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14. ABSTRACT
Oxidative DNA damage has been closely linked to cancer development. An active DNA repair system is critical to prevent the occurrence of mutations leading to carcinogenesis. It was the objective of this investigation to test the hypothesis that natural products such as flavonoids are able to stimulate the repair of oxidative DNA damage. For this purpose LNCaP prostate tumor cells were exposed to FeSO4 to induce oxidative DNA damage (8-hydroxydeoxyguanosine determined by HPLC). DNA repair was evaluated by following the decrease of oxidative DNA damage over time. We were able to demonstrate that in LNCaP cells exposed to naringenin (80 μmol/L) oxidative DNA repair activity was increased by 24% compared to media treated controls. RT PCR results demonstrated that the increase in DNA repair was associated with an increased mRNA expression of three BER repair enzymes important in the repair of oxidative DNA damage: 8-oxoguanine-DNA glycosylase 1 (hOGG1), apurinic/apyrimidinic endonuclease (APE) and DNA polymerase β (DNA pol-β). We observed the maximum stimulatory effect on mRNA expression at 24 hours of naringenin treatment. The tea flavonoid EGC and apigenin from parsley did not show any DNA repair-stimulatory activity.

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Introduction

Prostate cancer is a disease of aging. The oxidative stress hypothesis of aging postulates that oxidative damage to critical molecules accumulates over a life span leading to chronic disease and cancer. Reactive oxygen species are formed continuously in living cells as byproducts of normal cellular metabolism, as well as by exogenous sources. In case of a failure to remove DNA damage, mutations occur at a high rate and contribute to malignant transformation. A particular abundant lesion, 8-hydroxydeoxyguanine (8-OHdG), is highly mutagenic, yielding GC to TA transversions (1). The base excision repair (BER) pathway is responsible for the repair of oxidative DNA damage. Removal of the damaged base by 8-oxoguanine-DNA glycosylase 1 (hOGG1) comprises the first step followed by apurinic/apyrimidinic endonuclease (APE) activity (2). DNA polymerase β (DNA pol-β) activity is responsible to fill the gap created by the excision of 8-OHdG. There is in vitro evidence that some flavonoids such as myricetin and baicalin will stimulate DNA repair (3, 4). Flavonoid concentrations used in these in vitro experiments are usually higher than physiologically achievable. It was the primary objective of this study to investigate the DNA repair stimulatory effect of different flavonoids such as naringenin, apigenin, ECG and their metabolic transformation products. It was the secondary objective to investigate if the intracellular concentration of these flavonoids is physiologically achievable.

Body and Key Research Accomplishments

Task 1:

a) The concentration of iron sulfate was optimized to produce reproducible oxidative DNA damage. 200 µmol/L $FeSO_4$ was chosen for future experiments. At this concentration oxidative DNA damage was increased 4.6 fold compared to cells not treated with $FeSO_4$. At this concentration DNA damage is high enough to be measured reproducibly by HPLC without affecting the viability of the cells (Figure 2 and 3). At 200 µmol/L $FeSO_4$ we demonstrated that LNCaP cells were able to repair the oxidative DNA damage (Figure 4). We also determined the concentration of 5-α-dihydrotestosterone necessary to generated reactive oxygen species. However for the treatment with 5-α-dihydrotestosterone it is necessary to grow LNCaP cells in charcoal stripped fetal bovine serum (CS-FBS) (Figure 5). Since LNCaP cells grow very slowly in medium with CS-FBS compared to regular FBS we were not able to determine the effect of 5-α-dihydrotestosterone-induced oxidative stress on DNA repair. In addition the concentration of 5-α-dihydrotestosterone necessary to induce reproducible oxidative stress is very high (>1mmol/L).

Conclusion Task 1a: In summary exposure with 200 µmol/L $FeSO_4$ is the most appropriate mode to induce oxidative damage.

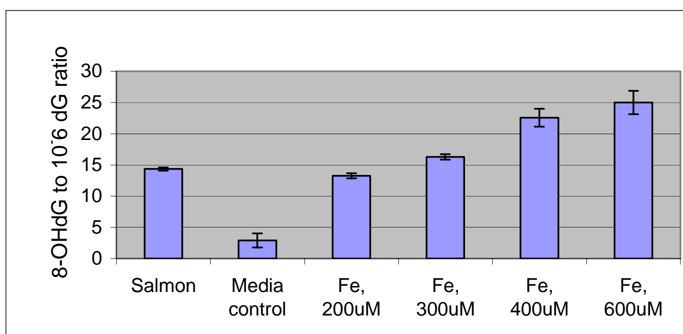


Figure 1. Oxidative DNA damage determined by HPLC in LNCaP P47 cells treated with different concentration of FeSO4 for 1 hour.

Figure 2. LNCaP P47 cell viability treated with different concentrations of FeSO4 and H_2O_2 for 1 hour and viability was tested immediately.

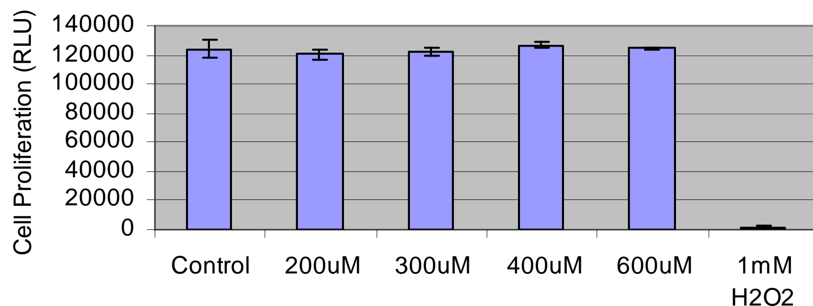


Figure 3. LNCaP P47 cell viability treated with different concentrations of FeSO4 and H_2O_2 for 1 hour and viability was tested after 24 hours.

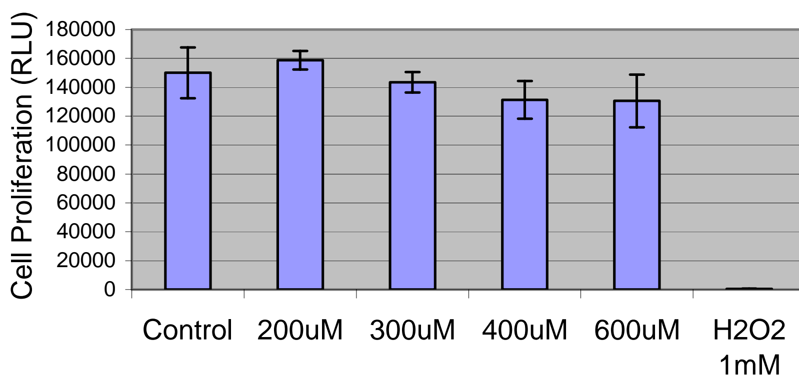


Figure 4. DNA repair in LNCaP P47 cells after FeSO4 (200 µmol/L) treatment for 24 to 72 hours.

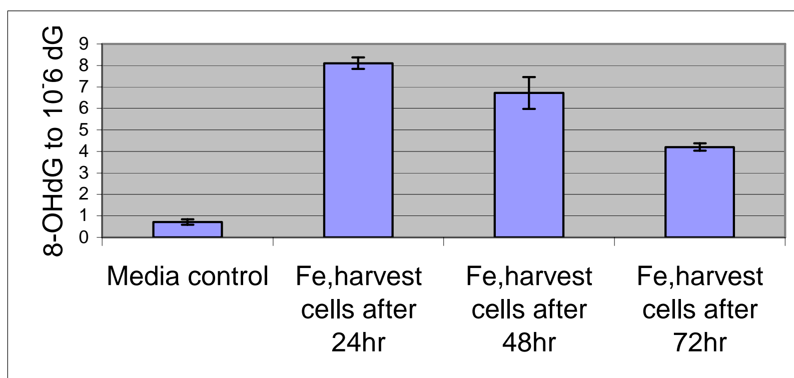
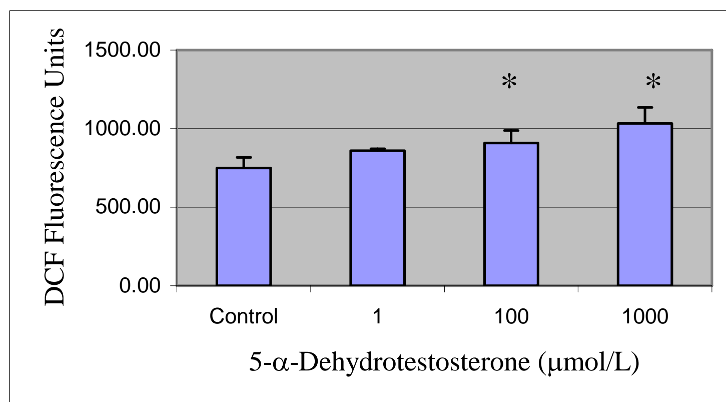


Figure 5. Intracellular concentration of reactive oxygen species (ROS) after 5α-dihydrotestosterone-treatment of LNCaP cells for 1 h. n=3, *significant different from control ($p<0.05$).

