In Vitro genotoxic and antigenotoxic studies of Thai Noni fruit juice by chromosomal aberration and sister chromatid exchange assays in human lymphocytes

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Abstract

The genotoxic and antigenotoxic effects of Noni fruit juice produced in Thailand have been studied in human lymphocytes for chromosome aberration assay and sister chromatid exchange (SCE) assay in vitro. Treatment of Noni fruit juice (3.1-50 mg/ml) alone for 3 h did not significantly induce chromosomal aberration or SCE (p<0.05). Noni fruit juice at 6.2 mg/ml is the optimum dose for cell survival and cell replication as demonstrated by the highest value of mitotic index and proliferation index (P.I.). Interestingly, pretreatment of Noni fruit juice at the same concentration of 6.2 mg/ml for 2 h followed by mitomycin C treatment at 3 µg/ml for 2 h significantly reduced SCE level induced by mitomycin C (p<0.05). However, these treatments did not show significant decrease in chromatid-type aberrations. Our data indicate that Thai Noni fruit juice is not genotoxic against human lymphocytes in vitro. In addition, pretreatment of Noni fruit juice at 6.2 mg/ml demonstrated no anticlastogenic effect while had some antigenotoxic effects as demonstrated by significant decrease in the SCE level induced by mitomycin C (p<0.05). Therefore, the optimum dose of Noni fruit juice used as a traditional medicine is required and needs to be studied further for the benefit of human health.

Keywords: chromosome aberration, sister chromatid exchange (SCE), Noni, Yor, Morinda citrifolia

1. Introduction

Noni is a common name of Indian Mulberry (Morinda citrifolia Linn.) while Yor is a common name used for Noni of Thailand. Noni fruit juice made from the fruit of Indian Mulberry has been reported to have a broad range of therapeutic effects; for example, antibacterial, antiviral, antifungal, anticancer, and anti-inflammatory activities including analgesic and immunomodulating effects (Akihisa et al., 2007). For the scientific studies, there are reports that polysaccharide-rich substances in Noni fruit juice have immunomodulating effects and contributed to anticancer activity (Hirazumi and Furusawa, 1999). Nayak et al. (2007) demonstrated that Noni fruit juice reduced blood sugar level and had wound-healing effects in diabetic rats (Nayak et al., 2007). Using swimming and rotarod tests in mice, Noni fruit juice intake could counteract fatigue, improves endurance and increases overall physical performance (Ma et al., 2007). Various chemical components in Noni fruits have already been identified such as luteolin, americanin A, 3,3′-bisdemethylpinoresinol, p-cresol, p-hydroxybenzaldehyde, 1,5,7-trihydroxy-2,6-dimethoxymethylantraquinone, p-hydroxyben-
Recently, Noni juice was reported to possess hepatotoxic effects (Yuce et al., 2006). However, it has been argued that hepatotoxic effects were due to interferon beta toxicity which had been used for treatment of multiple sclerosis in that patient (West, 2006), whereas Noni juice itself could protect the liver (Potterat and Hamburger, 2007; Westendorf et al., 2007). The European Commission (2006) reported that there is no convincing evidence for a causal relationship between the acute hepatitis observed in the case studies and the consumption of Noni juice (European Commission, 2006). Nevertheless, to evaluate the safety level of Noni juice intake data are needed. Concerning the genotoxicity of Noni juice, a Thai Noni fruit juice concentrate (Lot # J5B) was obtained from Noni fruits grown in the central part of Thailand. Ripe Noni fruits collected in October, close to the end of rainy season, and the juice produced at the end of January, was collected and used for the experiments. The quantities of carbohydrate, protein, ash, moisture, and calories were analyzed using AOAC methods (Official Methods of Analysis, 1995). Total fat was determined by the Caviezel method, based on a gas chromatographic technique (Pendl et al., 1998).

2. Materials and Methods

2.1 Thai Noni fruit juice

Fermented Thai Noni fruit juice concentrate (Lot # J5B) was obtained from Noni fruits grown in the central part of Thailand. Ripe Noni fruits collected in October, close to the end of rainy season, and the juice produced at the Research Center, Faculty of Medicine, Thammasat University, Thailand (Nandhasri et al., 2004). Noni fruit juice (NFJ) was achieved by fermentation of ripe Noni fruits with red sugar at the proportion of 3:1 for 3-6 months. Then, NFJ was separated from the residue by filtering through cotton cloth and kept in the refrigerator (4°C) until used.

