ABSTRACT

Cruciferae vegetables are widely studied due to their potential anticarcinogenic properties. In the present work we analyzed the antigenotoxic effect of watercress (*Nasturtium officinale*, W. Aiton), a member of this family. The vegetal was processed, and the juice obtained was evaluated in relation with genetic material protection and DNA repair capacity against two deleterious agents: mitomycin C and hydrogen peroxide. The antigenotoxic properties were analyzed through cytokinesis block micronucleus test and comet assay (standard and with endonuclease), respectively. Besides, we tested the functionality of P-glycoprotein in human lymphocytes exposed to the whole extract. Watercress had no effect on mitomycin cytostatic effect (p>0.05) and although a decrease trend in micronuclei frequency was observed no statistical difference was found. In relation to DNA repair, a reduction in damage index was observed (p<0.001) since 4 hours of incubation with the juice but it did not reduce the number of oxidized pyrimidines. Respect to P-glycoprotein transporter, our results indicate that the juice of watercress did not change the activity of protein. In conclusion, to better understand the real beneficial effect of watercress in vitro, further studies are needed to elucidate the mechanism behind the chemopreventive effect observed.

Key words: chemopreventive effects, comet assay, micronucleus test, transporters, *Nasturtium officinale*

RESUMEN

Las crucíferas son ampliamente estudiadas dado sus potenciales propiedades anticarcinogénicas. En el presente trabajo, analizamos el efecto antigenotóxico del berro (*Nasturtium officinale*, W. Aiton), un miembro de esta familia. El vegetal fue procesado y el jugo obtenido fue evaluado en relación con la protección y reparación del daño al material genético inducido por dos agentes deletéreos: mitomicina C y peróxido de hidrógeno. Las propiedades antigenotóxicas fueron analizadas a través del test del micronúcleo con bloqueo de la citocinesis y el ensayo cometa (estándar y con endonucleasa), respectivamente. Además, evaluamos la funcionalidad de la glicoproteína P en linfocitos humanos expuestos al extracto completo. El berro no modificó el efecto citostático de la mitomicina (p>0,05) y si bien se determinó una tendencia decreciente en la frecuencia de micronúcleos no se encontraron diferencias estadísticamente significativas. En relación a la reparación del ADN, se determinó una disminución del índice de daño (p<0,001) a partir de las cuatro horas de exposición al jugo sin existir cambios en el número de pirimidinas oxidadas. Respecto del transportador, nuestros resultados indican que el jugo de berro no modifica la actividad de la proteína. En conclusión, para lograr entender con mayor claridad el beneficio real del berro in vitro, es necesario realizar otros estudios con el fin de elucidar el mecanismo subyacente a los efectos quimiopreventivos observados.

Palabras clave: efectos quimiopreventivos, ensayo cometa, test del micronúcleo, transportadores, *Nasturtium officinale*
INTRODUCTION

Cruciferous vegetables are widely studied due to their potential anticarcinogenic properties attributed mainly to phytochemicals derived from glucosinolates. Different evidence derived from in vitro and in vivo studies has indicated that these vegetables may reduce oxidative DNA damage, induce phase 2 enzymes activities, cell cycle arrest and apoptosis in cancer cell lines and inhibit some members of CYP family (Hayes et al., 2008). However, controversial effects have been detected for isothiocyanates, their metabolites. These phytochemicals enhanced cellular protection against deleterious agents and environments, but also induced cytotoxic effects and oxidative stress in several cell lines (Huang et al., 1998; Zhang et al., 2005).

Previous reports of our laboratory illustrate that watercress juice (Nasturtium officinale, W. Aiton) does not act as a genotoxic agent to human lymphocytes in vitro (Casanova et al., 2010) furthermore it has a protective effect against DNA damage induced by hydrogen peroxide in vitro (Casanova and Carballo, 2011) and cyclophosphamide in vivo (mice) (Casanova et al., 2013). Meanwhile, other groups demonstrated that consumption of watercress modulates superoxide dismutase and glutathione peroxidase activities in vitro and in vivo (Hoffman et al., 2009) in addition to decrease of oxidative DNA damage (Gill et al., 2007). More recent, another study showed that diet supplementation with this cruciferous attenuates DNA damage and lipid peroxidation induced by exhaustive exercise (Fogarty et al., 2012).