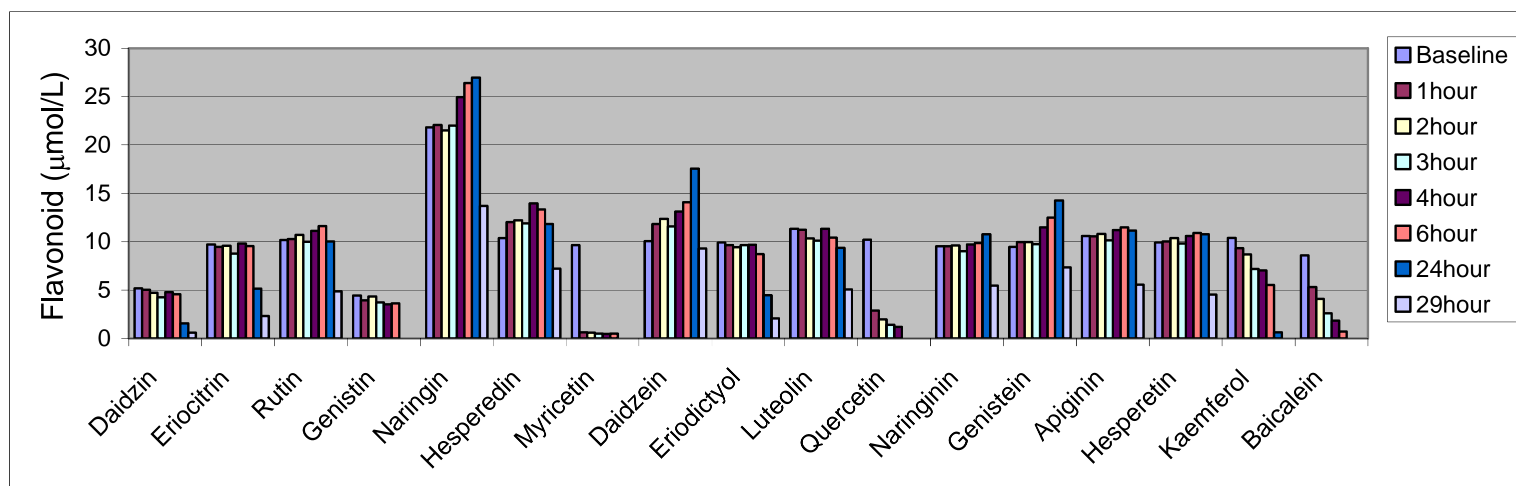


Task 1:

b) To screen different flavonoids for their ability to stimulate DNA repair we screened the pH stability at pH 7 as in culture medium and antiproliferative effect. Based on these results we selected the following three flavonoids to continue the investigations on the stimulatory effect on DNA repair:
   - -naringenin from citrus
   - -apigenin from parsley
   - -epicatechin gallate (ECG) from tea

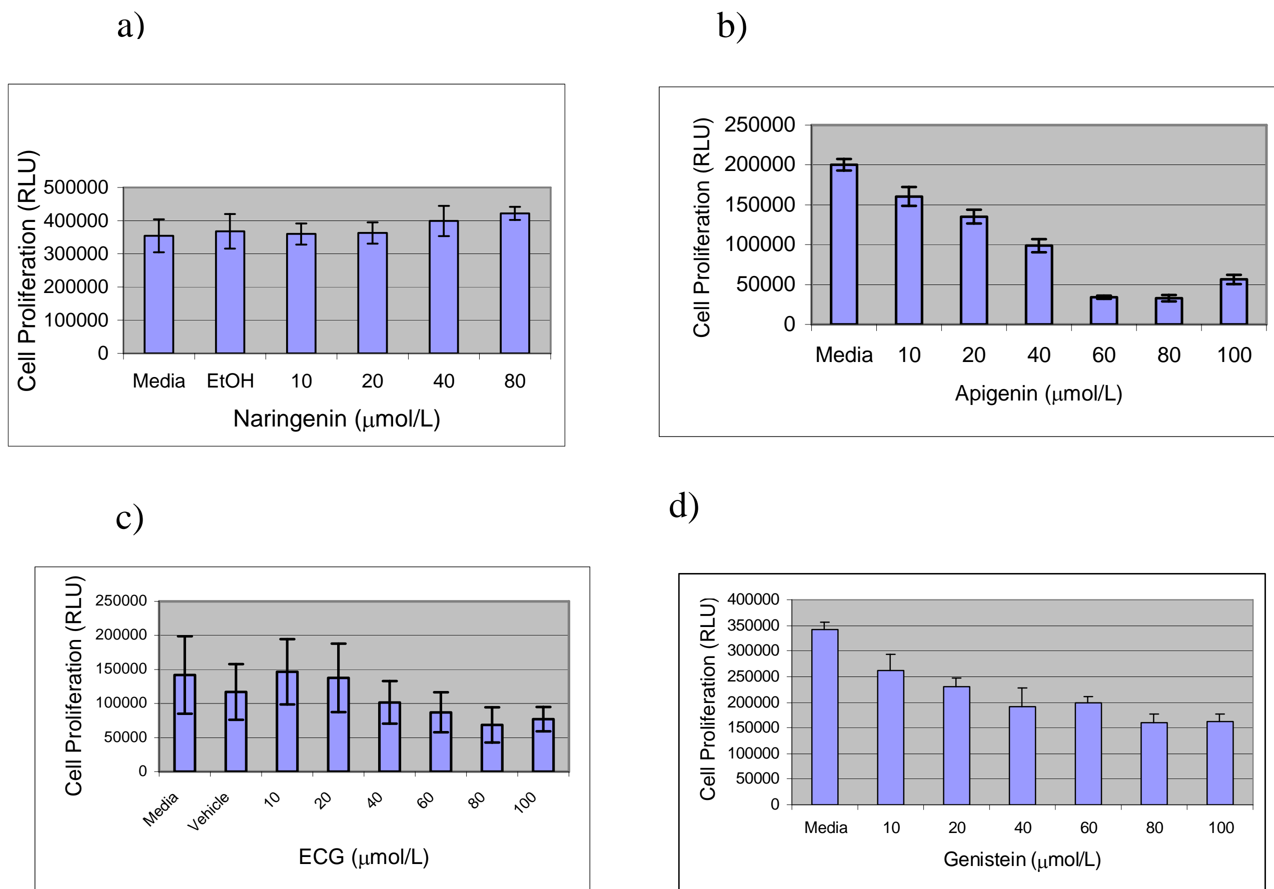
These three flavonoids are representative of the flavanone and flavanol chemical subgroups. Unfortunately many compounds of the other flavonoid subgroups are not pH stable and will be degraded in cell culture experiments (Figure 6).

Figure 6. Stability of different flavonoids in cell culture medium at 0 to 29 hours.



The antiproliferative activity of the naringenin, ECG, apigenin and genistein was determined in LNCaP cells using the (Figure 7). Cell viability was tested at 24 hours of incubation with the test compounds using the CellTiter-Glo™ Assay (Promega, Madison, WI).

Figure 7. Antiproliferative activity of a) naringenin, b apigenin, c) ECG and d) genistein at concentrations from 0 to 80 µmol/L.



<u>Conclusion from Task 1b):</u> We demonstrated that naringenin, apigenin and ECG were the best candidates to be tested for potential DNA repair-stimulatory activity based on the chemical pH stability and low antiproliferative activity. However, since apigenin did exhibit an antiproliferative activity we used a concentration of 20 µmol/L to test the DNA repair stimulatory activity.

Task 1:

c) The intracellular concentration of naringenin and tea polyphenols was determined in LNCaP cells cultured in RPMI 1640 medium after addition of 10 µmol/L of the individual flavonoid. The maximum intracellular concentration of naringenin was 40 pmol/10^6 cells (Figure 8). The same experiment was performed with all tea polyphenols (EC, ECG, EGCG, EGC) (Figure 9).

Figure 8. Decrease of naringenin in a) medium and uptake into b) LNCaP cells.