2.2 Analysis of the composition profile of Noni fruit juice

The quantities of carbohydrate, protein, ash, moisture, and calories were analyzed using AOAC methods (Official Methods of Analysis, 1995). Total fat was determined by the Caviezel method, based on a gas chromatographic technique (Pendl et al., 1998).

2.3 Genotoxic studies

2.3.1 Cell cultures

Fresh blood samples for all experiments were obtained by venipuncture from 4 healthy volunteers, age 25-35 years with no recent exposure to radiation or drugs. These studies were approved by our institutional ethics committee. Lymphocyte-enriched buffy coat (from 0.8 ml whole blood) was cultured in 5 ml culture medium containing RPMI1640 (Hyclone, U.S.A.), fetal bovine serum (Hyclone, U.S.A.), autologous plasma, penicillin-streptomycin (Seromed, Germany), phytohemagglutinin (Seromed, Germany) and L-glutamine (Hyclone, U.S.A.) using standard blood culture conditions as previously described (Chiewchanwit and Au, 1994). At 24 h after initiation of the culture, the lymphocyte cultures were centrifuged for packed cells and the supernatant medium was removed and saved for reuse after treatment. The remaining lymphocytes were treated with NFI at a concentrations of 3.1, 6.2, 12.5, 25, 50 and 100 mg/ml in plain RPMI 1640 culture medium at 37°C for 3 h. For positive control, the lymphocytes were treated with MMC (Sigma-Aldrich, U.S.A.) at concentration of 3 µg/ml for 3 h, while, for negative control, the lymphocytes were treated with plain RPMI 1640 for 3 h. After treatment, lymphocyte cultures were centrifuged for packed cells. The supernatant medium was discarded and the saved culture medium was re-used. Bromodeoxyuridine (BrdU) solution (Sigma-Aldrich, U.S.A.) was added to the culture medium for the final concentration at 5 mM and cell culture was continued at 37°C in the dark.

2.3.2 Cell culture harvest and staining

(1) For chromosomal aberration assay

Prior to harvest at 48 h after initiation, Colcemid solution (Seromed, Germany) was added to the cells for 1.5 h to block cells at the metaphase stage. Cells were harvested by centrifugation for packed cells and the supernatant was discarded. Cells were continually treated with hypotonic solution and fixed with Carnoy’s fixative solution. Slides were prepared and stained with the fluorescent plus Giemsa technique according to our previous protocol (Chiewchanwit and Au, 1994). First, slides were placed in aluminum foil-covered Coplin jars containing 40 ml of 12.5 mg/ml Bisbenzimide (Hoechst 33258) aqueous solution (Sigma-Aldrich, U.S.A.) for 30 min. Then, they were rinsed serially through distilled water and mounted with a few drops of McIIIvane’s buffer using a coverslip for each slide. Slides were then placed on a slide heater and exposed to UV light for 30 min. After the UV exposure, the covered slips were removed. Slides were...
washed thoroughly with distilled water and then stained with a 10% aqueous solution of Giemsa (Biotech reagents, Thailand) for 10 min. Slides were air-dried and mounted. Three independent experiments were performed. From each experiment, 50 cells showing the first metaphase-staining pattern (homogeneously stained chromosomes) were scored from coded slides for the frequencies of chromosome aberration including chromatid-type aberrations and chromosome-type aberrations. Mitotic indices determined as the total number of mitotic cells/1,000 cells) were also evaluated.

(2) For sister chromatid exchange (SCE) assay

To harvest cells for the SCE assay, a similar procedure to that for chromosome aberration assay was used except that harvesting was performed at 72 h after initiation. Fluorescent plus Giemsa technique was performed for staining and only cells having the second metaphase staining (MII phase) were analyzed for the frequencies of SCEs. Proliferation indices determined as (MI+2MII+3MIII)/100 cells were also evaluated.

2.4 Antigenotoxic studies

2.4.1 Cell cultures

Human lymphocytes were cultured for 24 h as described in 2.3.2(1) At 24 h after initiation of the lymphocyte culture, the supernatant was removed and saved. The remaining lymphocyte cultures were treated with Noni fruit juice at concentrations of 6.2, 12.5, 25, 50 and 100 mg/ml in plain RPMI 1640 culture medium for 2 h at 37°C, centrifuged, and the supernatant medium discarded. Then, the treated cells were further treated with MMC solution (3 µg/ml) for 2 h at 37°C. After treatment, the lymphocyte cultures were centrifuged for packed cells and the supernatant medium discarded. The treated lymphocytes continued to be cultured at 37°C in the dark with the previously saved medium, and BrdU solution was added to the final concentration at 5 mM.