The use of many components of the diet as supplements or therapeutic agents is not a totally safe practice. The chemicals could interact between them or even with drugs leading to toxic effects or therapeutic failure. The outcome will depend on characteristics of the plant, patient and drug. The membrane transporters, like P-glycoprotein or multidrug resistance proteins, play important roles in absorption, distribution and elimination of xenobiotics. P-glycoprotein is expressed in many normal tissues including blood cells and there are evidences that phenethyl isothiocyanate (PEITC) present in watercress inhibited P-glycoprotein activity in human breast cell lines but it may not be a substrate for this protein (Ji and Morris, 2005).

In the present study, we studied the antigenotoxic activity of watercress juice against two genotoxic agents with different mechanism of action: mitomycin C (MMC) and hydrogen peroxide-induced DNA damage using the cytokinesis-block micronucleus and standard and modified comet assays respectively. In addition, we evaluated the functionality of P-glycoprotein in human lymphocytes exposed to the extract.s.

MATERIAL AND METHODS

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

Watercress juice

Watercress was purchased from organic market garden located in Luján, Buenos Aires. The juice of cress plant (leaves) was prepared with a commercial processor on ice and protected from light exposure. Then it was centrifuged at 10,000 g, for 20 minutes at 4 ºC and the supernatants were clarified, sterilized by microfiltration using 0.22 µm pore membranes (Millipore) aliquoted. A single batch preparation with a concentration of 6.5 g watercress/ml juice was freezed until use.

Phytochemical screening

The juice was subjected to quantitative chemical screening for the identification of the major classes of active chemical constituents. The phytochemical profile was determined according to methodology described (Portmann et al., 2012) and includes determination of total phenols, tannins, and flavonoids content. All measurements were done in triplicate.

Total phenols were determined by Folin-Ciocalteu procedure as other have done (Makkar et al., 1993) at 725 nm. The amount of total phenols was expressed as µg tannic acid/g material. Calibration curve of tannic acid was developed.

Total tannin content was determined by Folin-Ciocalteu procedure, after the removal of tannin by precipitation with bovine serum albumin. Non absorbed phenolics were determined as described for total phenols. Calculated values were subtracted from total phenol contents and total tannin contents expressed as µg tannic acid/g material.

Flavonoids were measured adding aluminum chloride and sodium acetate (flavonoids reactive) to aliquots of juice. After incubation at room temperature, absorbance was recorded at 430 nm. Calibration curve of rutin was
developed. The amount of flavonoids was calculated as µg rutin/g material (Maksimovic et al., 2005).

The total intact glucosinolate content of juice has been studied by reverse-phase HPLC-UV (Gilson 170 chromatograph equipped with a diode array detector, wavelength 254 nm) using a Gemini S4 C18 column with mobile phase composed of 30% sodium monobasic phosphate 0.01M/70% methanol (flow rate: 1.5ml/min) and reported earlier (Casanova et al., 2013).

**Evaluation of protection capacity against DNA damage: cytome assay**

Human lymphocytes were isolated using Ficoll-Paque (GE Healthcare) density gradients (Boyum, 1964). Briefly, blood was diluted with phosphate buffered saline and layered onto Ficoll-Paque (4:3). The blood was centrifuged for 20 minutes at 800 g; the lymphocyte layer was removed and washed twice in phosphate buffered saline (PBS). Cell density was counted with a hemocytometer. The lymphocyte cultures (1×10^6 cells/ml) were grown in RPMI 1640 (Gibco BRL) supplemented with 15% fetal bovine serum (Gibco BRL) and phytohemagglutinin (10 µg/ml); PHA) in a humidified incubator with 5% CO₂ in polystyrene plates. The lymphocytes were incubated at 37 °C in polystyrene plates. The watercress juice or PBS were added to cultures at 20 h after PHA stimulation, followed by the addition of MMC (final concentration of 0.152 µM) at 44 h and cytochalasin B (4.5µg/ml) at 48 h. At 72 h, lymphocytes cultures were spun directly onto glass slides using a cytocentrifuge (200 g, 5 minutes; Shandon, Cytospin 3, Microlat). Slides were allowed to air dry before methanol fixation at room temperature for 10 min. Before scoring slides were stained with giemsa 10% (Merck).

