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Antigenotoxic properties of anthocyanin-enriched fraction of strawberry (cv. Romina) extract on DNA damage induced by H_2O_2 in human peripheral blood leukocytes

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Abstract

Strawberry fruit *Fragaria* × *ananassa* Duchesne, Rosaceae (cv. Romina), rich in anthocyanin polyphenols, has been demonstrated to have favorable effects on health due to its antioxidant properties. The present study investigated the antigenotoxic potential of anthocyanin-enriched fraction of Romina strawberry methanolic extract (ACY) against DNA damage on human peripheral blood leukocytes, induced by hydrogen peroxide *in vitro*. Five concentrations of the ACY extract were used in all experiments (2.5, 5, 10, 15, 20 μg/ml). The results of the alkaline comet assay showed no genotoxic effect of the ACY. After the pre-exposure of the leukocytes to the ACY, and subsequent incubation with H_2O_2, a decreased number of DNA damaged cells was recorded in all the tested concentrations, compared to controls. In the post-treatment, there was a concentration-dependent DNA damage reduction, while a statistically significant decrease was achieved with 15 and 20 μg/ml concentrations. The results indicate that ACY is efficient in oxidative DNA damage reduction, and it is more potent as a post-applicative than a protective agent.

***Key words**:* DNA damage, comet assay, strawberry (cv. Romina), anthocyanins, leukocytes

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Introduction

Oxidative stress has been implicated in numerous cellular mechanisms that underlie the development of different human diseases. During the various physiological processes in the body, reactive oxygen species are produced, and the endogenous antioxidant system represents an effective kind of defense. Oxidative damages of cellular macromolecules, lipids, proteins and nucleic acids are a consequence of the disturbance of balance between the generating of free radicals in the cell and the mechanisms of antioxidant defense. On the other hand, it is well known that a diet rich in fresh fruit and vegetables, as natural, exogenous antioxidants, has extremely positive effects on human health. It can be a form of prevention of various diseases such as cancer, cardiovascular diseases, type 2 diabetes mellitus, inflammatory and age-related diseases (1,2,3). Many purple and red-colored fruits and vegetables contain beneficial natural pigments, which are known as strong antioxidants and protectors of cells from oxidative damages (4,5). Berry fruits, especially members of several families, such as Rosaceae (strawberry, raspberry, blackberry), and Ericaceae (blueberry, cranberry), are among the best dietary sources of bioactive compounds. Berries contain mainly phenolic compounds (phenolic acids, flavonoids, such as anthocyanins and flavonols, and tannins) and ascorbic acid (6). There is thus an increased interest in explaining the role of specific bioactive compounds from berries, as natural antioxidants, and their mechanisms of action in cells. Anthocyanins are one of the largest and most important groups of water soluble plant pigments (7) and their concentration varies significantly among plant species, even among species of the same genus (8). The anthocyanin composition has previously been analyzed in strawberry fruit from many different cultivars (9). Romina fruit cultivar is recognized for its high content of anthocyanins and an evaluated antioxidant capacity (10). Romina strawberry also has a combination of a high content of vitamin C, folic acid and flavonols, so it has valuable nutraceutical and therapeutic potentials (11,12). The present study aimed to investigate the antigenotoxic potency of an anthocyanin-enriched fraction of strawberry, *Fragaria* × *ananassa* Duchesne, Rosaceae (cv. Romina) methanolic extract (ACY) on DNA damage induced by H_2O_2 *in vitro* in human peripheral blood leucocytes, using the comet assay.

Material and Methods

Subjects

Peripheral blood samples were collected from six healthy volunteers, aged between 25 and 30. Whole blood samples were used for analysis, since any additional manipulation of cells, like mechanical isolation, may result in increased DNA damage and substantial variability in results (13). The participants did not use medications, alcohol, cigarettes, or any food supplements for two months before the study. They gave their consent under the ethical standards of the Ethics Committee for Clinical Trials of the Faculty of Pharmacy, University of Belgrade.

Study design

The fruit of *Fragaria* × *ananassa* (cv. Romina) strawberry was collected in experimental fields of the Agricultural Faculty of the Polytechnic University of Marche Ancona, Italy. The anthocyanin fraction from ACY was obtained as previously described by Alvarez-Suarez et al. (14). The five concentrations of ACY: 2.5, 5, 10, 15, 20 μg/ml were prepared in phosphate buffered saline (PBS) and used for the experiments. The first experiment was the evaluation of the possible genotoxic effect; further, the antigenotoxic properties of ACY were tested. In all experiments, the comet assay was used.

