agarose, Triton X-100 and ethidium bromide were from Sigma (St. Louis, MO, USA); standard agarose was from Amresco (Solon, OH, USA); hydrogen peroxide solution (30% v/v) was from International Laboratory (South San Francisco, CA, USA) , sodium chloride was from BDH Chemicals Ltd (Poole, Dorset, UK) and sodium hydroxide from Riedel-de Haen (Seelze, Germany); phosphate buffered saline pH 7.2 (PBS) powder was from Euroimmun (Luebeck, Germany).

For the preparation of Giemsa stain, methanol was from RCI Labscan Ltd Bangkok, Thailand); PBS tablet (pH 6.8) and Giemsa were from Merck (Darmstadt, Germany).

2.1 Blood Sample Collection

Ethical approval was obtained from the Macau Society for the Study of Women's Health. The subjects recruited were absence from clinical disease and not taking of any medication and health supplements. All subjects provided written consent and arrived in the morning of testing with overnight fasting. Venous blood samples were collected in EDTA blood tube and kept at $4^{o}C$ until processing on the same day.

2.2 Blueberry/Bilberry Juice

Commercial available form of glass bottle blueberry/bilberry juice (Chabaa, 100% bilberry mixed blueberry juice, manufactured by Chabaa Bangkok Co. Ltd) was used in this study. Ingredients shown were 50% bilberry puree, 49.97% blueberry juice, and 0.03% vitamin C; and 100 mL juice contained 16 g of sugar, 16 mg of vitamin C, 279 µg of vitamin A, 10 mg of calcium and 22 mg of sodium.

2.3 In vitro Study of Blueberry/Bilberry Juice on Lymphocytes

Lymphocytes were isolated from venous blood samples as described previously [17]. A serial dilution of blueberry/bilberry juice was prepared using PBS as diluents and PBS alone was used as control. The blueberry/bilberry juice was tested at concentration between 1 x 10^{-2} to 1 x 10^{-6} (v/v) where the original bottle of juice was considered as neat. One mL of each juice dilutions or PBS was added to the 1.5mL microtube containing retrieved lymphocytes and cell suspensions were mixed gently. The cell suspensions were incubated at 37°C for 30 min and were spun at 1500 rpm for 5 min at room temperature after incubation. Supernatant was discarded and the cells were washed with 1 mL of cold PBS and the washing step was repeated once. After the pre-incubation, the washed lymphocytes were challenged with 1 mL of 50 µM freshly prepared hydrogen peroxide on ice for 5 min where oxidative stress was induced. The microtubes were spun at 1500 rpm, 5 min and the cell pellets were washed with 1 mL of cold PBS and the washing step repeated once. Supernatants were discarded. The cells were then subjected to comet assay.

2.4 Supplementation Study of Blueberry/Bilberry Juice on Lymphocytes

Nine healthy subjects (five males, four females) with the age of 35 ± 12 years old (mean ± SD) were recruited. All subjects consumed a bottle of blueberry/bilberry juice (200 mL) or control drink (200 mL) on one day and had the other drink on the other day.

Blood samples were taken before and 2 hours after consuming the drinks. They remained fasting during the 2-hour period. In other words, a total of 4 blood samples were taken from each subject in 2 separate occasions which at least 1 week apart.

Control drink contained 32 mg of vitamin C and 32 g of glucose in 200 mL water. Commercial available form of vitamin C tablets (Redoxon, double action effervescent tablet vitamin C plus zinc, PT Bayer, Indonesia) and glucose (Glucolin, The British Dispensary (L.P.), Thailand) were used. Each vitamin C tablet contained 1000 mg of vitamin C and 10 mg of zinc and 100 g of Glucolin contained 99.4 g

dextrose monohydrate, 60 mg calcium glycerophosphate and 10 µg vitamin D3.

2.5 Comet Assay and Slides Reading

The standard comet assay was performed as described previously with minor modification [17]. Alkaline treatment was conducted with two changes (2 x 10 min) of electrophoresis solution. The electrophoresis was run at constant voltage at 25V. Slides were examined under a fluorescence microscope (Microphot-Fx with Epi-fluorescence attachment, excitation filter G: Ex 510-560, Nikon, Tokyo, Japan) and 100 cells were scored per gel.

2.6 Supplementation Study of Blueberry Fruit Supplementation on Leucocytes

Nine healthy subjects (five males, four females) with the age of 40 ± 8 years old (mean ± SD) participated. Subjects ingested 40 g of blueberry from local supermarket with 100 mL water after overnight fasting. While only 100 mL of water was taken at the other occasion as control. Test and control trials were at least one week apart.

