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Original research

Determination of cytotoxic, genotoxic and oxidative effects of Naringenin

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Abstract: Naringenin is one of the most researched flavonoid members, it possesses majority of the biological activities of flavonoids. In this work, it is aimed to investigate the *in vitro* cytotoxic, genotoxic and oxidative effects of Naringenin, which is found in many foods in our diet. The cytotoxic effect of Naringenin on human umbilical cord vein endothelial cells were analysed with the lactate dehydrogenase release and cell proliferation methods; in human lymphocytes the oxidative properties against carbon tetrachloride were examined with the measurement of catalase, superoxide dismutase and malondialdehyde; genotoxic effects were investigated with single cell gel electrophoresis and micronuclei methods. Naringenin revealed cytotoxic and genotoxic properties especially at high concentrations (100 and 200 μM). The most effective results in terms of antigenotoxic and antioxidant effects for Naringenin was observed 8 μM concentration. Therefore, care should be taken that Naringenin does not consume food containing high levels unconsciously, and individual concentration adjustments should be made in the treatments applied.

Keywords: Naringenin, Comet, LDH, Micronuclei, WST-1

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Introduction

In humans, an important component of the diet is phenolic compounds, defined as naturally occurring antioxidants. Phenolic compounds can be found in the fruits, vegetables, seeds, flowers, leaves, branches and bolls of plants (Coşkun, 2006). Phenolic compounds constitute the most studied group of plant-derived substances. The phenolic compounds in the plants are separated into three groups; phenolic acids, flavonoids and phenolic polymers. Of these, phenolic acids and flavonoids in foods are important (Aydın and Üstün, 2007). Flavonoids have received increased interest in the field of medicine and medicine due to their anti-inflammatory, antitumor, antifuncal, antimicrobial, antiviral, antiallergic and antithrombotic properties as well as antioxidant properties (Mazid et al., 2011; Prabakaran et al., 2011; Kocyigit et. al., 2016). Antioxidant metabolism of flavonoids is mainly due to their large capacity to stabilize and delocalize unpaired electrons, to reduce and chelate redox-active metal ions like Fe, to quench singlet oxygen, and to stimulate other antioxidative defense enzyme activities (Masuoka et al., 2012; Garg and Singla, 2015).

Among the various types of flavonoids, Naringenin (5,7,4-trihydroxyflavanone, NG), a naturally occurring flavanone in grapefruits and tomatoes, has been demonstrated to elicit a wide range of pharmacological and biological activities, including anti-inflammatory, anti-oxidative and anti-apoptotic (Renugadevi and Prabu, 2009; Bai et al., 2014). It is also known that in addition to the positive features mentioned, NG can be linked by

DNA intercalation and cytotoxic (Wang et al., 2006; Li et al., 2007).

Since previous studies have reported the positive and negative aspects of NG, in our study, it is aimed at the investigation of in vitro cytotoxic, genotoxic and oxidative effects NG, which is found in many foods in our diet. The cytotoxic effects of NG on human umbilical cord vein endothelial cells (HUVECs) were analyzed with the lactate dehydrogenase (LDH) release and cell proliferation (WST-1) methods; in human lymphocytes the oxidative properties against carbon tetrachloride (CCL_4) were examined with the measurement of catalase, superoxide dismutase and malondialdehyde; genotoxic effects were investigated with single cell gel electrophoresis (Comet) and micronuclei (MN) methods.

Materials and Methods

Active compound

NG was purchased from Sigma (St. Louis, MO, USA) and 100 mg/ml solution was dissolved in DMSO (Dimetil sülfoksit) stored at −20◦C until use.

Cell culture

HUVECs isolated from umbilical cords were cultured in DMEM-LG together with 10% FBS and 1% penicillin/streptomycin in a Forma Direct Heat CO_2 incubator (Thermo) at 37°C and 5% CO_2. Cell culture flasks (Greiner) with a polylysine layer were used to grow the cell lines. Various final concentrations of NG were added.