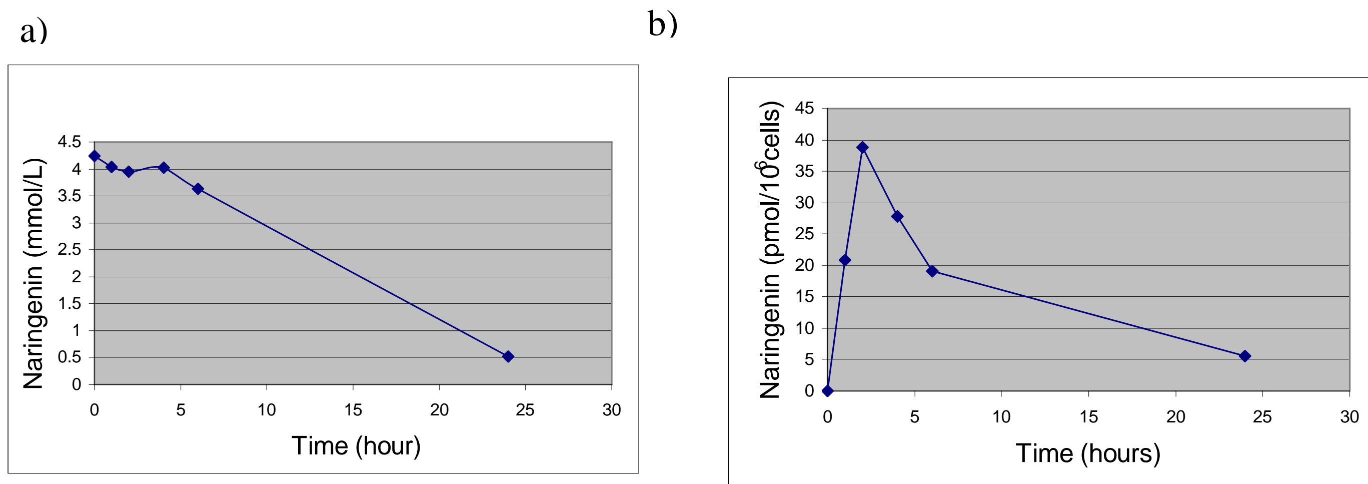
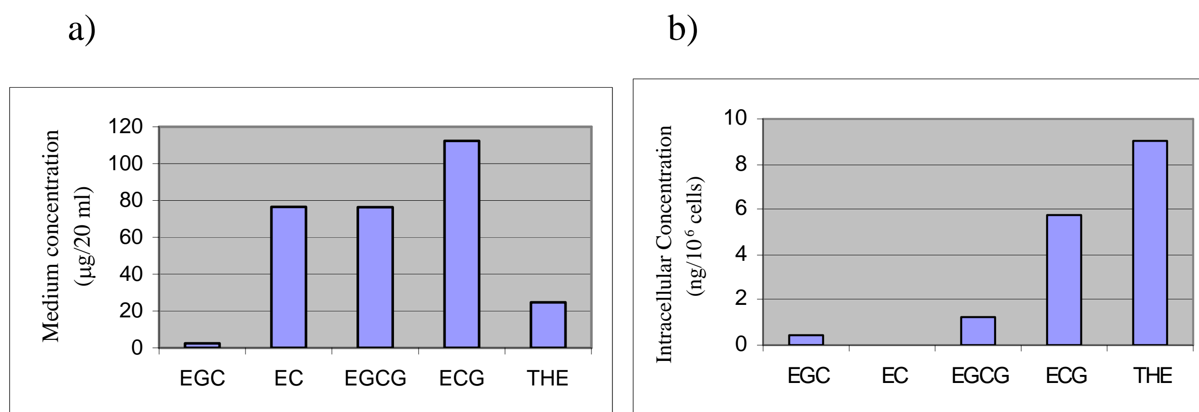


Figure 9. Medium (a) and intracellular (b) concentration of tea polyphenols (EGC, EC, EGCG, ECG and theaflavin-mix) after exposure of cells to 20 µmol/L of tea polyphenols for 2 hours.



Conclusion Task 1c: The intracellular concentration of flavonoids was about 500 to 1000-fold lower compared to medium concentration. Therefore the intracellular concentration reflects physiological concentrations achievable in the human body.

Task 1:

d) The DNA repair stimulatory effect of naringenin, apigenin and ECG was determined in LNCaP cells exposed for one hour to FeSO4 (200 µmol/L). After removal of the iron, cells were treated with increasing concentrations of naringenin (10 to 80 µmol/L), 20 µmol/L of agipenin or ECG for 24 hours. We demonstrated that naringenin stimulated DNA repair in turn leading to a decrease in oxidative DNA damage of 3-24% compared to media only treated cells (Figure 10), whereas apigenin and ECG did not decrease oxidative DNA damage (Figure 12). Using real time PCR we also demonstrated that mRNA expression of hOGG1, DNA pol-β and APE was increased at 8 hours compared to baseline and decreased at 24 hours (Figure 11a-c). At 24 hours

naringenin had the strongest stimulatory effect on mRNA expression of these BER enzymes. HOGG1, DNA pol-β and APE were determined using real-time PCR analysis. Total RNA was extracted using the RNAeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using Taq polymerase and oligo (dT) followed by PCR amplification using the following real-time PCR Assay-on-Demand custom-made kits (Applied Biosystems Inc., Foster City, CA): human OGG1: Hs00213454_m1; ; human polymerase beta: Hs00160263_m1 and human APE/ref-1: Hs00205565_m1. PCR reaction mix was prepared using Taqman Universal Master Mix (Applied Biosystems Inc., Foster City, CA), cDNA template in Rnase-free water, target assay mix or control assay mix. Samples were analyzed on the ABI 7700 (Taqman), which was available in the UCLA Sequencing and Genotyping Core Facility.

Figure 10. DNA repair stimulated by naringenin determined by the decrease in 8-OhdG/dG ratio in LNCaP cell DNA after treatment with 0 to 80 μmol/L naringenin.

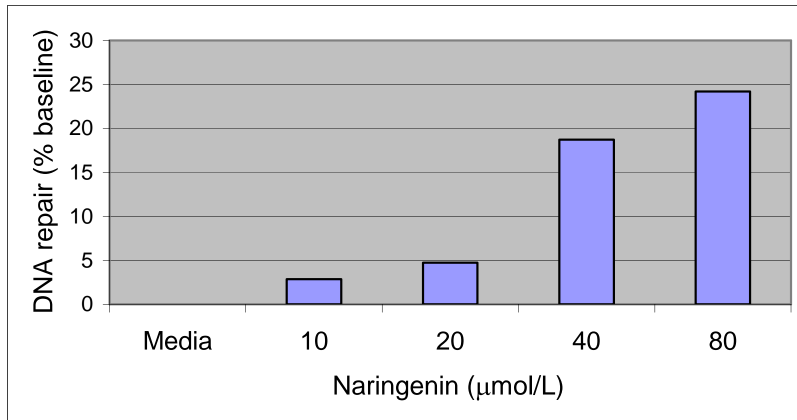
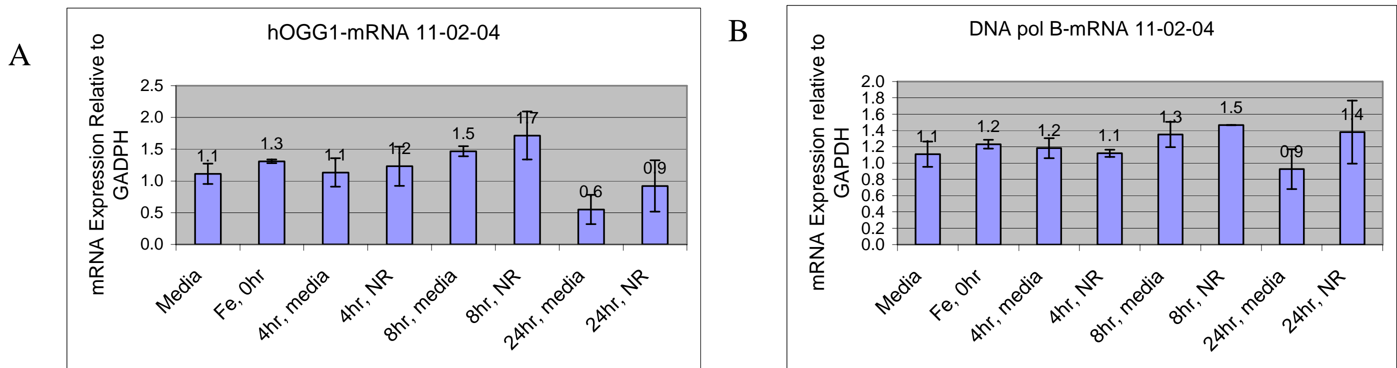


Figure 11. BER repair enzyme m-RNA expression determined by real time PCR after naringenin intervention for 0-24 hours. A) hOGG1; B) DNA pol-b; C) APE. HOGG1, DNA pol-β and APE mRNA expression in LNCaP cells was determined by RT-PCR and expressed in ratio to GAPDH housekeeping gene (n=2).