2.4.2 Cell culture harvest and staining

Cells were harvested as described in 2.3.2(1) except that harvesting was done at 53 h after initiation for chromosome aberration assay and at 77 h after initiation for SCE assay because of cell cycle delay resulting from the toxicity of MMC. The Fluorescent plus Giemsa technique was also performed for staining.

2.5 Cell scoring

Three independent experiments for each genotoxic/antigenotoxic study were performed. For chromosome aberration assay, 50 cells per dose per experiment showing the first metaphase-staining pattern were scored from coded slides for the frequency of chromosome aberrations. For sister chromatid exchange assay, 25 cells per dose per experiment showing a second metaphase staining pattern were scored from coded slides for the frequency of SCEs.

2.6 Statistical analysis

Raw data obtained from the chromosome aberration and SCE assays were transformed to stabilize the variance by the procedures of Whorton et al. (1984) (Whorton et al., 1984) as followings:

Transformed chromosome aberration (CA_t):

\[ CA_t = \frac{1}{2} \left( \frac{\sqrt{CA} + \sqrt{(CA + 1)}}{\sqrt{2}} \right) \]

Transformed sister chromatid exchange (SCE_t):

\[ SCE_t = \sqrt{SCE} \]

Dunnett’s t-test was performed to analyse the difference between the mean of the treated groups and of the control group using the transformed data.

3. Results

3.1 Composition profile of Thai Noni fruit juice (NFJ)

As comparison with Tahitian NFJ (European Commission, 2002), the composition profile of Thai NFJ is a little higher especially when concerning protein, fat, and carbohydrate content and with a little lower pH as shown in Table 1.

3.2 In vitro clastogenic studies of Thai Noni fruit juice by chromosomal aberration assay using human lymphocyte

As shown in Table 2, Figure 1 and Figure 2, treatment by NFJ at concentrations of 3.1-100 mg/ml for 3 h demonstrated that NFJ did not significantly induce structural chromosomal aberration in human lymphocytes. NFJ at concentrations of 50, 25 and 12.5 mg/ml showed significant decrease
in M.I. and P.I. when compared to the negative control (p<0.05). Interestingly, lower concentration of NFJ (6.2 mg/ml and 3.1 mg/ml) increased the level of M.I. and P.I. At the optimum dose of NFJ at 6.2 mg/ml, the highest increase in P.I. and M.I. was shown which was equal to that of the negative control. The positive control (treatment of MMC at concentration of 3 μg/ml for 3 h) showed a significant increase in chromosomal aberration and a significant decrease in the M.I. and P.I. compared to those of the negative control (p<0.05).

### 3.3 In vitro genotoxic studies of Thai Noni fruit juice by sister chromatid exchange assay using human lymphocytes

Treatment of NFJ at concentrations 3.1-100 mg/ml for 3 h demonstrated that NFJ did not significantly induce sister chromatid exchange in human lymphocytes (p>0.05) as shown in Table 3. Correspondingly as shown in chromosomal aberration studies in human lymphocytes induced by various concentrations of Thai Noni fruit juice (n=3)
somal aberration studies, treatment of NFJ at concentrations of 50, 25 and 12.5 mg/ml showed significant decrease in M.I. and P.I. as compared to the negative control (p<0.05), while treatments at lower concentration of NFJ (6.2 mg/ml and 3.1 mg/ml) increased the level of M.I. and P.I. with the optimum dose at 6.2 mg/ml. For the positive control, treatment of MMC at concentration of 3 µg/ml for 3 h showed significant increase in SCE level and significant decrease in the M.I. and P.I. compared to those of the negative control (p<0.05).

3.4 *In vitro* anticlastogenic studies of Thai Noni fruit juice against mitomycin C by chromosome aberration assay using human lymphocyte

As shown in Table 4, pretreatment of NFJ at concentrations of 6.2-100 mg/ml for 2 h followed by MMC treatment at 3 µg/ml for 2 h did not significantly decrease structural chromosomal aberration induced by MMC in human lymphocytes (p<0.05). No differences in M.I. and P.I. of the various treatments from that of the positive control (MMC treatment alone) were observed. In addition, a synergistic toxic effect between NFJ at the high dose of 100 mg/ml and MMC at 3 µg/ml was found. No mitotic cell was detected at this treatment.

