Effects of Bilberry on Deoxyribonucleic Acid Damage and Oxidant-Antioxidant Balance in the Lens, Induced by Ultraviolet Radiation

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Abstract

Background: This study investigated the possible protective effects of bilberry extract after exposing rat eyes to ultraviolet-B (UV-B) radiation.

Methods: Four groups of rats were included in this study, each consisting of 10 Wistar rats. The first group acted as the control, and the second group was exposed to UV-B, 5 KJ/m² (λm = 300 nm), for 15 minutes. The third group was orally administered bilberry extract (160 mg twice per day) for two weeks before exposure to the UV-B, while the fourth group was administered the same dose of bilberry extract for two weeks before euthanisation. A comet assay was used to examine DNA damage, while the malondialdehyde (MDA) level and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT), activities were measured in the lens.

Results: After exposing the rats to UV-B radiation, the mean percentage tail DNA and tail moment were significantly increased (P < 0.001) when compared to the control group. In the same context, the lens tissue MDA levels and CAT activity were also significantly increased (P < 0.001). The supplementation of the bilberry extract was found to improve the comet assay parameters and enzymatic activity of the rat lens tissue.

Conclusion: The administration of bilberry led to a decrease in the oxidative stress in the lens tissues and DNA damage induced by UV-B radiation in the lenses of Wistar rats.

Keywords: ultraviolet, rat, lens, comet assay, bilberry, malondialdehyde

Introduction

Ultraviolet (UV) irradiation represents a significant environmental hazard that can cause acute and chronic inflammatory changes in the cornea, lens, and retina of the eye. Sources of UV radiation are not merely from electric welding and tanning lamps, but also from sunny days on the sea, or in snowy mountains when eyes are left unprotected. In recent decades, the risk of acute photochemically-induced ocular damage has increased due to stratospheric ozone depletion (1). Although the energy is much less than that of ionizing radiation, ophthalmologists find that it is necessary to study ways to prevent the damage caused by UV radiation due to its association with clinical ocular diseases. Results from several epidemiological studies suggest that individuals with high exposure to UV radiation have an increased risk of cataracts in life (2,3). A particularly strong association has been observed between cataract development and exposure to radiation wavelengths of 290–320 nm (designated UV-B) (4). UV-B radiation is thought to contribute to cataract formation by directly damaging DNA (5,6), producing reactive oxygen species (ROS) (7,8), and generating cytotoxic products from actively translating ribosomes (9). In addition to UV-B, hydrogen peroxide is chronically present in the aqueous environment surrounding the anterior lens and may contribute to cataract development (10–12). Because many of the enzymatic cofactors and chemical constituents necessary for antioxidant activity are obtained only through the diet, adequate nutrition is likely to be important in preventing ROS-induced oxidative damage and maintaining the overall health of the eye (13–15). Some epidemiological and experimental studies suggest that the increased consumption of dietary antioxidants such as vitamin C, vitamin E, zinc, and carotenoids may reduce the incidence or progression of ocular diseases (16–19).

Bilberries, rich in polyphenols which belong to the flavonoid family (20,21), are generally recognised as low energy foods, containing little or no saturated fat, cholesterol, and sodium, and a good source of dietary fibre and the antioxidant vitamins E and C. The anthocyanins are the most abundant flavonoids present in bilberries, and have been shown to be potential antioxidants (22). These compounds can penetrate the nervous system...
system even after short-term feeding (23), and they have been reported to be able to reduce age-associated oxidative stress and the related cognitive decline (24). Furthermore, anthocyanins display the potential to ameliorate cardiovascular diseases (25), and obesity-related pathological changes in rodent models (26–29), but findings using whole bilberries as anthocyanin sources have not demonstrated similar efficacy (30–32). Due to their antioxidative and antiinflammatory properties (33,34), anthocyanins and their extracts may have potential benefits in eye health (35–37), neural functions (38), and age-related retinal stress (39).