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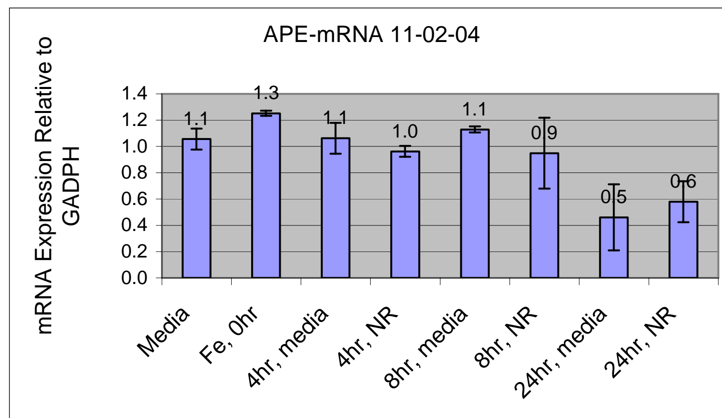
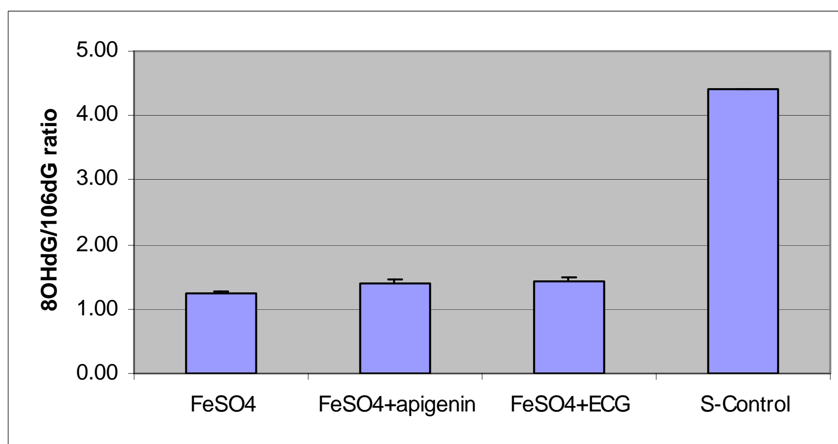


Figure 12. DNA repair stimulated by apigenin and ECG determined by the decrease in 8-OhdG/dG ratio in LNCaP cell DNA after treatment with 20 µmol/L apigenin and ECG.



Conclusion Task 1d: We were able to demonstrate that naringenin was the only flavonoid among the tested flavonoids that stimulated BER in oxidatively stressed LNCaP cells. The results have been published (see appendix) (5).

Task 2:

According to our results naringenin is the only flavonoid that stimulated DNA repair among the flavonoids stable at pH 7. Therefore we did not investigate possible synergistic effects of combinations of different flavonoids for their DNA repair enhancing activity. Instead we determined a possible synergistic effect on antiproliferative activity of tea polyphenols (Figure 13 and Table 1).

Figure 13. Antiproliferative activity of three tea polyphenols (epigallocatechin gallate EGCG, epigallocatechin ECG and theaflavin THE). N=3.

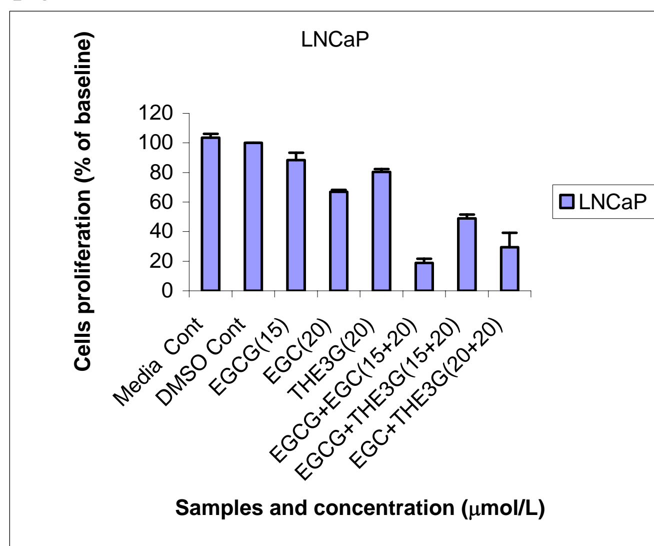


Table 1. Synergistic effect of green and black tea polyphenols on LNCaP cell culture proliferation. Data are mean ± std. n=3.

	measured	calculated	p-value
	% of control		
EGCG+EGC	18.8±2.8	55.3±6.3	0.0002
EGCG+THE	48.9±2.5	68.9±6.9	0.00032
EGC+THE	23.8±0.8	47.2±3.3	0.0359

Conclusion Task 2: We were able to demonstrate a synergistic effect in cell growth inhibition between combinations of EGCG+EGC, EGCG+THE and EGC +THE.

Task 3:

a) Commercially available human microsomes were purchased and exposed to naringenin. The incubation mixture was extracted with ethylacetate. Extracts were analyzed using HPLC after the incubation and peak area of naringenin and unknown new metabolite peaks were evaluated (Table 2). After incubation the peak area of naringenin was reduced by 53% and two new peaks were detected. Since human microsomes are very expensive and the amount of conjugated naringenin was not enough to perform any antiproliferative experiments we decided to use mouse liver homogenates to produce metabolites.

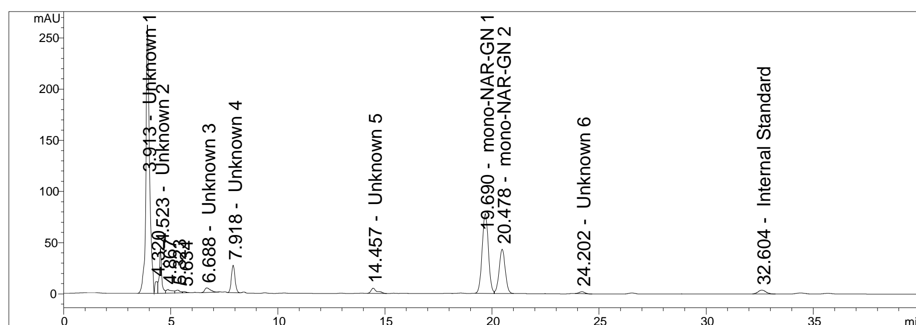
Table 2: Incubation of human liver microsomes incubated with 10 µmol/L of naringenin.

Retention Time	Compound	no microsomes	microsomes
		Peak area	
46.3	Unknown	4.2±1.3	61.9±0.6
48.7	Unknown	0.0	24.3±2.3
52.4	TRF Internal Std	31.4±0.7	33.8±1.3
57.4	Naringenin	317.9±4.5	151.8±0.8

b) Another set of experiments were performed by incubating mouse liver homogenates with different concentrations of naringenin following the method by Uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA) with liver homogenate. Homogenates were extracted with ethylacetate and extracts analyzed by HPLC. No glucuronides were found.

c) Another set of experiments were performed using cell free extracts of mouse liver homogenates, incubated with b-glucuronidase inhibitor D-saccharic acid 1,4-lactone (Sigma Chemicals,) and a pore forming fungal peptide (alamethicin, Sigma,) according to Williams et al and O'Leary et al (6, 7). Incubation mixtures were extracted with ethylacetate and in a second separate extraction with methanol. The parent compound naringenin was detected in the ethylacetate extract, wherease 2 metabolites were found in the methanol extract (Figure 14). Currently we are repeating this incubation in a larger scale to gain enough metabolite mix to perform a cell culture experiment.

Figure 14: HPLC chromatogram of methanol extract of liver homogenate incubation with naringenin. Only trace amounts of naringenin (Retention time 34.4) remain. Peaks at 18.6 and 20.5 are mono-glucuronide metabolites of naringenin.



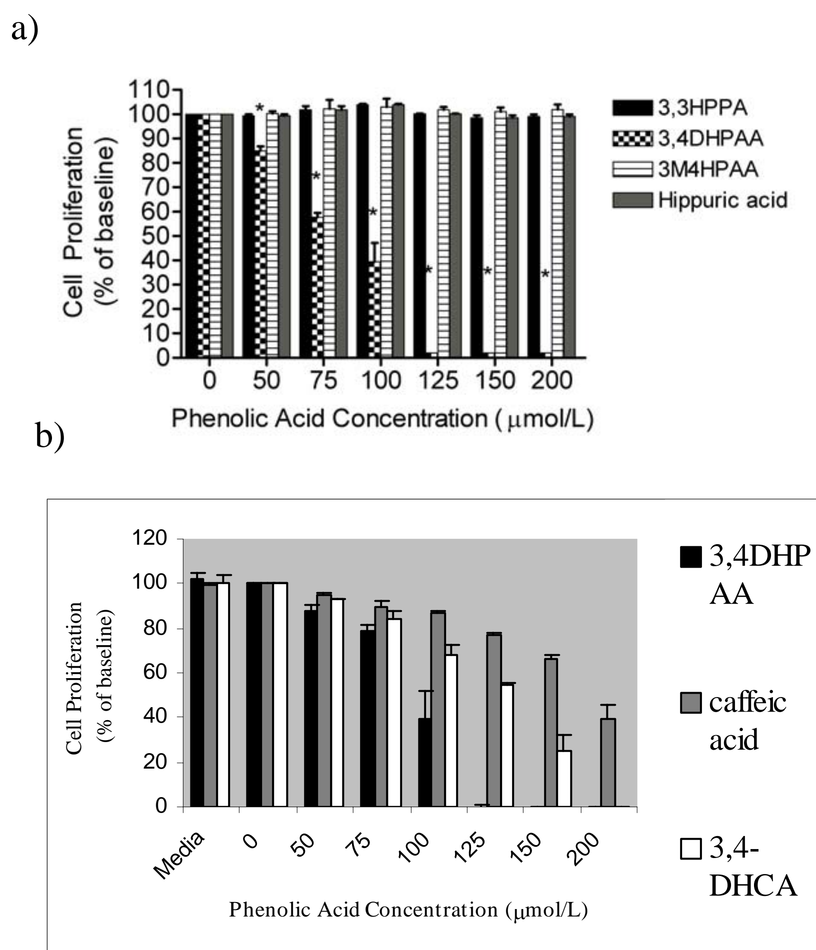
<u>Conclusion Task 3:</u> We were able to develop an incubation system to generate sufficient naringenin glucuronide mixture to test the growth inhibitory and DNA repair stimulatory activity of the metabolite mixture compared to the parent compound. However these experiments are still ongoing and an additional report will have to be submitted.