At least 2,000 binucleated lymphocytes were scored for the number of micronuclei, nucleoplasmic bridges and nuclear buds. The proportion of mono, bi and multinucleated cells was used to determine cytostatic effects through the nuclear division index (NDI) = (1 × n1) + (2 × n2) + (3 × n3) + (4 × n4)/N, where n1-n4 represent the number of cells in with 1, 2, 3 or 4 nuclei and N is the total number of viable cells evaluated. Percentage of necrotic and apoptotic cells were measured to establish cytotoxic effects (Fenech, 2007).

**Evaluation of in vitro repair capacity and endonuclease III sensitive sites using the comet assay**

The procedure described earlier (Bowden et al., 2003) was used with modifications. Heparinized samples of peripheral blood were obtained from healthy donors as a source of leucocytes. H₂O₂ treatment was done before cells were incubated with the juice. Briefly, cells (50 µl) were treated with H₂O₂ (25 µM) for 10 min on ice to induce DNA strand breaks. The reaction was quenched using 2% DMSO (Merck) solution in PBS. Each sample was washed and then incubated in RPMI 1640 medium supplemented with 15% fetal bovine serum and the watercress juice (13.2 mg/ml or 26.4 mg/ml) for 0, 2, 4 or 6 hours at 37 °C. Each sample was centrifuged; cell pellets were mixed with 200 µl of 1% low melting point agarose solution at 37 °C and spread onto slides precoated with 1% normal melting point agarose. The slides were submerged in cold, freshly prepared lysis solution (2.5M NaCl, 100mM Na₂EDTA, 10mM trizma, 1% Triton X-100 and DMSO 10%, pH 10) and left overnight at 4 °C. The slides were placed in cold electrophoresis alkaline buffer (10N NaOH, 200mM Na₂EDTA, pH >13) and the embedded cells were exposed for 20 min to allow DNA unwinding to occur, before electrophoresis at 25V and 300mA (0.75V/cm) for 20 min. The slides were then washed with neutralization buffer (Tris 0.4M, pH 7.5) and the DNA stained with ethidium bromide (0.02 mg/ml) and observed using a fluorescent microscope at 40× of magnitude. All procedures were carried out in the darkness and on ice to avoid additional DNA damage and inhibit DNA repair, respectively. Cell viability was determined by means of the ethidium bromide/acridine orange assay (Mercille and Massie, 1994).

One hundred randomly selected cells (for duplicate) were analyzed visually on a scale of 1-4, depending on the grade of damage, to calculate the Damage Index (DI)= (1 × n1) + (2 × n2) + (3 × n3) + (4 × n4), where n is the number of cells in each category evaluated.

The slides were washed twice with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0) for 5 minutes, to evaluate the levels of oxidized pyrimidines. Then, the gels were incubated for 30 minutes with 50 µl of Endo III or 50 µl of buffer at 37 °C. The following steps were done according to the standard protocol. The levels of Endo III sensitive sites (ESS) were calculated as the damage index obtained with enzyme minus the score without enzyme.

The correlation between visual scoring and computer-based image analysis has been established; there is a good correlation between visual score and image analysis parameters (Dusinska and Collins, 2008). All the slides were coded and scored by the same observer.
Modulation of P-glycoprotein activity

Human lymphocytes were isolated using Ficoll-Paque density gradients as described above. 5x10^5 cells were incubated with rhodamine 123 for 10 minutes in the dark and washed twice with cold PBS. Then, they were incubated at 37 °C with culture medium supplemented with fetal bovine serum and watercress juice for 3 hours. Cells were washed, resuspended in PBS and kept on ice until analysis. Verapamil (100 mM) was used as a positive control because it is an inhibitor of P-glycoprotein. Fluorescence intensity was measured in lymphocytes region (10,000 events) with a FAC-SCAN Ortho-Cytoron cytometer. Geometric mean fluorescence intensity (GMFI) of each sample was taken from the histogram. The results were expressed as a proportion of basal score.