To evaluate the potential genotoxic properties of ACY, cells were treated with the five above-mentioned concentrations of the extract for 30 min at 37°C. For negative control, cells were treated with solvent (PBS), while a positive control treatment was carried out with 50 μM hydrogen peroxide (H_2O_2), the smallest concentration that produced a significant level of DNA damage in treated cells vs. untreated controls.

In the antigenotoxic assessment of ACY, cells were incubated with the extract before their exposure to H_2O_2, in a pre-treatment experiment. In a post-treatment experiment, cells were treated with H_2O_2 before their exposure to ACY. In all the experiments, the duration of treatment with ACY was 30 min at 37°C, while the treatment with H_2O_2 was conducted for 15 min at 4°C.

The single cell gel electrophoresis assay (comet assay)

The comet assay was performed essentially as described by Singh et al. (14). From each sample, 6 μL of whole blood cells were suspended in 0.67% low-melting-point agarose (LMP) (Sigma-Aldrich, St. Louis, MO) and pipetted onto microscope slides previously coated with a 1% layer of normal-melting-point agarose (Sigma-Aldrich, St. Louis, MO), spread by a coverslip and left in a freezer for 5 min to solidify. The cell suspensions on slides were treated with tested concentrations of ACY and H_2O_2 as described above. Moreover, all the slides were covered with a third layer of 0.5% LMP agarose and once again left to solidify in the freezer for 5 min. After the removal of coverslips, the slides were placed in a cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X 100 and 10% dimethylsulfoxide, pH 10) at 4°C overnight. After 24 h, the slides were removed from lysing solution, placed in a horizontal gel electrophoresis tank (CHU2 manufacturer, connected to a Power Supplier EPS 601), and flooded with cold electrophoresis buffer (10 M NaOH, 200 mM EDTA), allowing the DNA to denature for 30 min before electrophoresis. The electrophoresis was conducted in a dark room at 25 V and 300 mA for 30 min. The slides were stained with ethidium bromide, performed as described by Singh et al., (15). The comets were analyzed using Olympus BX50 fluorescent microscope at 100 × magnification. The number of comets was used as a parameter of DNA migration. Depending on the extent of DNA damage, nuclei of cells, resembling comets, were graded into 5 classes, as described by Anderson et al. (16): class A- undamaged cells with no comet tail (< 5% damaged DNA); class B- low-level damage (5%–20%); class C- medium-level damage (20%–40%); class D- high level damage (40%–95%); and class E- destruction (> 95%). DNA migration was

characterized as DNA damage greater than 5% (B + C + D + E comet classes). The mean value was calculated for 100 comets per experimental condition. Necrotic and apoptotic cells were excluded from the analyses.

Statistical analysis

The data were expressed as mean values with a standard deviation (SD) of six experiments using GraphPad Prism (5.0) statistical software (GraphPad Software Inc, La Jolla, CA, USA). Statistical analyses of variance (one-way ANOVA) were performed with Tukey's post hoc test for comparison of different treatments versus the respective controls, and p-values of less than 0.05 were considered to be statistically significant.

Results

The genotoxic and antigenotoxic potential of anthocyanin fraction from ACY in human peripheral blood cells *via* alkaline comet assay was tested. The potential of the ACY to induce DNA damage was assessed at five concentrations (2.5, 5, 10, 15, 20 µg/ml). The ACY showed no genotoxic effect, since there was no significant difference in DNA damage levels between control cells treated with PBS and ACY treated cells in all concentrations used (**Figure 1)**.

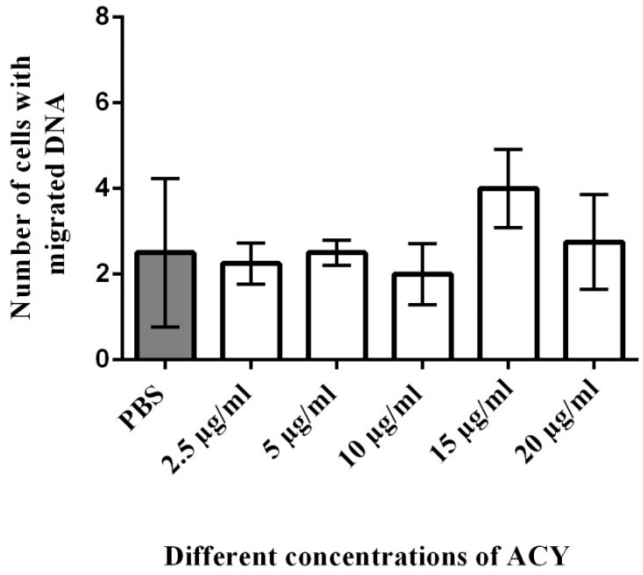

Figure 1. Number of peripheral blood leukocytes with migrated DNA induced by five different concentrations of ACY, evaluated by comet assay.