Task 4:

The following phenolic acid breakdown products have been determined as intestinal metabolites from green and black tea, citrus and soy: 4OH-phenylacetic acid, 3-(3hydroxy-phenyl)-proprionic acid, homovanillic acid, 3,4dihydroxyphenyl acetic acid, hippuric acid, 2,4,6-trihydroxybenzoic

acid; citrus: same phenolic acids, 3-hydroxyphenylacetic acid, 3-(4-hydroxy-3-methoxyphenyl) propionic acid, *p*-hydroxybenzoic acid. We tested the antiproliferative activity of all of the phenolic acid products found in the human colon simulator (8). Only 3,4DHPAA exhibited a significant inhibition of cell proliferation at 24 and 48 hours (IC50= 75 µmol/L) (Figure 15a) (8). Since only a dihydroxyphenolic acid was active we tested the antiproliferative activity of two other dihydroxyphenolic acids (caffeic acid and 3,4 dihydroxy-cinnamic acid) (Figure 15b). Both compounds exhibited lower antiproliferative activity compared to 3,4DHPAA.

Figure 15. Antiproliferative activity of a) 3-(3hydroxy-phenyl)-proprionic acid (3,3HPPA), 3,4dihydroxyphenylacetic acid (3,4DHPAA), 3-methoxy4-hydroxyphenylacetic acid (3M4HPAA) and hippuric acid; b) 3,4-dihydroxyphenyl acetic (3,4DHPAA), caffeic acid and 3,4 dihydroxy-cinnamic acid at 10 to 100 µmol/L for 24 hours.



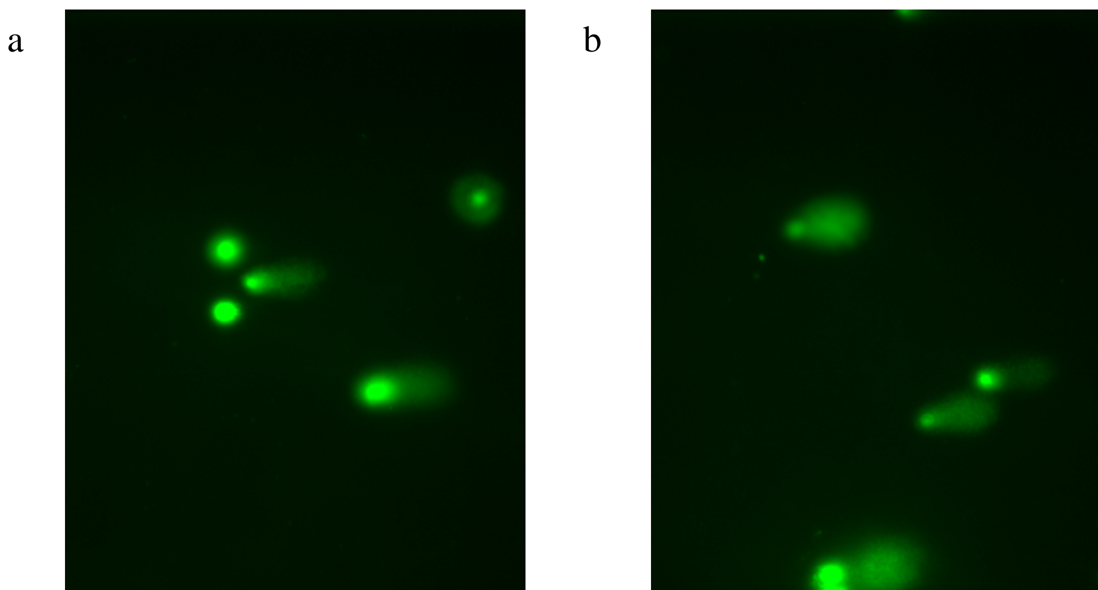
Conclusion Task 4: Investigations are ongoing in my laboratory to determine the chemical characteristics of flavonoid metabolites generated in the human colon. Among the phenolic acids identified by GC/MS only 3,4-dihydroxyphenylacetic acid (3,4DHPA) had antiproliferative activity. We are planning to determine whether 3,4DHPA has DNA repair stimulatory effects.

Additional work performed:

Since performing the HPLC determination of oxidative DNA damage is very labor intensive we set up the Comet assay to determine DNA strand breaks after exposure to oxidative stress (200 µmol/L $FeSO_4$). The alkaline comet assay detects alkali-labile DNA sites, which are a global assessment of DNA single-strand and double-strand breaks and DNA base damage. The cell/agarose suspension was dispersed on a layer of 2% agarose in running buffer on glass slide. Slides were incubated in lysis solution at 4°C for 1 h followed by eletrophoresis buffer (pH>13) for unwinding DNA. Finally single cells were subjected to electrophoresis. Nuclei were stained with SYBR®Gold (Molecular Probes, Eugene, OR) and length of the comet tail will were determined using a Zeiss fluorescent microscope (rhodamine filter). Digital pictures were taken and evaluated using the J 1,31 Image program. The length of comet tails were proportional to the number of single strand breaks (9) (Figure 16). This comet assay was applied to normal prostate cells (PrEC), LNCaP human prostate cancer cells and hi-myc mouse cancer cells (Figure 17).

All three cell lines suffered increasing DNA damage with exposure to increasing concentration of oxidative stress ($FeSO_4$). However at 800 µmol/L of $FeSO_4$ LNCaP cells developed a significantly higher amount of DNA strand breaks compared to PrEC cells. This is in good agreement with work by other investigators demonstrating that oxidative DNA repair is compromised in prostate cancer cell lines such as LNCaP and PC-3 (10). In future experiments we will measure DNA strand breaks after replacing $FeSO_4$-containing medium with control medium for 24 hours to be able to compare the repair capacity of the normal vs cancer cell lines.

Figue 16: Alkaline comet assay showing increasing DNA damage associated with increased comet length (a to b).



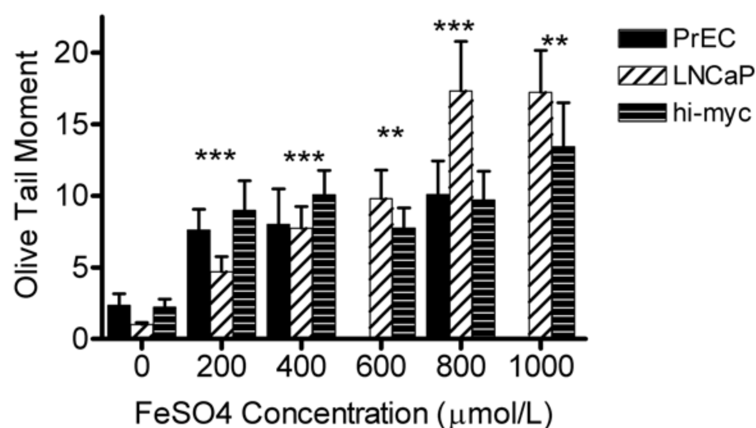


Figure 17: Determinaiton of oxidative DNA damage using the alkaline comet assay in PrEC, LNCaP and hi-myc prostate cells. Cells were incubated with FeSO4 for 1 hour and harvested and frozen in RPMI 1640 medium with 10% FBS to protect cells from damage. Comet assay was performed in all three cell lines in parallel.

Reportable Outcomes:

Henning, S.M., Gao, K., Xu, A. & Heber, D. The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells. FASEB J. 19(4), A1475, 2005.

Gao, K., **Henning,S.M**., Youssefian, A.,A., Seeram, N.P., Xu, A. and Heber, D. 2005. The Citrus Flavonoid Naringenin Stimulates DNA Repair in Prostate Cancer Cells. J. Nutr. Biochem. 2005 Aug 17; [Epub ahead of print]

Gao, K., Xu, A., Krul, C., Venema, K., Liu, Y., Niu, Y., Lu, J., Bensoussan, L., Seeram, N.P., Heber, D. & **Henning, S.M**. Of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements only 3,4-dihydroxy phenylacetic acid has antiproliferative activity. J. Nutr. (accepted for publication), January 2006.

Conclusions:

We were able to demonstrate that naringenin has DNA stimulatory activity in LNCaP cells. This increase in DNA repair was based on an increase in three DNA repair enzymes such as hOGG1, DNA pol-b and APE. Currently we are generating enough naringenin glucuronide mixture for cell culture experiments. In the future these flavonoid metabolites and 3,4dihydroxyphenylacetic acid will be tested for their DNA repair-stimulatory activity.