2.7 Comet Assay and Slides Reading

In this part of study, whole blood samples instead of isolated lymphocytes were used in comet assay. Pre- and post-treatment of blueberry and water (control) whole blood samples were challenged by UV light source (312 nm). Both challenged and unchallenged cells of whole blood were then subjected to comet assay.

Four µL of whole blood was mixed with 85 µL of low-melting-point agarose 1% (w/v) and pipetted the mixture onto the microscopic slide pre-coated with 1% standard agarose. The mixture was covered with a cover slip and chilled to solidify. Cover slip was then removed and slides were irradiated by UV light source at 18 cm height. Slides were transferred in a staining jar containing lysis solution (0.1 M Na_2EDTA, 2.5 M NaCl, 10mM Tris, 10% DMSO and pH 10) and kept at 4^{o}C for 1 hour in the dark. Afterwards, slides were put into a staining jar containing electrophoresis solution (pH>13) at 4ºC for 10 minutes twice to allow DNA unwinding and alkaline-liable sites expression. The slides were then put electrophoresis tank and electrophoresis was run at 25V constant voltage, 0.3A current for 30 minutes. The slides were air dried or dried at 37ºC for 30 minutes and fixed in 100% methanol for 10 minutes. Working Giemsa stain solution was prepared by diluting equal volume of commercially Giemsa stain with PBS (pH 6.8) according to manufacturer's recommendations and filtered with Whatman No. 1 filter paper. The slides were stained for 30 minutes with a working solution of Giemsa stain in the staining jar. Afterwards, slides were washed with PBS (pH 6.8) twice in other staining jar for 1 min each and air dried. Dried slides were then examined under light microscope.

2.8 DNA Damage Scoring

The level of DNA damage was represented in the comet size and tail length [18] and the degrees of DNA damage were graded from 0 to 4 where grade 0 represented no DNA damage and grade 4 represented most severe DNA damage. The total DNA damage score of each slide were calculated as follow:

Total DNA damage score = (0xG0) + (1xG1) + (2xG2) + (3xG3) + (4xG4)

Where G0 was the number of cell with no DNA damage and progressively increase to G4 which was most severely damaged. The sum of G0, G1, G2, G3, and G4 is equal to 100. The higher the comet score, the greater the DNA damage found in the leucocytes/lymphocytes.

2.9 Statistical Analysis

Prism 5.0 for Window (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis. For in vitro study, Kruskal-Wallis test followed by Dunn's Multiple comparison test was used to compare the geno protective effect (based on the DNA damage score) of different concentrations of blueberry juice with PBS as control. For in vivo study, Wilcoxon signed rank test was used to evaluate the differences in DNA damage before and after taking the blueberry drinks or control drinks. Results were considered as significantly different at P value < .05. Results were presented as the mean +SD of the total DNA damage score of comet assay.

3. RESULTS AND DISCUSSION

The mean comet score of H_2O_2 stressed lymphocytes (n=4; without pre-treatment of blueberry juice) was 313 in the in vitro study. Results of lymphocytic DNA damage with or without juice pre-treatment at different concentrations were shown in Fig. 1. It showed

that the diluted blueberry/bilberry juice at concentrations of 10^{-3}, 10^{-4} and 10^{-5} significantly lowered then comet scores hydrogen peroxide stressed cells.

In the in vivo supplementation study of bilberry/blueberry juice, the mean of comet scores (n=9) before and after taking control drinks with induced oxidative stress were 295 and 296 respectively which demonstrated no significant changed (Fig. 2; P>.05; Wilcoxon signed rank test). For the blueberry/bilberry juice trial, the mean of comet scores decreased from 319 to 259 after 2 hours of supplementation which was a 19% drop (P=.039). On the other hand, there was no statistical significant change in comet score of basal DNA damage (no H_2O_2 stress) and in control experiment (P>.05). For the supplementation of fresh blueberry fruit study, there was no statistically significant difference between pre and post ingestion blueberry basal DNA damage, i.e. in the absence of UV light stress (Fig. 3; P>.05; Wilcoxon signed rank test). Again, no significant change in DNA damage in control experiment in both UV irradiated and non-irradiated samples (P>.05). Nevertheless, there was a 28% (mean 254 vs 184) reduction in comet score in UV irradiated sample after 2 hour of blueberry intake (P=.004).