UV-B-induced ocular changes are well known and well documented in the literature. Wu et al. found that UV irradiation has a dose-dependent effect on DNA single strand breaks of lens epithelial cells (40). Another study stated that UV radiation caused the decreased activity of SOD, GSH-Px, and CAT, suggesting that elevated oxidative stress may lead to cell death, and may be responsible for the decrease in the cell density in all three zones of the lens epithelium (41). The functional role of SOD in apoptosis was examined in cultured human lens epithelial cells and it was found that cells with higher enzyme levels were more resistant to the cytotoxic effects of UV-B radiation (42).

This study was aimed at evaluating the in vivo protective effects of bilberry extract on the genetic material of the epithelial cells, and measuring its impact on the enzymatic activity of tissue in the lenses of Wistar rats.

Materials and Methods

All chemicals were purchased from Sigma Chemical Co (USA). The rats were randomly selected from the animal house facility at the Research Institute of Ophthalmology in Giza, Egypt. The experimental protocol was approved by the local ethical committee that applies ARVO (The Association for Research in Vision and Ophthalmology) statements for using animals in ophthalmic and vision research. A high pressure mercury lamp (200 W, Osram, Germany) was the radiation source, and a spherical reflector was placed behind the lamp to collect and concentrate the radiation. In the forward direction, the radiation passed through a water filter to absorb the infrared radiation, followed by a 300 nm interference filter (43). The mercury lamp was calibrated at the National Institute of Standards. Fifteen minutes before exposure, the rats were anesthetised with a mixture of Ketalar (ketamine, 90 mg/kg) and Rompun (xylazine, 10 mg/kg) by intraperitoneal injection. Mydriacil (tropicamide), 10 mg/mL, was instilled in both eyes of each rat to induce mydriasis.

Fresh bilberry was obtained from South Sinai, Egypt. The fruits were washed in water and dried. Then, they were electrically crushed in a blender with 100 mL of 70% methanol. The resultant mixture was kept at 4 °C for 24 hours. After filtration, the clear supernatant was freeze-dried and the resultant dry residue was weighed and solubilised in phosphate buffer saline (PBS) buffer (pH 7.4). The total anthocyanins were estimated using the pH differential absorbance method (44). Absorbance was measured in a spectrophotometer (Shimadzu UV-visible Recording 240 Graphical, Japan). The total anthocyanins were found to be 320 mg/100 gm (SD 35). For oral administration, the final concentration was adjusted to 160 mg/mL to be given twice a day.

Forty inbred Wistar rats of both sexes, weighing 200–250 g, were divided into four groups. Group one acted as the control group. In group two, the rats’ eyes were exposed to UV-B, 5 KJ/m$^2$ ($\lambda_{nm} = 300$ nm), for 15 minutes (UV group). The third group was administered bilberry in oral doses of 160 mg of extract twice per day for two weeks before exposure to the UV light (UV + bilberry group). Group 4 was administered bilberry in oral doses of 160 mg of extract twice per day for two weeks before euthanisation (bilberry group).

After euthanisation and decapitation, the whole eye was removed from the rat and placed corneal side down on sterile gauze, and held in place by forceps. Two incisions, at right angles to each other, were made across the surface where the optic nerve enters the eye, and the sclera was pulled back to expose the lens. The iris and ciliary muscles, which are attached on the equatorial plane of the lens, were gently teased away with forceps as the lens was removed from the eye. The orientation of the lens was maintained using the remnants of the pigmented equatorial plane where the iris was attached to the lens. The lens epithelial cells are located on the hemispherical surface oriented toward the cornea.