Statistical analysis

Differences between controls and treatments were analyzed by one-way analysis of variance and a post-hoc Student Newman Keuls test. The significance of correlation between Endo III sensitive sites and damage index was estimated by Pearson correlation test. A value of p<0.05 was considered as statistically significant for all the endpoints evaluated (Sigma Stat software).

RESULTS

The phytochemical profile of Nasturtium officinale juice indicated the presence of phenols, tannins and flavonoids (Table 1). Besides, the juice contains 8.9 µmol total intact glucosinolates/ml material.

In relation with antigenotoxic activity of watercress, we evaluated its ability to protect and repair DNA damage induced by two genotoxic agents. Respect to mitomycin C, Table 2 and Figure 1 show the results of protective potential obtained with the micronucleus test. Watercress had no effect on MMC cytostatic effect (p>0.05) and although a decreasing trend in micronuclei frequency was observed no statistical difference was found (p>0.05). There was no difference between watercress-treated and MMC-control group in relation with nucleoplasmic bridges and nuclear buds (p>0.05). In addition, neither apoptotic nor necrotic cells were detected.

The ability of human leucocytes to repair strand breaks induced by H_2O_2 was measured at different times (0, 2, 4 and 6 hours). Since no statistical difference was found between volunteers, the data were pooled. Figure 2 shows the effect of watercress juice on DNA repair. For both concentrations, a decrease in DI was observed (p<0.001); the antigenotoxic effect was observed after 4 hours of incubation with the juice. We continued the analysis of the repair process and detected that damage decreased with time so that after 24 hours the levels of breaks were not statistical different in relation to the control (treated with saline solution) (data not shown). Although our results show that watercress juice did not reduce the number of oxidized pyrimidines induced in leucocytes by exposure to hydrogen peroxide there was a positive association between ESS and DI (Table 3: p>0.05, Figure 3: p<0.001).

Respect to the functionality assay of P-glycoprotein in human lymphocytes, the expression of results suggests that addition of watercress juice to cellular suspension does not impact on the rhodamine transport (p>0.05) whereas verapamil has an inhibitor effect (p<0.05) (Table 4).

Table 1. Quantitative estimation of phytochemicals (µg/g).

<table>
<thead>
<tr>
<th>Phytochemical (X ± SD)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>285 ± 20</td>
</tr>
<tr>
<td>Tannins</td>
<td>42 ± 14</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>146 ± 3.54</td>
</tr>
</tbody>
</table>

Table 2. Nuclear division index of human lymphocytes exposed to watercress juice and/or mitomycin C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NDI (X ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (PBS)</td>
<td>1.66 ± 0.08 *</td>
</tr>
<tr>
<td>Positive control (MMC 0.152 µM)</td>
<td>1.53 ± 0.03</td>
</tr>
<tr>
<td>13.2 mg/ml WJ + MMC 0.152 µM</td>
<td>1.52 ± 0.07</td>
</tr>
<tr>
<td>26.4 mg/ml WJ + MMC 0.152 µM</td>
<td>1.52 ± 0.06</td>
</tr>
</tbody>
</table>

The phytochemical profile of Nasturtium officinale juice indicated the presence of phenols, tannins and flavonoids (Table 1). Besides, the juice contains 8.9 µmol total intact glucosinolates/ml material.
**Figure 1.** Micronucleus, nucleoplasmic bridges and nuclear buds frequencies of human lymphocytes exposed to watercress juice and/or mitomycin C.

X: Micronucleus, nucleoplasmic bridges or nuclear buds.

MMC: mitomycin C 0.152 µM.

WJ: watercress juice.

ANOVA NS

**Figure 2.** Antigenotoxic effect of watercress juice on DNA damage.

A + D: Watercress juice 13.2 mg/ml + H2O2 25µM.