Future Studies:

Set up ELISA assay to measure the concentration of 8OhdG excreted into the medium as a indication of DNA repair. With this assay it will be less labor intensive to determine the effect of different flavonoid metabolites on DNA repair.

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Appendix:

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The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells

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Abstract

As part of a systematic study of the effects of phytochemicals beyond antioxidation on cancer prevention, we investigated whether naringenin (NR), a citrus flavonoid, stimulates DNA repair following oxidative damage in LNCaP human prostate cancer cells. The 8-hydroxydeoxyguanosine (8-OH-dG) to deoxyguanosine (dG) ratio was measured after cells were treated with 200 μmol/L of ferrous sulfate in serum-free medium followed by NR exposure for 24 h in growth medium. The results demonstrated that exposure to 10–80 μmol/L of NR led to a significant decrease in the ratio of 8-OH-dG to 10^6 dG. Because cells were treated with NR after ferrous sulfate was removed, we conclude that we demonstrated an effect on DNA repair beyond antioxidation. In support of this conclusion, we determined the induction of mRNA expression over time after oxidative stress followed by NR administration of three major enzymes in the DNA base excision repair (BER) pathway: 8-oxoguanine-DNA glycosylase 1 (hOGG1), apurinic/apyrimidinic endonuclease and DNA polymerase β (DNA poly β). hOGG1 and DNA poly β mRNA expression in cells after 24-h exposure to NR was increased significantly compared with control cells without NR. The intracellular concentration of NR after exposure to 80 μmol/L was 3 pmol/mg protein, which is physiologically achievable in tissues. In conclusion, the cancer-preventive effects of citrus fruits demonstrated in epidemiological studies may be due in part to stimulation of DNA repair by NR, which by stimulating BER processes may prevent mutagenic changes in prostate cancer cells.
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Keywords: Naringenin; Antioxidant activity; 8-Hydroxydeoxyguanosine/10^6deoxyguanosine ratio; APE/Ref-1; hOGG1; DNA poly β

1. Introduction

Flavonoids are found in many plant-based foods including fruits and vegetables and compose the major dietary group of plant polyphenols. Among the various types of flavonoids in fruits and vegetables associated with cancer prevention, the flavanones naringenin (NR) and hesperidin are found in grapefruit and oranges [1]. Although the antioxidant effects of citrus flavonoids due to their capability to scavenge free radicals [2–4] have attracted a great deal of attention, there are effects beyond antioxidation that may be important in determining the anticancer activity of phytochemicals such as flavonoids, including effects on cell proliferation [5], inhibition of angiogenesis [6], inhibition of subcellular signaling [7] and stimulation of DNA repair enzymes [8,9].

Reactive oxygen species (ROS) formed endogenously or due to exogenous factors can cause oxidative damage to biologic macromolecules including nucleic acids [10]. Excessively damaged cells either undergo apoptosis [11] or survive. In the surviving cells, checkpoint pathways are activated to inhibit progression of cells through the G1 and G2 phases to permit removal of damage [12] and re-enter

Abbreviations: hOGG1, 8-oxoguanine-DNA glycosylase 1; APE/Ref-1, apurinic/apyrimidinic endonuclease; DNA poly β, DNA polymerase β; BER, base excision repair; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; 8-OH-dG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine.

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into the cell cycle. If the DNA damage is not repaired, then gene mutations occur at a high rate and can lead to malignant transformation. A particularly abundant lesion of oxidative DNA damage is 8-hydroxydeoxyguanosine (8-OH-dG), which is highly mutagenic as the result of GC to TA transversions [13]. The base excision repair (BER) pathway is responsible for the repair of oxidatively damaged DNA bases. Removal of the damaged base is a result of increased 8-oxoguanine-DNA glycosylase 1 (hOGG1) and apurinic/apyrimidinic endonuclease (APE/Ref-1) activities [14]. DNA polymerase β (DNA poly β) then fills the gap created by the excision of 8-OH-dG [15]. The flavonoids myricetin [16] and baicalin [9] have been shown to stimulate DNA repair at a physiologically achievable concentration of 100 μmol/L. The present study investigates whether NR at a physiologically relevant concentration in prostate cancer cells stimulates repair of oxidative DNA damage through the BER pathway.

2. Materials and methods

2.1. Materials

NR was obtained from LKT Laboratories (St. Paul, MN, USA). Tris–HCL, sodium acetate, ferrous sulfate, diethylenetriamine pentaacetic acid (DTPA) and deoxyguanosine (dG) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DNase I from bovine pancreas, nuclease P1 (NP1) from *Penicillium citrinum* and alkaline phosphatase (AP) were purchased from Roche Diagnostics (Indianapolis, IN, USA); phosphodiesterase (PDE) I from snake venom (*Crotalus adamanteus*) and PDE II from calf spleen were purchased from Worthington Biochemical (Lakewood, NJ, USA). All reagents were of highest commercial quality (Fisher Scientific, Tustin, CA, USA). Oligo(dT)$_{12-18}$ Primer and SuperScript III Reverse Transcriptase (RT) were from Invitrogen (Carlsbad, CA, USA); dNTP was obtained from Fisher Scientific.

2.2. Cell line and culture condition

A hormone-responsive human prostate cancer cell line (LNCaP) was obtained from America Type Culture Collection (Bethesda, MD, USA). The LNCaP prostate cancer cells were cultured in RPMI 1640 medium from VWR Scientific (San Francisco, CA, USA), supplemented with 10% FBS, 100 U/ml of penicillin and 100 ng/ml of streptomycin. Cells were grown at 37°C in a humidified atmosphere supplemented with 5% CO_2 in air. The doubling time for LNCaP was 36 h.

For the 8-OH-dG determination, cells were seeded in 100-mm cell culture dishes at the amount of 5×10^6 cells/dish and then cultured at 37°C for at least 24 h before treatment. After being treated with ferrous sulfate and NR, cells were rinsed with 5 ml of cold phosphate-buffered saline (PBS), dislodged with 1 ml of trypsin–EDTA at 37°C for 2 min, then suspended with 9 ml of growth medium and centrifuged at 2500 rpm for 5 min, washed with 5 ml of PBS and then stored at −80°C for DNA isolation. To test cell viability, cells were seeded in 96-well plates at a concentration of 50 cells/μl, 100 μl/well, and cultured at 37°C for 24 h before treatment. For the NR uptake experiment, cells were seeded in 150-cm^2 flasks, cultured at 37°C to reach 80% confluence, then cultured with NR spiked in growth medium for different times and then collected the same way as that for 8-OH-dG determination.

2.3. DNA ratio of 8-OH-dG to 10^6 dG determination by high-performance liquid chromatography

DNA was isolated from cells using the DNA isolation kit from Roche Diagnostics. The DNA was dried under the flow of N_2 and redissolved in 100 μl of AE buffer (Qiagen, Valencia, CA, USA), supplemented with 2 μl of 2-mmol/L iron chelator DTPA to prevent artificial oxidation. The digestion procedure was performed as previously described [17]. Briefly, the following incubation steps were performed: DNase I, 30 min at 37°C; NP1, 60 min at 37°C; AP, 30 min at 37°C; and PDE I and PDE II, 30 min at 37°C. The incubation mixture was filtered through a Millipore ultrafree 0.5 filter.

The high-performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 HPLC system consisting of a binary pump, thermostatted autosampler, variable wavelength detector, controlled by Chemstation Software 11.0 (Agilent Technology, San Diego, CA, USA), and ESA Coulochem II electrochemical detector (ESA, Bedford, MA, USA), a C_{18} Alltima guard column,7.5×4.6 mm, particle size of 5 μM (Alltech, Deerfield, IL, USA) connected to a YMC, and an ODS-AQ column, 4.6×15 cm, 120 Å, S-5 (Waters, Milford, MA, USA). The mobile phase consisted of 100 mmol/L of sodium acetate buffer (pH 5.2) supplemented with 10% aqueous methanol. Elution was isocratic at a flow rate of 0.8 ml/min. The dG concentration was monitored based on absorbance (245 nm) and 8-OH-dG based on the electrochemical reading (400 mV). Levels were quantified using the standard curves of each compound. The degree of DNA damage was expressed as 8-OH-dG per 10^6 dG.

2.4. Cell uptake of NR evaluation

Cells were mixed with 12 μl of 10% ascorbic acid−40 mmol/L NaH_2PO_4–0.1% EDTA, 20 μl of PBS (pH 7.4), 20 μl of 12.952-μmol/L 3,3′,4′-Trihydroxyflavone as internal standard, 500 U of β-D-glucuronidase type X-A from *Escherichia coli* (Sigma, St. Louis, MO, USA) and 4 U of sulfatase type VIII from abalone entrails (Sigma). The mixture was incubated at 37°C for 45 min followed by two extractions using 4 ml of ethyl acetate. The supernatant was vacuum concentrated for 2 h at low temperature with a Savant SC-100 Speed-Vac system (Savant Instruments, Farmingdale, NY, USA). The samples were reconstituted in a 200-μl mixture of mobile phase A and methanol (3:2, vol/vol), and 20 μl was injected into the HPLC column.