Cytotoxicity assay

Cells were seeded into 96-well plates at a density of $1x10^6$ cells/mL. After 24 h of seeding, cells were treated with different concentrations of acids or media alone as a control. The cytotoxicity of NG on human cultured lymphocytes was also assayed at 48 h by using following methods.

Lactate Dehydrogenase (LDH) assay

A Cytotoxicity Detection Kit (LDH; Roche) was used to measure the amount of LDH released by dead cells, according to the manufacturer's instructions. The conversion of tetrazolium salt into a red formazan dye product was measured calorimetrically. For this assay, a positive control leading to 100% cytotoxicity and lysing the cells completely was included in the assay. HUVECs were seeded into the 96-well plates at 1.0×10^4 cells/well and PBMCs at 5×10^3 cells/well in 100 μL of culture medium. After 24 h, wells discharged and 4, 8, 12,5, 25, 50, 100 and 200 μM concentrations of NG were added to the cells that had adhered to the bottoms of the wells. Experiments were performed three times per dose. Then, cells were cultured for up to 48 h following exposure to NG. For testing the released LDH activity, 100 mL of culture medium was transferred to a new 96-well plate at 24^{th} and 48^{th} hour. The 100 μL of reaction solutions from the kit, containing the detection dye and the catalyst were added to the wells and kept in the dark at room temperature for 30 min. Then, the absorbance at 490 nm was measured in a microplate reader [BioTek-Power Wave, Winooski, USA] (Kapuci et al., 2014).

Cell proliferation assay

Water Soluble Tetrazolium Salts (WST-1) assay

The WST-1 cell proliferation assay kit analyzes the viability and quantity of cells by solubilizing the tetrazolium salts by cellular enzymes. The growth of viable cells causes an increase of total mitochondrial dehydrogenase activity in the sample. This process also causes an increase in the amount of formazan dye formation that is correlated with the number of active cells in the culture. The same concentrations of HUVECs and PBMC cells as were used in the LDH assay were that cultured in microplates (tissue culture grade, 96-well and flat bottom) in a final volume of 100 μL/well culture medium in a humidified atmosphere (37°C, 5% CO_2); the cells were then treated with NG. After 48 h, the cell proliferation reagent WST-1 was added at a concentration of 10 μL/well. Cells were incubated for 4 h in a humidified atmosphere (37°C, 5% CO_2). The absorbances of the samples were measured at 450 nm with an ELISA reader (Kapuci et al., 2014).

Genotoxic assay

Micronuclei assay (MN)

Blood from four healthy nonsmoking donors between the ages of 23 and 25 were used for the MN assay. Heparinized whole blood (0.5 mL) was cultured in a final volume of 7 mL RPMI-1640 which contained 15% heat-inactivated fetal calf serum, 1% streptomycin, 1% penicillin, 2% glutamine and 2% phytohemagglutinin. To this solution the various agents to be tested was added and cultured at 37 °C for 72 h in a 5% CO_2 moist atmosphere.

Carbon tetrachloride (CCL_4) (5 µM), a carcinogen agent, was used as a positive control. The investigated NG was used at concentrations of 4, 8, 12, 5, 25, 50, 100 and 200 µM. CCL_4 and the NG were dissolved in 10% DMSO. CCL_4 and NG were added to the cultures just before incubation as indicated in Table. During the MN assay, cytochalasin B (3µg/mL) was added to the whole blood at 44 h incubation. After 72 h incubation, the cells were harvested by centrifugation (1000 rpm, 10 min), the supernatant collected and immediately assayed for biochemical analysis. To the cell pellet was added 6 mL of 0.05 M KCl and incubated at 37 °C for 7 min. Cells were harvested by centrifugation (1000 rpm, 10 min) and the supernatant was removed. To the pellet, 6 mL of fresh fixative (1: 3 acetic acid: methanol) was added drop by drop. The fixation procedure was repeated three times and the tube was centrifuged (1000 rpm, 10 min). The cell pellet was re-suspended in 1 mL of fresh fixative, dropped on to a clean microscope slide, incubated at room temperature for 72 h and stained with giemsa dye. The coded slides were air-dried and only bi-nucleated cells were scored for MN analysis. For each experimental group, approximately 1000 bi-nucleated cells were analyzed for the presence of MN (Orhan et. al., 2016).