B + D: Watercress juice 26.4 mg/ml + H2O2 25µM.

D: H2O2 25µM.

** p < 0.001 (ANOVA, post test: Student-Newman-Keuls).
Table 3. Level of Endonuclease III sensitive sites.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>A + D (X ± SD)</th>
<th>B + D (X ± SD)</th>
<th>D (X ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8 ± 1</td>
<td>7 ± 2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>7 ± 1</td>
<td>8 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>8 ± 4</td>
<td>6 ± 2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>6</td>
<td>11 ± 1</td>
<td>7 ± 3</td>
<td>10 ± 3</td>
</tr>
</tbody>
</table>

Table 4. Effect of watercress on P-glycoprotein activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GMFI (X ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control (verapamil 100 µM)</td>
<td>4.19 ± 0.94*</td>
</tr>
<tr>
<td>13.2 mg/ml WJ</td>
<td>1.02 ± 0.13</td>
</tr>
<tr>
<td>26.4 mg/ml WJ</td>
<td>1.08 ± 0.27</td>
</tr>
</tbody>
</table>

Figure 2. Association between Endonuclease III sensitive sites and damage index
p= 0.786; p<0.001
DISCUSSION

In the last decades, the use of medicinal plants and components of the diet as chemopreventive agents has increased. It is important to highlight that effects always depend on plant’s species, its origin and growing conditions, its composition, extract preparation, extraction method, doses and matrix analyzed. Nowadays, it is believed that the beneficial characteristics attributed to some vegetables are due to the presence of diverse phytochemicals which exert their effects by multiple mechanisms.

The micronucleus test is a useful method to detect genotoxic and cytotoxic effects and it can be adapt to establish a relationship between nutrition and DNA damage (Bull et al., 2011). In the present study, we evaluated potential capacity of a diet component to protect against MMC-induced damage. The treatment with watercress juice, at both concentrations, reduced micronucleus frequency compared to those obtained with the mutagenic agent. However, these trends had not statistical significance which could be due to the short period of intervention used or very different response detected in the human samples (individual susceptibility) that disables the statistical evaluation.

The results of repair capacity evaluated by comet assay show that the extract exerted antigenotoxic activity against oxidative damage after 4 hours of incubation at both concentrations used. This effect may be related to the antioxidant capacity of watercress and to a combination of bioactive substances, acting at different targets and via different mechanisms. The amount of oxidized pyrimidines in DNA was used as a specific biomarker of oxidative damage. Although no difference was found at level of modified bases between controls and treated groups we detected a positive correlation between ESS and DI. This indicates watercress enhanced DNA repair process and it could be related with the modification of several enzymes involved in oxidative stress process. Supplementary studies may explain this situation.

It has to be mentioned that other authors have reported that watercress supplementation reduces the level of oxidized purines in lymphocytes (Gill et al., 2007). The discrepancy may be ascribed to the presence of some constituents in the whole plant that may be absent, or in minor proportion in our juice. Otherwise the experimental design used is so different that the results are not equivalent.

The contradictory results of MN and comet assays are related to different end-points detected. The first one determines unrepaired DNA damage or spindle mistakes while the comet assay detects strand breaks and labile sites which could be easy repairable. Both methods are widely used as biomarkers of the relationship between diet and disease or health.

Taking account that isothiocyanates interact with P-glycoprotein and other transporters, consume of watercress could modulates their activities, influence the pharmacokinetics of drugs and change therapy outcomes (Rodrigues Fragoso et al., 2011). Our data suggest that the intake of the vegetal may be not inducing changes in the bioavailability of drugs in opposition with isolated PEITC.

In conclusion, to better understand the real beneficial effect of watercress in vitro, further studies are needed to elucidate the mechanism behind the chemopreventive effect observed.

REFERENCES


IN VITRO EVALUATION OF WATERCRESS CHEMOPREVENTIVE EFFECT


ACKNOWLEDGMENTS

The authors would like to acknowledge the University of Buenos Aires for grant [UBACYT 20020100100123 2011-2014] and to PhD Catalina Cortada for her help in the P-glycoprotein functionality assay.