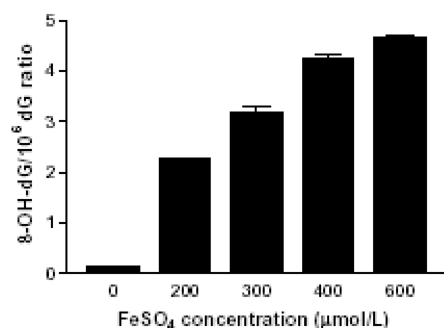

Fig. 1. 8-OH-dG/10^6 dG ratio in cells treated with 200–600 μmol/L of ferrous sulfate for 60 min. $n=2$, experiment repeated three times with similar results. Values are mean±S.D.

The HPLC-UV system consisted of an Agilent 1050 quaternary pump, autosampler controlled by Chemstation Software 7.01 (Agilent Technology, Wilmington, DE, USA), and Agilent 1050 multiple wavelength detector, a C_{18} Alltima guard column, 7.5×4.6 mm, particle size of 5 μm (Alltech), and an Aqua column, 250×4.6 mmol/L, 125 Å, particle size of 5 μm (Phenomenex, Torrance, CA, USA). The column was eluted at room temperature with a linear gradient from 95% mobile phase A [75 mmol/L citric acid–25 mmol/L ammonium acetate] and 5% mobile phase B [75 mmol/L citric acid–25 mmol/L ammonium acetate/acetonitrile (50:50)] at a flow rate of 0.8 ml/min. The gradient was linearly changed to 90% A/10% B in 4 min, 70% A/30% B in 4–12 min, 66% A/34% B in 12–17 min, 63% A/37% B in 17–20 min, 57% A/43% B in 20–29 min, 42% A/58% B in 29–35 min, 40% A/60% B in 35–40 min, 38% A/62% B in 40–68 min, 20% A/80% B in 68–72 min and 95% A/5% B in 72–80 min. The eluent was monitored at a detector setting of 260 nm.

2.5. Cell viability assay

The cells in 96-well plates were treated with ferrous sulfate for 60 min; after removal of ferrous sulfate, the viability was determined immediately or 24 h after cells were cultured at 37°C. Viability assay was performed with a CellTiter-Glo* Luminescent Cell Viability Assay (Promega, Madison, WI, USA). A 100-μl assay mix was added into each well after being equilibrated to room temperature. Plates were mixed on an orbital shaker for 2 min and further incubated at room temperature for 10 min. The content of each well was transferred into a new clean opaque 96-well plate, and the luminescence was determined for each sample using an Orion microplate luminometer from Berthold Detection Systems (Oak Ridge, TN, USA).

2.6. Real-time quantitative RT-polymerase chain reaction

The RNA was isolated using a Mini-Rneasy kit (Qiagen). The cDNA was synthesized from 5 μg of total RNA mixed with 1 μl of 0.5-μg/μl Oligo(dT)$_{12-18}$ primer and 1 μl of 10α-mmol/L dNTP. The relative mRNA quantity of APE/Ref-1, DNA poly β and hOGG1 was determined using Assay-on-Demand gene expression products (assay IDs: Hs00205565_m1, Hs00160263_m1 and Hs00213454_m1, respectively) from Applied Biosystems (Foster City, CA, USA), GAPDH gene (assay ID: Hs99999905_m1) as internal control and Taqman Universal PCR Master Mix. The real-time polymerase chain reaction (PCR) was performed on a PRISM 7700 Sequence Detection System (Applied Biosystems). The PCR was performed with the following conditions: 2 min, 50°C; 10 min, 95°C; 40 cycles of 15 s, 95°C; 1 min, 60°C.

2.7. Statistical analysis

PRISM statistical analysis software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data are expressed as mean±S.D. Means of outcome variables from cells undergoing treatment were compared with medium controls using Student's t test.

3. Results

3.1. Response of LNCaP cells to ferrous sulfate-induced oxidative stress

The optimum ferrous sulfate concentration that would induce enough oxidative damage to be detectable by HPLC

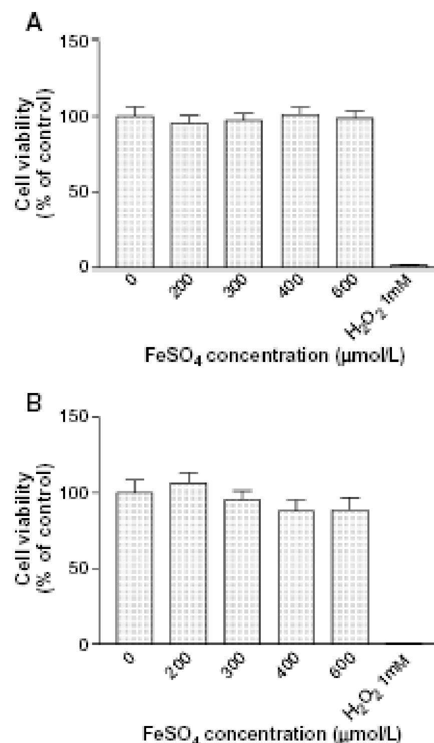

Fig. 2. Cell viability (A) after or (B) 24 h after cells were treated with 200–600 μmol/L of ferrous sulfate for 60 min. $n=3$, experiment repeated three times with similar results. Values are mean±S.D.

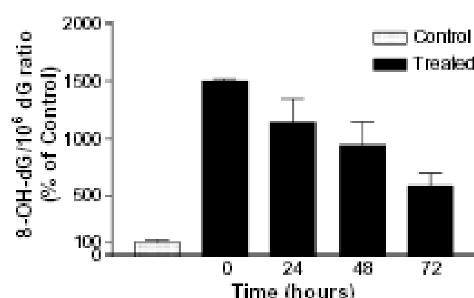

Fig. 3. 8-OH-dG/10^6 dG ratio in cells 24, 48 and 72 h after 200-μmol/L ferrous sulfate treatment for 60 min. $n=2$. Values are mean±S.D.

in a reproducible manner but not so high as to induce cell death in LNCaP cells was determined. Our results demonstrated that the oxidative DNA damage expressed as the 8-OH-dG/10^6 dG ratio occurred in a ferrous sulfate concentration-dependent manner. The 200–600-μmol/L ferrous sulfate treatment of LNCaP cells caused a 15- to 30-fold increase of the 8-OH-dG/10^6 dG ratio compared with media control (Fig. 1). The cell viability after treatment with 200–600 μmol/L of ferrous sulfate was measured immediately or 24 h after ferrous sulfate removal. The results indicated that treatment with up to 600 μmol/L of ferrous sulfate did not affect cell viability (Fig. 2).

In addition, we tested the DNA repair capacity of LNCaP cells up to 72 h after treatment with 200 μmol/L of ferrous sulfate. As shown in Fig. 3, DNA repair progressed slowly. Approximately 24% of DNA damage was repaired after 24 h, and approximately 40% of DNA damage was left unrepaired 72 h after treatment. Based on these facts, we decided that 200-μmol/L ferrous sulfate treatment of LNCaP cells was an appropriate cell culture model to investigate the DNA repair stimulatory effect of NR.

3.2. The effect of NR on the repair of oxidative DNA damage

Because the intention of this investigation was to determine the effect of NR on DNA repair and not its ability to scavenge free radicals, the ferrous sulfate generator of

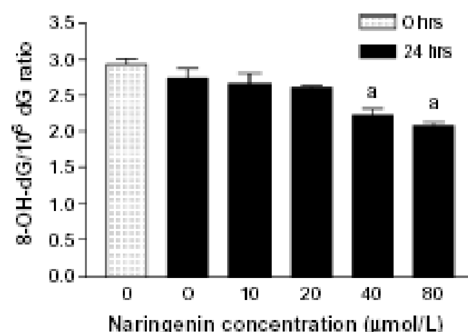

Fig. 4. Decrease of 8-OH-dG/10^6 dG ratio in cells exposed to 10–80 μmol/L of NR after 200-μmol/L ferrous sulfate treatment. $n=2$, experiment repeated three times with similar results. *a* indicates significant difference from control cells treated with the same concentration of ferrous sulfate, $P<.05$ (Student's *t* test).