Comet assay

The comet assay also known as single cell gel electrophoresis (SCGE) was performed and scored according to Singh and others (1988) and Prabhavathy Das and others (2006) with the following modifications:10 µL of fresh mononuclear leukocyte cell suspension (roughly 10.000 cells) were mixed with 75 µL of 0.7 % low melting agarose in PBS at 37 ◦C. Subsequently, 75 µl of the mixture was layered onto a slide pre-coated with thin layers of 1 % normal melting point agarose (NMA), and immediately covered with a cover slip. Slides were left for 5 min at 4 ◦C to allow the agarose to solidify. After removing the cover slips, the slides were submersed in freshly prepared cold (4 ◦C) lysing solution for at least 2h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer at 4 ◦C for unwinding (30 min) and then electrophoresed (24V/300mA, 30min). The whole procedure was carried out in dim light to minimize artifactual. After electrophoresis, gently lift the slides to the buffer and placed on a drain tray. The slides were drop wise coated with neutralization buffer, let sit for at least 5 minutes. Drain slides and repeated two more times. The dried microscope slides were stained with ethidium bromide covered with a cover slip and analyzed with a fluorescence microscope. Fifty cells were randomly scored by eye in each sample, on a scale of 0 – 4, based on fluorescence outside the nucleus as previously described by Collins (2004). The scale used was as follows: 0, no tail; 1, comet tail < half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus; 4, comet > twice the width of the nucleus. The visual score for each class was calculated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score characterizing the degree of DNA damage in the entire study groups was the sum of the scores in the five comet classes. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU), as reported by Collins (2004). The comets were scored independently by two investigators.

Oxidative Stress Parameters

Superoxide dismutase (SOD) assay

Cu-Zn-SOD activity of the whole blood cell culture supernatant was evaluated by the method of Sun and others (1988). In the assay, 2.45 mL of assay reagent [0.3mM xanthine, 0.6mM Na_2EDTA, 0.15mM nitroblue tetrazolium (NBT), 0.4 M Na_2CO_3, and 1 g/L bovine serum albumin] was combined with equal amount of protein from each experimental group and 50µL xanthine oxidase was added to initiate the reaction. The reduction of NBT by superoxide anion radicals was determined by measuring the absorbance at 560 nm. Cu, Zn-SOD activity was expressed in units of SOD per mg protein, where 1 U was determined as the amount of enzyme causing half-maximal inhibition of NBT reduction.

Catalase (CAT) assay

Catalase activity was determined by measuring the decrease in absorbance at 240 nm (Aebi, 1984). The reaction mixture contained 0.5 ml of enzyme extract and 2.0 ml of 0.1 M sodium phosphate buffer (pH 6.8) and the reaction was started by the addition of 0.5 ml of 10 mM hydrogen peroxide. The decrease of absorbance was recorded. Decrease of absorbance was recorded in every 15 sec up to 3 min. The values of the 1-minute linear absorbance reduction are based on the calculations.

Malanoaldehyde (MDA) assay

MDA levels in the whole blood cell culture supernatant were determined spectrophotometrically according to the method described by Ohkawa and others (1979). A mixture of 8.1% sodium dodecyl sulphate, 20% acetic acid and 0.9% thiobarbituric acid was combined with equal amount of protein from each experimental group (Paglia and Valentine, 1967). Distilled water was added to the mixture to make the total volume 4 mL. This mixture was incubated at 95° C for 1 h. After incubation, the samples were left to cool under cold water, 1 mL distilled water and 5 mL n-butanol/pyridine (15:1, v/v) were added to the solution and mixed thoroughly. The samples were centrifuged at 4000 rpm for 10 min. The supernatants were separated and measured at 532 nm. The level of MDA was calculated from a standard graph made by using different concentrations (1-10 nmol) of 1,1,3,3-tetramethoxypropane and was expressed as μmol of formed MDA in one mL of serum.