3.5 *In vitro* antigenotoxic studies of Thai Noni fruit juice against mitomycin C by sister chromatid exchange assay using human lymphocytes

Pretreatment of Thai Noni fruit juice at concentrations of 12.5-100 mg/ml for 2 h followed by MMC treatment at 3 µg/ml for 2 h did not significantly reduce SCE level induced by MMC (p<0.05) (see Table 5 and Figure 5). However, pretreatment by NFJ at a concentration of 6.2 mg/ml demonstrated significant decrease in SCE level induced by MMC (p<0.05). For the M.I. and P.I. levels, there was no significant difference between the treated group and the positive control (MMC treatment alone). Nevertheless, pretreatment by NFJ at concentrations of 12.5 mg/ml and 6.2 mg/ml tended to have higher P.I. and M.I. as compared to higher doses of NFJ pretreatment.

We also extended our experiments to test the effect of

Table 4. *In vitro* anticlastogenic studies of various concentrations of Thai Noni fruit juice against mitomycin C (MMC) at concentration of 3 µg/ml by chromosome aberration assay (n=3)

<table>
<thead>
<tr>
<th>Concentration of Thai Noni fruit juice (mg/ml)</th>
<th>% Abnormal cells ±S.E.M.</th>
<th>Chromatid breaks/100 cells ±S.E.M.</th>
<th>Chromatid exchanges/100 cells ±S.E.M.</th>
<th>Total Chromatid-type aberrations/100 cells ±S.E.M.</th>
<th>P.I. +S.E.M. (53 h)</th>
<th>M.I. +S.E.M. (53 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 100</td>
<td>0±0*</td>
<td>0±0*</td>
<td>0±0*</td>
<td>0±0*</td>
<td>6.3±1.2*</td>
<td>38.2±5.5*</td>
</tr>
<tr>
<td>50</td>
<td>87.3±4.0</td>
<td>247.3±12.2</td>
<td>168.0±40.1</td>
<td>415.3±39.6</td>
<td>0.3±0.1</td>
<td>3.5±0.6</td>
</tr>
<tr>
<td>25</td>
<td>76.7±10.0</td>
<td>178.0±20.0</td>
<td>128.0±45.5</td>
<td>306.0±50.7</td>
<td>0.7±0.2</td>
<td>6.6±1.5</td>
</tr>
<tr>
<td>12.5</td>
<td>87.3±7.0</td>
<td>218.7±25.4</td>
<td>147.3±67.8</td>
<td>366.0±84.0</td>
<td>0.5±0.1</td>
<td>4.8±1.1</td>
</tr>
<tr>
<td>6.2</td>
<td>81.0±6.7</td>
<td>204.7±39.6</td>
<td>130.7±40.2</td>
<td>335.3±22.2</td>
<td>0.6±0.1</td>
<td>5.7±0.7</td>
</tr>
</tbody>
</table>

MMC 3 µg/ml 83.3±5.2 190.0±9.4 129.3±19.5 319.3±15.1 0.6±0.2 6.3±1.6

* significantly different from the positive control (MMC 3 µg/ml) (p<0.05)

Abbreviation: P.I.: proliferation index = (MI+2MII+3MIII)/100 cells;
M.I.: mitotic index = number of mitotic cells/1,000 cells
Table 5. *In vitro* antigenotoxic studies of various concentrations of Thai Noni fruit juice against mitomycin C (MMC) at concentration of 3 µg/ml by sister chromatid exchange assay (n=3)

<table>
<thead>
<tr>
<th>Concentration of Thai Noni fruit juice (mg/ml)</th>
<th>SCE/cell ± S.E.M. (77 h)</th>
<th>P.I. ± S.E.M. (77 h)</th>
<th>M.I. ± S.E.M. (77 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.6 ± 0.3*</td>
<td>9.3 ± 1.2*</td>
<td>38.8 ± 5.1</td>
</tr>
<tr>
<td>100</td>
<td>50.2 ± 2.5</td>
<td>4.0 ± 0.4</td>
<td>32.0 ± 3.2</td>
</tr>
<tr>
<td>50</td>
<td>42.7 ± 2.1</td>
<td>3.8 ± 0.6</td>
<td>30.1 ± 4.8</td>
</tr>
<tr>
<td>25</td>
<td>40.7 ± 2.0</td>
<td>4.7 ± 0.5</td>
<td>30.1 ± 1.9</td>
</tr>
<tr>
<td>12.5</td>
<td>45.0 ± 2.5</td>
<td>6.3 ± 0.5</td>
<td>43.8 ± 4.4</td>
</tr>
<tr>
<td>6.2</td>
<td>37.0 ± 2.7*</td>
<td>5.8 ± 0.9</td>
<td>36.7 ± 5.2</td>
</tr>
<tr>
<td>MMC 3 µg/ml</td>
<td>45.2 ± 2.7</td>
<td>5.1 ± 0.3</td>
<td>36.1 ± 1.6</td>
</tr>
</tbody>
</table>