Slika 1. Broj leukocita periferne krvi sa migriranom DNK izazvanom sa pet različitih koncentracija ACY, procenjen komet testom.

The same ACY concentrations were used for antigenotoxic effect against H_2O_2 induced DNA damaging effects in two procedures: pre- and post-treatment. After the pre-exposure of the leukocytes to the ACY for 30 minutes and subsequent incubation of the same cells with H_2O_2, a decrease in the number of DNA damaged cells could be observed in all the tested concentrations compared to controls. However, there was no statistically significant difference between ACY treated cells compared to positive controls exposed only to H_2O_2 (**Figure 2)**.

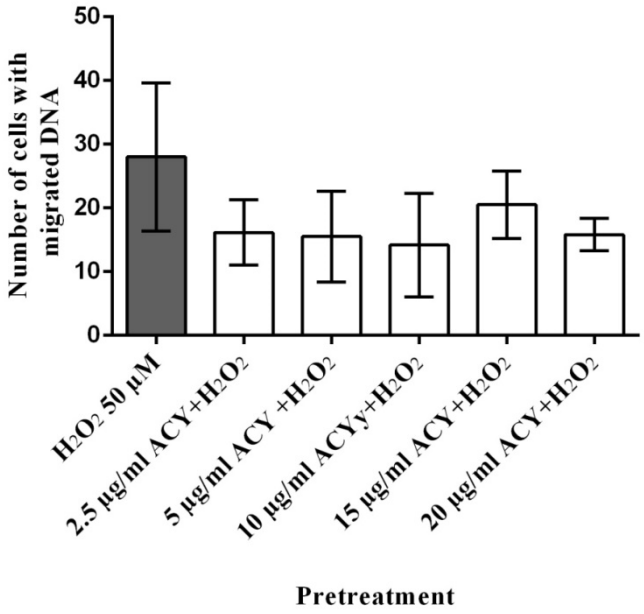

Figure 2. Number of peripheral blood leukocytes with migrated DNA in pretreatment experiment. Five different concentrations of the ACY were added before H_2O_2 cells treatment.

Slika 2. Broj leukocita periferne krvi sa migriranom DNK u eksperimentu predtretmana. Pet različitih koncentracija ACY je dodato pre tretmana ćelija sa H_2O_2.

In the post-treatment, the ACY exhibited concentration-dependent antigenotoxic potential by attenuating H_2O_2 induced-DNA damage at all concentrations tested, while a statistically significant DNA damage reduction was achieved with the two highest concentrations used: 15 and 20 µg/ml. A decrease in the number of DNA damaged cells was observed 30 minutes after the application of the ACY in post-treatment experiment (**Figure 3).** The presented results indicate that ACY is efficient in oxidative DNA damage reduction, and it is more potent as a post-applicative than protective agent.

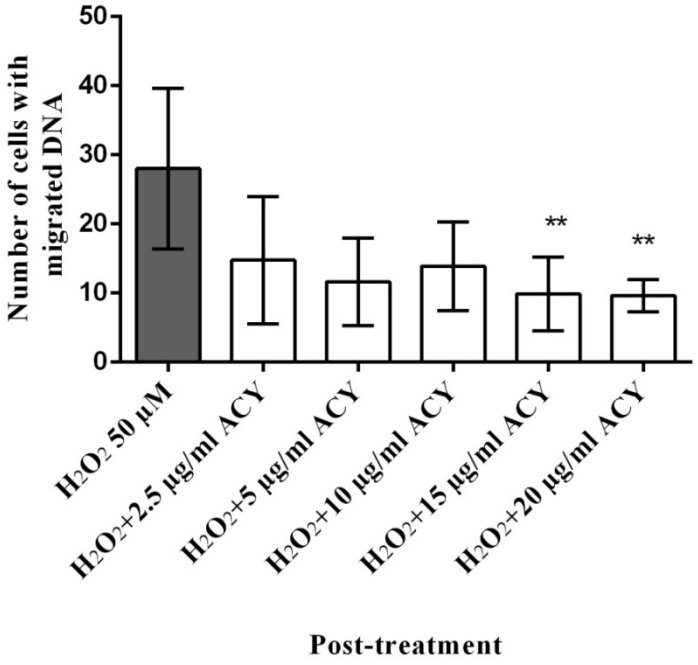

Figure 3. **Number of peripheral blood leukocytes with migrated DNA in post-treatment experiment. Five different concentrations of the ACY were added after H_2O_2 cells treatment. $p< 0.05$ was considered to be statistically significant.**

Slika 3. **Broj leukocita periferne krvi sa migriranom DNK u eksperimentu posttretmana. Pet različitih koncentracija ACY je dodato nakon tretmana ćelija sa H_2O_2. $p <0.05$ je smatrano statistički značajnim.**

Discussion

Genotoxicity is the ability of different agents to produce damage to DNA. The compounds that reduce the DNA damage are called antigenotoxic agents. Research of different fruit and vegetables, as well as medicinal plants and their antioxidative and antigenotoxic properties, is a great challenge. Comet test is a sensitive, rapid, and relatively simple assay, able to evaluate genotoxic and antigenotoxic effects in different cell types.