Statistical analysis

The statistical analysis of the LDH, WST-1, Comet, MN and oxidative stress parameter results data were performed using the Mann–Whitney U-test and one-way ANOVA. A level of probability of $p<0.05$ was taken as indicating statistical significance. All experiments were performed in three replicates and data was compared for reproducibility. For these procedures, SPSS 18.0 version for Windows (SPSS Inc, Chicago, Illinois, USA) was used. The results are expressed as the mean ± SE.

Results

In the present study, different concentrations of NG were performed with the Micronuclei (MN) test which widely used as a short term testy system. From each group, approximately 1000 bi-nucleate cells are analyzed for presence of MN. In MN test used positive control (CCL_4) significantly increased the MN frequency on peripheral lymphocytes when compared with the control as seen in Table. Such an increase was found to be statistically significant ($p < 0.05$). NG revealed cytotoxic and genotoxic properties especially at high concentrations (100 and 200 μM). The most effective results in terms of antigenotoxic and antioxidant effects for NG was observed 8 μM concentration (Table).

It was observed that the effect of NG on DNA damage in human lymphocytes induced by CCL_4 was determined by Comet assay. As shown from the results presented in Table. Especially low concentrations of NG, there were significant ($p < 0.05$) inhibitory effects on DNA damage. This result shows that the DNA damage in human lymphocytes induced by CCL_4 was reduced by the NG application. As an indicator of cell membrane damage, the LDH assay was used for detecting the cytotoxic activity of NG on HUVECs 24, 48, 72 and 96 h of incubation of the cells with 25 μM, 50 μM, 100 μM and 200 μM concentrations.

After 96th, absorbance of LDH was measured at 490 nm, cytotoxicity was then calculated according to the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{(Experimental value – Negative control)}}{\text{(Positive control – Negative control)}} \text{ X 100}$$

Table. The effects of CCL_4 and NG on MN, Comet and Oxidative Stress Parameters (SOD, CAT and MDA).

Test Items	Concentrations	MN numbers ± S. E	Visual Score (AU) ± S. E	CAT ± S. E (k/g protein)	SOD ± S. E (U/mL)	MDA ± S. E (n mol/mL)
Control		2.34 ± 0.03^{a}	7.40 ± 2.62^{a}	1974.5 ± 40.2^{a}	5,12 ± 1,16^{a}	2,32 ± 0,54^{a}
CCL_4	5 μM	4.53 ± 0.19^{e}	116.32 ± 2.13^{e}	1657.3 ± 14.2^{e}	3,28 ± 1,46^{e}	5,34 ± 1,72^{e}
NG	50 μM	2.85 ± 0.09^{bc}	42.20 ± 4.16^{b}	1890.4 ± 22.7^{b}	4,81± 0,17^{b}	2,70 ± 1,24^{ab}
CCL_4 + NG	5 μM + 4 μM	2.72 ± 0.14^{b}	86.13 ± 4.16^{d}	1873.4 ± 17.7^{b}	4,96 ± 0,17^{ab}	2,92 ± 1,17^{b}
CCL_4 + NG	5 μM + 8 μM	2.51 ± 0.16^{a}	81.18 ± 4.16^{c}	1904.4 ± 28.7^{ab}	5,01± 0,17^{a}	2,61 ± 1,19^{a}
CCL_4 + NG	5 μM + 12.5 μM	3.23 ± 0.23^{c}	98.12 ± 4.16^{d}	1821.4 ± 23.7^{c}	4,95 ± 0,17^{ab}	2,83 ± 1,32^{b}
CCL_4 + NG	5 μM + 25 μM	3.84 ± 0.06^{cd}	105.24 ± 1.82^{d}	1756.2 ± 36.8^{d}	4,38 ± 0,10^{c}	3,60 ± 1,14^{c}
CCL_4 + NG	5 μM + 50 μM	3.93 ± 0.04^{d}	108.73 ± 0.40^{d}	1710.4 ± 2.3^{de}	4,10 ± 0,13^{cd}	3,80 ± 2,32^{cd}
CCL_4 + NG	5 μM + 100 μM	---	118.10 ± 0.03^{e}	---	---	---
CCL_4 + NG	5 μM + 200 μM	---	120.50 ± 1.20^{e}	---	---	---

CCL_4 was used as positive controls for human lymphocytes.