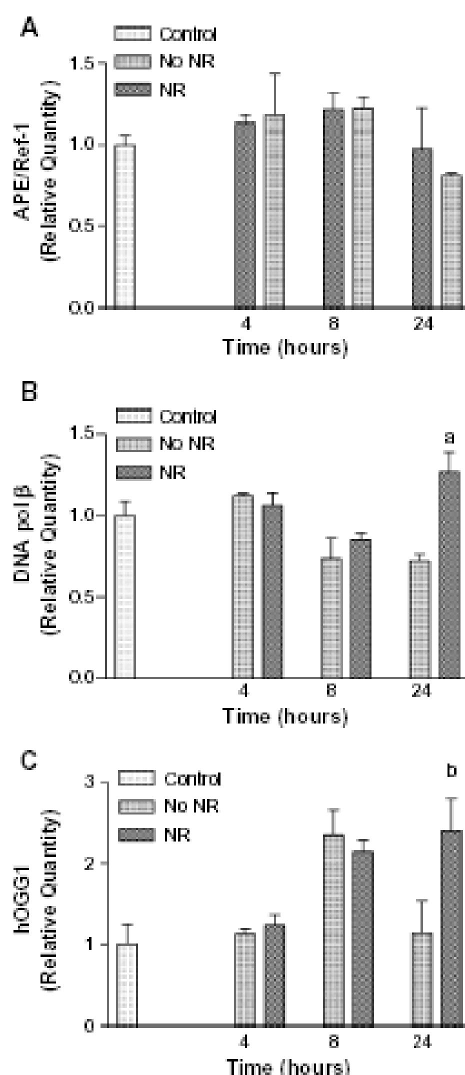

Fig. 5. The NR effect on mRNA expression of three main enzymes in the BER pathway — (A) APE/Ref-1, (B) DNA poly β and (C) hOGG1 — was determined using real-time PCR relative to GAPDH, a reference housekeeping gene. *a* and *b* indicate significant difference from control cells not treated with NR, $P<.05$ (Student's *t* test).

ROS was removed prior to the exposure of LNCaP cells to different concentrations of NR (10–80 μmol/L) over a subsequent 24-h period of study. DNA repair during the latter 24-h period was assessed by measured reductions in the 8-OH-dG/10^6 dG ratio. The 8-OH-dG/10^6 dG ratio was decreased in a concentration-dependent manner up to 24% compared with control cells after NR treatment (Fig. 4).

3.3. The effect of NR on gene expression of BER enzymes

The three main enzymes in the BER pathway — hOGG1, APE/Ref-1 and DNA poly β — were investigated. The mRNA levels for each enzyme were compared with the control cells not exposed to ferrous sulfate. The APE/Ref-1 mRNA expression increased slightly by 13% and 17% after 4 h and 21% and 22% after 8 h with or without NR

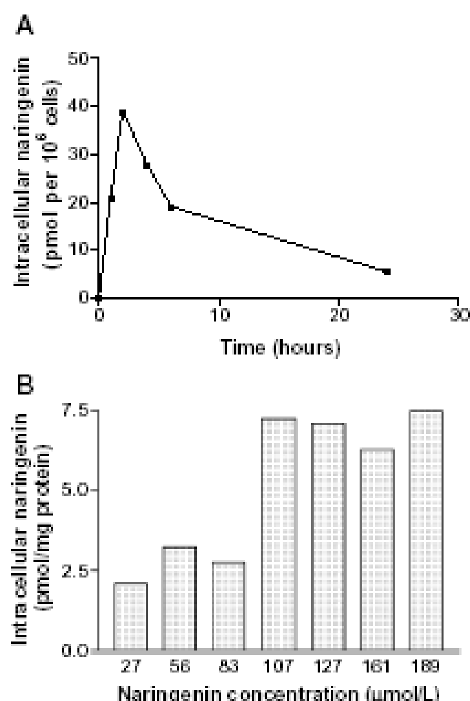

Fig. 6. NR uptake by cells. (A) NR uptake with a concentration of 5 μmol/L at 1, 2, 4, 6, and 24 h. (B) NR uptake with different concentrations at 4 h.

treatment and returned to baseline levels after 24 h. The hOGG1 mRNA expression was increased by 23% and 13% after 4 h and 114% and 135% after 8 h with or without NR treatment compared with the control only treated with ferrous sulfate for 60 min. At 24 h, the gene expression of cells not treated with NR returned to baseline levels, whereas hOGG1 expression in cells treated with NR was significantly increased by 140% over baseline levels in cells not treated with NR (Fig 5C). The relative quantity of DNA poly β was not increased significantly compared with controls not treated with ferrous sulfate for up to 8 h. However, at 24 h, the DNA poly β mRNA expression was increased significantly by 85% when comparing cells treated with NR with media controls (Fig. 5B).

3.4. Cell uptake of NR

As shown in Fig. 6, the intracellular NR concentration reached the highest concentration after 4 h, with a concentration of 40 pmol/10^6 cells. After 24 h, 10% of NR remained inside the cells compared with the peak concentration. Cell uptake of NR from 25 to 200 μmol/L at 4 h was also determined by the same method. The results indicated that the uptake reached a plateau at approximately 7.0 pmol/mg protein.

4. Discussion

Prostate cancer is the most commonly diagnosed male malignancy in the United States and the second leading cause of male cancer death after lung cancer, with an estimate of 220,900 new cases for 2003 [18]. Unlike many other neoplasms, prostate carcinoma is slow growing initially and often remains subclinical for an extended period. The incidence of prostate cancer increases rapidly with age, and multiple factors such as ethnicity, genetics, environment, hormones and diet have been implicated in its oncogenesis [19]. Loss of glutathione S-transferase P1 is an early change in prostate cancer cells leading to the production of ROS, which in turn can stimulate prostate cancer cell growth in the LNCaP cell line, one of the most widely studied models of prostate carcinogenesis [20].

In the present study, we clearly demonstrated that LNCaP cells are highly resistant to oxidative stress. Cell viability was not compromised by ferrous sulfate treatment at concentrations as high as 600 μmol/L. The present results also demonstrated that the capacity to repair oxidative DNA damage was limited in LNCaP cells (Fig. 3). Our results in this regard confirmed those of Fan et al. [21] in demonstrating that LNCaP cells have reduced DNA repair enzyme activity compared with normal prostate cells. Therefore, LNCaP cells provided a relevant model for studying the effects of NR on DNA repair. In the present study, the baseline level of 8-OH-dG/10^6 dG in the intact LNCaP cells was <1.0. These results matched well with a recently published validation of background levels of DNA oxidation by the European Standards Committee on Oxidative DNA Damage or ESCODD, which demonstrated a median value of 2.78 8-OH-dG/10^6 dG in HeLa cells [22].

In previous studies, NR has been reported to inhibit cell proliferation in HMEC and MCF-7 cells with IC_{50}s of 17 μg/ml (63 μmol/L) and 51 μg/ml (187 μmol/L), respectively [23]. In comparison with other flavonoids such as rhamnetin and apigenin, NR exhibited a lower anti-proliferative effect on human cancer cell lines [24–26]. In the present study, the IC_{50} of NR on LNCaP proliferation at 24 h was >300 μmol/L (data not shown) and NR did not exhibit any antiproliferative or cytotoxic effect on LNCaP cells at the 80-μmol/L concentration used. Ferrous sulfate was used to induce oxidative stress in LNCaP cells via the Fenton reaction, which in turn led to increased oxidized DNA base lesions [27]. To separate the antioxidant and DNA repair effects of NR, ROS generation via ferrous sulfate exposure of the cells was terminated prior to exposure to NR for the subsequent 24 h. Using this study design, we clearly demonstrated that NR stimulated induction of DNA repair enzymes. In addition, gene expression of the three major enzymes of the BER pathway was determined and showed differential responses to the NR stimulatory effect. In the present study, hOGG1 and DNA poly β were increased significantly after NR treatment for 24 h. The mRNA level of hOGG1 and DNA poly β remained high in cells exposed to NR. A similar stimulatory effect on DNA poly β mRNA was previously demonstrated by myricetin exposure at a concentration of 100 μmol/L [8]. Because LNCaP cells are known to have reduced ability of

DNA repair [21], the sustained increased levels of hOGG1 and DNA poly β mRNA in response to NR exposure may be important in mediating the potential cancer-preventive effects of this flavonoid in prostate cancer.

On the other hand, there were no significant changes in APE/Ref-1 mRNA after NR exposure. The stability of APE/Ref-1 mRNA expression may be due to the central role of this enzyme in signal transduction as it mediates the DNA binding of a number of transcription factors [28]. This may explain why NR had no effect on APE/Ref-1 mRNA.

An additional consideration in the interpretation of these data is whether GAPDH is suitable as a reference gene [29,30]. It has been reported that GAPDH can be affected by many factors such as different stages of pathology [31], oxidative stress [32] or food deprivation [33]. However, under the conditions of our study, the control samples were also treated with ferrous sulfate; therefore, a possible stimulation of GAPDH gene expression by oxidative stress would have occurred in the control samples as well.

An important characteristic of flavonoids is their limited bioavailability [34]. Only 2–15% of flavonoids are absorbed from the gastrointestinal tract. Plasma concentrations of flavonoids after consumption of a flavonoid-rich food are in the range of 0.5–1 μmol/L [35]. Although exposing the cultured cells with 80 μmol/L might appear unphysiological, we demonstrated that the intracellular concentration is 0.5% of the NR concentration in the medium. Therefore, the intracellular concentration at which these changes occur is in an achievable physiological range.

In summary, we demonstrated the effects of NR beyond antioxidation by showing that it can stimulate the induction of BER enzyme gene expression in LNCaP prostate cancer cells following an oxidative stress. Moreover, this stimulation in turn led to enhanced DNA repair as determined by quantitation of 8-OH-dG/10^6 dG levels. We conclude that induction of DNA repair enzyme expression by NR may contribute to the cancer-preventive effects associated with an increased dietary intake of fruits containing flavonoids.

Acknowledgment

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