In the present study, for the first time, the antigenotoxic potential of antocyanin-enriched fraction of the Romina strawberry extract as a natural antioxidant was tested and a high degree of protection of DNA molecules from oxidative damage induced by hydrogen peroxide was shown. Taking into account the results of previous studies on this extract, the results of the present work confirmed its beneficial antioxidative effects at the cellular level. Namely, previous results obtained on Romina strawberry whole methanolic extract and its anthocyanin-enriched fraction, showed that the anthocyanin family was

definitely the major active component of both fractions, representing 86-94% of the total phenolic compound identified by HPLC (17). The results of the phytochemical and antioxidant characterization of anthocyanin-enriched fraction also revealed higher values of total polyphenols and flavonoids content, total antioxidant capacity and ferric-reducing antioxidant power (FRAP), compared to the whole methanolic Romina extract (18). The high level of antioxidant effect can also be attributed to the fact that Romina strawberry variety extract has also been shown to contain high levels of vitamin C (38,5mg/100g fresh weight) (19). Results of other human trials demonstrated that supplementation with 320 mg/day of purified anthocyanins from bilberries and black currants for 12 weeks decreased LDL-cholesterol (20,21), and that ingestion of 500 mg/day of elderberry extract rich in anthocyanins over the same period resulted in lower cardiovascular risk in postmenopausal women (21). Antioxidative properties of anthocyanins arise from their high reactivity as hydrogen or electron donors, from the ability of the polyphenol-derived radicals to stabilize and delocalize the unpaired electron, and from their ability to chelate transition metal ions (termination of the Fenton reaction) (22). A study about mechanisms of action of natural antioxidants *in vivo* in health and disease is needed (19). The results obtained in this study showed no genotoxic potential of ACY in any of the tested concentrations. Comparing the obtained results of two differently designed experiments, pre-treatment and post-treatment with ACY, concerning the time of induction of oxidative damage with hydrogen peroxide, before or after addition of ACY, we can conclude that the results obtained in post-treatment had a more pronounced effect. The two highest concentrations of ACY were the most effective against DNA damage induced in whole blood cells by hydrogen peroxide. It means that ACY acts better on interventional than on prevention level.

Conclusion

Anthocyanin-enriched fraction of the Romina strawberry variety extract (ACY), as a natural antioxidant, is effective against *in vitro* induced oxidative DNA damage on human peripheral leukocytes and it is more potent as a post-applicative agent.

Acknowledgement

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Antigenotoksična svojstva ekstrakta jagode obogaćenog antocijaninom (cv. Romina) na oštećenja DNK izazvana H_2O_2 u leukocitima periferne krvi čoveka

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Kratak sadržaj

Plodovi jagode *Fragaria* × *ananassa* Duchesne, Rosaceae (cv. Romina), bogati antocijaninima, pokazali su povoljne efekte na zdravlje zahvaljujući svojim antioksidativnim svojstvima. U ovoj studiji ispitivan je antigenotoksični potencijal frakcije metanolnog ekstrakta jagode Romina koji je obogaćen antocijaninom (ACY) na oštećenja DNK na leukocitima humane periferne krvi, izazvanih vodonik-peroksidom *in vitro*. U svim eksperimentima je korišćeno i testirano pet koncentracija ekstrakta ACY (2.5, 5, 10, 15, 20 μg /ml). Rezultati ispitivanja alkalnim komet testom nisu pokazali genotoksični efekat ACY. Nakon izloženosti leukocita ACY i potom njihovog tretmana i inkubacije sa H_2O_2, smanjen je broj ćelija sa oštećenom DNK, u svim ispitivanim koncentracijama u poređenju sa kontrolama. U post-tretmanu, došlo je do smanjenja oštećenja DNK u zavisnosti od koncentracije ekstrakta, dok je statistički značajno smanjenje postignuto sa koncentracijama od 15 i 20 μg/ml. Rezultati ukazuju da je ACY efikasan u smanjenju oksidativnih oštećenja DNK i da pokazuje snažniji efekat kao postaplikativno nego kao zaštitno sredstvo.

Ključne reči: oštećenje DNK, komet test, jagoda (cv. Romina), antocijanini, leukociti