Single cell gel electrophoresis (comet assay)

The comet assay is a simple and inexpensive method for the detection of different types of DNA damage, including single- and double-strand breaks, DNA adducts, cross-links, and alkaline-labile sites, in a low throughput format. For the assay, the lens epithelial cells
of the control and experimental groups were homogenised in a chilled homogeniser buffer, pH 7.5, containing 75 mM NaCl and 24 mM Na₂ EDTA (ethylenediaminetetraacetic acid) to obtain a 10% tissue solution. A potter-type homogeniser was used and samples were kept on ice during and after homogenisation. 6 μL of lens homogenate was suspended on a 0.5% low-melting agarose and sandwiched between a bottom layer of 0.6% normal-melting agarose and a top layer of 0.5% low-melting agarose on fully-frosted slides. The slides were kept on ice during the polymerisation of each gel layer. After the solidification of the 0.6% agarose layer, the slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na₂ EDTA, 10 mM Tris-HCl, 1% triton X-100, and 10% DMSO) at 4 °C. After 1 hour, the slides were placed in an electrophoresis buffer (0.3 M NaOH, 1 mM Na₂ EDTA, pH 13) for 10 minutes at 0 °C to allow the DNA to unwind. Electrophoresis was performed for 10 minutes at 300 mA and 1 V/cm. The slides were neutralised with a Tris-HCl buffer, pH 7.5, and stained with 20 μg/mL ethidium-bromide for 10 minutes. Each slide was analysed using a fluorescence microscope (with excitation filter of 420–490 nm [issue 510 nm]). One-hundred cells were analysed on each slide. We used the Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a charge-coupled device (CCD) camera to assess the quantitative and qualitative extent of DNA damage in the cells. Tail length (μm) is the distance of DNA migration from the centre of the body of the nuclear core and is used to evaluate the extent of DNA damage. The tail moment is defined as the product of the tail length and the fraction of total DNA in the tail (tail moment = tail length x % of DNA in the tail). Both the tail length and tail moment were measured automatically using image analysis software (45).

Oxidant / antioxidant measurements

The lenses without their capsules were weighed and homogenised in deionised water, and the upper clear part of the tissue homogenate (supernatant) was used in the measurements. The protein level of the clear supernatants was studied by using the Lowry method (46). Malondialdehyde (MDA) levels (nmol/mg), superoxide dismutase (SOD) (U/mg), glutathione peroxidase (GSH-Px) (mIU/mg) and catalase (CAT) (IU/mg) enzyme activities were measured in the supernatants as given below.

Malondialdehyde levels were measured using the thiobarbituric acid reactive substances (TBARS) method (47). SOD activity was measured as described before (48). One unit of SOD activity was expressed as the enzyme protein amount causing 50% inhibition in the nitro blue tetrazolium (NBT) reduction rate. CAT activity was determined by measuring the absorbance decrease of H₂O₂ at 240 nm (49). GSH-Px activity was measured by following the changes in the nicotinamide adenine dinucleotide phosphate (NADPH) absorbance at 340 nm (50). In the activity calculations, extinction coefficients of H₂O₂ and NADPH were used for CAT and GSH-Px enzymes, respectively.

Statistical analysis

Data were presented as the mean standard deviation (SD). In order to compare between multiple groups, the analysis of variance (ANOVA), followed by the paired t test, was employed using a commercially available software package (SPSS-11 for windows, SPSS Inc, Chicago, IL, USA). The results were considered to be significant at P values less than 0.05 (2-sided).

Results

The analysis of the comet assay photographs given in figure 1 show the red round spot of the intact DNA without migration, while the comet-shaped area adjacent to the nucleus represents DNA breaks that are small enough to move in the gel. In figure 1a, which represents the undamaged control cells, the DNA was tightly compressed and maintained the circular disposition of a normal nucleus. Figure 1b, the comet photo for the UV group, indicates the profile of the nuclear DNA that was altered with the appearance of a fluorescent streak extending from the nucleus. Cells containing damaged DNA appeared as a comet with a bright head and tail after exposure of the rats to UV-B. Figure 1c, the UV + bilberry group, reflects the appearance of some repair and less damage to the cells after bilberry supplementation. Meanwhile, in figure 1d, the bilberry group showed no observed changes between the bilberry group cells and control cells.