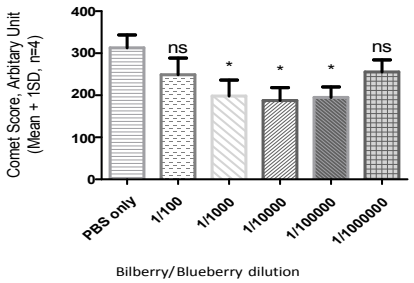

Fig. 1. Mean total comet score with standard derivation (n=4) in the presence of bilberry/blueberry juice from 10^{-2} to 10^{-6} of original juice in comet assay

*Where * = p<.05 compared to positive control, Dunn's multiple comparison test*

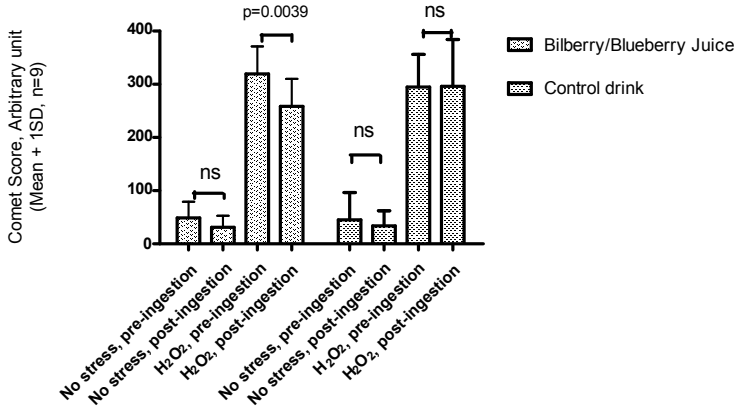

Fig. 2. Effects of pre- and post-ingestion of 200 mL bilberry/blueberry juice and control drinks on lymphocytes with and without 50 µM H_2O_2 incubation measured by comet assay

Data are expressed as mean+SD of comet score, Wilcoxon matched-paired signed rank test

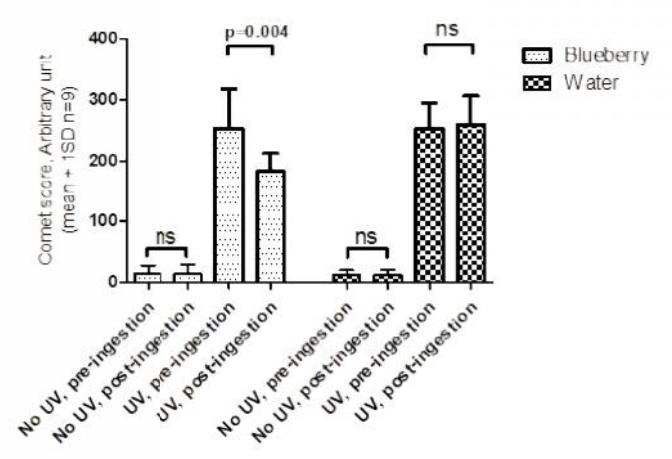

Fig. 3. Effects of pre- and post-ingestion of 40 g fresh blueberry and 100 mL water on leucocytes with and without UV irradiation measured by comet assay

Data are expressed as mean+SD of comet score, Wilcoxon matched-paired signed rank test

Our results showed that blueberry/bilberry juice exerted a genoprotective effect on human lymphocytes, both in vitro and in vivo, against induced exogenous oxidative stress. Blueberry/bilberry juice pre-treatment showed a protective effect on lymphocytic DNA in vitro study from the concentration of 10^{-3} to 10^{-5}. This was the common U-shaped dose-effect demonstrated in other in vitro experiment [18]. Since in vivo activity could not be solely supported by in vitro data, a normal dose of commercial available juice was consumed and the effect on peripheral blood cell DNA was investigated. In the in vivo study, lymphocytic DNA damage induced by oxidative stress was diminished (19% reduction) with ingestion of blueberry/bilberry juice but not in control drinks.

Previous studies have demonstrated 4-week or longer supplementation of blueberry juice or bilberry extract are able to lower oxidative DNA damage in human [19]. Our results showed both juice and fresh fruit of blueberry/bilberry contributed to the protection on peripheral leucocytes in a very short time frame. This supported long term supplementation data since acute effect was free from dietary interference as the subjects remained fasting over the 2-hour period. Those active ingredients were absorbed by gastrointestinal tract, reached cellular level and exerted the effect on nuclear DNA within 2 hours. Although the identification of active ingredients of blueberry/bilberry was not the scope of this study, we could exclude vitamin C and sugar as the candidate of DNA protective agent as no effect was shown in control drinks.

4. CONCLUSION

In conclusion, we showed that blueberry supplementation conferred fast protection on human leucocyte DNA in circulation and the protective effect was not mediated by vitamin C.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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