$^{a, b, c, d, e, f}$ Statistically significant differences in the same column are indicated by the different superscripts ($\alpha = 0.05$).

As seen in Figure, the concentrations of NG (25, 50, 100 and 200 μM) caused a significant increase in the LDH level when compared with negative control groups. This increase was found as statistically significant ($p < 0.05$). Cell viability was evaluated on HUVECs, treated with four doses of NG. WST-1 assay, a similar experimental set-up as used in the LDH assay, was used and findings were compatible with LDH results. Statistical analysis demonstrated an important reduction in cell viability in the all concentrations of NG compared with control group at 96th hour ($p < 0.05$) (Fig.).

In the oxidative stress parameter study, it is evident that CCL_4 exposure significantly decreased the activities of enzymes (SOD and CAT) while it increased the level of MDA as shown in Table.

On the other hand, co-treatments of CCL_4 with different concentrations of NG increased the activities of CAT, SOD and decreased the MDA level. The statistical analysis (Table) showed a significant difference in CAT, SOD and MDA between all treated samples with CCL_4 and simultaneous treatment of CCL_4 with NG ($p < 0.05$).

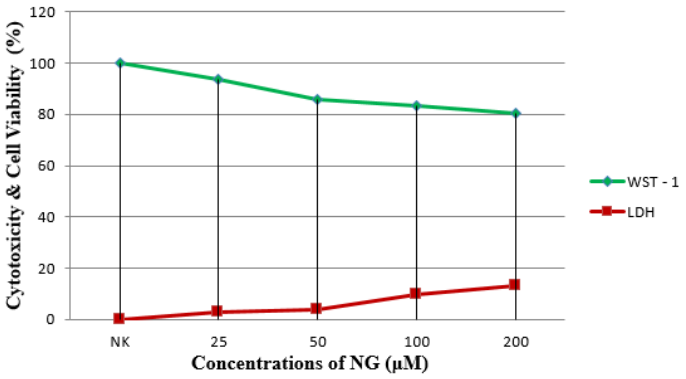

Figure. Results of LDH and WST-1

Discussion

The results of our study with HUVECs showed that concentrations of NG (100 and 200 μM) decreased cell viability and cell index and increased LDH release. Additional concentrations of NG (4, 8 and 12.5 μM) were applied in lymphocyte cells due to the cytotoxic effect of NG at high concentrations on HUVECs. Antimutagenic and antioxidant properties of NG were determined at low concentrations studied.

Although the mechanism of the mutagenic activities of flavonoids including NG is not fully known, it has been claimed that flavonoids may exhibit mutagenic effects with an intermediate product that interfere with DNA, intercalate with P450 cytochrome enzyme, and stimulate enzymes responsible for activation of mutagenic substances used in the study (Snijman et al., 2007). It has also been expressed that flavonoids in which NA is involved may induce DNA damage depending on concentration and duration, and genotoxic effects may be due to prooxidant activities (Rusak et al., 2010). In our experiments, the presence of cytotoxicity in the concentrations of NG, in particular at concentrations of 100 and 200 μM, suggests that high concentrations of NG may have resulted from the use of the above-mentioned mechanisms to exhibit genotoxicity.

As a result of our studies, it has been found that NG in abundant amounts in foodstuffs such as strawberries, tomatoes, citrus fruits and cocoa in our daily diet has beneficial results at certain concentrations but prooxidant, genotoxic and cytotoxic effect at high concentrations. In this context; care should be taken not to unconsciously consume foods containing high levels of NG, and individual concentration adjustments should be made in the treatments applied. In this way, organ toxicity, which can be caused by the prooxidant and genotoxic properties of NG in humans, can be prevented.

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