Table 1 indicates the comet assay parameters (percentage tailed cells, tail length, percentage tailed DNA, and tail moment) for the control and post-treatment groups (UV, UV + bilberry, and bilberry group), and the differences between the control and the post-treatment groups. The results indicated that all comet assay parameters for the UV group were significantly increased (P < 0.001) compared to the control values. For the UV + bilberry group, the tail length,
percentage tailed DNA, and tail moment values were significantly increased compared to the control, but they were significantly decreased compared to the UV group values, meaning that there is some improvement toward mimicking the control. There were no significant differences between the bilberry group and the control group.

In the results obtained for the lens tissue given in table 2, there were significant increases in the MDA level and CAT activity in the UV group compared with the control \((P < 0.001)\), whereas the SOD activity was significantly decreased \((P < 0.001)\). In the UV + bilberry group, the MDA level, and CAT activity were significantly lower, and the SOD activity was significantly higher compared with the corresponding values in the UV group \((P < 0.001)\). In the UV + bilberry group, the MDA level was significantly higher \((P < 0.001)\) and SOD activity was significantly lower \((P < 0.001)\) relative to the control group. As for the CAT activity, no significant difference was found between the two groups. Additionally, no significant differences were seen between any of the groups in terms of GSH-Px activity. Finally, there were no significant differences between the bilberry group and control group.

**Discussion**

Cataracts are the leading cause of blindness worldwide. The World Health Organization defines cataract as a clouding of the lens of the eye which impedes the transfer of light. Cataract is a multi-factorial disease associated with diabetes, smoking, ultraviolet radiation, alcohol, ionizing radiation, steroids, and hypertension. There is strong experimental \((43)\) and epidemiological evidence \((2,51)\) that ultraviolet radiation causes...
cataracts. The only cure for cataracts is surgery, but this treatment is not accessible to all. It has been estimated that a delay of the onset of a cataract for 10 years could reduce the need for cataract surgery by 50% (52).

Oxidative stress is caused by the presence of a number of reactive oxygen species (ROS) where the cell is unable to counterbalance, resulting in damage to one or more biomolecules, including DNA, RNA, proteins, and lipids. Oxidative stress has been implicated in the natural aging process as well as a variety of diseases. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is a major oxidant in the human eye and increased levels are observed in patients with cataracts (12). The lens is equipped with antioxidant defence mechanisms designed to protect against the harmful effects of UV radiation and ROS (13). These mechanisms consist of enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and low-molecular-weight compounds (vitamins C and E, and glutathione) that metabolise and/or conjugate ROS, thereby rendering them inactive. MDA is the breakdown product of the major chain reactions leading to the oxidation of polyunsaturated fatty acids and, thus, serves as a reliable marker of oxidative stress-mediated lipid peroxidation.

There is a long historical precedent for developing medical drugs from living things, often in the form of plant extracts. For example, digitalis, a cardiac drug, was first discovered in the leaves of the digitalis flower (Digitalis purpurea Linnaeus) (53). Here, we focus on an edible berry, the bilberry (Vaccinium myrtillus), which contains an abundant amount of anthocyanins. Anthocyanins are the water-soluble glycosides of anthocyanidins, which are flavylum cation derivatives. Fifteen different anthocyanins are found in bilberries (36). Previous reports have proposed that the anthocyanins are the molecules responsible for the bilberry’s main pharmaceutical effects, which include antioxidant activity (54,55), and its free-radical scavenging property (22). The anthocyanin-rich bilberry extract has long been a popular treatment for various eye conditions, and as this antioxidant may have a tissue-protecting effect, it could have pharmaceutical applications in human preventive therapy.