*significantly different from positive control (MMC 3 µg/ml) (p<0.05)

Abbreviation: P.I.: proliferation index = (MI+2MII+3MIII)/100 cells;
M.I.: mitotic index = number of mitotic cells/1,000 cells

pretreatment with NFJ at lower doses of 3.1, 1.5 and 0.8 mg/ml (data not shown) and found that pretreatment of NFJ at these lower levels still could not protect cells from clastogenicity induced by MMC. Only pretreatment with NFJ at the optimum dose of 6.2 mg/ml could significantly reduce the frequency of SCEs induced by MMC. No significant difference of M.I. and P.I. between the NFJ pretreatment and the MMC treatment alone was observed.

Figure 5. Sister chromatid exchange (SCE) levels in terms of transformed SCE (SCEt) induced by various concentrations of Noni fruit juice followed by mitomycin C 3 µg/ml (*significantly different from positive control (MMC 3 µg/ml) at p<0.05)

4. Discussion and Conclusion

Nowadays, NFJ is one of the popular products in the market of food supplements. Therapeutic potentials of NFJ are being vastly advertised and promoted. Therefore, concern of the safety level of NFJ intake needs attention. Our data demonstrate that NFJ at concentrations of 3.1-100 mg/ml is not genotoxic against human lymphocytes *in vitro*. No dose response was observed. Interestingly, NFJ at the dose of 6.2 mg/ml was the optimum dose of stimulation of cell proliferation (equal to that of the negative control). In addition, pretreatment of NFJ at this specific dose followed by MMC treatment (3 µg/ml), could protect cells from genotoxicity as shown by a significant decrease in the frequencies of SCEs induced by MMC. However, under our experimental protocol, pretreatment by NFJ could not protect cells from clastogenicity induced by MMC. Pretreatment with NFJ at the highest dose of 100 mg/ml followed by MMC synergistically induced extensive cytotoxicity to human lymphocytes since no mitotic cells were found. These data confirm previous studies that Tahitian NFJ does not have genotoxic potential in mammalian cells (Westendorf et al., 2007). However, cytotoxic potential of NFJ has not been reported in normal human cells before, despite cytotoxic effects being found against tumor cells (Arpornsuwan and Punjanon, 2006). Noni fruit juice, on the other hand, at a specific dose showed some protective activity against genotoxicity induced by MMC. A previous report also demonstrated the preventive effect of Noni fruit juice at the initiation stage of carcinogenesis. 10% Tahitian NFJ could prevent DMBA-DNA adduct formation in rats (Wang and Su, 2001). Another report showed that treatment of a polysaccharide-rich fraction from Noni juice, known as Noni-ppt, together with MMC demonstrated additive beneficial effects of antitumor activity on ascites tumor in mice (Furusawa et al., 2003). Nevertheless, further investigations are needed for our study. Co-treatment or post-treatment studies with NFJ using different chemotherapeutic agents or irradiation to induce genotoxicity could produce different results. Individual genetic polymorphism, genetic susceptibility and metabolism are of concern for *in vivo* studies. Therefore, scientific studies of NFJ in human are necessary to clarify the use of NFJ as a therapeutic medicine in human.

In conclusion, our study reveals that NFJ at concentrations of 3.1-100 mg/ml has no genotoxic potential to human cells. Pretreatment of NFJ at the specific dose of 6.2 mg/ml could provide some antigenotoxic effect against MMC. In contrast, high dose pretreatment by NFJ (100 mg/ml) followed by MMC could provide a synergistically extensive cytotoxic effect. Optimum dosage of NFJ used as a traditional medicine is strongly required and more research is needed. In addition, NFJ product produced from various resources and different protocols could also provide different results. Good manufacturing practice for the NFJ products are needed to be controlled as well.
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