By means of the comet assay, the present study has elucidated some of the molecular changes subsequent to UV-B irradiation, (5 KJ/m\textsuperscript{2} and \(\lambda\text{m} = 300\) nm). In the comet assay, a damaged cell takes on the appearance of a comet, with head and tail regions. A variety of geometric and densitometric parameters are provided by the image analysis software, which allows an estimation of the amount of DNA in the head and tail regions, and the extent of migration into the tail region. Because the tail length and density reflect the number of single-strand breaks in the DNA, the percentage of DNA in the tail provides a quantitative measure of the damaged DNA. Also, the elevated mean tail moment is indicative of DNA damage. After supplementation with bilberry, all comet assay parameters were significantly decreased compared with the UV group, meaning that cells are able to repair sublethal DNA, so that most cells show decreased tail moments, which is indicative for minimizing the introduction of strand breaks arising from DNA repair by the cells.

MDA as an indicator of lipid peroxidation, has a very significantly increased level, SOD activity was significantly reduced, and CAT activity was significantly increased after UV exposure. The

| Table 2: Malondialdehyde (MDA) level and superoxide dismutase (SOD), glutathione peroxidase (GSH P x), and catalase (CAT) activities in lens tissues for all studied groups |
|----------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                                  | Control group  | Post-treatment | UV             | UV +           | Bilberry       | UV             | UV +           | Bilberry       |
|                                  |                |                | UV +           | Bilberry       | UV             | UV +           | Bilberry       |
| MDA (nmol/mg)                    | 0.074          | 2.800          | 0.320          | 0.082          | 2.726          | 0.246          | 0.008          |
|                                  | (SD 0.010)     | (SD 0.400)***  | (SD 0.021)***  | (SD 0.003)     | (SD 0.170)     | (SD 0.066)     | (SD 0.001)     |
| SOD (U/mg)                       | 3.600          | 1.110          | 2.199          | 3.504          | −2.490         | −1.401         | −0.096         |
|                                  | (SD 0.214)     | (SD 0.050)***  | (SD 0.074)***  | (SD 0.114)     | (SD 0.129)     | (SD 0.059)     | (SD 0.003)     |
| GSH P x (mIU/mg)                 | 5.030          | 4.700          | 5.112          | 5.244          | −0.330         | 0.082          | 0.214          |
|                                  | (SD 0.513)     | (SD 0.713)     | (SD 0.487)     | (SD 0.300)     | (SD 0.011)     | (SD 0.010)     | (SD 0.010)     |
| CAT (IU/mg)                      | 0.504          | 1.934          | 1              | 0.513          | 1.430          | 0.496          | 0.099          |
|                                  | (SD 0.070)     | (SD 0.080)***  | (SD 0.050)***  | (SD 0.065)     | (SD 0.016)     | (SD 0.002)     | (SD 0.001)     |

(*** The difference is relative to the control.
(*** Statistically significant \( P < 0.001 \).
cause of increased MDA is the increase in lipid damage by the oxygen ($O_2$) radicals arising from the decrease in SOD activity. As for the decrease in SOD activity and the increase in CAT activity, one possibility is that of the $H_2O_2$ produced apart from the SOD, thereby providing a balance mechanism for the organism. Bilberry’s effects as an antioxidant appeared clearly in minimising the changes induced by UV radiation. The data also showed that bilberry extracts had no cytotoxicity to the lens tissues that appeared from the absence of any significant difference between the bilberry group and control group.

**Conclusion**

In conclusion, exposing rats to UV-B radiation may act as a risk factor for the occurrence of oxidative stress-based lens opacification. The potent free radical scavenger and antioxidant, bilberry, may protect rats’ lens tissues from oxidative damage, minimise DNA strand breaks, and prevent the disruption of the oxidant/antioxidant balance in the lens tissue, thus preventing organ dysfunction. Although bilberry shows a protective effect against UV-B induced cataracts, extrapolation from animal studies to human subjects requires further investigation.

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**Conflict of Interest**

None.

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**Authors’ Contributions**

Conception and design, analysis and interpretation of the data, drafting of the article, statistical expertise, collection and assembly of data: EMA  
Critical revision of the article for the important intellectual content, final approval of the article, provision of study materials or patient, obtaining of funding and administrative, technical or logistic support: MAA

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